NOVEL PROCESSES AND PRODUCTS FOR RECOMBINANT PRODUCTION OF BIOPHARMACEUTICALS

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Theory is when you know all and nothing works.
Practice is when all works and nobody knows why.
We have put together theory and practice:
nothing works... and nobody knows why!

Albert Einstein

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ABSTRACT

The monoclonal antibody market represents the fastest-growing segment within the biopharmaceutical industry (Evans and Das 2005). Indeed, recombinant antibodies and antibody fragments are widespread tools for research, diagnostics and therapy (Joosten *et al*., 2003). Large-scale production of recombinant antibodies and antibody fragments requires a suitable expression system which has to be cheap, accessible for genetic modifications, easily scaled up for greater demands and safe for use in consumer applications. However, the established eukaryotic systems are expensive, time consuming and sometimes inefficient (Farid, 2007). Although prokaryotic expression systems can reduce production costs, recombinant antibody production in conventional bacterial hosts, such as *E. coli,* often results in inclusion bodies formation (Baneyx and Mujacic 2004). Since the lowering of the expression temperature can increase product solubility facilitating its correct folding (Sahdev *et al*., 2008), a novel process for recombinant antibody fragments production at low temperatures was developed based on the use of the Antarctic Gram-negative bacterium *P. haloplanktis* TAC125 as recombinant expression host. To test the versatility of the new developed process, the production of three aggregation prone model proteins was evaluated corresponding to the most common formats of antibody fragments: Fab, ScFv and VHH. The construction of an *ad hoc* genetic expression system for each model protein followed a rational design where several critical aspects were considered including the selection of molecular signals for periplasmic protein addressing and the choice of optimal gene-expression strategy. For Fab fragment production in heterodimeric form an artificial operon was designed and constructed. Moreover, a new defined minimal medium was developed to maximise bacterial growth parameters and recombinant production yields. The production of model antibody fragments has been evaluated in lab-scale bioreactor and the effect of different cultivation operational strategies on production yields has been investigated. All model proteins were produced in soluble and biologically competent form in optimised conditions. About 5 mg L^{-1} of biologically active ScFv were obtained in *P. haloplanktis* TAC125 batch fermentation and up to 4 mg L^{-1} of soluble Fab in C-limited chemostat cultivation with a volumetric productivity of 0,2 mg L^{-1} h^{-1}. Moreover, a comparison of Fab fragment production by different microbial hosts including yeasts, filamentous fungi and bacteria was performed revealing that the psychrophilic expression system leads to the highest Fab specific productivity and best quality of recombinant product (Dragosit *et al*., submitted). In order to further optimise the novel process for recombinant production of biopharmaceuticals by the psychrophilic expression system, new genetic tools for recombinant gene expression and protein addressing in *P. haloplanktis* TAC125 were searched out and characterised. Transcriptional analysis of several target genes was performed in a cheap defined medium in the presence of inexpensive compounds of physical stimuli as inducers in order to identify new psychrophilic promoters for regulated expression of recombinant proteins at low temperatures. The analysis led to the identification of the gluconate permease PSHAb0479 and the alginate lyase PSHAa1748 promoters, strongly up-regulated by the presence of D-gluconic acid and alginic acid respectively. In order to improve extracellular secretion of recombinant proteins in psychrophilc expression system a deep investigation was performed on protein secretion machineries evolved by *P. haloplanktis* TAC125. Molecular characterisation of canonical T2SS was carried out and a deep study was performed on the still uncharacterised psychrophilic secretion system (PSS) responsible for the secretion of the recombinant alpha-amylase used as carrier for recombinant protein secretion in the psychrophilic bacterium. The function of *pss*A gene was related to the new secretion system. Data collected have provided important information which can be used for the construction of engineered *P. haloplanktis* TAC125 strains with improved ability in recombinant proteins extracellular secretion.

RIASSUNTO

Processi e prodotti innovativi per la produzione di farmaci ricombinanti per la terapia e la diagnostica molecolare

Premesse scientifiche e metodologiche

I recenti sviluppi nel campo della genetica molecolare e nella tecnologia del DNA ricombinante hanno dato un forte contributo all'affermazione di proteine ricombinanti nel mercato dei biofarmaci. In particolare, oltre il 30% del mercato biofarmaceutico è attualmente occupato dagli anticorpi monoclonali (mAbs) (Evans and Das 2005). L'alta affinità e specificità di legame con l'antigene, infatti, fanno degli anticorpi monoclonali uno strumento molecolare di enorme valore applicativo. La recente disponibilità di frammenti anticorpali ricombinanti di ridotte dimensioni e ridotta complessità strutturale apre la strada a all'impiego di mAbs in numerose nuove applicazioni soprattutto in campo diagnostico e terapeutico (Joosten *et al.*, 2003). L'impiego di mAbs e frammenti anticorpali ricombinanti in campo medico è però ad oggi limitato dall'alto costo della terapia. A causa del grande numero di pazienti e delle alte dosi richieste, sono necessari processi produttivi molto costosi per far fronte all'alta richiesta del mercato (Farid, 2007). L'impiego di anticorpi monoclonali ricombinanti su larga scala richiede dunque la disponibilità di un sistema di espressione adeguato che ne consenta la produzione in maniera rapida, efficiente ed economica. Il processo classico di produzione di mAbs prevede l'impiego di cellule di mammifero, in particolare cellule CHO ricombinanti, i cui costi di processo molto alti e rese contenute (1-2 mg/L) rendono il prezzo di mercato del prodotto finale molto poco competitivo (300-3000 USD/g) (Farid, 2007). Piante e animali transgenici mostrano rese più elevate e un sensibile abbattimento dei costi di produzione ma la bassa riproducibilità del processo e i potenziali rischi di contaminazione da parte di agenti infettivi limita l'utilizzo di questi sistemi (Ferrer-Miralles *et al*., 2009). L'impiego di sistemi di espressione procariotici può ridurre significativamente i costi di produzione di anticorpi monoclonali grazie alla sensibile riduzione dei tempi del processo, la maggiore riproducibilità e la minore richiesta di nutrienti (Arbabi-Ghahroudi *et al.* 2005). Anche i sistemi di espressione procariotici convenzionalmente utilizzati per la produzione di proteine ricombinanti, primo tra tutti *Escherichia coli,* mostrano però delle limitazioni. Nonostante infatti sia spesso possibile ottenere alte concentrazioni di prodotto, altrettanto spesso esso si presenta sottoforma di aggregati proteici insolubili che prendono il nome di corpi di inclusione (Baneyx and Mujacic 2004). Nonostante siano molte le strategie messe a punto per limitare questo fenomeno, resta ancora da definire il sistema di espressione ideale per la produzione di frammenti anticorpali ricombinanti. Uno dei parametri che maggiormente influenza la resa di prodotto solubile è la temperatura di crescita dei batteri produttori (Sahdev *et al*., 2008). La diminuzione della temperatura infatti riduce il contributo entropico sfavorendo le interazioni idrofobiche che sono tra le cause principali della formazione dei corpi inclusi. Il recente sviluppo di sistemi di espressione innovativi che utilizzano batteri adattati al freddo rappresenta dunque una valida alternativa per la produzione su larga scala di frammenti anticorpali ricombinanti in forma solubile e biologicamente attiva. A tal proposito risulta particolarmente interessante il batterio psicrofilo Gram negativo *Pseudoalteromonas haloplanktis* TAC125. Questo batterio, per il quale sono già disponibili sistemi genetici per l'espressione e secrezione di proteine ricombinanti (Parrilli *et al*., 2008), è in grado di crescere in un ampio intervallo di temperature (4-28°C) e concentrazioni saline (0,5-45 g/L NaCl) raggiungendo alte densità cellulari. Inoltre la disponibilità della sequenza del suo genoma (Medigue *et al.,* 2005) rende *P. haloplanktis* TAC125 un possibile bersaglio per strategie di *strain improvement.*

Obiettivi

L'obiettivo principale di questo progetto di ricerca è lo sviluppo e la messa a punto di un processo per la produzione di frammenti anticorpali ricombinanti nel batterio psicrofilo *Pseudoalteromonas haloplanktis* TAC125. L'efficienza del nuovo processo sarà valutata attraverso la produzione di tre proteine modello corrispondenti ai più comuni formati di anticorpi monoclonali, in particolare:

-Fab (*fragment antigen binding*): nell'ambito del progetto europeo GENOPHYS il frammento Fab anti-idiotipo Ab2/2H5 Fab3H6 (Kunert *et al.,* 2002) è stato scelto come proteina modello per l'analisi comparativa della produzione ricombinante di una proteina complessa in diversi organismi ospiti (lieviti, funghi filamentosi e batteri). Inoltre, il Fab3H6 è una molecola di grande interesse biofarmaceutico in quanto costituisce un elemento fondamentale nella formulazione di un vaccino contro un ampio spettro di ceppi del virus dell'immunodeficienza umana di tipo 1 (HIV-I) (Kunert *et al.*, 2002). Il frammento Fab è un eterodimero di circa 50 kDa costituito da una catena leggera completa (C_L + V_L) legata mediante un ponte disolfurico ad un frammento della catena pesante che comprende il dominio variabile V_H e il dominio costante C_H1.

-ScFv (*Single chain variable Fragment*): l'anticorpo a singola catena anti 2-fenil-5-ossazolone ScFvOx (Fiedler and Conrad, 1995) è stato scelto come modello di questa classe di frammenti anticorpali in quanto si presenta come una proteina molto insolubile ed è utilizzata come modello per l'ottimizzazione di protocolli di *refolding* di corpi di inclusione (Patil *et al*., 2008). L'anticorpo a singola catena è un frammento contenente le sole regioni variabili della catena pesante e leggera di un anticorpo monoclonale legate da un *linker* flessibile in modo da costituire un'unica catena polipeptidica di circa 30 kDa.

-VHH *(heavy chain antibody fragment):* come modello per questa classe di frammenti anticopali che consiste nella sola regione variabile di anticorpi di camelidi caratterizzati dalla naturale mancanza della catena leggera, è stato scelto il VHH D6.1 diretto contro il fattore di crescita dei fibroblasti umano 1 (FGFR1). Questo anticorpo ricombinante è stato isolato da una *library naive* di lama (Monegal *et al*., 2009) ma la sua produzione su larga scala in *E. coli* è limitata a causa della formazione dei corpi di inclusione. Il frammento anticorpale VHH consiste di una singola catena polipeptidica di circa 15 kDa con un solo ponte disolfurico.

Allo scopo di realizzare un efficiente sistema per la produzione di farmaci ricombinanti in forma solubile e biologicamente attiva nel nuovo sistema di espressione psicrofilo, altri obiettivi di questo lavoro saranno lo sviluppo di nuovi prodotti per la produzione di proteine ricombinanti alle basse temperature. In primo luogo saranno identificati nuovi e più efficienti sistemi genetici (promotori regolati e costitutivi) per l'espressione ricombinante alle basse temperature. Inoltre sarà effettuato lo studio dei meccanismi di secrezione proteica extracellulare evoluti dal batterio psicrofilo al fine di ottimizzare i sistemi per l'espressione e secrezione di proteine ricombinanti a freddo.

Risultati e discussione

Parte 1. Nuovi processi per la produzione di frammenti anticorpali ricombinanti in P. haloplanktis TAC125

Allestimento e validazione del sistema genetico di espressione: il primo passo necessario allo sviluppo di un processo per la produzione di frammenti anticorpali ricombinanti alle basse temperature è stato la messa a punto di opportune cassette di espressione utilizzando i sistemi genetici per l'espressione ricombinante a freddo già disponibili (Parrilli *et al*., 2008). In primo luogo, dal momento che per tutti i frammenti in esame la formazione di ponti disolfurici intramolecolari è necessaria per la corretta strutturazione dei domini immunoglobulinici e dunque per l'assunzione della struttura biologicamente attiva, si è reso necessario l'indirizzamento dei prodotti ricombinanti nello

spazio periplasmatico. Essendo disponibili due diversi peptidi segnale per la traslocazione periplasmatica, uno isolato dalla proteina periplasmatica endogena DsbA (Madonna *et al*., 2005) e l'altro dalla proteina psicrofila eterologa α-amilasi da *Ph*TAB23 (Feller *et al.,*1998), la proteina modello ScFvOx è stata utilizzata per valutare l'efficienza di tali segnali nel promuoverne la secrezione nel periplasma. Il ScFvOx è stato scelto in quanto risulta avere una particolare tendenza ad aggregare se non messo in condizione di strutturarsi correttamente. Inoltre è stata valutata a possibilità di indirizzare il prodotto ricombinante nel mezzo extracellulare mediante la fusione con la proteina *carrier* α-amilasi, già dimostratasi in grado di promuovere la secrezione extracellulare di diverse proteine ricombinanti (Cusano *et al*., 2006). Il gene codificante l'anticorpo scFvOx è stato dunque fuso a valle delle due diverse sequenze segnale per la traslocazione periplasmatica e al gene codificante l' α-amilasi e posta sotto il controllo di un promotore costitutivo forte. L'analisi della localizzazione cellulare del prodotto ricombinante è stata condotta in terreno ricco attraverso esperimenti di *Western blotting* su estratti proteici citoplasmatici, periplasmatici e sul mezzo di coltura. L'analisi ha rivelato che il ScFvOx viene prodotto e correttamente traslocato nel compartimento periplasmatico solo quando fuso al peptide segnale isolato dalla DsbA. Non si osserva invece alcuna produzione quando la proteina è dotata del peptide segnale isolato dalla α-amilasi. Inoltre, l'analisi della produzione del prodotto di fusione con la proteina *carrier* per la secrezione extracellulare ha rivelato un accumulo intracellulare e una conseguente degradazione proteolitica del prodotto ricombinante. Le ragioni di quanto osservato possono risiedere nel diverso meccanismo per la traslocazione periplasmatica guidato dai due diversi peptidi segnale. L' α-amilasi infatti viene traslocata nel periplasma attraverso il sistema post-traduzionale Sec (Wickner *et al*., 1996). Il peptide segnale della DsbA invece presenta caratteristiche comuni a proteine riconosciute dal sistema di traslocazione SRP-like che invece segue un meccanismo co-traduzionale (Schierle *et al*., 2003). Probabilmente, la cinetica di aggregazione del ScFvOx nel citoplasma è maggiore di quella di secrezione periplasmatica guidata dal sistema Sec da cui consegue l'accumulo e la degradazione proteolitica del prodotto nel citoplasma. L'utilizzo di un sistema di secrezione co-traduzionale impedisce invece alla proteina di strutturarsi prima di raggiungere il compartimento periplasmatico. Per questi motivi si è scelto di utilizzare lo stesso segnale molecolare, PsDsbA, per la traslocazione periplasmatica di tutti i frammenti anticorpali in esame. Inoltre tutti i prodotti ricombinanti sono stati dotati di *tag* C-terminali (6xHis tag e c-Myc tag) per l'immunorivelazione e la successiva purificazione. Per quanto riguarda il frammento Fab, la sua natura eterodimerica richiede che le due catene siano sintetizzate all'interno del batterio ospite in maniera stechiometrica. A questo scopo è stato costruito un operone sintetico capace di esprimere i geni codificanti la catena leggera e il frammento V_H + C_H1 della catena pesante in un unico trascritto bicistronico, in modo da realizzare il *coupling traduzionale* dei due geni. L'operone artificiale è stato disegnato in base all'analisi in *silico* dei *cluster* genici di *Ph*TAC125, focalizzando l'attenzione sulle regioni di sovrapposizione tra geni potenzialmente in *coupling* traduzionale. La costruzione ha dunque previsto una sovrapposizione tra il codone di *stop* del gene a monte (LC) con l'*atg* del gene a valle (HC) e di conseguenza l'inserimento della sequenza *Shine-Dalgarno* (SD), necessaria alla traduzione della HC, all'interno del gene LC attraverso l'introduzione di una mutazione silente. Dal momento che la lunghezza, la composizione e la posizione della sequenza SD hanno effetti sull'efficienza di inizio della traduzione, si è scelto di utilizzare una sequenza identica a quella che garantisce la traduzione del primo gene ed alla medesima distanza dal codone di *start*. Allo scopo di validare l'operone artificiale costruito è stata condotta un'analisi della produzione costitutiva e della localizzazione cellulare del prodotto il terreno ricco. Attraverso esperimenti di RT-PCR è stata verificata la stabilità del trascritto *in vivo* mentre mediante esperimenti di immunorivelazione condotti con anticorpi in grado

di riconoscere selettivamente la catena pesante e leggera del Fab è stata verificata la produzione bilanciata delle due catene e la loro corretta localizzazione periplasmatica.
Al fine di utilizzare i costrutti ottenuti per l'espressione ricombinante di ScFvOx, Fab 3H6 e VHH D6.1 in bioreattore, essi sono stati infine posti sotto il controllo del promotore psicrofilo inducibile da L-malato (Papa *et al.,* 2007) in quanto esso risulta essere il promotore più efficiente tra quelli disponibili nel promuovere la trascrizione in terreno minimo.
Sviluppo di un nuovo terreno di coltura sintetico per la produzione di rAbs a freddo: al fine di massimizzare le rese di produzione dei costrutti ricombinanti realizzati, si è scelto di agire sulla composizione del mezzo di coltura. Inoltre, la disponibilità di un terreno minimo e definito, ottimale per la crescita del microrganismo ospite, rende possibile lo sviluppo di processi in chemostato. Dati pregressi hanno rivelato una preferenza da parte di *Ph*TAC125 per substrati di natura peptidica o amminoacidica (Medigue *et al*., 2005) e sono state ottenute buone rese di produzione di proteine ricombinanti, alla temperatura di 15°C, in terreno minimo contenente una base salina SCHATZ e casaminoacidi come fonte di carbonio (Papa *et al.,* 2007). Per questi motivi si è scelto di valutare diversi terreni sintetici contenenti singoli amminoacidi come unica fonte di carbonio le cui quantità sono state bilanciate sulla composizione del terreno di riferimento. Dall'analisi preliminare sono stati selezionati quattro terreni contenenti rispettivamente gli amminoacidi L-alanina, L-aspartato, L-glutammato e L-leucina come fonte di carbonio. Su questi terreni sono state allestite delle colture cellulari in beuta di ceppi di *Ph*TAC125 esprimenti una β-galattosidasi psicrofila sotto il controllo dello stesso promotore, inducibile da L-malato, scelto per la produzione dei frammenti anticorpali. La scelta è ricaduta su questo ceppo in quanto la produzione dell'enzima psicrofilo permette di quantizzare più rapidamente la produzione ricombinante e avere così indicazioni importanti sull'efficienza del promotore nei terreni formulati. Dall'analisi dei dati ottenuti è emerso che la produzione della β-galattosidasi nel terreno contenente L-Leu risulta aumentata di circa 5 volte rispetto al riferimento (casaminoacidi). Le rese di produzione ottenute con gli altri terreni analizzati risultano invece poco soddisfacenti. L'analisi ha inoltre permesso di valutare e comparare alcuni parametri di crescita del microrganismo sui terreni formulati. In particolare è stata osservata una maggiore velocità specifica di crescita nei terreni contenenti L-Glu e L-Asp rispetto al riferimento. Dal momento che il terreno sintetico contenente L-Leu come unica fonte di carbonio ha mostrato le maggiori rese di biomassa e di prodotto ricombinante si è scelto di utilizzare tale substrato nella formulazione del terreno per l'allestimento della colture di *Ph*TAC125 in bioreattore. In un fermentatore STR sono state allestite colture cellulari in terreni contenenti oltre che la sola L-Leu anche la stessa in combinazione con gli amminoacidi con i quali è stata osservata una elevata velocità di crescita (L-Glu, L-Asp, L-Glu+L-Asp, L-Glu+L-Ala), alla temperatura di esercizio di 15°C. Ancora una volta è stata osservata una resa di prodotto ricombinante molto più elevata nel terreno contenente la sola L-Leu. La persistenza di una lunga fase di latenza e i lunghi tempi di duplicazione che caratterizzano la crescita del microrganismo rende però questo terreno inadeguato per un utilizzo in un processo industriale. Inoltre l'aumento della concentrazione di leucina nel mezzo di coltura causa un'inibizione della crescita del batterio psicrofilo. Dati di letteratura (Quay *et al.,*1977) hanno evidenziato in *E. coli* la dipendenza della velocità di *uptake* della L-Leu dalla presenza degli altri amminoacidi ramificati (L-Ile e L-Val) nel mezzo di coltura. È stato dunque formulato un nuovo terreno di coltura contenente i tre amminoacidi ramificati L-Ile, L-Leu e L-Val (LIV) in un rapporto molare 1:1:2. In tale terreno è stato possibile osservare una drastica riduzione della fase di latenza della crescita cellulare e un aumento della velocità specifica di crescita a valori paragonabili a quelli ottenuti nel terreno di riferimento. Sono inoltre state condotte analisi del consumo dei substrati nel terreno di coltura per valutare la cinetica di consumo dei singoli componenti durante la

fase di crescita esponenziale e la loro concentrazione residua al termine della crescita. La curva del consumo dei substrati evidenzia che i tre amminoacidi sono consumati velocemente all'inizio della fase di crescita esponenziale mentre la concentrazione di acido L-malico resta pressoché invariata. Dopo 24 ore di crescita la velocità di consumo degli amminoacidi diminuisce sensibilmente mentre si ha un rapido consumo del malato la cui concentrazione si riduce a valori prossimi allo zero. Alla fine della fase di *batch* la concentrazione degli amminoacidi residua nel mezzo di coltura è ancora non trascurabile. Alla luce di quanto osservato, la insufficiente conversione dei substrati in biomassa (Yx/s=30%) potrebbe essere causata da uno squilibrio di nutrienti nel mezzo di coltura e potrebbe essere aumentata riformulando il brodo di coltura in base alla composizione elementare del microrganismo. È stata infine valutata la produzione di ammonio nel mezzo di coltura ed è stata osservata una cinetica di accumulo sovrapponibile a quella osservata per la curva di crescita. Questo dato concorda con l'osservazione di una forte tendenza del batterio a rendere più basico il pH del mezzo di crescita e potrebbe essere dovuto ad un eccesso di azoto nel terreno di coltura. Sulla base di quanto osservato, è stato inoltre allestito un processo per la crescita di *Ph*TAC125 in continuo. Dopo una fase *batch* in terreno LIV è stato mantenuto lo *steady state* per 5 tempi di residenza addizionando L-Leu con una velocità di diluizione pari a 0,05 h^{-1}.

<u>*Scale up* del processo di produzione dei frammenti anticorpali in bireattore</u>: la produzione del frammento anticorpale Fab3H6 è stata effettuata in primo luogo in modalità *batch* in un bioreattore STR nel terreno di crescita precedentemente ottimizzato e in presenza dell'induttore L-malato. L'analisi della produzione, effettuata mediante saggio ELISA su campioni prelevati a diversi tempi di crescita, ha rivelato una accumulo del Fab3H6 fino a una resa di circa 4 mg L^{-1}. Attraverso esperimenti di *Western blotting* è stata inoltre dimostrata la completa traslocazione del prodotto ricombinante nel periplasma. Nell'ambito del progetto europeo GENOPHYS, inoltre, la produzione del frammento Fab3H6 è stata valutata in chemostato utilizzando terreno LIV sia per la fase di crescita in *batch* che per l'alimentazione. Nelle condizioni scelte è stato possibile stabilizzare una produttività volumetrica di Fab3H6 periplasmatico di circa 200 μg L^{-1} h^{1}. Inoltre dall'analisi comparativa della produzione del Fab3H6 in diversi microrganismi ospite è emerso che *Ph*TAC125 mostra la più alta resa di prodotto per grammo di biomassa e rese di produzione paragonabili a quelle ottenute nel consolidato *Pichia pastoris*. Il processo di produzione del Fab3H6 in continuo è stato ulteriormente ottimizzato attraverso l'utilizzo della sola L-Leu nel terreno di *feeding*. Le rese di prodotto ottenute con il nuovo processo sono paragonabili a quelle in terreno LIV ma i costi di processo risultano sensibilmente ridotti.

Il processo per la produzione del frammento anticorpale ricombinante ScFvOx è stato esercito in modalità *batch* nelle condizioni precedentemente descritte per il Fab3H6. L'analisi della produzione, effettuata attraverso esperimenti di ELISA su estratti cellulari solubili prelevati a diversi tempi di crescita, ha rivelato un accumulo del prodotto ricombinate con una resa di produzione massima in tarda fase esponenziale (48h) di circa 4,7 mg L^{-1}. La verifica della corretta localizzazione cellulare è stata effettuata mediante SDS-PAGE su estratti periplasmatici, citoplasmatici e totali di aliquote di coltura cellulare prelevate dopo 48 ore di crescita. Dall'analisi è emerso che l'anticorpo ScFvOx è prodotto in forma solubile e correttamente localizzato nel periplasma nelle condizioni analizzate. Il prodotto ricombinante è stato dunque purificato su piccola scala mediante cromatografia di pseudo affinità sfruttando la coda di poli-istidine presenta all'estremità C-terminale del prodotto di interesse. È stata infine condotta un'analisi dell'attività biologica del prodotto ricombinante ottenuto mediante esperimenti di ELISA in presenza dell'aptene 2-fenil-ossazolone. L'analisi ha rivelato una resa di proteina pura e biologicamente attiva di circa 4 mg per litro di coltura. L'analisi della produzione del

frammento ScFvOx è stata effettuata inoltre in colture cellulari in continuo. La stabilizzazione del processo in continuo è stata ottenuta in terreno LIV con $D=0,05^{-1}$ e la produttività volumetrica di ScFvOx osservata è stata pari a 230 $\mu g\ L^{-1}\ h^{-1}$.
La produzione del frammento anticorpale VHH D6.1, infine, è stata effettuata in un processo in *batch* nelle condizioni già ottimizzate per gli altri frammenti. La verifica della produzione, effettuata attraverso esperimenti di immunorivelazione ha rivelato la presenza di una banda specifica di peso molecolare apparente di circa 30kDa, superiore alla massa attesa (≈15KDa), in corrispondenza degli estratti periplasmatici ottenuti da aliquote di coltura cellulare prelevati a diversi tempi di crescita. Il prodotto ottenuto è stato parzialmente purificato mediante cromatografia di pseudo affinità sfruttando la coda di poli-istidine presenta all'estremità C-terminale ma non è stato possibile effettuare un saggio di attività biologica in quanto il prodotto purificato è risultato poco stabile in soluzione.

Parte 2. Nuovi prodotti per la produzione di farmaci ricombinanti in P. haloplanktis TAC125

Identificazione di nuovi promotori regolati e costitutivi per l'espressione genica ricombinante in *Ph*TAC125

Allo scopo di migliorare l'efficienza del nuovo sistema di espressione a freddo e di rendere i processi di produzione di farmaci ricombinanti in *Ph*TAC125 più competitivi in campo industriale si è scelto di sfruttare la conoscenza della sequenza del genoma del batterio psicrofilo per l'identificazione di nuovi e più efficienti sistemi per l'espressione regolata di geni ricombinanti. Il processo per la produzione di frammenti anticorpali ricombinanti precedentemente ottimizzato, basato sull'utilizzo del promotore indotto dalla presenza di L-malato nel mezzo di coltura, può risultare infatti economicamente vantaggioso solo se il prodotto presenta un elevato valore aggiunto, considerati l'alto costo dei substrati richiesti per massimizzare l'efficienza trascrizionale e quello dell'induttore stesso. Per questo motivo, mediante analisi *in silico* del genoma batterico, sono stati identificati alcuni promotori potenzialmente regolati e ne è stata valutata l'efficienza trascrizionale *in vivo*, in presenza ed in assenza di induzione, in un terreno di crescita minimo, molto economico, contenente L-glutammato come unica fonte di carbonio. In questo terreno infatti *Ph*TAC125 mostra un'alta velocità specifica di crescita e una discreta resa in biomassa ($Yx/s \geq 40\%$). In particolare, attraverso esperimenti di *Real Time* PCR, è stata valutata la variazione del numero di copie dei trascritti in esame nelle condizioni di induzione rispetto alla condizione *standard* (terreno con L-Glu, T=15°C). E' stata dunque valutata la variazione del numero di copie dei seguenti trascritti: il gene *galT* appartenente all'operone del galattosio, in assenza e i presenza di galattosio; la CDS PSHAa1748 codificante una putativa alginato liasi, in presenza e in assenza di alginato; la CDS PSHAb0479 codificante una gluconato permeasi, in presenza e assenza di gluconato. Tutti i potenziali induttori sono stati addizionati al terreno *standard* a t=0 alla concentrazione di 4,0 $g\ L^{-1}$. E' stata inoltre valutata la variazione dell'efficienza trascrizionale del promotore del gene PSHAa2063 codificante il *Trigger Factor* (TF) alle temperature di 15°C e 4°C. Il TF è stato infatti precedentemente identificato, mediante analisi di proteomica differenziale, tra le proteine di *Ph*TAC125 sovraespresse a basse temperature. L'analisi ha rivelato un effetto di induzione solo da parte dell'alginato (circa 10 volte rispetto alle condizioni *standard*) e del gluconato (circa 20 volte) sui rispettivi promotori che potranno dunque essere utilizzati per la costruzione di nuovi vettori per l'espressione regolata di geni ricombinanti a freddo.

Studio dei sistemi di secrezione extracellulare nel batterio *Ph*TAC125

La comprensione dei meccanismi di secrezione sfruttati dal batterio antartico *Ph*TAC125 è un presupposto indispensabile per poter ottimizzare i processi di produzione di farmaci

ricombinanti a basse temperature. La collocazione extracellulare del prodotto di interesse consente infatti di agevolare notevolmente i processi di *downstream* data la minore concentrazione e complessità del contenuto proteico nel mezzo extracellulare. La recente disponibilità della sequenza completa del genoma di *Ph*TAC125 ha facilitato lo studio della secrezione nel batterio antartico. Dall'analisi in *silico* del genoma è emerso che, nonostante il gran numero di proteine secrete dal batterio, *Ph*TAC125 possiede solo uno dei sistemi di secrezione finora caratterizzati nei Gram negativi, il sistema di tipo II GSP (*General Secretory Pathway*). Attraverso la costruzione di un mutante genomico in cui il gene *gsp*E, codificante l'ATPasi del sistema GSP, è stato inattivato, è stato dimostrato che il T2SS è responsabile della secrezione della maggior parte delle esoproteasi in *Ph*TAC125. Il dato più interessante emerso da questo studio è che, nonostante l'unico sistema di secrezione del batterio risulti inattivato, il mutante *Ph*TAC125 (*gsp*E$^-$) è ancora in grado di secernere proteine nel mezzo extracellulare indicando la presenza di almeno un altro sistema di secrezione, non ancora caratterizzato, evolutosi nel batterio psicrofilo. Inoltre, il sistema GSP non risulta essere in alcun modo responsabile della secrezione della α-amilasi eterologa, utilizzata come *carrier* per la secrezione di proteine ricombinanti nel batterio psicrofilo (Parrilli *et al.,* 2008). Allo scopo di identificare il nuovo sistema di secrezione responsabile della localizzazione extracellulare dell'α-amilasi nel batterio psicrofilo, il sistema PSS (*Psychrophilic Secretion System*), è stata realizzata una strategia di complementazione che ha previsto la costruzione di una genoteca cosmidica di *Ph*TAC125 e il suo trasferimento in cellule di *E. coli* ricombinanti in grado di produrre l'α-amilasi psicrofila ma non di secernerla nel mezzo di coltura. Tale strategia ha permesso di isolare un solo clone cosmidico, contenente una regione di circa 37Kb del cromosoma b di *Ph*TAC125, in grado di conferire al batterio mesofilo la capacità di secernere l'α-amilasi nel mezzo extracellulare. Dall'analisi della sintenia della porzione genica di *Ph*TAC125 contenuta nel cosmide selezionato con genomi di organismi ad esso vicini filogeneticamente è emerso che la regione comprendente le CDS dalla PSHAb0134 alla PSHAb0142 è molto conservata nei microrganismi selezionati tranne per la CDS PSHAb0140. Inoltre non è presente un omologo di tale gene in *E. coli*. Per comprendere il ruolo della funzione codificata dal gene PSHAb0140 nella secrezione dell'α-amilasi è stato costruito un mutante genomico di *Ph*TAC125 in cui tale gene è stato deleto. Tale mutante non è in grado di secernere l'α-amilasi nel mezzo di coltura dimostrando che il prodotto della CDS PSHAb0140, d'ora in avanti chiamato PssA, è necessario per il corretto funzionamento del nuovo sistema di secrezione PSS. Dall'analisi *in silico* si evince che PssA è un'ipotetica lipoproteina costituita da un peptide *leader* caratteristico di questa famiglia di proteine che la colloca nello spazio periplasmatico ancorata alla membrana esterna, da tre domini TPR (*Tetratricopeptide Reapeat Domain*) presenti all'estremità N-terminale e da due domini LysM di legame al peptidoglicano all'estremità C-terminale. Queste caratteristiche insieme ai risultati sperimentali ottenuti suggeriscono per la proteina PssA un ruolo di adattatore molecolare incaricato di reclutare altri gli componenti cellulari necessari alla secrezione dell'α-amilasi e degli altri substrati del sistema PSS nel mezzo di coltura.

INTRODUCTION

Monoclonal antibodies and antibody fragments as biopharmaceuticals

According to recent reports, it is clear that recombinant antibodies have come of age as biopharmaceuticals. The global sales of monoclonal antibodies were $33 billion in 2008 as compared to $27 billion in 2007 and total revenues are predicted to increase in the next years (Canadian Corporate News Report, 2008).

Antibodies are glycoproteins which specifically recognise foreign molecules (antigens). IgG antibodies are large molecules of about 150 kDa composed of 4 peptide chains. They contain two identical heavy chains of about 50 kDa and two identical light chains of about 25 kDa, thus tetrameric quaternary structure. The two heavy chains are linked to each other and to a light chain each by disulfide bonds. The resulting tetramer has two identical halves which together form the Y-like shape. Each end of the fork contains an identical antigen binding site (Fig. 1). The unique ability of antibodies to specifically recognise and bind with high affinity to virtually any type of antigen, made them interesting molecules for medical and scientific research. In 1975 Köhler and Milstein developed the monoclonal antibody technology (Köhler *et al*. 1975) by immortalising mouse cell lines that secreted only one single type of antibody with unique antigen specificity, called monoclonal antibodies (mAbs). With this technology, isolation and production of mAbs against protein, carbohydrate, nucleic acids and hapten antigens was achieved resulting in a rapid development of the use of antibodies in diagnostics, human therapeutics and as fundamental research tools. The development and applications of recombinant DNA technology led to the design of several formats of recombinant antibody fragments (Fig.2). Smaller recombinant antibody fragments (for example, classic monovalent antibody fragments (Fab, scFv) and engineered variants (diabodies, triabodies, minibodies and single-domain antibodies) retain the targeting specificity of whole mAbs and possess other unique and superior properties for a range of diagnostic and therapeutic applications. Indeed, for some clinical applications small antibody fragments have advantages over whole antibodies. Firstly, the lack of Fc regions reduces the risks of immune response. Secondly, the small size permits them to penetrate tissues and solid tumours more rapidly than whole antibodies (Yokota *et al*., 1992). Furthermore smaller antibody fragments have a much faster clearance rate in the blood circulation, which leads to differences of selectivity (Yokota *et al*., 1992).

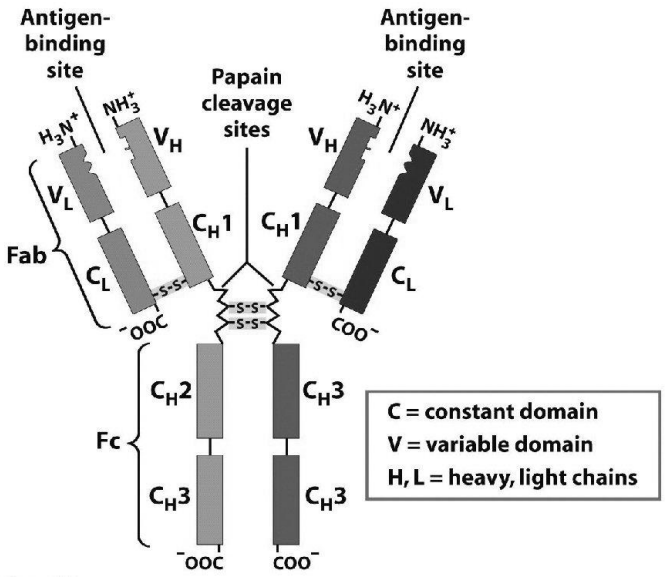


Figure 1: Structural regions of an antibody molecule. Lehninger, Principles of Biochemistry Fifth Edition. Figure 5.21a. © 2008 W.H. Freeman and Company

By recombinant DNA technology, antibody fragments have been forged into multivalent and multispecific reagents and engineered for enhanced therapeutic efficacy. A new use of the binding capacity of antibody fragments is the design of a fusion approach, in which an antigen recognising antibody fragment is coupled to a

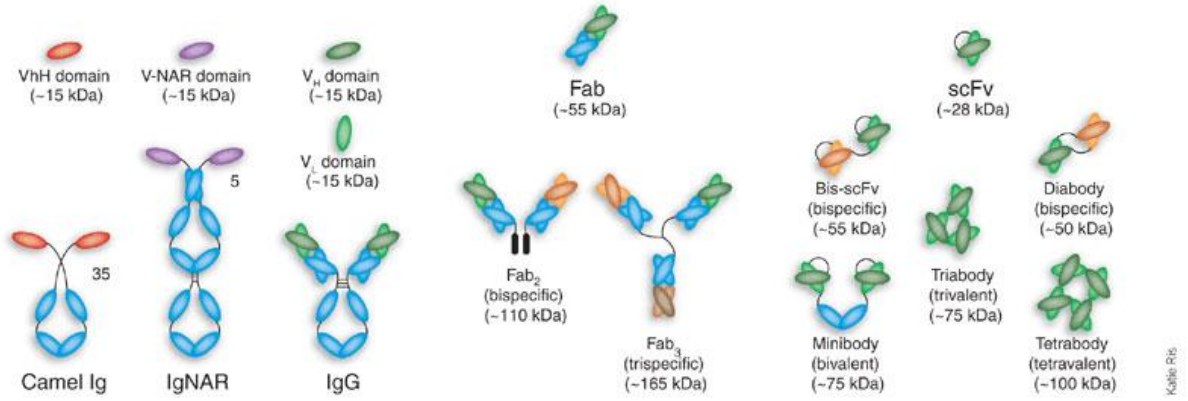


Figure 2: Antibody fragments overview (taken from Hollinger & Hudson, 2005)

range of effector molecules (Fig.3) including enzymes for prodrug therapy, toxins for cancer treatment (Schrama *et al*., 2006), viruses for gene therapy, cationic tails for DNA delivery, liposomes for improved drug delivery and biosensors for real-time detection of target molecules (Spooner *et al*., 1994). The use of bi-functional antibodies in medicine is aimed at delivery of an effecter which is only active where it is required. It thereby limits the dose of the drug, resulting in less side effects of the drug towards healthy tissue and/or less immunogenic response to the drug itself. Also the physical interaction between the target and the effector molecule increases the potency of the effecter.

More applications outside research and medicine can be considered, such as consumer applications. Single-domain antibodies are anticipated to significantly expand the repertoire of antibody-based reagents against the vast range of novel biomarkers being discovered through proteomics. Examples are the use in biosensors, treatment of wastewater (Graham *et al*., 1995), industrial scale separation processes such as separation of chiral molecules (Got *et al*., 1997), purification of specific components like proteins from biological materials or the use as abzymes (Wade *et al*., 1997). They have also been considered as components of novel consumer goods with new improved functionalities like the use of antibodies in shampoos to prevent the formation of dandruff or in toothpaste to protect against tooth decay caused by caries (Frenken *et al*., 1998).

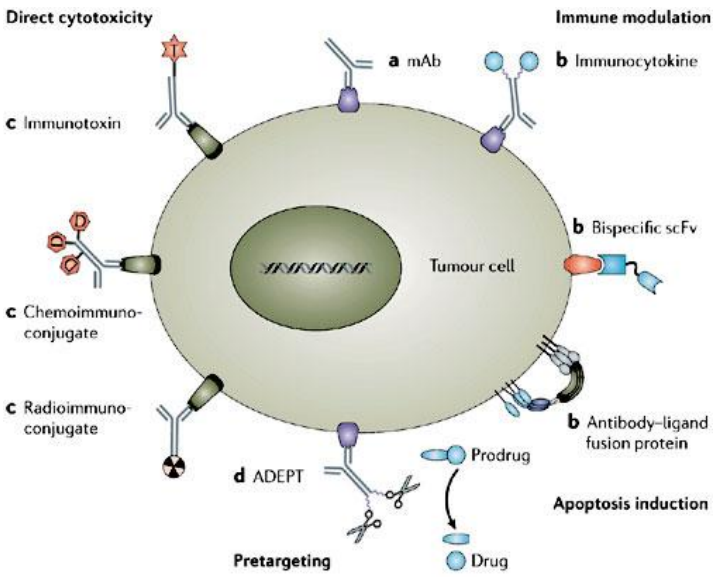


Figure 3: Multispecific antibody fragments (Schrama *et al.*, 2006).

Antibody therapeutics are already a multi-billion dollar a year market and a large number of monoclonal antibodies and antibody fragments are at various stages of clinical trials (Evans and Das 2005). However, they are amongst the most expensive of all drugs where the annual cost per patient can reach $35,000 for treating cancer

(Farid, 2007). Indeed antibody therapies involve most often high doses (>1 g per patient per year) for a large number of patients and this comes up to a total production demand in the range of multi-tons per year (Farid, 2007). Consequently, expensive large-scale production capacity is required to fulfill market demand.

Industrial production of monoclonal antibodies

To be able to use monoclonal antibodies, antibody fragments and antibody fusion proteins in large scale applications, a suitable expression system has to be chosen. The possibility of large-scale production of antibodies and fragments requires that the production system is cheap, accessible for genetic modifications, easily scaled up for greater demands and safe for use in consumer applications. Several expression systems are available, both from eukaryotic (Table 1) and prokaryotic (Table 2) origin.

Eukaryotic expression systems

Several eukaryotic systems can be envisaged for large-scale production of monoclonal antibodies and fragments like mammalian cells, insect cells, plants, transgenic animals and lower eukaryotes. Most of the approved monoclonal antibodies are manufactured in mammalian cells. The majority of them use batch/fed batch cultures followed by purification steps that rely primarily on chromatography with intermediate filtration and viral clearance operations. However, large-scale production is expensive and time-consuming (Farid, 2007). Cultured insect cells are used as hosts for recombinant baculovirus infections. The production of a recombinant viral vector for gene expression is time-consuming, the cell growth is slow when compared with former expression systems, the cost of growth medium is high and each protein batch preparation has to be obtained from fresh cells since viral infection is lethal. (Ferrer-Miralles 2009). Plants show several advantages as large-scale antibody production systems, like the ease and low costs of growing plants, even in large quantities. However, the generation of transgenic plants that express antibodies is a time consuming process and the downstream processing to isolate the recombinant antibodies from the plant parts is relatively expensive and laborious (Joosten *et al*., 2003). An attractive possibility for the cost-effective large-scale production of antibody fragments and antibody fusion proteins are yeast or fungal fermentations. Large-scale fermentation of these organisms is an established

Table 1

Eukaryotic expression systems for heterologous protein production and possible advantages and disadvantages of the expression system.

Expression systems	Ease of molecular cloning	upscaling	Economic feasibility[1]	Pathogenic contaminants[2]
Mammalian cells	+	+/-	+	+
Insect cells	++	+	+	+
Plants	++	+++	++	++
Transgenic animals*	+/-	+++	+/-	+/-
Yeasts	+++	+++	+++	+++
Filamentous fungi	+++	+++	+++	+++

+++ = excellent, ++ = good, + = sufficient, +/- = poor. * With transgenic animals in this context is mentioned the production of antibodies or antibody fragments in the milk of transgenic animals, for example rabbits, sheep, goats or cows [1] With economical feasibility is mentioned the time and cost of molecular cloning, upscaling and downstream processing (purification). [2] Pathogenic contaminants like viruses or pyrogens. Modified from: Joosten *et al.* 2003

technology already used for bulk production of several other recombinant proteins and extensive knowledge is available on downstream processes. Besides that, yeasts and filamentous fungi are accessible for genetic modifications and the protein of interest may be secreted into the culture medium. The main pitfall of this expression system is related to N-linked glycosylation patterns which differ from higher eukaryotes, in which sugar side chains of high mannose content affect the serum half-life and immunogenicity of the final product (Ferrer-Mirales *et al*., 2009). Furthermore, although less studied than in bacteria, the production of recombinant proteins in yeasts also triggers conformational stress responses and produced proteins fail sometimes to reach their native conformation (Gasser *et al*., 2008).

Prokaryotic expression systems

Bacterial expression systems have also been investigated for their potential to produce mAbs and different mAb fragments. While many bacterial strains, either Gram positive and Gram negative, have been tested for recombinant antibodies production with different extents (Tab.2), most experience has been gathered with *Escherichia coli* (Ferrer-Mirales *et al*., 2009). Incentives for the use of *E. coli* expression systems include simple fermentation conditions, ease of genetic manipulation, ease of scale-up, relatively short duration between transformation and protein purification, no concerns about viruses that are harmful to humans and relatively low capital costs for fermentation (Arbabi-Ghahroudi *et al.* 2005). However, several obstacles to the production of quality proteins limit its application as a factory for recombinant pharmaceuticals. Indeed, heterologous proteins over-expression in *E. coli* often results in insoluble aggregates production as cytoplasmic or periplasmic inclusion bodies (Baneyx and Mujacic 2004). Inclusion body (IB) proteins need elaborate and cost-intensive solubilisation, refolding and purification procedures to recover functionally active product (Vallejo *et al.,* 2004). Hence, the final yield of fragments is only a small percentage of the protein that was initially present in the inclusion bodies and this causes a huge increase of process costs. In order to face the challenge to achieve a finer balance between the quality and the yield of recombinant proteins many strategies have been explored including expression of chimerical proteins (Park *et al.,* 2008) and co-expression with chaperones (Kolaj *et al*., 2009). Expression of “difficult” proteins has also been carried out by lowering the temperature at the physiological limit allowed for the growth of mesophilic host organism (between 15 and 18°C). Lowering the temperature, in fact, has a pleiotropic effect on the folding process, destabilizing the hydrophobic interactions needed for intermediates aggregation (Sahdev *et al*., 2008). Although in some cases this approach has been reported to increase yields of soluble and active recombinant protein products, the major drawback in *E. coli* cultivation at sub-optimal temperatures is the decrease in biomass production which reduces the global process productivity. Therefore the exploitation of an industrial process performed in this conditions might hardly be considered. A rational alternative to improve the quality or recombinant products is to explore the potential of naturally cold-adapted bacteria as hosts for protein production at low temperature (even at around 0°C). In this context, a few cold adapted species are under early but intense exploration as cold cell factories, among them, *Pseudoalteromonas haloplanktis* being a representative example. The development of a cold expression system for recombinant proteins production in *P. haloplanktis* TAC125 was already described (Parrilli *et al.,* 2008) and examples of fully soluble and biologically competent production of several thermal-labile and aggregation-prone proteins were extensively

reported (Vigentini *et al.,* 2006; Papa *et al.,* 2007). Furthermore, with respect to *E. coli*, *P. haloplanktis* TAC125 is extremely efficient in secreting proteins in the culture medium. By the use of a psychrophilic α-amylase as secretion carrier for the extracellular targeting of recombinant proteins an efficient gene-expression system was set up (Cusano *et al*., 2006b). Observed efficiency of the cold-adapted system (secretion yield was always above 80%) placed it amongst the best heterologous secretion systems in Gram-negative bacteria reported so far.

Therefore, the use of psychrophilic bacteria as alternative expression hosts is the compelling choice towards the exploitation of industrial processes for the production of soluble and biologically competent recombinant antibody fragments at low temperatures.

Table 2

Antibody fragments production in prokaryotic expression systems

Strains	Characteristics	Food grade	Production yields
Lactobacillus zeae	$Gram^+$	yes	scFv, ND* (secreted/cell-bound)
Bacillus subtilis	$Gram^+$	yes	10 to 15 mg/L scFv (secreted)
Streptomyces lividans	$Gram^+$	yes	1 mg/L Fv fragment (secreted)
Staphylococcus carnosus	$Gram^+$	yes	5–10 mg/L V_H domain (secreted)
Proteus mirabilis	$Gram^-$	no	40 to 200 mg/L scFv (secreted)
Escherichia coli	$Gram^-$	no	Several fragments (inclusion bodies/periplasmic space)

*ND = not determined. Modified from: Joosten *et al.,* 2003

The psychrophilic host: *Pseudoalteromonas haloplanktis* TAC125

P. haloplanktis TAC125 is a Gram-negative bacterium isolated from an Antarctic coastal seawater sample collected in the vicinity of the French Antarctic station Dumont D'Urville, Terre Adélie. It can be classified as a Eurypsychrophile (i.e. a bacterium growing in a wide range of low temperatures; Atlas and Bartha, 1993) and was the first Antarctic Gram-negative bacterium of which the genome was fully sequenced and carefully annotated (Médigue *et al*., 2005). Genomic and metabolic features of this bacterium, accounting for its remarkable versatility and fast growth compared with other bacteria from aqueous environments, were discovered by combining genome sequencing and further *in silico* and *in vivo* analyses. *P. haloplanktis* TAC125 is able to duplicate in a wide range of temperatures (0-30°C), with an apparent optimal growth temperature a t 20°C, where the observed duplication time in rich medium is 31 minutes (Tutino *et al.,* 1999). However, the bacterium still duplicates at fast speed even at lower temperatures (at 4°C, one cell division is completed in about 100 min; unpublished results from this laboratory) and, when provided with sufficient nutrients and aeration, it grows to very high density (up to A_{600}=20) under laboratory settings, even at 0°C. This growth performance makes it one of the faster growing psychrophiles characterised so far. Fast growth rates, combined with the ability of *P. haloplanktis* TAC125 to reach very high cell densities even under laboratory growth conditions and to be easily transformed by intergeneric conjugation (Duilio *et al*., 2004), made this bacterium an attractive host for the development of an efficient gene expression system at low temperatures.

The psychrophilic expression system

The psychrophilic expression vector

A few other reported examples of recombinant protein production in psychrophiles made use of molecular signals (such as the origin of replication and the transcriptional promoter) derived from mesophiles. A different philosophy inspired the construction of our gene-expression systems, which derived from the proper assembly of true psychrophilic molecular signals into a modified *E. coli* cloning vector. By combining mesophilic and psychrophilic genetic signals a collection of gene-expression vectors was set up to produce recombinant proteins in *P. haloplanktis* TAC125 (Fig. 4). The mesophilic signals consist of the pUC18-derived origin of replication (OriC) and a selection marker gene (a β-lactamase encoding gene), allowing the plasmid to replicate either in *E. coli* or in the psychrophilic host. Another crucial mesophilic signal is represented by the OriT sequence, the conjugational DNA transfer origin from the broad host range plasmid pJB3 (Blatny *at al*., 1997). Structural and functional studies led to the isolation of the psychrophilic origin of replication (OriR) from the *P. haloplanktis* TAC125 endogenous plasmid pMtBL (Tutino *et al*., 2001).

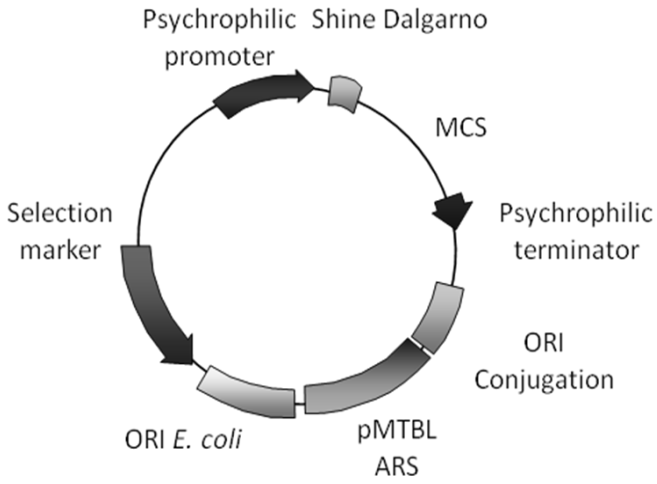


Figure 4: The psychrophilic expression vector

Psychrophilic promoters

○ Constitutive expression

The structural/functional characterisation of *P. haloplanktis* TAC125 promoters was carried out by random cloning of genomic DNA fragments and identification of promoter sequences by evaluating their capability to express a promoter-less reporter gene (Duilio *et al*., 2004). By this promoter-trap strategy, a collection of constitutive psychrophilic promoters showing different strengths at different temperatures was identified. The implementation of the above described psychrophilic promoters in the pMtBL-derived shuttle vectors resulted in the set up of cold-adapted gene-expression systems, characterised by the constitutive production of the recombinant protein.

○ Regulated expression

Sometimes efficient production can only be achieved by fine tuning the recombinant gene expression. This goal can be reached by using regulated promoters and efficient induction strategies. Recently, by using a differential proteomic approach, the isolation and characterisation of a two-component system has been carried out. This regulatory system is responsible for the transcriptional regulation of the gene coding for an outer protein porine, and it is strongly induced by the presence of L-malate in the medium (Papa *et al*., 2006). The regulative region of the porine gene

was used for the construction of an inducible cold expression vector, where the recombinant protein expression is under L-malate control (Papa *et al*., 2007).

Molecular signals for protein addressing

○ Periplasmic secretion

Although the production of recombinant protein in the host cytoplasm is the preferred strategy many processes due to higher production yields, this approach cannot be pursued when the wanted product requires the correct formation of disulphide bonds to attain its catalytic competent conformation. Indeed as for all Gram-negative bacteria, *P. haloplanktis* TAC125 cytoplasm is a reducing environment and the formation of disulphide bridges is confined in the periplasmic space. From the genome analysis, we know that *P. haloplanktis* TAC125 contains all the canonical periplasmic export machineries (Medigue *et al*., 2005). Therefore, gene fragments encoding two signal peptides from psychrophilic secreted proteins following different translocation mechanisms have been cloned in the psychrophilic expression vectors, under the control of different promoters in order to allow the signal peptides N-terminal fusion for periplasmic addressing of recombinant proteins.

○ Extra-cellular secretion

In order to combine the effects of low temperatures on the recombinant product solubility with the advantages linked to extra-cellular protein targeting, a gene expression system for the production and extra-cellular secretion of recombinant proteins in psychrophilic bacteria was set up. The novel system makes use of the psychrophilic α-amylase from *P. haloplanktis* TAB23 (Feller *et al*., 1992) as a secretion carrier. This exo-protein is produced and secreted as a larger precursor with a long C-terminal pro-peptide that is not mandatory for the α-amylase secretion when it is produced by recombinant cold-adapted bacteria the propeptide (Tutino *et al*., 2002; Cusano *et al.,* 2006a). Starting from the latter observation, the secretion of chimeric proteins obtained by the replacement of α-amylase C-terminal propeptide with a passenger protein was studied (Cusano *et al.,* 2006b). The novel genetic system (Fig. 5) allows the easy in-frame cloning of any gene downstream of the mature psychrophilic α-amylase encoding region. The spacer between the carrier and passenger proteins contains the motif -Ala-Ser-Ser-Thr- recognised and cleaved by a *P. haloplanktis* TAC125 secreted protease that allows the separation of the protein of interest from the secretion carrier when it reaches the extra-cellular medium.

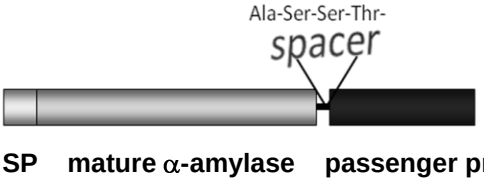


Figure 5: Schematic representation of recombinant proteins fusion to the secretion carrier

Aim of the study

The aim of this study is the development of novel processes, involving *P. haloplanktis* TAC125 as expression host, for large scale production of recombinant antibody fragments in soluble and biologically competent form and new products for recombinant production of biopharmaceuticals at low temperatures.

New processes

Based on the previously described genetic tools for recombinant protein production and cellular addressing in *P. haloplanktis* TAC125, an *ad hoc* expression system for recombinant antibody fragments soluble production will be constructed and validated.
By a rational approach, a new defined medium will be developed in order to maximise the expression system efficiency and biomass productivity.
Antibody fragments production processes will be scaled up to lab scale bioreactors and tested in batch and continuous fermentation modes.
To test the versatility of the psychrophilic expression system three different formats of recombinant antibody fragments will be considered:

Fab (fragment antigen binding)

Fab fragments (fragment antigen binding) are the antigen binding domains of an antibody molecule, containing the light chain (CL + VL) and the portion VH + CH1 of antibody heavy chain (Fig.5). Besides the four intramolecular disulfide bounds, one in each immunoglobulin domains, an additional interchain disulfide bridge is present between CL and CH1. Due to the heterodimeric structure, Fab fragments are quite difficult to produce in biologically active form in microbial expression systems. Indeed, the correct assembly of recombinant light and heavy chain in functional Fab fragments can only be achieved if the two subunits are synthesized by the host cell in stoichiometric ratio. Unbalanced production can lead to formation of soluble homodimers (Hotta *et al*., 2004), very difficult to separate from the desired product without an antigen-affinity purification, or misfolded insoluble aggregates (Wu *et al*., 1998). Therapeutic Fab fragments are mostly produced in mammalian expression systems or in yeasts while just a few examples are reported of Fab expression in bacteria (Peterson *et al.*, 2006).

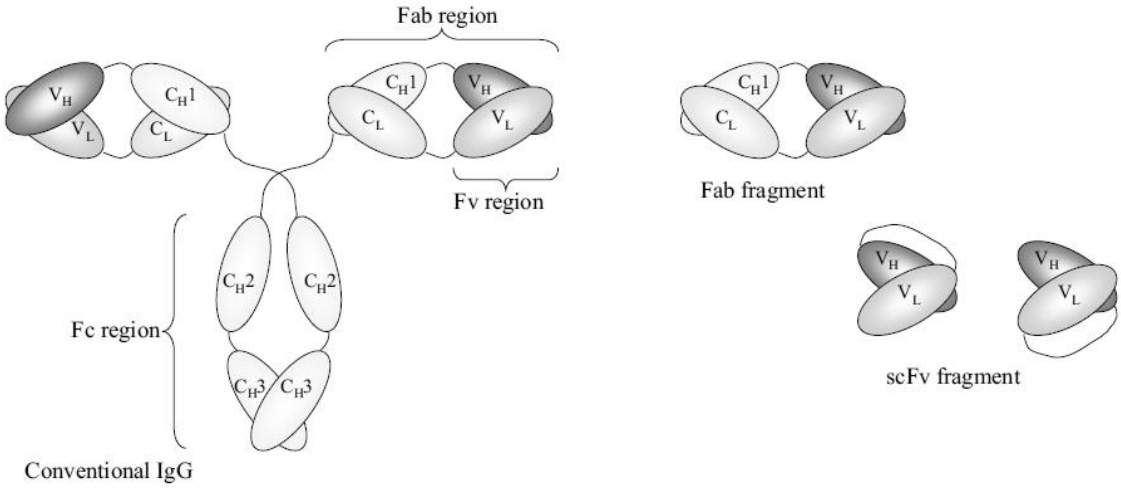


Figure 5: Schematic representation of the structure of a conventional IgG and fragments that can be generated thereof and used in this work. The constant heavy-chain domains CH1, CH2 and CH3 are shown in yellow, the constant light-chain domain (CL) in green and the variable heavy-chain (VH) or light-chain (VL) domains in red and orange, respectively. Modified from Joosten *et al.,* 2003

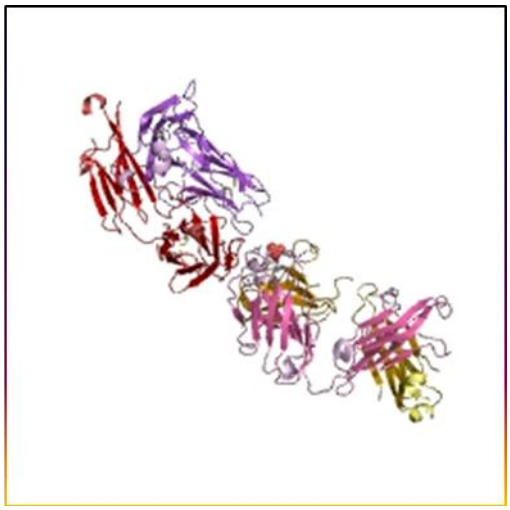


Figure 6: Crystal structure of the 2F5 Fab'-3H6 Fab complex. S.Bryson *et al*. 2008)

In the framework of the European project GENOPHYS the anti-idiotypic antibody Ab2/2H5 Fab 3H6 fragment (Fig. 6) was chosen as a protein model for genome-wide comparison of physiological bottlenecks in multi-subunit protein production in prokaryotic and eukaryotic microbial hosts.
The mAb 2F5 is one of the very few antibodies with the ability to neutralize a wide spectrum of type 1 human immunodeficiency virus (HIV-1) strains and primary isolates. However, neutralizing 2F5 like antibodies are rarely detected in patients sera. The anti-idiotypic antibody Ab2/3H6 Fab is able to recognize the paratope of mAb 2H5 bearing a structural miming of ELDKWA , the core epitope of gp-41, which is only exposed during the fusion of the virus to cells. It can therefore provide the means to induce a broadly neutralizing anti HIV-1 antibody response and could represent a key component in anti HIV vaccines formulation (Kunert *et al*., 2002).
The Ab2/2H5 Fab 3H6 represents an interesting model of complex therapeutic protein to validate the new developed processes for recombinant biopharmaceuticals production by the psychrophilic expression system. Furthermore, in the framework of GENOPHYS European project, a comparison of Fab 3H6 production in different microbial hosts (bacteria, yeasts and filamentous fungi) will be performed in chemostat cultivations.

ScFv (Single chain variable Fragment)

ScFv (Single chain variable Fragment) is the minimal fragment (~30 kDa) that still contains the whole antigen-binding site of a IgG antibody, composed of both the variable heavy chain (V_H) and variable light chain (V_L) joined by a hydrophilic and flexible linker peptide (Fig.4). The length of the linker (15–25 amino acids) is determinant for protein stability (Bird *et al*., 1988). Single chain antibodies contain two immunoglobulinic domains each with one disulfide bond. ScFvs have the tendency to form aggregates especially when over-produced in *E. coli* expression system (Baneyx and Mujacic 2004). To test the ability of psychrophilic expression system in recombinant biopharmaceuticals production in soluble form, the anti-2-phenyl-5-oxazolone single chain variable fragment (ScFvOx) was chosen as model (Fiedler and Conrad, 1995). ScFvOx (Fig. 7) is a typical example of aggregation-prone ScFv and it has been used for years as model for IB refolding protocol development (Patil *et al*., 2008).

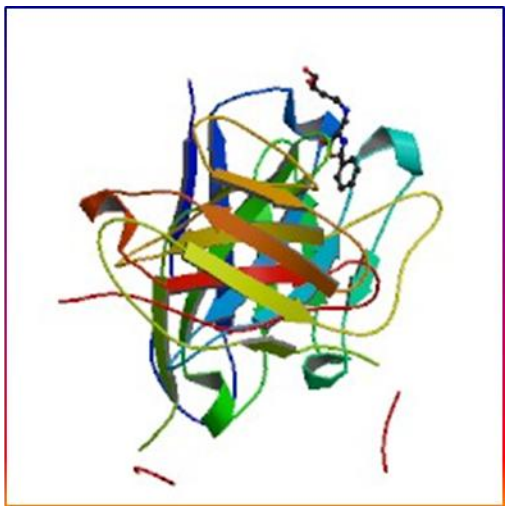


Figure 7: Crystal structure of scFvOx in complex with its ligand (Scotti *et al*., 2006)

VHH (heavy chain antibody fragment)

In 1993 Hamers-Casterman *et al.* discovered a novel class of IgG antibodies in *Camelidae* (camels, dromedaries and llamas). These antibodies are devoid of light chains and therefore called 'heavy-chain antibody" or HCAb (Fig. 8). HCAbs have a molecular weight of ~95 kDa instead of the ~150 kDa for conventional IgG antibodies. Their binding domains consist only of the heavy-chain variable domains, referred to as VHHs (Muyldermans *et al*., 1999) to distinguish it from conventional VHs. VHH is the smallest available intact antigen-binding fragment (~15 kDa) and it has a great potential in therapeutic and diagnostic application as multispecific fusion product (Joosten *et al*., 2003).

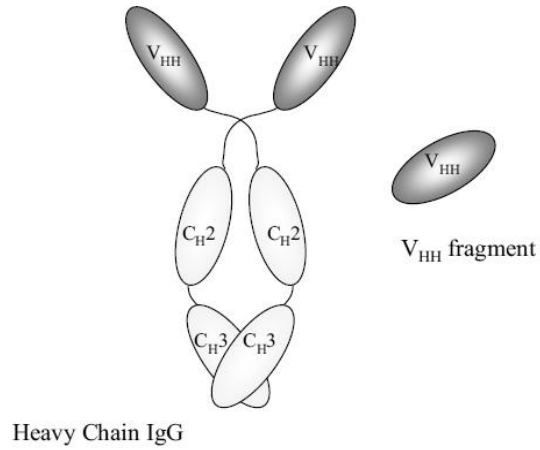


Figure 8 : Schematic representation of a heavy-chain IgG antibody and the variable heavy-chain antibody fragment (VHH) that can be generated of the latter. The constant heavy-chain domains CH1, CH2 and CH3 are shown in yellow, the constant light-chain domain (CL) in green and the variable heavy-chain (VH or VHH) or light-chain (VL) domains in red and orange, respectively. Modified from Joosten *et al*., 2003)

To validate the new optimised process for recombinant antibody fragments production at low temperatures, it will be also tested for VHH format production.

An anti- human fibroblast growth factor receptor 1 (FGFR1) VHH D6.1 was chosen as model protein. It has been selected by phage display from a pre-immune llama library (Monegal *et al*., 2009) but its large scale production in conventional *E. coli* expression systems was unsatisfactory due to inclusion bodies formation (De Marco A., personal communication). A new production process leading to improve soluble production of VHH D6.1 is therefore required for its further characterization.

New products

In order to further optimise the novel processes for recombinant production of biopharmaceuticals by the psychrophilic expression system, new genetic tools for recombinant gene expression and protein addressing in *P. haloplanktis* TAC125 will be searched out and characterised.

By *in silico* analysis of *P. haloplanktis* TAC125 genome several potentially regulated promoters will be identified and their transcriptional efficiency will be tested in minimal defined media in different conditions. The analysis will lead to the construction new expression vectors for regulated expression of recombinant proteins at low temperatures.

In order to improve extracellular secretion of recombinant proteins in psychrophilc expression system and to identify new molecular signals for recombinant proteins addressing in culture medium, a deep investigation will be performed on protein secretion machineries evolved by *P. haloplanktis* TAC125. Molecular characterisation of canonical type II secretion system (T2SS) GSP (General Secretory Pathway) will be carried out and a deep study will be performed on the still uncharacterised psychrophilic secretion system (PSS) responsible for the secretion of the recombinant alpha-amylase used as carrier for recombinant protein secretion.

RESULTS AND DISCUSSION

Part I: New Processes

Section 1: Antibody fragments expression vector design and validation

The first step for antibody fragments production in *P. haloplanktis* TAC125 was the construction of a suitable expression vector based on the available genetic tools for recombinant gene expression at low temperatures. The construction of an *ad hoc* genetic expression system for each model protein followed a rational design where several critical aspects were considered including the selection of molecular signals for recombinant products cellular addressing, the choice of optimal gene-expression strategy and the addition of molecular tags for subsequent protein immunodetection and purification.

Choice of molecular signals for antibody fragments secretion

Antibody fragments, as well as all antibody molecules, contain disulfide bonds in their tertiary structure each in every immunoglobulin domain. Moreover, Fab fragments contain an additional disulfide bridge which joins together light and heavy chains thus forming heterodimeric quaternary structure. To achieve soluble and biologically competent production of recombinant antibody fragments in *P. haloplanktis* TAC125 a useful option is to address the recombinant proteins into the periplasmic compartment where the oxidising environment and the enzymatic repertoire facilitate disulfide bonds formation.

Two different psychrophilic signal peptides were tested far for periplasmic secretion of recombinant proteins in *P. haloplanktis* TAC125: one (PsA) isolated from a psychrophilic *P. haloplanktis* TAB23 α-amilase and the other (PsD) from the endogenous periplasmic protein DsbA (Disulfide bond oxidoreductase I). In order to assess the ability of the available signal peptides to promote recombinant antibody fragments translocation across bacterial inner membrane, the sequences encoding the two different leader peptides were fused to the *scFvOx* gene. The protein ScFvOx was chosen because it shows the highest tendency to aggregate in non-native conditions and therefore represents a valid model to test the secretion systems efficiency. The PCR amplified gene was cloned into the cold adapted periplasmic gene expression vectors pPM13psA and pPM13psD respectively (unpublished results) under the control of a strong constitutive psychrophilic promoter (Duilio *et al*., 2004). The resulting fusion proteins, PsA-ScFvOx and PsD-ScFvOx, both contained the c-Myc tag at their C-terminal end to allow the product immunodetection. In addition, extracellular secretion of recombinant ScFvOx was evaluated by a fusion approach involving the psychrophilic α-amylase as secretion carrier (Cusano *et al.,* 2006b). Hence, the *scFvOx* gene was cloned into pFF*amy** vector for constitutive expression of α-amylase-passenger protein chimeras.

Recombinant *P. haloplanktis* TAC125 strains were grown in rich medium at 15°C in shaken flasks. Protein patterns of the soluble cell extracts were analysed by SDS-PAGE to evaluate the production of the ScFvOx by the psychrophilic expression host. Interestingly, the analysis revealed that no ScFvOx production is obtained when its periplasmic translocation is driven by the α-amylase signal peptide PsA (data not shown). On the contrary, the periplasmic protein PsD-ScFvOx was produced in soluble form by *P. haloplanktis* TAC125 recombinant cells (Fig. 9A).

Cellular localisation of recombinant ScFvOx was verified by cellular fractionation (cytoplasm and periplasm). The periplasmic extraction efficiency was evaluated looking at the distribution of the periplasmic alkaline phosphatase activity between

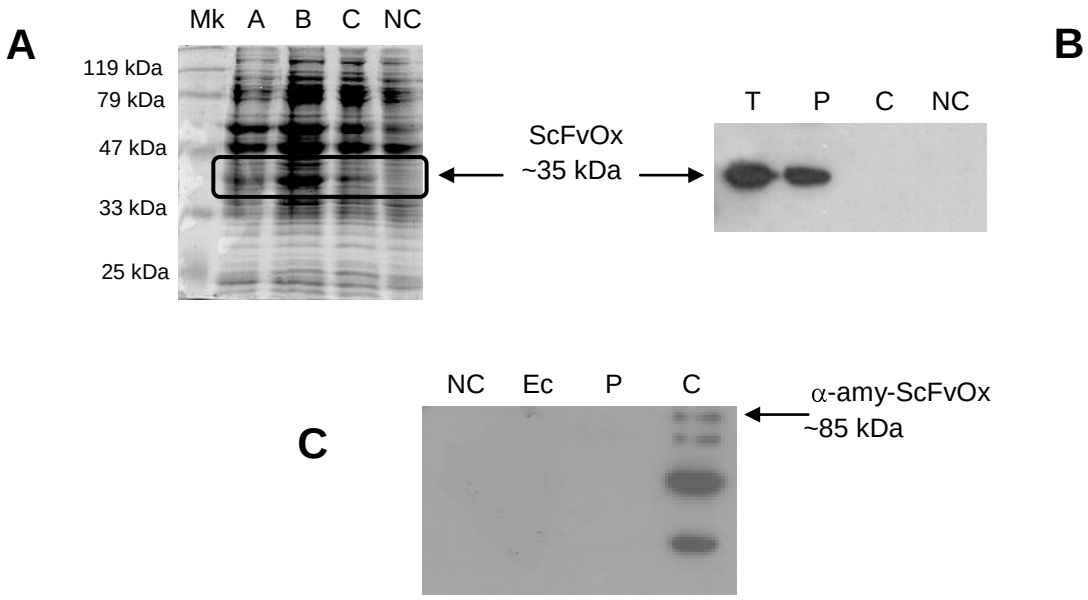


Figure 9: Panel A: SDS-PAGE analysis of ScFvOx production. A, B and C are total soluble protein extracts from recombinant *Ph*TAC125 (pPM13*psDscFvOx)* strain at respectively 24, 36, 48 hours cultivation. Panel B: anti c-Myc Western blotting analysis of ScFvOx cellular localization on total soluble proteins (T), periplasmic (P) and cytoplasmic fraction (C). Panel C: anti α-amilase Western blotting analysis of α-amilase-ScFvOx chimera cellular localization on extracellular medium (Ec), periplasmic (P) and cytoplasmic (C) extracts. Total soluble protein extract of *P. haloplanktis* TAC125 wild type strain was used as negative control (NC). Mk: molecular weight protein ladder.

cytoplasmic and periplasmic fractions. 98% of total alkaline phosphatase was found in periplasmic fraction. Total cellular soluble extracts and corresponding periplasmic and cytoplasmic fractions of the recombinant and wild type *P. haloplanktis* TAC125 strains were analysed by Western blotting using specific anti c-Myc monoclonal antibodies. As shown in figure 9 panel B, a specific signal is present in total extract of recombinant cells showing an apparent molecular weight corresponding to the expected one for recombinant ScFvOx-c-Myc fusion protein (~35kDa). The same specific signal is present in the recombinant periplasmic fraction and completely absent in the corresponding cytoplasm indicating that PsD-ScFvOx-c-Myc protein is not only successfully produced in soluble form but also totally translocated in the bacterial periplasmic space.

The analysis of α-amylase-ScFvOx chimera cellular localisation was performed on cytoplasmic soluble protein extracts, periplasmic proteic fraction and extracellular medium by Western blotting experiments using specific polyclonal anti α-amylase antibodies (Fig. 9C). Surprisingly, the analysis revealed specific signals, probably corresponding to proteolysis products, only in cytoplasmic fraction, indicating that the chimera is not able to reach the extracellular medium neither the periplasmic space but totally accumulates into bacterial cytoplasm.

Due to the observed results, the correct ScFvOx translocation across the inner membrane seems to occur only when the PsD signal peptide is used as secretion leader. Either if fused to the carrier protein α-amylase or to its signal peptide PsA alone, the recombinant antibody fragment accumulates into bacterial cytoplasm where it cannot fold properly and it is subjected to proteolytic degradation which results in complete degradation in the case of PsA-ScFvOx.

This can be due to the different translocation mechanism driven by the two tested signal peptides. The PsA leader peptide is a canonical molecular determinant

recognised by Sec secretion machinery which promotes protein secretion across the inner membrane by a post-translational mechanism (Wickner *et al.,* 1996). To allow the recognition of the preprotein by the Sec machinery the unfolded state of protein is required and therefore some translocation problems could be experienced by those proteins whose folding kinetic is faster than recognition event by the export system. Indeed, if the protein acquires any three-dimensional structure, it becomes an inadequate substrate for the translocation machinery and it is retained in the cytoplasm. On the other hand, the PsD leader peptide is predicted to be a SRP-dependent molecular signal for periplasmic secretion which follows a co-translational mechanism. The SRP system (Schierle *et al*., 2003) recognises the nascent polypeptide during its synthesis and its translation and translocation result to be simultaneous.

Data reported demonstrate that only the co-translational molecular signal (PsD) allows the ScFvOx production, its periplasmic translocation and accumulation in soluble form. The total absence of protein in the cytoplasmic fraction indicates a proper recognition and an efficient translocation mechanism. This result is suggestive that the choice of a translocation system that drives periplasmic export of the protein during its synthesis allows to overcome the incorrect folding problems and the physiological barrier due to a fast folding kinetic and therefore the PsD signal peptide will be used for the periplasmic addressing of all the model antibody fragments used in this work.

Construction of an artificial operon for Fab 3H6 expression

The correct assembly of recombinant light and heavy chain in functional Fab fragments can only be achieved if the two subunits are synthesized by the host cell in stoichiometric ratio. In order to achieve a balanced soluble production of Fab 3H6 in *P. haloplanktis* TAC125 an artificial operon was constructed for the co-expression of Fab 3H6 light and heavy chains coding genes (Fig.10).

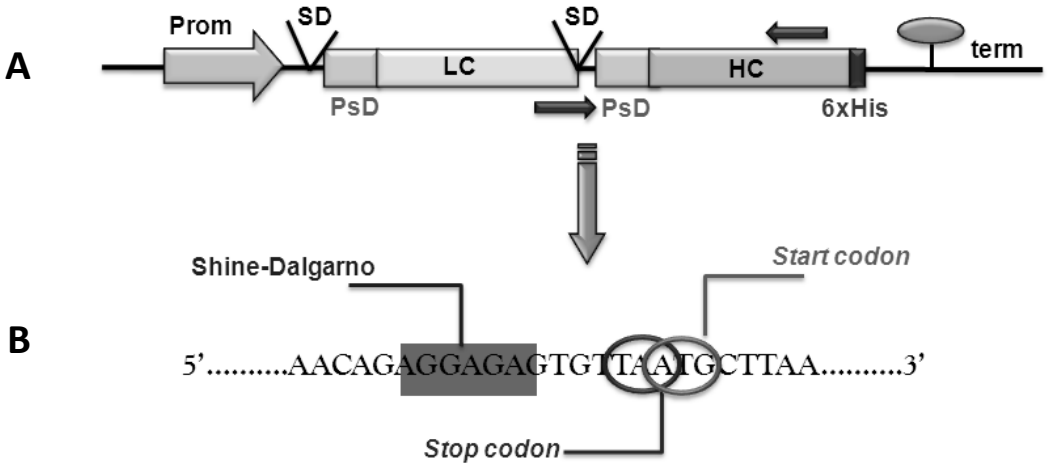


Figure 10: A: Schematic representation of Fab 3H6 artificial operon. LC, Light chain gene; HC, Heavy chain gene; PsD, *Ph*DsbA signal peptide; SD, Shine Dalgarno sequence; Prom, generic psychrophilic promoter; term, psychrophilic terminator. Red arrows indicate the position of primers used for cDNA synthesis and amplification. B: Fab 3H6 operon intergenic region.

Each gene was fused to the sequence encoding the psychrophilic signal peptide PsD for periplasmic secretion and a 6xHis tag coding sequence was added downstream of the heavy chain encoding gene. Moreover, particular attention was paid on the Fab 3H6 operon intergenic region design. Indeed, bacterial genes are commonly

transcribed to form polycistronic mRNAs bearing reading frames whose respective translational efficiencies are controlled by a fine regulation. Normally, genes products which are associated with common cellular processes such as a metabolic pathway are synthesized in equimolar amounts from polycistronic messenger RNA molecules and the complete translation of the preceding gene is necessary for efficient translation of a distal gene. Therefore, translational coupling can be used to control the relative stoichiometry of proteins expression. Coupled genes are characterised by a particular structure at their intercistronic junction, identified for the first time in tryptophan operon of *E. coli* (Oppenheim and Yanofsky, 1980). In order to achieve balanced translation of the two Fab 3H6 chains through translational coupling, an *in silico* analysis of the structural organisation of naturally coupled operons was performed on *P. haloplanktis* TAC125 genome. As reported in Table 3, most of the analysed operons show the same organisation where the start codon of the second gene of the cluster overlaps to the previous gene stop codon, sharing one base pair. Such structure was then used for Fab 3H6 artificial operon construction (Fig.10B). Since a ribosome binding site sequence is necessary for efficient expression of the distal gene of a translationally-coupled gene pair, the same Shine Dalgarno sequence placed upstream to the *lc* gene was added for HC translation by silent mutagenesis of the *lc* gene 3' region.

Table 3: Intergenic regions comparison of natural coupled operons in *P. haloplanktis* TAC125

GENES	SEQUENCE	PROTEINS
bcs B/*bcs* Z	.....AGGAGATAAATAATG.....	indologlucanase precursor
suc C/sucD	.....GGAGGGCAAATAATG.....	succinyl-CoA synthetase a and b
gsp H/*gsp* I	.....GAGAGCCCGAATAATG....	gsp components
gsp J/*gsp* K	.....GAAGGTGGGCACTAATG.	gsp components
trp CF/*trp* B	.....GAGGTAACTAATG.....	anthranilate isomerase
trp D/*trp* CF	.....GAGGTAACTAATG.....	anthranilate phosphoribosyl-transferase
tes A/*yil* J	.....AGAGGAGTCCTAATG.....	testosterone metabolism
trp B/*trp* A	.....AAGGAGGTCAAATATGA...	tryptophan synthetase a and b
trp E/*trp* G	.....GAGGCGGGTTAACATG...	anthranilate synthase I and II
nag K/*nan* E	.....GAGAACTAAAATG.....	N-acetylglucosamine kinase

In blue are indicated the Shine Dalgarno sequences. Red circles: stop codons, Green circles: start codons.

In order to assess the stabily of the bicistronic Fab 3H6 mRNA, the artificial operon was cloned into the psychrophilic expression vector pPM13 under the control of a strong constitutive psychrophilic promoter and reverse transcription experiments were carried out on total RNA extracted from recombinant and wild type bacterial cells after 48h cultivation in rich media at 15°C. PCR amplification of the synthesized cDNA was then performed by using specific primers annealing to the mRNA intergenic region and to the 3' of *hc* gene respectively (Fig.10A). The analysis revealed a specific amplification of a fragment of the expected size of about 770bp length (Fig.11) demonstrating that Fab 3H6 artificial operon is correctly transcribed and its corresponding mRNA is stable within bacterial cell.

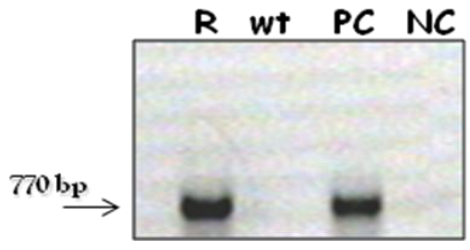


Figure 11: Amplification of cDNA synthesized from *Ph*TAC125 (pPM13-*fab*) (R) and wild type (wt) total RNA after growth in rich medium up to stationary phase. Plasmidic DNA (*pPM13-Fab*) was used as template in positive control (PC), and total RNA in negative control (NC).

A qualitative estimation of Fab 3H6 light and heavy chain relative production was also performed in order to evaluate the expression system efficiency at translational level. Recombinant pPM13-*fab* and wild type *P. haloplanktis* TAC125 strains were grown in rich media at 15°C and total soluble proteins were extracted from samples collected after about 48h cultivation and subjected to Western blotting analysis using specific antibodies directed against either the Fab light chain and the His-tag (present at the C-terminus of Fab heavy chain) in mild reducing condition (Fig.12 A-B). In these conditions, both heterodimeric Fab and free Fab chains should be detected in the analysed samples. Immunodetection performed with antibodies anti light chain revealed the presence of two specific signals, one showing an apparent molecular weight of about 50kDa which is the expected mass for Fab 3H6 heterodimer, and the other corresponding to free light chain of about 25kDa (Fig.12 A). The same experiment carried by anti His tag antibodies (Fig. 12B) revealed the same immunodetected signals indicating the presence of heavy chain either in monomeric and dimeric form. The collected data demonstrate that both light and heavy Fab chains are synthesised in soluble form by the host cells and suggest their correct assembly in soluble and heterodimeric form.

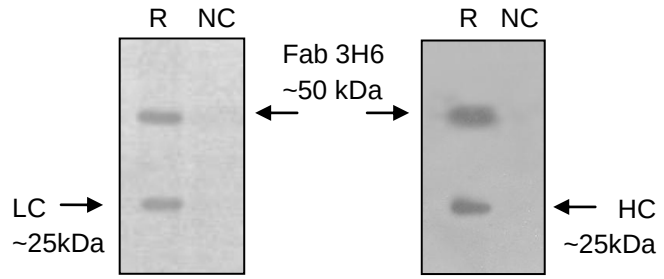


Figure 12: Western blotting analysis of recombinant Fab 3H6 production on total proteins soluble extracts of recombinant (R) and wild type (NC) *P. haloplanktis* TAC125 cells. Immunodetection was performed with anti-human-κ-light chain monoclonal antibodies (Panel A) and anti His tag monoclonal antibodies (Panel B). LC, Fab light chain; HC, Fab heavy chain.

Expression cassettes for antibody fragments production in P. haloplanktis TAC125

The psychrophilic expression vector pUCRP (Papa *et al*., 2007) (Fig. 13) was chosen for large scale expression of recombinant antibody fragments in *P. haloplanktis* TAC125. It contains a regulated psychrophilic promoter which is strongly induced by the presence of L-malate in the culture medium and it shows the highest efficiency in minimal media among the available psychrophilic promoters (unpublished results). The pUCRP vector was previously modified by inserting a C-terminal 6xHis tag (unpublished result). The *psD-scFvOx-c-myc* gene was inserted into the modified pUCRP vector in frame with the His tag coding sequence thus creating a recombinant fusion protein containing, besides the N-terminal leader peptide for periplasmic secretion PsD, two consecutive C-terminal tags: the c-Myc, for protein immunodetection and the 6xHis tag for affinity purification of recombinant product (Fig. 14 A).

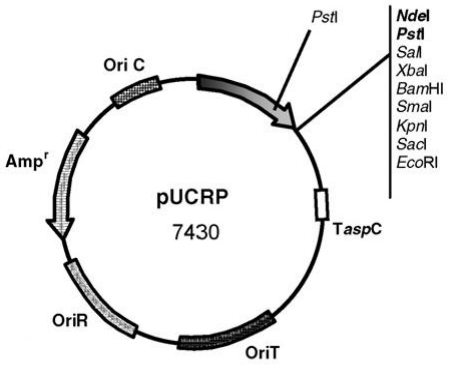


Figure 13: pUCRP expression vector map. Modified from Papa *et al.,* 2007

The same modified expression vector was also used for VHH D6.1production by replacing the *scFvOx* gene with the PCR amplified *vhh*D6.1 one (Fig 14B).

Fab 3H6 operon, already containing all the needed tags and signals for periplasmic addressing (Fig. 10) was instead directly digested from the previously constructed pPM13-*fab* and inserted into a non modified pUCRP vector.

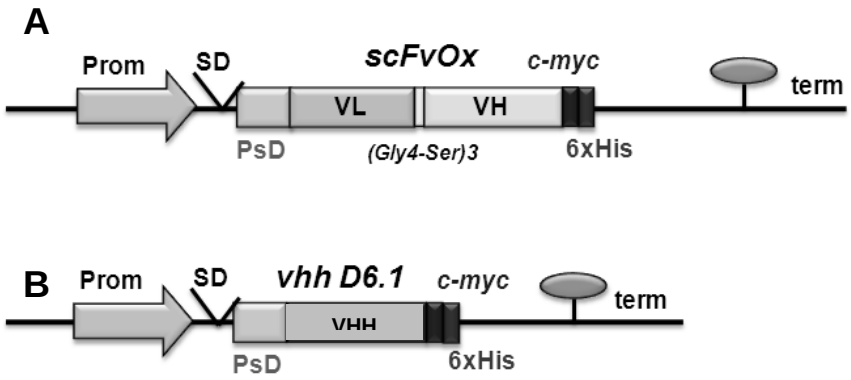


Figure 14: Schematic representation of ScFvOx (A) and VHH D6.1 (B) expression cassettes. SD, Shine Dalgarno sequence; Prom, psychrophilic L-malate inducible promoter; term, psychrophilic terminator; PsD, DsbA signal peptide; VL, variable light chain; VH, variable heavy chain.

Section 2: Medium optimization for recombinant biopharmaceuticals production in *P. haloplanktis* TAC125

The new regulated expression system recently developed for recombinant protein production at low temperatures, strongly induced by the presence of L-malate in culture medium (Papa *et al*., 2006) was chosen for recombinant antibody fragments production in *P. haloplanktis* TAC125. By this system, in optimised conditions, yields of 620 mg L^{-1} of a psychrophilic β-galactosidase and 27 mg L^{-1} of a mesophilic α-glucosidase were obtained in a fully soluble and catalytically competent form (Papa *et al*., 2007). However, performances of the new inducible expression system are still unsatisfactory for large scale recombinant protein production due to the low biomass yield (Papa *et al*., 2007) and consequently low recombinant protein productivity.
The understanding of the growth physiology and the optimisation of cultivation strategies are essential factors to achieve the goal of high protein production by recombinant organisms. Indeed, when the product is cell-associated, the productivity is correlated to biomass level. In this case, the optimisation of the protein production is strictly depending on media composition and, in turn, closely related to the choice of the more suitable cultivation strategy to be used.
To set up a new process for recombinant antibody fragments production in *P. haloplanktis* TAC125, the influence of medium composition and cultivation operational strategies on final biomass concentration, growth rate and recombinant protein production yields was investigated.

Analysis of P. haloplanktis TAC125 growth profiles in amino acid-containing synthetic media

According to the composition of the optimised medium for psychrophilic β-galactosidase production, a mineral medium supplemented with 0,5%w/v *Casaminoacids* (Difco™), different growth media were formulated containing the same mineral medium supplemented with each amino acid as sole carbon source.
In contrast to many γ-proteobacteria, *P. haloplanktis* TAC125 does not possess a phosphoenolpyruvate-dependent phosphotransferase system for the transport and first metabolic step of carbohydrate degradation (Medigue *et al.,* 2005) but the genome analysis reveals the presence of all metabolic pathways for amino acids biosynthesis and degradation. This evidence makes theoretically possible *P.*

haloplanktis TAC125 growth even in presence of a single amino acid as sole carbon source.

Table 4: Sinthetic media composition

Carbon source	Mol/L	Carbon source	Mol/L
L-alanine	0,0385	**L-Lysine**	0,0192
L-arginine	0,0096	**L-methionine**	0,0385
L-aspartate	0,0385	**L-phenylalanine**	0,0385
L-glutamate	0,0304	**L-proline**	0,0385
Glycine	0,0385	**L-serine**	0,0385
L-histidine	0,0130	**L-threonine**	0,0385
L-isoleucine	0,0385	**L-valine**	0,0385
L-leucine	0,0385	**Ref. CAA**	0,0400

A total of 15 synthetic media were formulated according to *Casaminoacids* composition (BD Bionutrients™ Technical Manual). Indeed, due to acid hydrolysis of caseins, several amino acids are absent or present in concentrations lower than 0,5% w/w in *Casaminoacids* and were not tested for *P. haloplanktis* TAC125 growth. In order to avoid a carbon/nitrogen unbalance, the concentration of each amino acid in synthetic media was calculated keeping the total nitrogen concentration at the same value contained in the *Casaminoacids* medium when supplemented at 0,5% w/v (0,038M) (Tab.4). *P. haloplanktis* TAC125 growth was tested in shaken flask cultures at the optimal growth temperature of 15°C. Surprisingly, no significant growth was observed in most of the analysed media (data not shown) and only 4 amino acids were selected for further analysis (Fig.15).

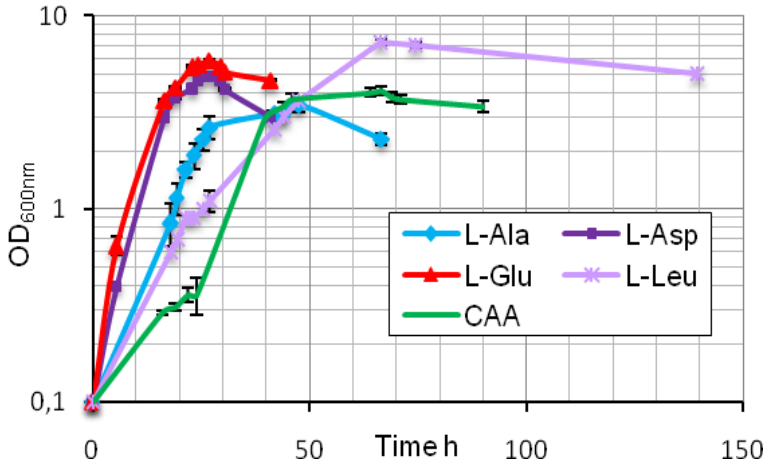


Figure 15: *P. haloplanktis* TAC125 growth profiles in selected synthetic media

Compared to the growth parameters observed in *Casaminoacids* medium, media containing L-glutamate and L-aspartate showed about two folds increase of specific growth rate and biomass yield (Tab. 5). *P. haloplanktis* TAC125 fast growth on acidic amino acids is probably due to the presence of an efficient uptake system and their rapid metabolism through transamination to TCA cycle intermediates. Growth parameters are also improved in L-alanine containing medium. L-alanine is rapidly metabolised through transamination to piruvate and several genes are annotated as putative Na^{+}-alanine symporter in *P. haloplanktis* TAC125 genome. More interestingly, biomass yield in the medium containing L-leucine as carbon source was almost three folds increased compared to that obtained in the same amount of *Casaminacids* while specific growth rate are comparable. L-leucine catabolism is complex and genes predicted to encode for metabolic enzymes involved in different pathways for branched amino acids degradation are present in *P. haloplanktis* TAC125 genome.

Table 5: Growth parameters in selected media

Carbon source	μmax	Final biomass g L^{-1}
L-Ala	0,202	2,1 ± 0,3
L-Asp	0,252	3,6 ± 0,4
L-Glu	0,340	4,4 ± 0,2
L-Leu	0,126	5,6 ± 0,3
Ref. CAA	0,194	2,2 ± 0,2

Furthermore no clear information are available regarding branched amino acids uptake systems in *P. haloplanktis* TAC125.

Recombinant psychropilic β-galactosidase production in synthetic media

The next step for a new defined medium development for recombinant proteins production in *P. haloplanktis* TAC125 was to assess the transcriptional efficiency of the psychrophilic expression system in selected synthetic media. For this purpose, a recombinant strain of *P. haloplanktis* TAC125 was used harbouring the plasmid pUCRP-*β-gal* in which the gene encoding a psychrophilic β-galactosidase is under the control of L-malate inducible promoter. β-galactosidase was chosen as reporter enzyme because its production is easily detected and quantified by enzymatic assays. Recombinant cells were cultivated in shaken flasks at 15°C in different synthetic media supplemented with L-malate and samples were collected from each culture at late exponential growth phase. Interestingly, specific β-galactosidase activity detected in protein extracts of cells grown in L-leucine-containing medium was about five folds higher than that obtained in *Casaminoacids* medium while a huge decrease in β-galactosidase production was observed in all other tested media (Fig. 16).

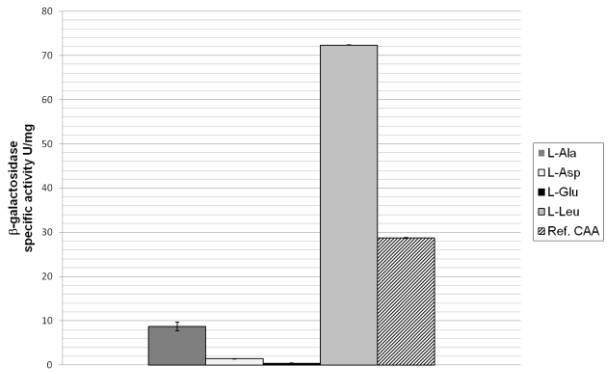


Figure 16: β-galactosidase production yield in selected media

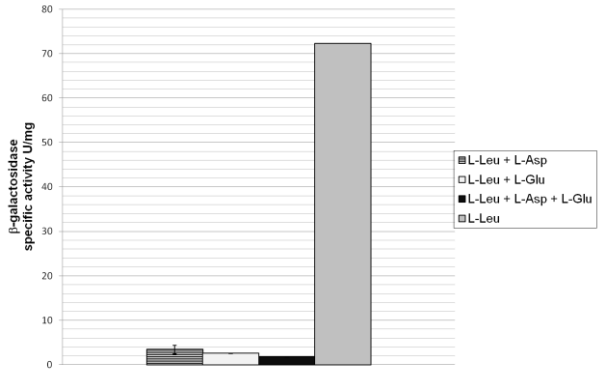


Figure 17: β-galactosidase production yield in combined synthetic media

The observed inhibition effect on recombinant protein production particularly in L-aspartate and L-glutamate containing media is likely to occur at transcriptional level. Indeed, L-malate induction involves a two component system (Papa *et al.*, 2009) where a periplasmic binding protein is responsible for L-malate sensing and, as a consequence of ligand binding, an altered state of the *cis*-acting gene regulation element is directed to the appropriate gene(s) by the protein's DNA binding activity The structural analogy between the acidic amino acids and the L-malate can be the reason for the observed inhibition of L-malate activation on the outer membrane porine PSHAb0363 promoter. When acidic amino acids are used in combination with L-malate a cross regulation can occur where L-malate uptake results to be repressed. Further investigation has been carried out in order to clarify the transcriptional regulation of PSHAb0363 promoter in L-glutamate containing media and it will be reported in the next sections.

Transcription activation by L-malate is also inhibited when L-glutamate and/or L-aspartate are used in combination with L-leucine (Fig. 17) even if specific growth rate is increased compared to that observed in L-leucine containing medium (data not shown).

Effect of L-leucine concentration on P. haloplanktis TAC125 growth

According to previous results, L-leucine represents a key component for the development of a new synthetic medium optimised for recombinant protein production in *P. haloplanktis* TAC125. In order to improve the biomass yield, different media containing increasing amount of L-leucine as carbon source were tested for *P. haloplanktis* TAC125 growth in a lab-scale STR bioreactor. Surprisingly, as shown in Figure 18, a clear inhibition effect is observed on *P. haloplanktis* TAC125 growth when higher L-leucine concentration is added in the medium in the range tested (0,5-1,0% w/v). This effect is likely to occur at the level of substrate uptake system.

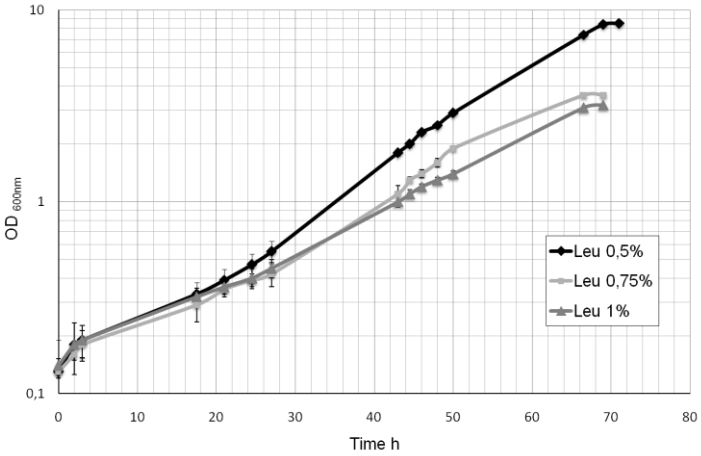


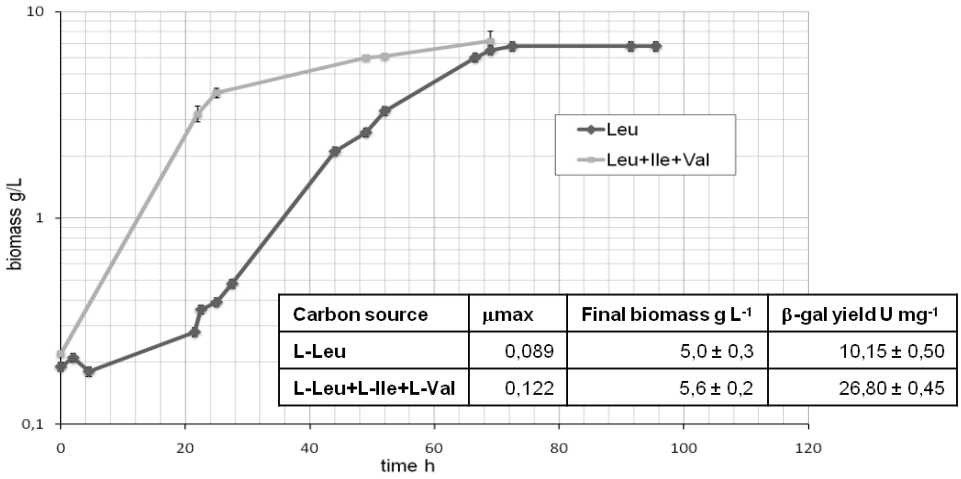
Figure 18: *P. haloplanktis* TAC125 growth profiles in synthetic media containing increasing L-leucine concentrations.

Different systems have been described for branched amino acids uptake in bacteria. Among them, the regulation mechanism of the ATP-dependent high affinity branched amino acids transport system LIV-I in *Escherichia coli* has been deeply investigated and it was found that the rate of transport responds only to changes in concentrations of leucine in the medium. In the presence of high concentrations of leucine the transport activity is repressed (Penrose *et al*., 1968; Rahmaniam *et al*., 1973; Oxender and Quay 1976; Quay and Oxender 1976) by the indirect action of a leucine-responsive regulatory protein Lrp on *liv*J and *liv*KHMGF operon encoding all the components of the high affinity branched amino acids transport system (Haney *et al.,* 1992; Landgraf *et al*., 1999). Actually it is not possible to univocally identify the *liv* gene cluster in *P. haloplanktis* TAC125 genome by an *in silico* analysis. However, the presence of several genes encoding putative ATP Binding Cassette (ABC) transporters and two *lrp* homologues (PSHAa1717 and PSHAa2263) and the experimentally observed inhibition on *P. haloplanktis* TAC125 growth by L-leucine addition in culture media suggest the presence of a mechanism similar to LIV-I for branched amino acids uptake in the psychrophilic bacterium. If L-leucine concentration could not be increased in the new developed medium, other carbon sources should be tested in combination with L-Leu in order to improve the biomass yield and growth kinetic of *P. haloplanktis* TAC125. Moreover a very long lag phase is observed in *P. haloplanktis* TAC125 growth on L-leucine alone even if it is added in lowest concentration. Quay and co-workers in 1976 proposed an alternative explanation for *E. coli* growth inhibition in leucine-containing minimal medium. Based on the observation of a huge decrease of intracellular levels of L-valine and L-Isoleucine during growth starvation, they hypothesised that mechanism of leucine sensitivity involved transport-mediated exchange of intracellular isoleucine and valine for leucine (Quay *et al*., 1976). In this situation, when leucine is the only carbon source available, LIV-I or any other similar system functionally uptakes extracellular leucine in exchange for intracellular isoleucine and valine that can become limiting for cellular protein synthesis. This leads to a period of restricted growth, corresponding to the long lag phase observed in *P. haloplanktis* TAC125 growth in leucine-containing media. When leucine concentration in the medium further decreases, *ilv* operon, also regulated by Lrp, can be completely derepressed

and the biosynthetic enzymes for isolecine and valine encoded by *ilv* genes are produced sufficiently to compensate the isoleucine and leucine consumption by protein synthesis and loss from the cell for solutes exchange and the growth can proceed normally.
According to this model, a new synthetic medium was developed containing L-leucine in combination with L-isoleucine and L-valine in stoichiometric ratio.

Analysis of P. haloplanktis TAC125 growth and recombinant β-galactosidase production in branched amino acids (LIV) synthetic medium

A new medium containing L-leucine, L-isoleucine and L-valine in 1:1:2 ratio, hereafter called LIV medium, was developed and it was tested for *P. haloplanktis* TAC125 growth in lab-scale STR bioreactor. As shown in Fig. 19, the new medium leads to a very short lag phase and a slight increase of specific growth rate and biomass yield compared to the previously optimised L-leucine containing medium. Furthermore,



Carbon source	μmax	Final biomass g L^{-1}	β-gal yield U mg^{-1}
L-Leu	0,089	5,0 ± 0,3	10,15 ± 0,50
L-Leu+L-Ile+L-Val	0,122	5,6 ± 0,2	26,80 ± 0,45

Figure 19: *P. haloplanktis* TAC125 growth profiles, kinetics, biomass and recombinant protein production yield in L-leucine and LIV synthetic media. β-galactosidase induction was performed by L-malate 0,4%addition at t=0.

recombinant β-galactosidase production results to be above two folds increased in the new growth conditions.
The observed results confirm the hypothesis that major factor in leucine sensitivity is ability of the transport system to carry out exchange of pool amino acids for extracellular leucine. The presence of all three branched amino acids in culture medium in stoichiometric ratio overcomes the loss of intracellular isoleucine and valine that was limiting for bacterial growth and for recombinant protein synthesis.
When a substrate consumption analysis was performed (Fig. 20) it revealed a significant residual amino acid concentration in the spent medium at the end of exponential growth leading to a low $Y_{x/s}$ (≤0,30). The amino acidic substrates are rapidly consumed during the first 20 hours of cultivation, corresponding to exponential bacterial growth at maximum specific growth rate, while L-malic acid concentration remains almost constant. From 20 to 48 hours of cultivation the amino acids concentration in the extracellular medium slightly decreases while a rapid consumption of L-malic acid is observed which results to be completely depleted from the culture medium at about 24 hours of cultivation. This doesn't seem to affect bacterial growth whose specific growth rate follows the rate of amino acids consumption. Furthermore a strong ammonia accumulation was observed at same rate of biomass production. This is not surprising considering that amino acids are

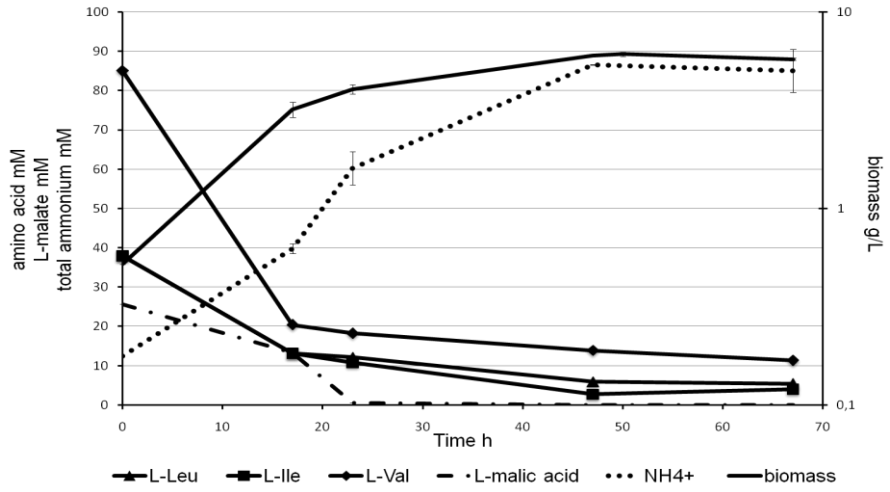


Figure 20: Substrate consumption analysis, biomass and ammonia accumulation during *P. haloplanktis* TAC125 batch cultivation in bioreactor.

also nitrogen sources and the LIV medium is unbalanced containing an excess of organic nitrogen. The role of ammonia accumulation in *P. haloplanktis* TAC125 growth inhibition was experimentally investigated (data not shown) and it was demonstrated that up to 150 mM ammonia does not affect *P. haloplanktis* TAC125 growth. According to the data collected, an explanation for the lower Yx/s in the new optimised medium must be searched out more deeply in bacterial physiology in this behaviour.

Set-up of chemostat cultivation process

The obtained results paved the way to the possibility to set up a chemostat process where a feeding medium containing L-leucine as sole carbone source will be used after a batch cultivation in LIV medium. Due to the residual concentration of substrates observed at the end of the exponential growth of *P. haloplanktis* TAC125 in LIV medium and to the hypothesis of a total derepression of *ilv* operon in those conditions, the addition of leucine only after exponential growth in LIV medium should lead to bacterial growth at steady state.

P. haloplanktis TAC125 was grown in LIV medium at 15°C in STR bioreactor until the highest biomass yield was reached (about 48 h of cultivation). The L-leucine containing medium was fed to the culture at a dilution rate of 0,05 h^{-1}, calculated on the base of the maximum specific growth rate observed in batch cultivation in LIV medium. As shown in Figure 21, in selected conditions a steady state is established in which the cell density remains constant for at least five resident times.

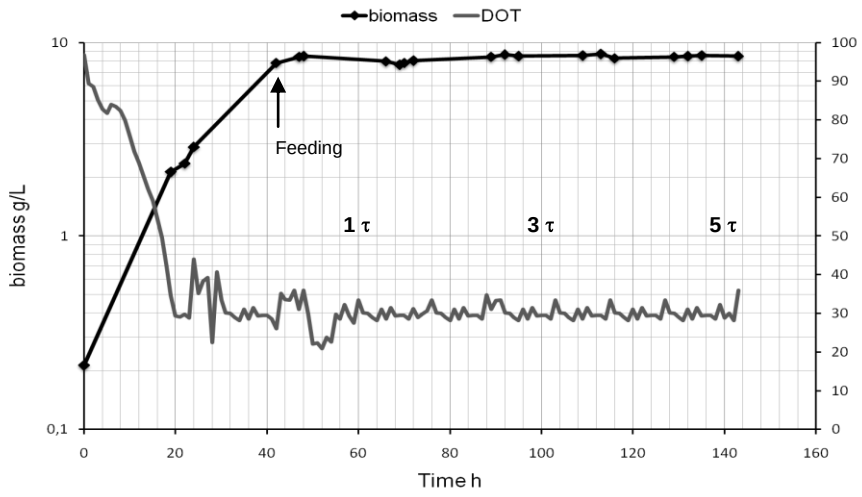


Figure 21: Growth and oxygen consumption profiles of *P. haloplanktis* TAC 125 in chemostat cultivation at 15°C. The black arrow indicates the end of batch phase in LIV medium and the beginning of the feeding with L-Leu. Resident times are indicated on

Section 3: Process scale up: recombinant antibody fragments production in bioreactor

After the construction of an *ad hoc* genetic expression system for each model protein and the development of a fermentation process optimised for recombinant proteins production by the selected psychrophilic expression system, the recombinant production of antibody fragments by *P. haloplanktis* TAC125 has been evaluated in bioreactor.
The production of the three model proteins Fab 3H6, ScFvOx and VHH D6.1 has been performed at 15°C and pH 7 in LIV medium in presence of L-malate 0,4% as inducer and dissolved oxygen concentration was kept always above 30% by adjusting air flow inlet and stirring speed.
Different cultivation operational strategies have been screened for each production process in order to maximize recombinant proteins yield.

Fab fragment recombinant production

○ Batch cultivation:

The analysis of Fab 3H6 production was performed by ELISA experiments on total soluble protein extracts from *P. haloplanktis* TAC125-pUCRP-*fab* samples collected at different times of cultivation. As shown in Figure 22, recombinant Fab 3H6 nicely accumulates during fermentation reaching the highest production yield of 3,99 ± 0,11 mg L^{-1} in late exponential phase corresponding to about 48 h of cultivation thus demonstrating that our model protein Fab 3H6 is produced in soluble form by the psychrophilic expression system in the optimised conditions. The highest rate of Fab 3H6 accumulation is observed between 20 and 25 hours of cultivation corresponding to the range in which the inducer L-malate is rapidly consumed (Fig. 22).The

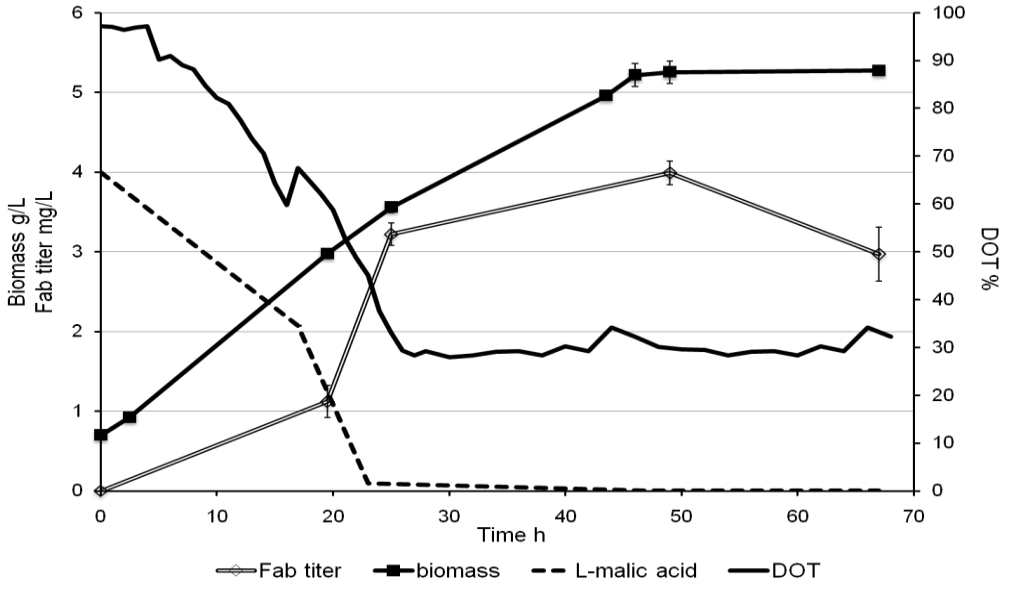


Figure 22: Fab 3H6 titer, biomass yield, oxygen and L-malate consumption profiles in *P. haloplanktis* TAC125 pUCRP-*fab* batch cultivation in LIV medium at 15°C.

observed yield of soluble Fab 3H6 is quite high compared to that obtained by other conventional microbial recombinant hosts (Gach *et al*., 2007). Furthermore, a high $Y_{P/X}$ of 0,89 ± 0,15 mg $g_{biomass}^{-1}$ was also observed suggesting that a further optimisation of culture media and/or cultivation strategies could represent a valid approach to increase the yields of recombinant Fab fragments production.

Analysis of Fab 3H6 cellular localisation and quaternary structure was performed by Western blotting experiments on periplasmic and cytoplasmic extraxts from samples collected at different times of cultivation, using specific anti-light chain antibodies in non-reducing conditions (Fig. 23). The analysis revealed a specific signal corresponding to Fab 3H6 in heterodimeric form (≈50kDa) exclusively in periplasmic fractions, demonstrating that soluble Fab 3H6 is not only effectively produced but also correctly and totally translocated into the periplasmic space. Moreover, the apparent molecular weight of the detected signal (≈50 kDa) and the complete absence of signals corresponding to free light chains (≈25 kDa) suggest that the recombinant product is fully assembled in heterodimeric quaternary structure.

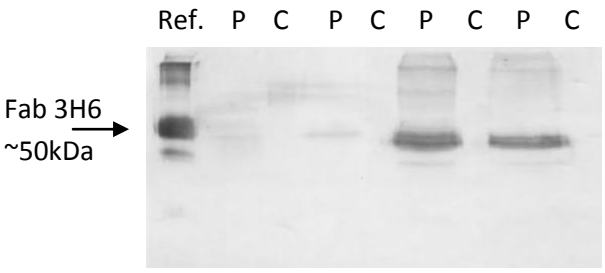


Figure 23: anti human κ light chain immunodetection on periplasmic (P) and cytoplasmic (C) fractions of bacterial samples collected at different times of cultivation. The black arrow indicates the increasing time of sampling. Ref, positive control, pure Fab 3H6 100ng.

○ Continuous cultivation:

Fab 3H6 recombinant production was also performed by a chemostat cultivation process. After 48 hours of batch growth in LIV medium the culture was fed with a medium containing L-leucine as sole carbon source at a dilution rate of 0,05 h^{-1}.

As shown in Figure 24, in the selected conditions, the steady state was achieved for at least 5 resident times in which both the cell density and the product titer remain constant. By the developed process a specific Fab 3H6 productivity of 0,19±0,02 mg L^{-1} h^{-1} was achieved with a constant production yield of about 3,8 mg L^{-1}.

Data collected demonstrated that a continuous fermentation process can be suitable for soluble production of recombinant Fab fragments at low temperatures.

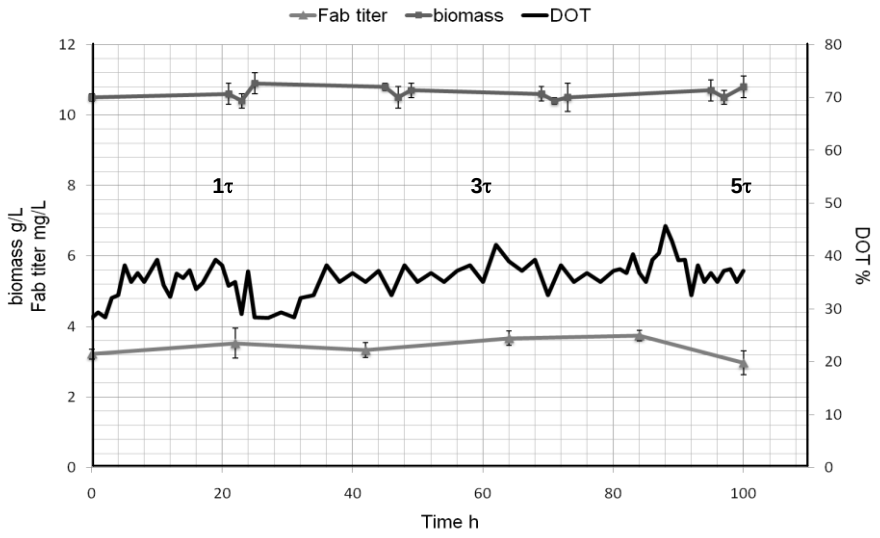


Figure 23: Growth, Fab titer and oxygen consumption profiles during *P. haloplanktis* TAC125 pUCRP-*fab* chemostat cultivation. Resident times are indicated on the graph.

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Comparative analysis of recombinant antibody fragment production in diverse microbial host cells

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ABSTRACT

While efficient heterologous protein production has proven to be feasible in general terms, the production of complex proteins has turned out to have a low success rate. A lot of molecular, biochemical and biological information about different host cells has been progressively accumulated, but such data has been gained through a variety of non-comparable approaches, leading to a poor base for generalisation.

Therefore, this study aimed at a comparative analysis of five different host species with proven capacities for protein expression to adress functional, structural and regulatory processes involved in expression of complex proteins. Another aim was to investigate the efficiency of folding, solubility and secretion and the physiological response in different host organisms at different critical environmental conditions.

Expression of a heterodimeric antibody Fab fragment was assessed in five microbial host systems, namely *Saccharomyces cerevisiae*, *Pichia pastoris*, *Trichoderma reesei*, *Escherichia coli* and *Pseudoalteromonas haloplanktis*. The comparative analysis of the specific production and the effect of cultivation temperature and oxygenation as examples for production-relevant environmental parameters highlight fundamental differences but also common features of these protein production platforms. The current study highlights *P. haloplanktis* as a high potential candidate for the expression of recombinant proteins. Furthermore it could be shown that lowering temperature had a positive effect on heterologous protein secretion in 3 out of 4 analyzed microorganisms.

Keywords

Saccharomyces cerevisiae, *Pichia pastoris*, *Trichoderma reesei*, *Escherichia coli*, *Pseudoalteromonas haloplanktis*, comparison, antibody Fab fragment, 3H6, heterologous protein production, environmental conditions

Introduction

Microbial host cells, most notably *Escherichia coli* and *Saccharomyces cerevisiae*, represent the most common model and host organisms for basic research and recombinant protein production. Of the few protein based biopharmaceuticals on the market, the majority is still produced in these two host organisms (10). Apart from these two working horses of

biotechnological research, many different organisms are currently used or tested for the production of technically and therapeutically relevant proteins, including bacterial and fungal host cells, insect and mammalian cell lines and transgenic plants. Since post-translational modifications (PTMs), such as glycosylation (6), play a major role for product quality and suitability for human administration, higher eukaryotic expression systems are commonly applied, because they can often produce human or human-like PTMs and generally achieve high product titers. Furthermore, the production of large proteins is often difficult in microbial, especially bacterial hosts (10). Nevertheless, detailed research has enabled substantial progress in producing PTMs such as glycosylation in bacterial cells (54) and fungal hosts such as *Pichia pastoris* (18, 20, 29, 41). Large proteins such as full length antibodies have also already been successfully produced in microbial hosts (35, 42, 48). In addition to these advances and the strong molecular biology knowledge background of microbial hosts, there are also the benefits of simple growth requirements on defined mineral growth media, high growth rates and consequently short fermentation and process times. Thus there is motivation to further develop and improve microbial host systems.

In the current study, different microbial host systems for recombinant protein production were compared. A complex model protein, the Fab 3H6 (12, 26), was expressed and secreted either into the periplasm or into the culture broth in *S. cerevisiae*. *P. pastoris*, *Trichoderma reesei*, *E. coli* and *Pseudoalteromonas haloplanktis*. These organisms represent well established platforms for heterologous

protein production in academia and industry or highly prospective new host systems, as in the case of *P. haloplanktis* (39). The general characteristics of these organisms are summarized in Table. 1. There are big differences in the capability to express specific

proteins even among yeast species, indicating the importance to identify differences as well as common features even in more closely related species. For each species a commonly used vector system with an inducible or a constitutive promoter for recombinant protein production in combination with chemostat cultivation was applied. Generally, secretion of the recombinant product into the periplasm or culture supernatant results in relatively pure protein and facilitates downstream processing, although periplasmic secretion of

Table 1. Host organisms used in this study and their general protein production characteristics. *na* - not applicable

host organism	cytoplasmic production	secretory production	periplasmic production	disulfide bond formation	glycosylation
S. cerevisiae	Yes – may result in poor product titers	Yes - generally low titers	*Na*	Yes	Yes – protein often hyperglycosylated
P. pastoris	Yes	Yes – generally lower titers than for intracellulary production	*Na*	Yes	Yes – smaller glycan-structures than *S. cerevisiae*
T. reesei	Yes	Yes	*Na*	Yes	Yes
E. coli	Yes – often highly pure protein in the form of inclusion bodies (IBs)	Yes – but inefficient and the underlying mechanisms are poorly understood	Yes	Not for intracellulary produced proteins, but can occur in the periplasm	No
P. haloplanktis	Yes	Yes	Yes	Can occur in the periplasm	No

recombinant polypeptides in bacteria such as *E. coli* may result in relatively low protein concentrations and filamentous fungi such as *T. reesei* secrete a vast amount of host protein into the culture supernatant (33). Codon optimization for the specific host organism may also

be applied to increase the product yield (7). In the current study non-codon optimized 3H6 Fab was used to facilitate comparison of the organisms and because codon optimization would not have been applicable to all organisms.
It is often still unpredictable which host systems will be the most suitable for the production of a particular protein, thus a trial and error approach is necessary to find the best suited host cell. The results of this study confirm that there is a great variability in the production of Fab 3H6 among different host organisms. Furthermore microorganisms encounter diverse stress conditions during fermentation processes, which may interfere with process performance (34). These environmental stresses and the consequently triggered physiological reactions, including changes of the protein folding/secretion machinery, are highly interrelated (16), thus strongly supporting the investigation of environmental factors influencing protein expression and secretion. Of all the possible stresses, temperature, oxidative stress, osmolarity and pH appear particularly important. Therefore the impact of temperature and oxygenation on recombinant protein production was analysed at steady-state using chemostat cultivation. As microorganisms encounter rather long-term suboptimal conditions during production processes rather than short-term perturbations, the analysis of environmental factors at steady-state conditions was the method of choice. Furthermore growth rate related effects can be avoided by applying chemostat cultivation (45).
The results obtained in the current study highlight common features and differences in the response to key fermentation parameters and the resulting product yields in different microbial species.

Materials and Methods

Strain construction

Saccharomyces cerevisiae – The 3H6 heavy and light chain (12) coding sequences were both integrated on a pYX integrative expression vector. For constitutive expression the *S. cerevisiae TPI* (triose phosphate isomerase) promoter was used and the *S. cerevisiae* α-factor leader sequence served as secretion signal (4). A polyA sequence served as terminator. HIS3 and URA3 were used as selection markers and expression vectors were integrated into the genome of the reference *S. cerevisiae* CEN.PK strain (52).

Pichia pastoris – Heavy and light chain coding sequences were both inserted into a single pGAPzαA expression vector. Both chains were expressed under the control of the *GAP* (glyceraldehyde-3-phosphate dehydrogenase) -promoter and the AOX terminator (2). The *S. cerevisiae* α-factor secretion signal was used to target the protein into the supernatant. The plasmid was linearized by restriction digest and integrated into the genomic GAP promoter locus of *P. pastoris* X-33.

Trichoderma reesei – Two expression cassettes were constructed, where the 3H6 light and heavy chain fragments were between the *cbh1* promoter and terminator. The c*bh1* signal sequence was fused with the antibody chain sequences. The expression cassettes were subsequently cloned to a final expression plasmid where the light and heavy chain cassettes were in tandem, followed by the acetamidase selection marker gene and a *cbh1* 3´flanking region fragment. The fragment carrying these elements was transformed into the *T. reesei* strain RutC-30 (36). The Fab production strain studied in this work had the Fab fragment expression cassettes and the acetamidase gene integrated into the *cbh1* locus.

Escherichia coli – 3H6 Fab heavy and light chain expression cassettes were present on the pET27b vector, using T7 promotor and terminator sequences and the pelB secretion signal for secretion into the periplasm. *E. coli* HMS174(DE3) was transformed with the obtained plasmid.

Pseudoalteromonas haloplanktis – An artificial operon was constructed for 3H6 heavy and light chain recombinant expression in *P. haloplanktis* strain TAC125. The operon was cloned in the psychrophilic vector pUCRP containing a strong L-malate inducible promoter (38) and each gene was fused to a psychrophilic signal peptide encoding sequence for periplasmic secretion. 6xHis tag was fused at the C-terminus of recombinant 3H6 heavy chain.

Secretion / Periplasmic expression of the 3H6 Fab fragment was verified by Western Blotting and quantified by ELISA (see section Analytical procedures for details) in all the hosts.

Growth conditions

Generally, cultivations were all performed in triplicate and for temperature and oxygenation experiments, the combinations for the temperature and oxygenation regimes were different for each chemostat to avoid the effect of evolutionary adaption. Samples were taken at steady state, indicated either by constant RQ and biomass yield or as in the case of *T. reesei* also by monitoring the expression of 13 genes(*rpl16a*, *hen6*, *hsp70*, *rps16b*, *gcn4*, *chs1*, *acs1*, *bgl2*, *bga1*, *egl1*, *vpa1*, antibody light chain, antibody heavy chain, and *nth1*) using TRAC analysis (44).

Detailed media recipes for all model-organisms of this study can be found in Supplemental data 1.

Saccharomyces cerevisiae – Shake flask cultivations of *S. cerevisiae* were performed in mineral medium with 2% (w/v) glucose as carbon source (batch medium, Supplemental data 1). Fermentation was performed in a BIOSTAT B (B-Braun) bioreactor as follows: after a batch phase of approximately 24 hours (initial OD660 was 20), chemostat cultivation was performed on 2% (w/w) glucose and 0.05% ethanol (w/w) as carbon sources under fully aerobic conditions at pH 5.0 and at a dilution rate of D = $0.1h^{-1}$ and a working volume of 1.25L with an expected yeast dry mass of 10g L^{-1}. Ethanol was added to avoid culture oscillation (40). The total gas flow was kept constant for all experiments at 1 vvm (volume gas per volume batch medium and minute) and the agitation was set at 1000 rpm. Chemostat cultivation was performed at 3 different temperatures: 23, 26 and 30°C. For cultivation at different oxygenation conditions, chemostat medium was used, with 2% (w/w) glucose, at a dilution rate of D = $0.1h^{-1}$. No ethanol was added in the oxygen experiment because no culture oscillations were observed without ethanol in the growth medium. The temperature was set at 26°C and the air concentration in the inlet gas stream was changed in a stepwise manner by partial replacement with an equivalent volume of nitrogen. The corresponding oxygen concentrations in the inlet air were 20.97 %, 5% and 2 %, resulting in normoxic, oxygen limiting and respiro-fermentative (hypoxic) conditions, respectively. The dissolved oxygen tension (DOT) in oxygen limiting condition was about 2-5%, in hypoxic conditions it was zero, whereas in fully aerobic conditions it was about 80%.

Pichia pastoris – Shake flask cultivations of *P. pastoris* were performed in YPD medium (2% (w/v) peptone, 1% (w/v) yeast extract, 2% (w/v) glucose). Bioreactor cultures were performed in a MBR 3L Bioreactor at a working volume of 1.5L as follows. After a batch phase of approximately 24 hours on mineral medium with glycerol as carbon source, chemostat cultivation was performed on mineral medium and glucose as carbon source under fully aerobic conditions (20% dissolved oxygen, DO), pH 5.0 and at a dilution rate of D = $0.1h^{-1}$ with an expected yeast dry mass of 25g L^{-1}. Chemostat cultivation was performed at 20, 25 and 30°C, respectively. For cultivation at different oxygenation conditions, the chemostat medium was used, the temperature was set to 25 °C and the oxygen concentrations in the inlet air were: 20.97% (normoxic condition), 10.91% (oxygen limitation) and 8.39% (hypoxic condition). The total gas flow was kept constant for all experiments at 1.5 vvm with a headspace overpressure of 0.2 bar, 700 rpm and 25 °C. In both limited and hypoxic conditions the dissolved oxygen tension (DOT) was zero, whereas in fully aerobic conditions DOT was about 45%.

Trichoderma reesei – *T. reesei* was grown in Braun Biostat CT2 bioreactors (B. Braun Biotech International GmbH) with a working volume of 2 L, with 0.5 vvm aeration at 800 rpm and pH 4.8 ± 0.1. Cultures were inoculated with 200 ml (10% final culture volume) filamentous pre-cultures inoculated with 1 x 10^6 conidia ml^{-1} and grown for approximately 72 h at 28°C in 50 ml volumes in 250 ml flasks which were shaken at 200 rpm. Nitrogen, oxygen and carbon dioxide were monitored online with an OmniStar mass spectrometer (Pfeiffer Vacuum, Germany). Chemostats were maintained at D = 0.03 h^{-1}. Cultivations were carried out at 28, 24 and 21.5°C.

Escherichia coli – Chemostat cultivations were performed in a 2L bench-top bioreactor (Biostat B, Braun Biotech, Melsungen, Germany) as follows. During the batch phase, the temperature was set to 33ºC and the dissolved oxygen concentration was maintained above 20% saturation by cascade automatic control of the stirrer speed between 700 and 1000 rpm and the air-flow between 1.5 and 4 L min^{-1}. pH was controlled at 7.0. After approximately 10 h of batch growth, continuous medium flow was started at a dilution rate of D = 0.10 h^{-1}. Induction was performed by addition of IPTG after the continuous process began. IPTG concentration was maintained constant at 25 µM throughout the process. Chemostat cultivations were carried out in glucose limited cultures at 37, 33 and 30 °C. Fully aerobic conditions were maintained by means of constant aeration and stirring.

Pseudoalteromonas haloplanktis – Shake flask cultivations of *P. haloplanktis* were performed in complex TYP medium (1.6% (w/v) bacto-triptone, 1.6% (w/v) yeast extract, 1% (w/v) NaCl pH 7.5). Fermentation was performed in a STR 3L Bioreactor (Applikon) connected to an ADI 1030 Bio Controller (Applikon) in SCHATZ mineral medium (38) containing L-leucine 0.5% (w/v), L-isoleucine 0.5% (w/v) and L-valine 1% (w/v) as carbon sources and supplemented with ampicillin 100µg ml^{-1} for plasmid selection and L-malate 0.4% (w/v) for promoter induction. After a batch phase of approximately 48 hours, chemostat cultivation was performed on same medium under fully aerobic conditions (≥30% DOT) at a dilution rate of D = 0.05h^{-1} and a working volume of 1.0L. Chemostat cultivation was performed at 15°C (µmax=100%).

Analytical procedures

Biomass determination - For biomass determination adequate sample volumes were washed in demineralised water and either dried to constant weight in pre-weight beakers at 105°C until constant weight (2) or collected and dried on pre-weighed filter discs and dried at 110°C until constant weight (43).

Periplasmic protein preparation for bacterial hosts - Bacterial pellets were resuspended in 1/20 of culture volume of borate buffer ($Na_2B_4O_7$ 200mM, NaCl 130mM, EDTA 5mM, pH 8) and incubated 18h at 4°C. The suspension was centrifuged at 8000rpm for 15min at 4°C and the supernatant used for ELISA.

Product quantification by ELISA – A sandwich ELISA was performed as described in previous studies (12, 14).

Western Blot Analysis – For Western Blot analysis, samples were subjected to standard polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions to avoid reduction of disulfide-bonds and blotted onto a nitrocellulose membrane (Biorad). After blocking the membrane in PBS 2% (w/v) dry milk for at least 2 hours, 3H6 Fab was detected using either anti-human IgG (light chains) or anti-human Fab specific alkaline phosphatase conjugate (Sigma). Blots were developed using the AP-substrate kit (Biorad).

Results

Fab 3H6 could be successfully produced and in all bacterial and fungal host cells. A band of approximately 50 kDa in size was obtained in all host organisms and indicated full length Fab 3H6 (Figure 1). Additionally, it can be seen that besides the correctly assembled heterodimeric Fab 3H6, free unassembled light chains, of approximately 25 kDa in size, were released in nearly all host organisms, to a different extent with exception of *P. haloplanktis*. Furhermore a small fraction of degradation products was detectable in *P. pastoris* and *E. coli* cultures. Moreover, during *E. coli* chemostat cultivation, presence of the 3H6 Fab was not only observed in the periplasmic space but also in the culture broth (Figure 1).

Productivities at temperatures optimal for growth For comparative purposes, the standard operating conditions for each microorganism were defined as an aerobic carbon limited continuous culture with a temperature setpoint at which μ_{max} was 100%. However, it should be noted that chemostat cultivations were not performed at a dilution rate corresponding to

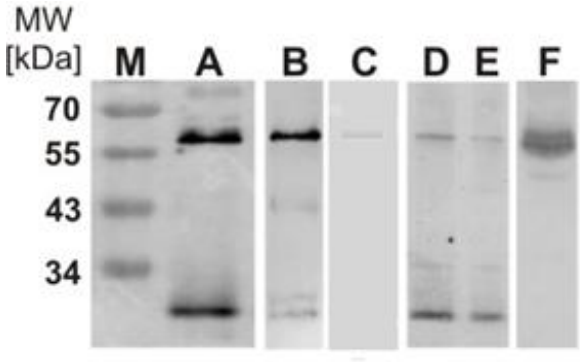

Fig.1 : Representative, non - quantitative Western Blot images to verify the successful production of antibody 3H6 Fab fragment. Molecular weight marker (M), *S. cerevisiae* culture supernatant (A), *P. pastoris* culture supernatant (B), *T. reesei* culture supernatant (C), *E. coli* periplasma preparation (D), *E. coli* culture supernatant (E) and *P. haloplanktis* periplasma preparation (F).

μ_{max} but at a dilution rate that corresponds to ≤ 60 % μ_{max} depending on the host organism. Cultivations were not performed at μ_{max} to allow subsequent cultivation at different temperatures that would not support growth at μ_{max} and to enable the analysis of the impact of growth temperature on specific production in a growth rate independent manner. Final product titers differed between different host organisms, but also specific production varied between the expression hosts (Table 2). *P. haloplanktis* and *P. pastoris* were able to produce the 3H6 Fab very effectively and showed the highest specific production, followed by *S. cerevisiae* and *E. coli*. For *T. reesei* product yields were low compared to the other 4 organisms.

Table 2. Final product titers and specific Fab production ($Y_{P/X}$) at optimal growth temperature, grown in carbon limited chemostat cultures at constant dilution rates (D). * For *E. coli* the combined (periplasmic + extracellular product) values are shown. +/- represents the standard error of the mean.

host organism	growth temperature [°C]	D[h^{-1}]	product titer [mg L^{-1}]	$Y_{P/X}$ [mg g biomass $^{-1}$]
S. cerevisiae	30	0.10	0.22 +/- 0.05	0.02 +/- 0.005
P. pastoris	30	0.10	5.4 +/- 0.2	0.21 +-/ 0.01
T. reesei	28	0.03	$3.3\ 10^{-3}$ +/- $2\ 10^{-4}$	$9.4\ 10^{-4}$ +/- $7\ 10^{-4}$
E. coli *	37	0.10	0.21 +/-0.043	0.017+/- 0.003
P. haloplanktis	15	0.05	4.14 +/- 0.16	0.93 +/- 0.05

The Effect of Temperature on biomass yield and specific production Temperatures below the optimal growth value have already been reported to have a beneficial effect on heterologous protein production, and specifically on the Fab 3H6 secretion in *P. pastoris* (3, 9, 15, 27, 47). Therefore we investigated if decreased temperature had an impact on 3H6 Fab production in the other host species as well. Therefore, continuous cultivations were performed at 3 different temperatures and data were collected after steady-state was reached. For each host organism temperature setpoints, referring to 60, 80 and 100% of μ_{max} were chosen. The impact of temperature was analysed for *S. cerevisiae*, *P. pastoris*, *T. reesei* and *E. coli*, whereas no data are available for *P. haloplanktis*.Interestingly the biomass yield during chemostat cultivation was affected by growth temperature in some of the host organisms (Table 3). *T. reesei* and *P. pastoris* had a higher biomass yield at lower growth temperature, whereas *S. cerevisiae* had a lower biomass yield at the lower temperature. No significant differences were observed for *E. coli*. Furthermore, growth temperature had a significant effect on specific production during

Table 3: Biomass yield coefficient $Y_{x/s}$ [g g^{-1} biomass per carbon source] in carbon limited chemostat cultures at constant dilution rate at different growth temperatures. T represents the temperature at which the indicated percentage of μ_{max} could be reached in batch culture. +/- represents the standard error of the mean. *nd* no data available.

	$Y_{x/s}$		
Host organism	**T = 100% μ_{max}**	**T = 80% μ_{max}**	**T = 60% μ_{max}**
S. cerevisiae	0.48 +/- 0.006	0.46 +/- 0.012	0.41 +/- 0.010
P. pastoris	0.52 +/- 0.01	0.53 +/- 0.01	0.55 +/- 0.01
T. reesei	0.355 +/- 0.006	0.378 +/- 0.006	0.437 +/- 0.018
E. coli	0.37 +/- 0.004	0.38 +/- 0.012	0.37 +/-0.015
P. haloplanktis	0.22 +/- 0.00	*nd*	*nd*

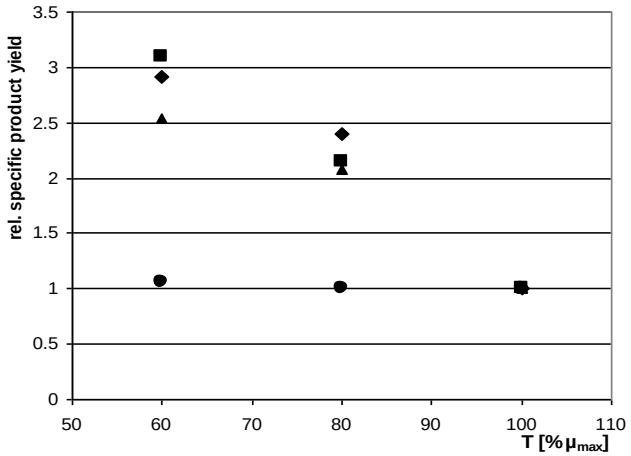

Fig.2: Relative specific production in carbon-limited chemostat cultures (D ≤ 60% μ_{max}) at different temperatures for *S. cerevisiae* (●), *T. reesei* (▲), *P. pastoris* (■) and *E. coli* (♦).The relative temperature values indicate the temperatures at which μ_{max} in batch culture would be reduced, relative to growth at the optimal temperature, by the percentages indicated.

chemostat cultivation in 3 out of 4 host organisms. As can be seen in Figure 2, *E. coli* showed a 2.4-fold increase while *P. pastoris* and *T. reesei* a showed a 2-fold increase in specific production when the growth temperature was decreased to a setpoint equivalent to 80% of μ_{max} in batch culture. At the temperature setpoint, which referred to 60% of μ_{max} in batch culture, these 3 organisms showed an even higher increase in specific production (3 fold for *P. pastoris* and *E. coli* and 2.5 fold for *T. reesei* compared to the maximum temperature setpoint, respectively). In contrast, *S. cerevisiae* showed no change of specific production with temperature.

Additionally an interesting effect was observed in *E. coli*. As already mentioned, during *E. coli* cultivations product leakage into the culture broth occurred. 3H6 Fab was found both in the periplasma and in the culture medium. It was observed that the amounts found in both fractions varied with temperature and hence with the amount of Fab produced (data not shown). At the lowest Fab 3H6 concentration, more protein was found in the periplasma but as the amount of Fab increased, it was mainly found in the culture broth.

As mentioned above for *P. pastoris* and *E. coli* a small amount of apparently degraded Fab was detectable on Western Blots. However the amount of degraded Fab did not accumulate or decrease at growth at different temperatures (data not shown).

The Effect of Oxygenation on biomass yield and specific productivity

It has already been reported in a previous study that decreased oxygen supply during chemostat cultivation of *P. pastoris* can lead to an increased specific productivity (2). A 2.5 fold increase in specific productivity was achieved by shifting from normoxic to hypoxic growth conditions. Based on these data, an oxygen limited fed batch strategy was developed (2). This shift was also accompanied by a 2-fold decrease in biomass yield and increased ethanol formation.

However, to check whether this is a general effect that also appears in other yeasts, the same experiment was performed with *S. cerevisiae.* The obtained data showed that no positive effect on specific production could be achieved by decreasing oxygen availability in *S. cerevisiae* (Table 4). As for *P. pastoris* a reduction of oxygen supply resulted in a nearly 2-fold reduction of biomass yield and ethanol accumulation in *S. cerevisiae*, but the specific Fab production showed no changes at any of the three oxygenation set points.

Table 4. The effect of oxygen provision on biomass yield, Fab 3H6 yield and ethanol production of recombinant *P. pastoris (2)* and *S. cerevisiae,* grown in glucose - limited chemostat cultures at D = 0.1h^{-1}. N – normoxic conditions, L – limited oxygenation, H – hypoxic conditions. +/- represents the standard error of the mean.

organism	oxygenation	$Y_{X/S}$ [g g carbon source $^{-1}$]	$Y_{P/X}$ [mg g biomass $^{-1}$]	ethanol in culture broth [g L^{-1}]
S. cerevisiae	N	0.46 +/- 0.01	0.027 +/- 0.0046	0.004 +/- 0.0004
	L	0.45 +/- 0.00	0.029 +/- 0.0046	0.008 +/- 0.0004
	H	0.31 +/- 0.01	0.023 +/- 0.0039	2.16 +/- 0.11
P. pastoris	N	0.48 +/- 0.01	0.27 +/- 0.02	0.31 +/- 0.17
	L	0.45 +/- 0.02	0.36 +/- 0.06	0.71 +/- 0.19
	H	0.25 +/- 0.01	0.69 +/- 0.02	5.72 +/- 0.14

No data are available for *E. coli* and *T. reesei*. For *E. coli* no stable cultivations could be established at decreased oxygen concentration because of excessive foaming at oxygen-limited conditions. Hypoxic conditions could not be applied due to the fact that media such as the one used in this study, do not support anaerobic cultivations. Standard media for aerobic cultivation of *E. coli* lack the co-factors selenium, nickel and molybdenum which are necessary for the formate hydrogen lyase complex (FLC). The FLC itself is induced under anaerobic conditions and serves to remove formate, which acts toxic on cells at high concentrations (49).

Discussion

The complex protein, Fab 3H6 was produced in 5 microbial host organisms with different success. The results demonstrate variation in the specific production between different expression hosts and platforms (Table 2). *P. haloplantkis* showed the highest specific production, demonstrating its potential as novel high yield protein expression host. Nevertheless, further optimization is required to improve *P. haloplanktis* growth rate, biomass yield and also to reduce medium complexity. Without these optimizations elaborate effort might be necessary to perform large scale cultivations.

The methylotrophic yeast *P. pastoris*, which showed the second highest specific production, has already been used for a long time as a well suited host for heterologous protein production. Apart from its advantages to grow on methanol and also to grow to extremely high cell densities (>100g L^{-1} yeast dry mass), many proteins have already been produced at high titers in this yeast (12, 13, 57). The pGAP expression system may have contributed to the high specific production of Fab 3H6 in *P. pastoris*. Integration into the host genome results in a stable expression phenotype and application of high antibiotic concentrations (e.g 100µg mL^{-1} Zeocin) enables the selection of multicopy clones with high gene copy number and consequently high recombinant protein mRNA levels (53).

S. cerevisiae showed rather moderate product levels, which may be partially explained by the pYX integrative vector systems, which generally may result in lower gene copy number / mRNA level, than the *P. pastoris* pGAP vector. Furthermore, *S. cerevisiae* is generally known as a non high performance secretor. However, the 3H6 Fab is a glycosylated protein (12) and *P. pastoris* and *S. cerevisiae* differ in the extent to which they glycosylate proteins (Table 1). Extensive clone characterization at the level of transcript and gene copy number will be necessary to better understand the reasons for these differences between *S. cerevisiae* and *P. pastoris*.

E. coli also showed low levels of heterologous Fab, similar to the amounts produced in *S. cerevisiae*, but it is well established that large proteins with an MW of >50kDa are difficult to express in bacterial host systems without extensive host/vector fine tuning. Specific production in *E. coli* might be increased by strictly controlling the ratio of heavy and light chain mRNA levels (48). In contrast, in the current study no strain engineering was applied and the heavy and light chain mRNA ratio was expected to be near 1:1 as they were both expressed under the control of the same promoter.

Interestingly *T. reesei* showed the lowest specific production although other proteins could be produced at high yields in this filamentous fungus (24, 25). TRAC analysis for *T. reesei* revealed that mRNA levels for both chains were very low (data not shown). Therefore, a bottleneck at the transcript level might exist. Low levels of Fab secretion (< 1 mg L^{-1}) in *T. reesei* have been reported previously. A more than a 50-fold increase in Fab production was achieved by fusing the heavy chain to *T. reesei* cellobiohydrolase CBHI, a strategy commonly applied for heterologous protein production in filamentous fungi (37). This fusion-strategy was not applied in the present study to enable the comparison with the other host organisms, but not producing a fusion protein most likely contributed to the low secretion levels of the recombinant Fab in *T. reesei*.

Generally, codon optimization might also increase product yields. Codon optimization has already been successfully applied in various host organisms to increase production of heterologous proteins (5, 7, 11, 31, 51). Nevertheless, it has been shown previously that codon optimization had no effect on the production of Fab 3H6 in *P. pastoris* (12).

Furthermore, codon usage analysis revealed that there are no significant differences in the general codon preferences of *P. pastoris* and *S. cerevisiae* (Supplemental data 3), indicating that for *S. cerevisiae* no improvement can be expected by codon optimization. In contrast codon usage analysis for *E. coli* revealed that many codons of the 3H6 Fab heavy and light expression cassettes represent less frequent codons in this bacterial host (Supplemental data 3). A great effect of codon optimization on productivity have been observed for *E. coli* (7), including our own unpublished data on Fab 3H6 expression, which showed that the productivity was increased by a factor of >10 when codon optimized sequences were used. With no data at hand for *T. reesei* and *P. haloplanktis* it remains unclear whether codon optimization can have a positive effect on Fab 3H6 secretion in these hosts.

Product formation and secretion correlates with cellular growth (19). Generally, higher growth rates may increase the specific production in all organisms due to higher ribosomal / translational activity. In *S. cerevisiae* and *P. pastoris*, systems which use glycolytic or TCA-cycle derived promoters, increased specific production may be also achieved at higher growth rate due to increased transcript levels of the heterologous protein.

Reducing the cultivation temperature showed a positive effect on specific production in 3 out of 4 species analysed, namely *E. coli*, *P. pastoris* and *T. reesei* but had no significant effect on specific production in *S. cerevisiae*. An increase of cell viability and lower amount of proteases in the culture broth was considered to be responsible for the increased product yield in *P. pastoris* (21, 30). For example, a temperature controlled methanol fed-batch strategy was developed for *P. pastoris* to increase product yield by reducing cellular lysis and contamination with intracellular proteases (21). Along with the increased specific production at decreased temperature *T. reesei* and *P. pastoris* responded with an increase of biomass yield at temperatures below the optimal growth temperature, whereas no such increased biomass yield was observed in *E. coli* and *S. cerevisiae*. In *S. cerevisiae* a decrease of temperature resulted in a decrease of biomass yield, although a positive effect of a reduction of growth temperature below the so-called optimum growth temperature has already been reported in several studies for a wide variety of microorgansisms (23, 27, 32, 56). It was concluded, that reduced protein folding stress and therefore lower maintenance energy were responsible for the increase in biomass yield. This conclusion is in good agreement with transcript data and proteome data available for *S.cerevisiae* and *P. pastoris.* It has been reported that environmental stress response (ESR) gene transcription was reduced and ribosome biogenesis related gene transcription increased at low temperature steady state growth of *S. cerevisiae* (50). Proteome data for *P. pastoris* showed that transcript and protein levels for stress induced chaperones such as SSA4 were reduced during chemostat cultivation at 20°C, whereas no change of mRNA levels for recombinant Fab 3H6 was observed (8, 15). Taken together it seems plausible that physiological changes related to protein folding and energy demand lead to increased specific production and / or increased biomass yield in *T. reesei*, *P. pastoris* and *E.coli*. The fact that *S. cerevisiae* showed no temperature dependent change (in the tested range) of biomass yield, although it was observed in batch culture studies (32) and no change of specific production occurred, may highlight strain dependency of the physiological response to temperature. Furthermore, differences in protein folding and processing between *S. cerevisiae* compared to *T. reesei* (1) or *P. pastoris* (17) have been reported, which may also account for the different behaviour observed in this study. The behaviour of *S. cerevisiae* may also be simply dependent on the low production level of Fab 3H6 resulting in no protein folding / secretion bottleneck that can be relieved by decreased temperature like in the other species.

E. coli additionally responded with increased leakage of recombinant Fab into the culture broth at lower temperature. This effect has already been described in previous studies (46), but it remains unclear, whether this effect was caused by increased recombinant protein amount itself or if low temperature triggered physiological changes that enhanced product leakage into the culture broth.

Nevertheless, this work demonstrates that producing recombinant proteins at temperatures below the optimal growth temperature improves protein production in many if not all hosts,

although the reasons for this are not well understood and are beyond the scope of this investigation.
Although oxygen availability and its strong effect on cellular physiology have been analysed for several species (22, 28, 43, 55), its potential impact on heterologous protein production and secretion had not been reported until recently (2). In oxygen-limited conditions metabolism is respirofermentative rather than purely respirative, as reflected in increased ethanol levels in *S. cerevisiae* and *P. pastoris* (Table 4). Increased transcript levels of the 3H6 Fab heavy and light chain during hypoxic cultivation conditions in *P. pastoris* (unpublished data) might contribute to the increased productivity as during respirofermentative growth the flux and transcript levels for the glycolytic machinery increased, also boosting heterologous protein mRNA levels under the control of the GAP promoter. Still, this has to be further investigated, especially as there occurred no improvement of specific production in *S. cerevisiae*, although similar physiological events (22), happen in this yeast during growth at depleted oxygenation conditions. As in *P. pastoris*, the flux through glycolysis increases as the amount of oxygen available for the cellular metabolism is reduced (unpublished data). However, in *S. cerevisiae* (22) the mRNAs of genes involved in glycolysis are not increased in a comparable experimental setup (55). The different response of these two yeast species may highlight substantial differences in cellular control regimes, indicating massive regulation at the transcript level *in P. pastoris* and largely post-transcriptional regulation in *S. cerevisiae*. Nevertheless, it is still unclear whether recombinant protein transcript levels can be considered the sole explanation for increased production of 3H6 Fab in *P. pastoris*.
The presented data show that common features as well as differences exist in the well-established protein production factories used in this study. Although eukaryotic systems are thought to be better equipped for the production of complex, disulfide bonded proteins such as Fab fragments we could show that also prokaryotic systems such as *P. haloplanktis* have a great potential as host systems as high production could be achieved without elaborate strain and vector engineering. In contrast, the filamentous fungus *T. reesei* showed only a weak performance at the secretion of the 3H6 Fab, without applying the commonly used method of generating fusion proteins to enhance heterologous protein production.
However, to analyse the host / platform dependencies of protein production more closely, other secreted model proteins, various vector systems and strains should be included in future studies. Additionally, with affordable high through-put transcript and proteome analysis available nowadays, proteome and transcript profiling will be applied to fully understand the influence of environmental factors on heterologous protein secretion and to shed more light onto the discrepancies that appeared among different host organisms.

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Author's contributions
MD performed cloning procedures for *P. pastoris* and *E. coli*. MD and KB performed cultivations for *P. pastoris*. FV and ER planned and performed cultivations for *E. coli*. PB performed cloning procedures for the *S. cerevisiae* studies. GF planed and performed cultivations for *S. cerevisiae*. JT constructed the *T. reesei* production strains, and LB, MGW and TP carried out the *T. reesei* cultivations and their analysis. MM assisted in media design and fermentation for *S. cerevisiae*, *P. pastoris* and *E. coli*. MG performed cloning procedures and cultivations for *P. haloplanktis.* EP assisted in media design for *P.*

haloplanktis. RK developed Fab 3H6 and provided vectors, ELISA and initial data on *P. pastoris* fermentations. BG, MiS, TP, MaS, AV and PB participated in the design of the study. AV, PF, DP, MLT, MP and DM conceived of the study.

References

1. Arvas, M., T. Pakula, K. Lanthaler, M. Saloheimo, M. Valkonen, T. Suortti, G. Robson, and M. Penttilä. 2006. Common features and interesting differences in transcriptional responses to secretion stress in the fungi Trichoderma reesei and Saccharomyces cerevisiae. BMC Genomics 7:32.
2. Baumann, K., M. Maurer, M. Dragosits, O. Cos, P. Ferrer, and D. Mattanovich. 2008. Hypoxic fed-batch cultivation of Pichia pastoris increases specific and volumetric productivity of recombinant proteins. Biotechnol Bioeng 100:177-83.
3. Blackwell, J., and R. Horgan. 1991. A novel strategy for production of a highly expressed recombinant protein in an active form. FEBS Lett 295:10-2.
4. Brake, A., J. Merryweather, D. Coit, U. Heberlein, F. Masiarz, G. Mullenbach, M. Urdea, P. Valenzuela, and P. Barr. 1984. Alpha-factor-directed synthesis and secretion of mature foreign proteins in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 81:4642-6.
5. Brat, D., E. Boles, and B. Wiedemann. 2009. Functional expression of a bacterial xylose isomerase in Saccharomyces cerevisiae. Appl Environ Microbiol 75:2304-11.
6. Brooks, S. 2004. Appropriate glycosylation of recombinant proteins for human use: implications of choice of expression system. Mol Biotechnol 28:241-55.
7. Burgess-Brown, N., S. Sharma, F. Sobott, C. Loenarz, U. Oppermann, and O. Gileadi. 2008. Codon optimization can improve expression of human genes in Escherichia coli: A multi-gene study. Protein Expr Purif 59:94-102.
8. Dragosits, M., J. Stadlmann, J. Albiol, K. Baumann, M. Maurer, B. Gasser, M. Sauer, F. Altmann, P. Ferrer, and D. Mattanovich. 2008. The Effect of Temperature on the Proteome of Recombinant Pichia pastoris. Journal of Proteome Research submitted.
9. Dragosits, M., J. Stadlmann, J. Albiol, K. Baumann, M. Maurer, B. Gasser, M. Sauer, F. Altmann, P. Ferrer, and D. Mattanovich. 2009. The Effect of Temperature on the Proteome of Recombinant Pichia pastoris. J Proteome Res 8:1380-92.
10. Ferrer-Miralles, N., J. Domingo-Espín, J. Corchero, E. Vázquez, and A. Villaverde. 2009. Microbial factories for recombinant pharmaceuticals. Microb Cell Fact 8:17.
11. Fletcher, S., M. Muto, and S. Mayfield. 2007. Optimization of recombinant protein expression in the chloroplasts of green algae. Adv Exp Med Biol 616:90-8.
12. Gach, J., M. Maurer, R. Hahn, B. Gasser, D. Mattanovich, H. Katinger, and R. Kunert. 2007. High level expression of a promising anti-idiotypic antibody fragment vaccine against HIV-1 in Pichia pastoris. J Biotechnol 128:735-46.
13. Gasser, B., and D. Mattanovich. 2007. Antibody production with yeasts and filamentous fungi: on the road to large scale? Biotechnol Lett 29:201-12.
14. Gasser, B., M. Maurer, J. Gach, R. Kunert, and D. Mattanovich. 2006. Engineering of Pichia pastoris for improved production of antibody fragments. Biotechnol Bioeng 94:353-61.
15. Gasser, B., M. Maurer, J. Rautio, M. Sauer, A. Bhattacharyya, M. Saloheimo, M. Penttilä, and D. Mattanovich. 2007. Monitoring of transcriptional regulation in Pichia pastoris under protein production conditions. BMC Genomics 8:179.
16. Gasser, B., M. Saloheimo, U. Rinas, M. Dragosits, E. Rodríguez-Carmona, K. Baumann, M. Giuliani, E. Parrilli, P. Branduardi, C. Lang, D. Porro, P. Ferrer, M. Tutino, D. Mattanovich, and A. Villaverde. 2008. Protein folding and conformational stress in microbial cells producing recombinant proteins: a host comparative overview. Microb Cell Fact 7:11.
17. Graf, A., B. Gasser, M. Dragosits, M. Sauer, G. Leparc, T. Tüchler, D. Kreil, and D. Mattanovich. 2008. Novel insights into the unfolded protein response using Pichia pastoris specific DNA microarrays. BMC Genomics 9:390.
18. Hamilton, S., R. Davidson, N. Sethuraman, J. Nett, Y. Jiang, S. Rios, P. Bobrowicz, T. Stadheim, H. Li, B. Choi, D. Hopkins, H. Wischnewski, J. Roser, T. Mitchell, R. Strawbridge, J. Hoopes, S. Wildt, and T. Gerngross. 2006. Humanization of yeast to produce complex terminally sialylated glycoproteins. Science 313:1441-3.
19. Hensing, M., R. Rouwenhorst, J. Heijnen, J. van Dijken, and J. Pronk. 1995. Physiological and technological aspects of large-scale heterologous-protein production with yeasts. Antonie Van Leeuwenhoek 67:261-79.
20. Jacobs, P., S. Geysens, W. Vervecken, R. Contreras, and N. Callewaert. 2009. Engineering complex-type N-glycosylation in Pichia pastoris using GlycoSwitch technology. Nat Protoc 4:58-70.

21. Jahic, M., F. Wallberg, M. Bollok, P. Garcia, and S. Enfors. 2003. Temperature limited fed-batch technique for control of proteolysis in Pichia pastoris bioreactor cultures. Microb Cell Fact 2:6.
22. Jouhten, P., E. Rintala, A. Huuskonen, A. Tamminen, M. Toivari, M. Wiebe, L. Ruohonen, M. Penttilä, and H. Maaheimo. 2008. Oxygen dependence of metabolic fluxes and energy generation of Saccharomyces cerevisiae CEN.PK113-1A. BMC Syst Biol 2:60.
23. Kasemets, K., I. Nisamedtinov, T. Laht, K. Abner, and T. Paalme. 2007. Growth characteristics of Saccharomyces cerevisiae S288C in changing environmental conditions: auxo-accelerostat study. Antonie Van Leeuwenhoek 92:109-28.
24. Kiiskinen, L., K. Kruus, M. Bailey, E. Ylösmäki, M. Siika-Aho, and M. Saloheimo. 2004. Expression of Melanocarpus albomyces laccase in Trichoderma reesei and characterization of the purified enzyme. Microbiology 150:3065-74.
25. Kontkanen, H., A. Westerholm-Parvinen, M. Saloheimo, M. Bailey, M. Rättö, I. Mattila, M. Mohsina, N. Kalkkinen, T. Nakari-Setälä, and J. Buchert. 2009. Novel Coprinopsis cinerea polyesterase that hydrolyzes cutin and suberin. Appl Environ Microbiol 75:2148-57.
26. Kunert, R., R. Weik, B. Ferko, G. Stiegler, and H. Katinger. 2002. Anti-idiotypic antibody Ab2/3H6 mimics the epitope of the neutralizing anti-HIV-1 monoclonal antibody 2F5. AIDS 16:667-8.
27. Lee, J., Y. Jeoin, D. Kim, J. Seo, and B. Hur. 2006. Eicosapentaenoic acid (EPA) biosynthetic gene cluster of Shewanella oneidensis MR-1: Cloning, heterologous expression, and effects of temperature and glucose on the production of EPA in Escherichia coli. Biotechnol. Bioprocess Eng 11:510-515.
28. Levanon, S., K. San, and G. Bennett. 2005. Effect of oxygen on the Escherichia coli ArcA and FNR regulation systems and metabolic responses. Biotechnol Bioeng 89:556-64.
29. Li, H., N. Sethuraman, T. Stadheim, D. Zha, B. Prinz, N. Ballew, P. Bobrowicz, B. Choi, W. Cook, M. Cukan, N. Houston-Cummings, R. Davidson, B. Gong, S. Hamilton, J. Hoopes, Y. Jiang, N. Kim, R. Mansfield, J. Nett, S. Rios, R. Strawbridge, S. Wildt, and T. Gerngross. 2006. Optimization of humanized IgGs in glycoengineered Pichia pastoris. Nat Biotechnol 24:210-5.
30. Li, Z., F. Xiong, Q. Lin, M. d'Anjou, A. Daugulis, D. Yang, and C. Hew. 2001. Low-temperature increases the yield of biologically active herring antifreeze protein in Pichia pastoris. Protein Expr Purif 21:438-45.
31. Lim, L., H. Li, N. Cheong, B. Lee, and K. Chua. 2004. High-level expression of a codon optimized recombinant dust mite allergen, Blo t 5, in Chinese hamster ovary cells. Biochem Biophys Res Commun 316:991-6.
32. Margesin, R. 2009. Effect of temperature on growth parameters of psychrophilic bacteria and yeasts. Extremophiles 13:257-62.
33. Martinez, D., R. Berka, B. Henrissat, M. Saloheimo, M. Arvas, S. Baker, J. Chapman, O. Chertkov, P. Coutinho, D. Cullen, E. Danchin, I. Grigoriev, P. Harris, M. Jackson, C. Kubicek, C. Han, I. Ho, L. Larrondo, A. de Leon, J. Magnuson, S. Merino, M. Misra, B. Nelson, N. Putnam, B. Robbertse, A. Salamov, M. Schmoll, A. Terry, N. Thayer, A. Westerholm-Parvinen, C. Schoch, J. Yao, R. Barabote, R. Barbote, M. Nelson, C. Detter, D. Bruce, C. Kuske, G. Xie, P. Richardson, D. Rokhsar, S. Lucas, E. Rubin, N. Dunn-Coleman, M. Ward, and T. Brettin. 2008. Genome sequencing and analysis of the biomass-degrading fungus Trichoderma reesei (syn. Hypocrea jecorina). Nat Biotechnol 26:553-60.
34. Mattanovich, D., B. Gasser, H. Hohenblum, and M. Sauer. 2004. Stress in recombinant protein producing yeasts. J Biotechnol 113:121-35.
35. Mazor, Y., T. Van Blarcom, B. Iverson, and G. Georgiou. 2009. Isolation of full-length IgG antibodies from combinatorial libraries expressed in Escherichia coli. Methods Mol Biol 525:217-39, xiv.
36. Montenecourt , B., and D. Eveleigh. 1977. Selective screening methods for the isolation of high yielding cellulase mutants of Trichoderma reesei on various carbon sources, p. 289-301, Adv Chem Ser.
37. Nyyssönen, E., and S. Keränen. 1995. Multiple roles of the cellulase CBHI in enhancing production of fusion antibodies by the filamentous fungus Trichoderma reesei. Curr Genet 28:71-9.
38. Papa, R., V. Rippa, G. Sannia, G. Marino, and A. Duilio. 2007. An effective cold inducible expression system developed in Pseudoalteromonas haloplanktis TAC125. J Biotechnol 127:199-210.
39. Parrilli, E., A. Duilio, and M. Tutino. 2008. Heterologous protein expression in psychrophilic hosts. Springer-Verlag Berlin Heidelberg.
40. Porro, D., E. Martegani, B. Ranzi, and L. Alberghina. 1988. Oscillations in continuous cultures of budding yeast: A segregated parameter analysis.Biotechnol Bioeng 32:411-7.
41. Potgieter, T., M. Cukan, J. Drummond, N. Houston-Cummings, Y. Jiang, F. Li, H. Lynaugh, M. Mallem, T. McKelvey, T. Mitchell, A. Nylen, A. Rittenhour, T. Stadheim, D. Zha, and M. d'Anjou. 2009. Production of monoclonal antibodies by glycoengineered Pichia pastoris. J Biotechnol 139:318-25.

42. Rakestraw, J., S. Sazinsky, A. Piatesi, E. Antipov, Wittrup, and KD. 2009. Directed evolution of a secretory leader for the improved expression of heterologous proteins and full-length antibodies in Saccharomyces cerevisiae, Biotechnol Bioeng.
43. Rautio, J., M. Bailey, T. Kivioja, H. Söderlund, M. Penttilä, and M. Saloheimo. 2006. Transcriptional monitoring of steady state and effects of anaerobic phases in chemostat cultures of the filamentous fungus Trichoderma reesei, p. 247, BMC Genomics.
44. Rautio, J., K. Kataja, R. Satokari, M. Penttilä, H. Söderlund, and M. Saloheimo. 2006. Rapid and multiplexed transcript analysis of microbial cultures using capillary electophoresis-detectable oligonucleotide probe pools. J Microbiol Methods 65:404-16.
45. Regenberg, B., T. Grotkjaer, O. Winther, A. Fausbøll, M. Akesson, C. Bro, L. Hansen, S. Brunak, and J. Nielsen. 2006. Growth-rate regulated genes have profound impact on interpretation of transcriptome profiling in Saccharomyces cerevisiae. Genome Biol 7:R107.
46. Rinas, U., and F. Hoffmann. Selective leakage of host-cell proteins during high-cell-density cultivation of recombinant and non-recombinant Escherichia coli. Biotechnol Prog 20:679-87.
47. Shi, X., T. Karkut, M. Chamankhah, M. Alting-Mees, S. Hemmingsen, and D. Hegedus. 2003. Optimal conditions for the expression of a single-chain antibody (scFv) gene in Pichia pastoris. Protein Expr Purif 28:321-30.
48. Simmons, L., D. Reilly, L. Klimowski, T. Raju, G. Meng, P. Sims, K. Hong, R. Shields, L. Damico, P. Rancatore, and D. Yansura. 2002. Expression of full-length immunoglobulins in Escherichia coli: rapid and efficient production of aglycosylated antibodies. J Immunol Methods 263:133-47.
49. Soini, J., K. Ukkonen, and P. Neubauer. 2008. High cell density media for Escherichia coli are generally designed for aerobic cultivations - consequences for large-scale bioprocesses and shake flask cultures. Microb Cell Fact 7:26.
50. Tai, S., P. Daran-Lapujade, M. Walsh, J. Pronk, and J. Daran. 2007. Acclimation of Saccharomyces cerevisiae to low temperature: a chemostat-based transcriptome analysis. Mol Biol Cell 18:5100-12.
51. Tokuoka, M., M. Tanaka, K. Ono, S. Takagi, T. Shintani, and K. Gomi. 2008. Codon optimization increases steady-state mRNA levels in Aspergillus oryzae heterologous gene expression. Appl Environ Microbiol 74:6538-46.
52. van Dijken JP, J. Bauer, L. Brambilla, P. Duboc, J. Francois, C. Gancedo, M. Giuseppin, J. Heijnen, M. Hoare, H. Lange, E. Madden, P. Niederberger, J. Nielsen, J. Parrou, T. Petit, D. Porro, M. Reuss, van Riel N, M. Rizzi, H. Steensma, C. Verrips, J. Vindeløv, and J. Pronk. 2000. An interlaboratory comparison of physiological and genetic properties of four Saccharomyces cerevisiae strains. Enzyme Microb Technol 26:706-714.
53. Vassileva, A., D. Chugh, S. Swaminathan, and N. Khanna. 2001. Expression of hepatitis B surface antigen in the methylotrophic yeast Pichia pastoris using the GAP promoter. J Biotechnol 88:21-35.
54. Wacker, M., D. Linton, P. Hitchen, M. Nita-Lazar, S. Haslam, S. North, M. Panico, H. Morris, A. Dell, B. Wren, and M. Aebi. 2002. N-linked glycosylation in Campylobacter jejuni and its functional transfer into E. coli. Science 298:1790-3.
55. Wiebe, M., E. Rintala, A. Tamminen, H. Simolin, L. Salusjärvi, M. Toivari, J. Kokkonen, J. Kiuru, R. Ketola, P. Jouhten, A. Huuskonen, H. Maaheimo, L. Ruohonen, and M. Penttilä. 2008. Central carbon metabolism of Saccharomyces cerevisiae in anaerobic, oxygen-limited and fully aerobic steady-state conditions and following a shift to anaerobic conditions. FEMS Yeast Res 8:140-54.
56. Wittmann, C., J. Weber, E. Betiku, J. Krömer, D. Böhm, and U. Rinas. 2007. Response of fluxome and metabolome to temperature-induced recombinant protein synthesis in Escherichia coli. J Biotechnol 132:375-84.
57. Zhao, H., Q. He, C. Xue, B. Sun, X. Yao, and Z. Liu. 2009. Secretory expression of glycosylated and aglycosylated mutein of onconase from Pichia pastoris using different secretion signals and their purification and characterization. FEMS Yeast Res 9:591-9.

Single chain antibody fragment production

○ Batch cultivation:

The anti 2-phenyl-5-oxazolone single chain antibody fragment ScFvOx production was firstly carried out by batch cultivation in LIV medium in optimised conditions. The production yield was evaluated by ELISA assays in presence of the hapten 2-phenyl-5-oxazolone on total soluble extracts from samples collected at different times of cultivation. As show in Figure 24, growth profiles of *P. haloplanktis* TAC125 pUCRP-*scfv* strain show a longer lag phase compared to that observed for the wild type and the Fab 3H6 producing strains. During the first 25 hours bacterial growth proceeds at a lower rate while just a few amount of recombinant ScFvOx is detected. After 30 hours of cultivation a huge increase of bacterial growth rate and ScFvOx production titer are observed reaching the highest yield after about 48h.

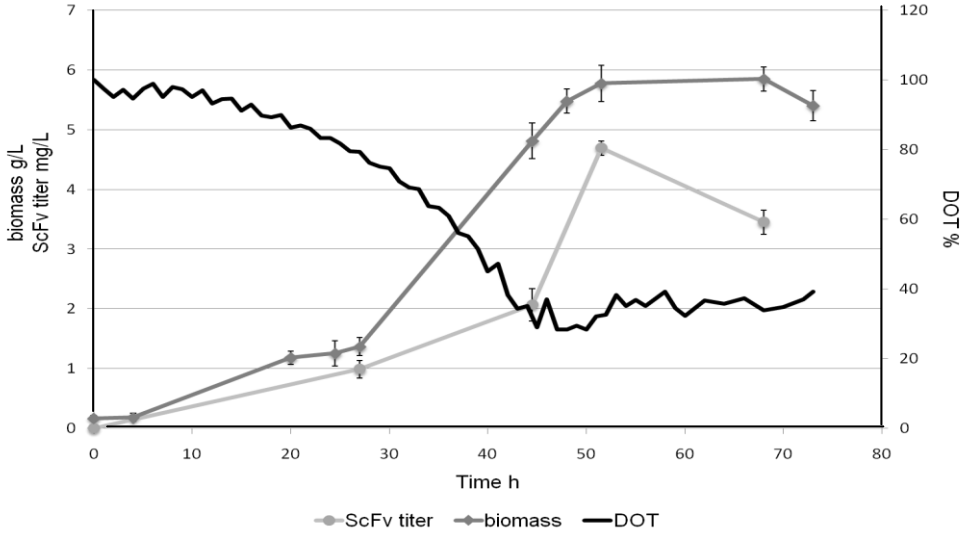


Figure 24: ScFvOx titer, biomass yield and oxygen consumption profiles in *P. haloplanktis* TAC125 pUCRP-*scfv* batch cultivation in LIV medium at 15°C.

By our optimised psychrophilic expression system, a maximum yield of of 4,69 ± 0,12 mg L^{-1} of soluble and biologically active ScFvOx was obtained which is the highest reported so far by conventional prokaryotic expression systems even after inclusion bodies refolding (Patil *et al*., 2008). Moreover, performances of our optimized process led to a high specific productivity ($Y_{P/X}$= 0,94±0,03 mg gX^{-1}) suggesting that a further increase in specific biomass yield would lead to higher ScFvOx production titers.

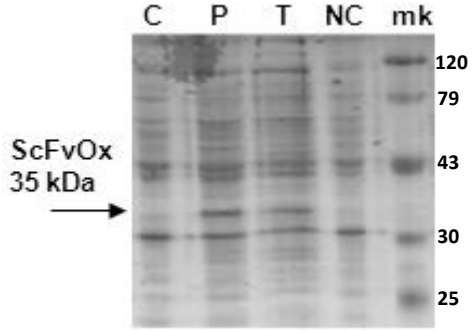


Figure 25: SDS-PAGE analysis of recombinant ScFvOx cellular localisation on cytoplasmic (C), periplasmic (P) and total (T) protein extracts of *P. haloplanktis* TAC125 pUCRP-*scfv* cells. Total soluble proteins of P. haloplanktis wild type strain was used as negative control (NC). Mk, molecular weight ladder.

Cellular localisation of recombinant ScFvOx was also investigated by cellular fractionation and SDS PAGE analysis of total soluble protein extracts, cytoplasmic and periplasmic fractions (Fig. 25). The analysis revealed that a band corresponding to recombinant ScFvOx-c-Myc-6xHis (about 35kDa) is present only in total and periplasmic extracts of recombinant *P. haloplanktis* TAC125 cells. The latter observation demonstrates that ScFvOx antibody fragment is not only nicely produced in soluble and active form but it is also efficiently and

totally translocated in the periplasmic compartment.

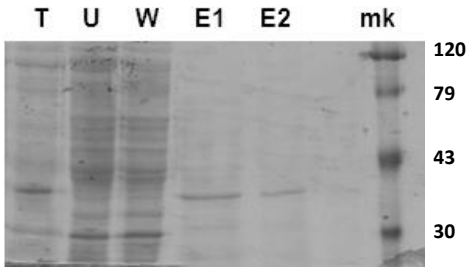


Figure 26: SDS-PAGE analysis of ScFvOx affinity purification. T, total soluble extract; U, unbound proteins fraction; W, wash fraction; E1, elution 250mM imidazole; E2, elution 500mM imidazole; mk, molecular weight protein ladder.

Recombinant ScFvOx was affinity purified from total cellular extracts collected after 48h of batch cultivation in optimised conditions by using the C-terminal 6xHis tag (Fig. 26). The binding activity of purified ScFvOx was verified by ELISA assay in the presence of the hapten 2-phenyl-5-oxazolone. The analysis revealed a yield of 4,05 ± 0,18 mg L^{-1} of pure biologically active ScFvOx. Data collected indicate that, apart from a slight loss due to the purification step, all the recombinant product obtained by the optimised psychrophilic expression system is correctly folded and biologically competent.

○ Continuous cultivation:

ScFvOx production at low temperatures was also performed by a continuous cultivation strategy. In particular, a C-limited chemostat cultivation process was set-up by using the optimised LIV medium for either batch growth phase and feeding. By a dilution rate of 0,05 h^{-1}, the steady state was achieved for at least 5 resident times in which both the cell density and the product titer remain constant (Fig. 27). By the developed process a specific active ScFvOx productivity of 0,23 ± 0,05 mg L^{-1} h^{-1} was achieved with a constant production yield of about 4,5 mg L^{-1}.

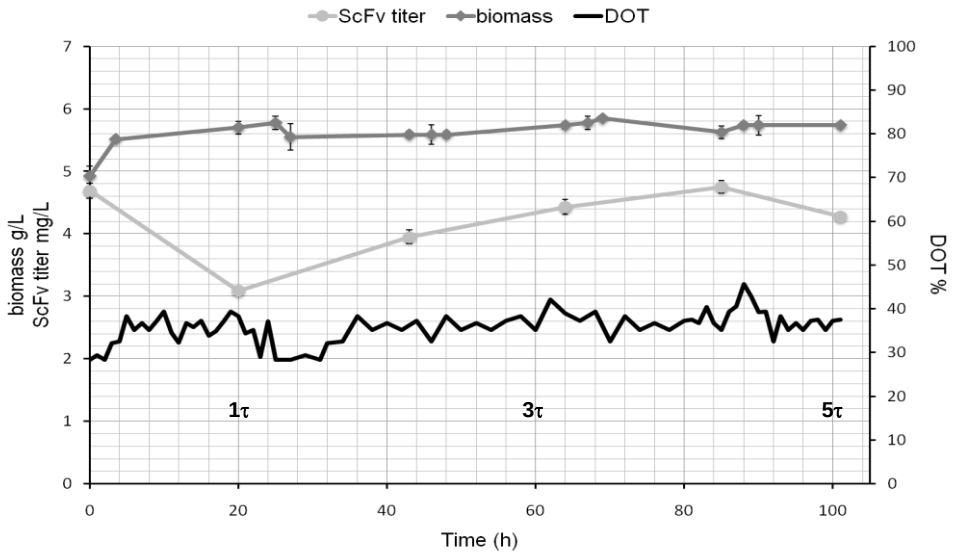


Figure 27: ScFvOx titer, biomass yield and oxygen consumption profiles in *P. haloplanktis* TAC125 pUCRP-*scfv* chemostat cultivation.

VHH antibody fragment production

The anti- human fibroblast growth factor receptor 1 (FGFR1) VHH D6.1 production was performed by *P. haloplanktis* TAC125 pUCRP-*vhh* batch cultivation in LIV medium in optimised conditions. Registered growth profiles (Fig. 28) are comparable to those observed for *P. haloplanktis* TAC125 wild type strain.

Analysis of production and cellular localisation of recombinant anti hFGFR1 VHHD6.1 was carried out by semi-quantitative Western blotting analysis on total soluble protein extracts and periplasmic fractions of samples collected at different

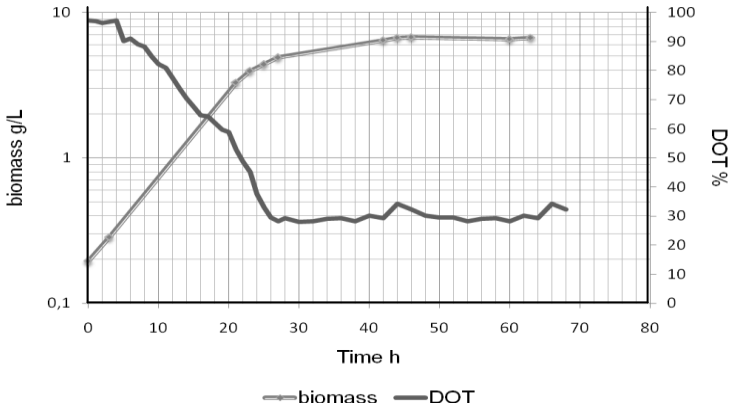


Figure 28: *P. haloplanktis* TAC125 pUCRP-*vhh* growth profiles in batch cultivation in LIV medium at 15°C

times of cultivation (Fig. 29). Immunodetection performed with anti c-Myc antibodies revealed VHHD6.1 soluble production during all fermentation and its correct periplasmic localisation during early (22h) and middle (29h) exponential growth phase. In contrast, no recombinant VHHD6.1 was found in periplasmic fraction extracted from samples collected at late exponential growth phase (42h) and stationary phase (60h) while production titers seem to increase during exponential growth reaching the highest yield at late exponential phase (42h). Furthermore, another specific signal showing an apparent molecular weight of about 30 kDa was detected in total soluble protein extracts probably corresponding to VHHD6.1 dimers. It is not surprising since a strong tendency of multimerisation has been reported for this and other formats of antibody fragments in *vivo* when their local concentration in recombinant host cells reaches a critical value (Hollinger and Hudson, 2005).

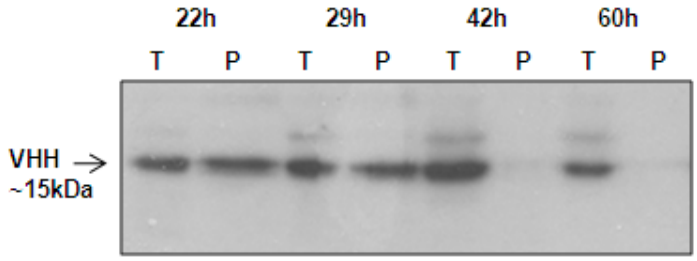
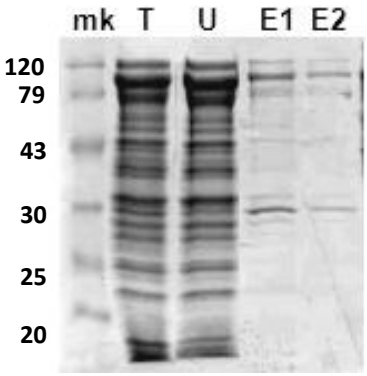


Figure 29: Western blotting analysis of VHHD6.1 soluble production and cellular localisation on total protein extracts (T) and periplasmic fraction (P) of recombinant *P. haloplanktis* TAC125 pUCRP-*vhh* cells collected at different times of batch cultivation.

It is worth to notice that as far as the high molecular weight signal relative intensity increases the secretion efficiency of recombinant product into periplasmic space seems to decrease. One explanation can be found in VHHs dimerisation kinetics that could be faster than the product recruitment by the periplasmic secretion system. On the other hand, VHH dimers formation could be a consequence of its cytoplasmic localisation. If protein secretion does not occur, its correct folding can not be achieved and hydrophobic interactions can take place among partly folded intermediates thus causing protein molecules aggregation in soluble dimeric complexes.

Although the co-translational SRP-mediated secretion system was successfully employed for Fab and ScFv formats model proteins, VHHD6.1 translocation across the inner membrane results to be somehow inhibited at high cell densities. Further investigation has to be carried out in order to find out the reason of this phenomenon and the best strategy to overcome it.

Recombinant anti hFGFR1 VHHD6.1 was partially purified from total soluble cell extracts by small scale His tag affinity chromatography (Fig. 30). Surprisingly, the purified product showed an apparent molecular weight of about 30 kDa corresponding to dimeric VHHD6.1. The total absence of purified proteins of the expected mass of about 15 kDa confirms the huge tendency of aggregation shown by this antibody fragment format even in soluble form. Moreover, the purified product resulted to be very poorly stable in solution when stored either at 4°C and -20°C



(data not shown). The low protein stability prevented the quantification of its production yield and the evaluation of its binding activity. Further experiments are still ongoing in collaboration with Dr. A. De Marco of Ifom-IEO campus in Milan in order to clarify the recombinant VHH structure and to stabilise the product in order to be able to get information regarding its binding activity.

Figure 30: SDS-PAGE analysis of VHHD6.1 affinity purification. T, total soluble cell extract; U, unbound proteins fraction; E1, elution 250mM imidazole; E2, elution 500mM imidazole; mk, molecular weight protein ladder.

Conclusions

The number of candidate proteins used as biopharmaceuticals or in industrial processes is rapidly increasing in recent years and, in particular, the biopharmaceutical market is currently dominated by monoclonal antibodies and antibody fragments (Evans and Das 2005). However, efficient expression of genes in homologous/heterologous expression systems and rapid purification steps are actually major bottlenecks for industrial production of recombinant antibody-based biopharmaceuticals. In fact, although many recombinant proteins have been successfully produced by common prokaryotic (*Escherichia coli*) and eukaryotic (yeasts and CHO cells) hosts, these conventional systems have often proved to be unproductive due to the peculiar properties of the protein to be produced. One of the main limitations experienced while producing proteins in conventional mesophilic expression systems is the need to operate at their optimal growth temperature (usually above 30 ºC) for the production process. Since temperature has a general negative impact on protein folding due to the strong temperature dependence of hydrophobic interactions that mainly drive their aggregation in form of inclusion bodies, the production of recombinant proteins at low temperatures represents an exciting model to improve the quality of the products. Recombinant protein production in psychrophilic bacteria, i.e. at temperature as low as 4°C, may minimise undesired hydrophobic interactions during protein folding, desirably resulting in enhancing the yield of soluble and correctly folded products. In this context , a few cold adapted species are under early but intense exploration as cold cell factories, among them, *Pseudoalteromonas haloplanktis* being a representative example. The efficiency of cold-adapted expression systems was tested by fully soluble and biologically competent production of several thermal-labile and aggregation-prone products (Parrilli *et al.,* 2008).

The aim of this work was the development of a new process for recombinant production of soluble antibody fragments by the psychrophilic bacterium *P. haloplanktis* TAC125. To test the versatility of the new developed process, the production of three aggregation prone model proteins was evaluated representing examples of different formats of antibody fragments: Fab, ScFv and VHH.

The first part of this work was the development of an *ad hoc* expression system for antibody fragments genes based on the available genetic tools for recombinant genes expression at low temperatures. A rational approach was applied for the choice of promoter, molecular signal for periplasmic protein addressing and fusion tags for immunodetection and purification of products. Moreover an artificial operon was designed and constructed for Fab fragment production in heterodimeric form.

By an experimental approach, a new defined minimal medium was developed to maximise bacterial growth parameters like specific growth rate and biomass yield and promoter transcription efficiency.
The production of model antibody fragments has been evaluated in lab-scale bioreactor and the effect of different cultivation operational strategies on production yields has been investigated.
Up to 4,0 mg L^{-1} of soluble and heterodimeric Fab 3H6 production were obtained by recombinant *P. haloplanktis* TAC125 batch cultivation in optimised conditions. The production yield was also stabilised in C-limited chemostat cultivation reaching a constant volumetric productivity of about 0,2 mg L^{-1} h^{-1}. Moreover Fab 3H6 production by continuous cultivation was also performed in comparison with other conventional microbial hosts including yeasts, filamentous fungi and bacteria in the framework of the European project GENOPHYS. The analysis revealed that Fab 3H6 is produced very effectively by the psychrophilic expression system in optimised conditions leading to highest production yields together with *Pichia pastoris* expression system and to the highest specific productivity (Dragosit *et al*., submitted). Similar results were obtained for single chain antibody fragment model protein ScFvOx. About 5 mg L^{-1} of soluble and biologically active recombinant protein were obtained by recombinant *P. haloplanktis* TAC125 batch cultivation in optimised conditions. A volumetric productivity of above 0,2 mg L^{-1} h^{-1} of soluble and biologically active ScFvOx was stabilised in C-limited chemostat cultivation in optimised conditions.
The production of recombinant VHHD6.1 heavy chain antibody fragment was also investigated in the optimised psychrophilic expression system. By batch cultivation in optimised conditions soluble and periplasmic production of recombinant VHHD6.1 was achieved. Nevertheless, the low stability in solution of recombinant product has prevented the analysis of its binding activity.
It is worth to mention that when a codon usage analysis was performed on a set of genes highly expressed in *P. haloplanktis* TAC125 (*http://www1.unifi.it/scibio/bioinfo/caiap/results/cut/Pseudoalteromonas_haloplanktis_TAC125_35%20set.cut*) a number of rare codons were identified and most of them were found in antibody fragments coding genes (data not shown) reflecting the different origin of recombinant genes from the host strain. The presence of rare codons in recombinant genes, especially if consecutive, may lead to amino acid misincorporation and/or truncation of the polypeptide, thus affecting the recombinant protein expression levels and quality (Rosano and Ceccarelli, 2009). A codon optimisation of recombinant antibody fragments coding genes can therefore be a good choice to further improve products yields.
In conclusion, all tested antibody fragments were successfully produced in soluble and catalytically competent form. Our results demonstrated that the production of recombinant proteins in psychrophilic bacteria is not only a mature and reliable technology but it is also a successful strategy to overcome the product solubility problems often occurring in conventional systems such as in *E. coli*. In this context, *P. haloplanktis* TAC125 and the gene expression systems set up have a valuable biotechnological potential as non-conventional systems for the production of “difficult” proteins.and biopharmaceuticals such as recombinant antibodies and antibody fragments.

Part II: New Products

Section 1: New regulated promoters identification for recombinant gene expression at low temperatures.

Recombinant protein production at low temperatures is right now a mature and reliable technology. As previously described, there are already several tools available for recombinant genes expressions in the psychrophilic host *P. haloplanktis* TAC125. However, an effective cold expression system needs to be finely tuned possibly using *ad hoc* promoters. Indeed, physical separation between bacterial growth phase and expression of the desired proteins can not only improve the productivity of the entire system but can also play an important role in the production of proteins toxic for the host cells. These goals can only be achieved by using regulated promoters and efficient induction strategies.

Recently, by a proteomic approach, a two-component system has been isolated and characterized. The regulatory system (PSHAb0361–PSHAb0362) is involved in the transcriptional regulation of the gene coding for an outer membrane porin (PSHAb0363) and it is strongly induced by the presence of L-malate in the medium (Papa *et al*., 2008). The regulative region including the two-component system located upstream the PSHAb0363 gene has been used to construct the first inducible psychrophilic expression vector, named pUCRP, that is under the control of L-malate (Papa *et al*., 2007).

The L-malate inducible expression system was successfully used in this work for recombinant production of soluble antibody fragments in *P. haloplanktis* TAC125. Nevertheless, good performances of the regulated promoter can be only achieved when branched amino acids are used as sole carbon sources for bacterial growth. The use of such substrates is unprofitable for a large scale production process due to their high cost and their very poor solubility in water. Moreover, the inducer itself, the L-malate, is also expensive and, since it is also consumed as carbon source by the psychrophilic bacterium, it is required in significantly high amounts (up to 4 g L^{-1}) to ensure sufficient induction of recombinant gene expression during all the cultivation.

As described in Part I-Section 2, a screening for suitable carbon sources for *P. haloplanktis* TAC125 growth revealed that the psychrophilic bacterium fast duplicates in minimal media containing amino acids like L-glutamate, L-aspartate and L-alanine. In particular, the synthetic medium containing L-glutamate as sole carbon source led to the highest bacterial specific growth rate either at the optimal (15°C) growth temperature (Tab. 5) and at temperatures as low as 4°C (data not shown). Moreover, the price of sodium glutamate, which is commonly used as food additive and flavour enhancer, is much more affordable than that of branched amino acids and it has a very high solubility in water (up to 74% w/v at RT).

Unfortunately, none of the psychrophilic promoters available so far shows high transcriptional efficiency in minimal media when L-glutamate is used as sole carbon source (data not shown).

In this section the identification of *P. haloplanktis* TAC125 potentially regulated genes and the evaluation of their transcription rates in L-glutamate containing defined medium is described in the presence of inexpensive compounds or physical stimuli as inducers. The identification of new regulated psychrophilic promoters with high transcriptional efficiency in cheap minimal media could pave the way to the use of the novel psychrophilic expression system even at industrial level.

Analysis of L-malate inducible promoter activity in different minimal media

In order to verify if the low production yields obtained in L-glutamate containing medium by the L-malate inducible expression system was really due to a low promoter efficiency in those conditions a relative quantification of PSHAb0363 transcription level was performed by Real Time PCR experiments. *P. haloplanktis* TAC125 wild type cells were grown in L-glutamate and in branched amino acids (LIV) containing media with or without L-malate. The inducer was added at t=0 at the concentration optimised for recombinant antibody fragments production (0,4% w/v). The analysis was performed on cDNA synthesised from total RNA extracted after 24h of cultivation in shaken flasks at 15°C. As shown in Figure 31, L-malate addition to the L-glutamate containing medium does not affect PSHAb0363 transcription thus confirming negative interference of L-glutamate or one of its catabolic derivatives on PSHAb0363 promoter transcriptional activation. On the contrary, in LIV medium, a 75 folds increase in PSHAb0363 mRNA copy number is observed when L-malate is added to the culture. Data collected confirm that the L-malate regulated expression system for recombinant proteins production at low temperatures can be effectively used only when branched amino acids are used as carbon sources for bacterial cultivation and therefore the identification of new regulated promoters is needed in order to set up a feasible process for large scale recombinant proteins production in psychrophilic bacteria.

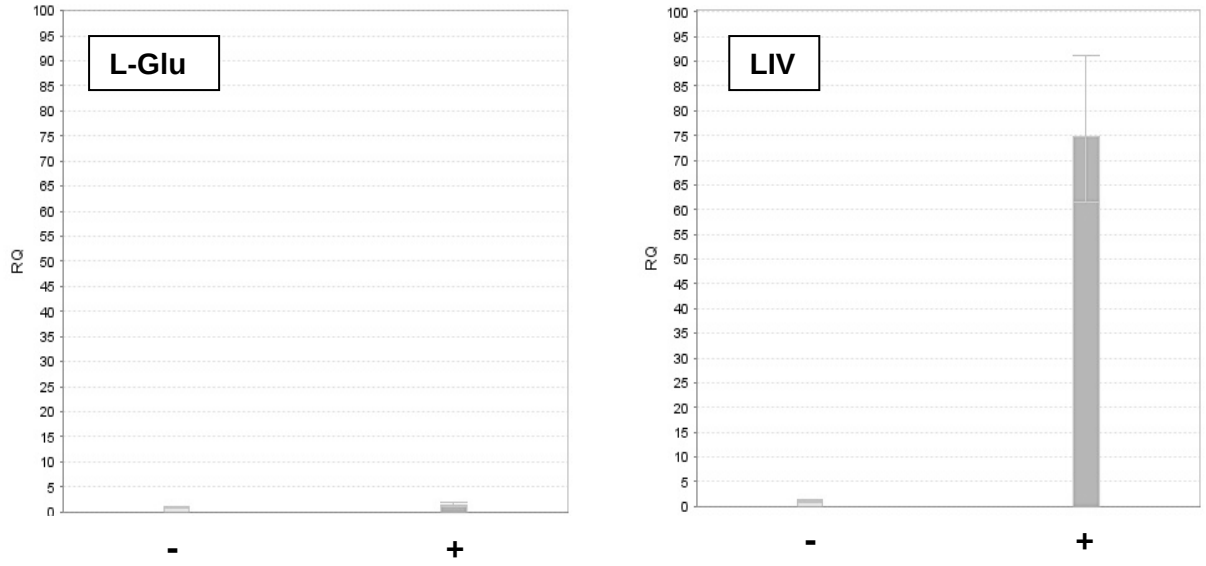


Figure 31: Real Time PCR relative quantification of *P. haloplanktis* TAC125 PSHAb0363 mRNA in L-glutamate and LIV media in the presence (+) or absence (-) of L-malate. RQ: relative quantification.

In silico analysis of P. haloplanktis TAC125 regulons involved in primary metabolism

An *in silico* analysis of *P. haloplanktis* TAC125 genome was firstly carried out in order to identify genes and/or operons whose transcription is expected to be up-regulated in the presence of particular carbon sources.

In bacteria, genes involved in primary metabolism processes are generally finely regulated in response to the nutrients availability. Marine bacteria are facing a medium generally unbalanced in terms of carbon, nitrogen, and phosphorous supply (Moran *et al.* 2004). Excess of several easily metabolised carbon sources present simultaneously is unlikely, making catabolite repression the exception rather than the rule. Indeed, *P. haloplanktis* TAC125 is lacking the cAMP-CAP complex that regulates carbon availability in related organisms such as *Vibrio* and *Shewanella*

(Medigue *et al*., 2005). Furthermore, in contrast to many γ-proteobacteria, it does not possess the glycolytic pathway and a PTS system for the transport and first metabolic step of carbohydrate degradation. This accounts for its lack of growth on glucose, likely to be phosphorylated by glucokinase (PSHAa1364).
P. haloplanktis TAC125 main metabolic pathway for pyruvate supply in the cell is the Entner-Doudoroff pathway according to the use of gluconate as a preferred carbon source (Medigue *et al*., 2005). Furthermore *P. haloplanktis* TAC125 is also able to grow on galactose thus indicating the presence of a functional Leloir pathway.
According to the latter observations, the regulation of genes involved in gluconate and galactose catabolism in *P. haloplanktis* TAC125 was investigated.

- Gluconate catabolism

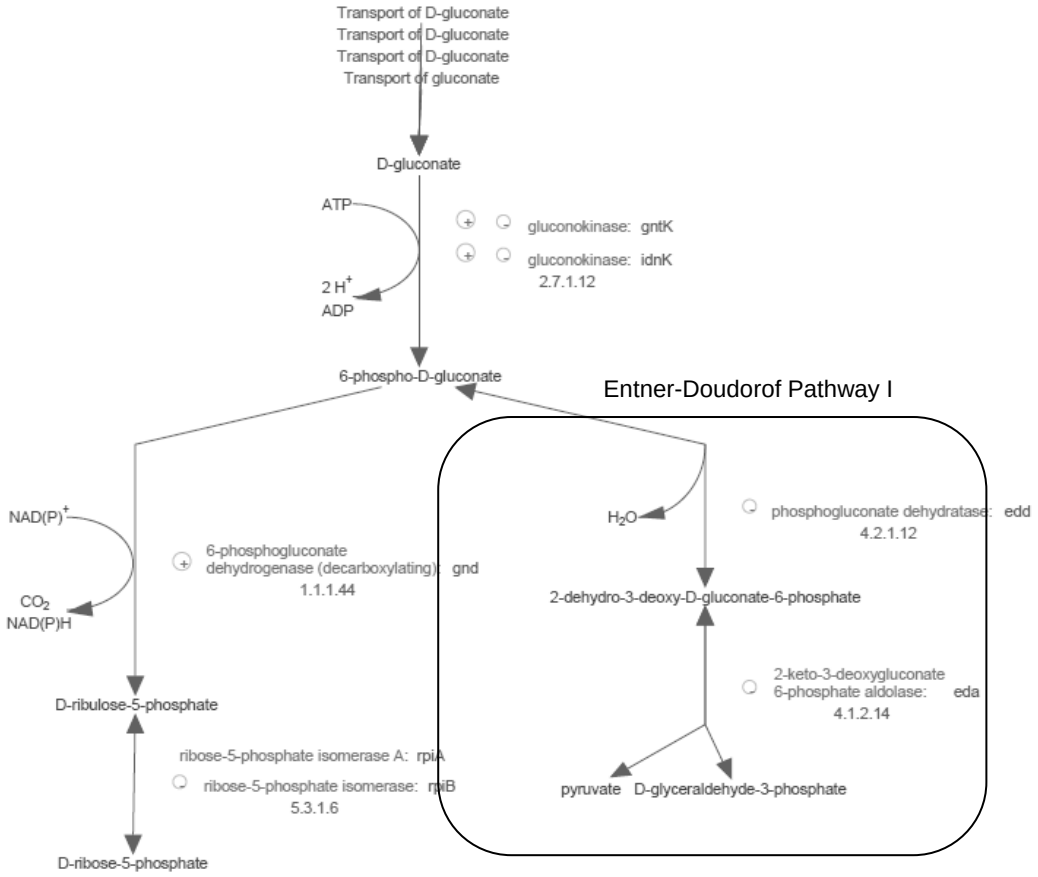


Figure 32: The superpathway of gluconate degradation including the Entner-Doudoroff pathway I. (Klemm *et al.,* 1996)

The initial steps of gluconate metabolism in *E. coli* are its entry into the cell and its subsequent phosphorylation to 6-phosphogluconate. Then, two enzymes of the Entner-Doudoroff pathway, 6-phosphogluconate dehydratase (EC 4.2.1 .12) and 6-phospho-2-dehydrogluconate aldolase (EC 4.1 .2.14), convert 6-phosphogluconate to pyruvate and glyceraldehyde 3-phosphate (Fig. 32).
Two sets of genes are involved in transport and phosporylation of gluconate in *E. coli*. The main system, GntI, contains *gnt*T and *gnt*U, encoding high- and low-affinity gluconate transporters respectively, and gntK, a thermoresistant gluconokinase (Porco *et al*., 1998). The GntII system, which was discovered in a GntI deletion mutant, contains *gnt*W and *gnt*V, encoding another high-affinity gluconate transporter and a thermosensitive gluconokinase (Istúriz *et al*., 1986). Thus, in *E. coli* there are four known gluconate transporters, including GntP another gluconate permease

(Klemm *et al.*, 1996). Previous genetic studies indicated that the GntI system is, together with the EDP genes *edd* and *eda*, negatively regulated by the *gnt*R gene product (Izu *et a*l., 1997). GntR belongs to the GalR-LacI family of regulators and it is highly conserved between bacteria (Haydon and Guest, 1991). In Figure 33 is shown the schematic representation of *gnt*KU regulon. The *gnt*R gene product which is constitutively expressed, binds to the operator site on *gnt*KU promoter when gluconate is not present.

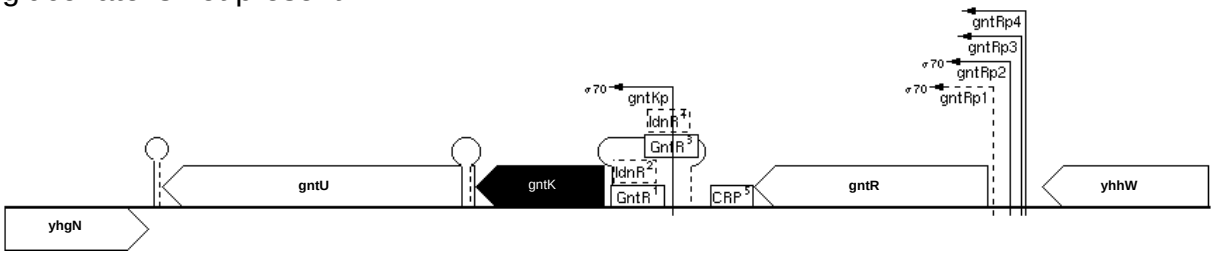


Figure 33: *E. coli gnt*KU gene local context. GntR and IdnR boxes indicate repressor binding sites and CRP box indicates cyclic AMP receptor protein activator binding site. Arrows and loops indicate promoters and terminators sites respectively. EcoCyc: *Nucl. Acid. Res*. 37:D464-70 2009

Gene orthologs for all enzymatic functions required in Entner-Doudoroff pathway are present in *P. haloplanktis* TAC125 genome. In particular, orthologs of *gnt*R and *gnt*U, PSHAb0478 and PSHAb0479 respectively, were found in *P. haloplanktis* TAC125 chromosome II (Fig. 34). However structural organisation of GntI locus is not conserved in *P. haloplanktis* TAC125. The gluconate permease encoding gene is indeed a separate transcriptional unit in the psychrophilic bacterium and the *gnt*K gene is replaced by a *idn*K homologue encoding a putative D-gluconate kinase active at low temperature. When an in *silico* analysis on PSHAb0479 promoter was performed it didn't revealed the presence of conserved consensus sequence for the operator site in the permease transcription unit. However the PSHAb0479 gene was selected for further investigation on its transcription regulation in the presence of D-gluconic acid.

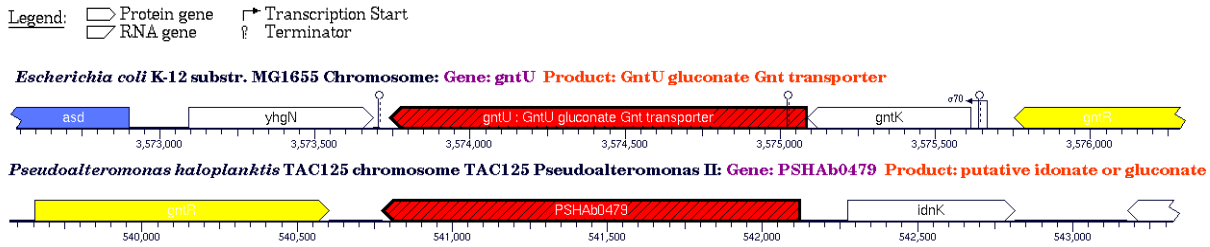


Figure 34: Multi-genome alignment of *gnt*U local gene context. Genes colour indicates orthologous groups.

- Galactose catabolism

D-galactose catabolism goes through Leloir pathway (Fig. 35). The enzymes of the Leloir pathway catalyze the conversion of D-galactose to the more metabolically useful version of the sugar, D-glucose-6-phosphate. In most organisms five enzymes are required to catalyze this conversion: a galactose-1-epimerase, a galactokinase, a galactose-1-phosphate uridylyltransferase, a UDP-glucose 4-epimerase, and a β-phosphoglucomutase. D-glucose-6-phosphate in *E. coli* goes to glycolytic pathway while in *P. haloplanktis* TAC125 it is converted in 6-phosphogluconate which is recruited by the Entner-Doudoroff pathway.
In *E. coli*, the genes responsible for D-galactose metabolism (the galactose operon *gal*ETKM) and for high affinity transport of methylgalactosides, glucose, and

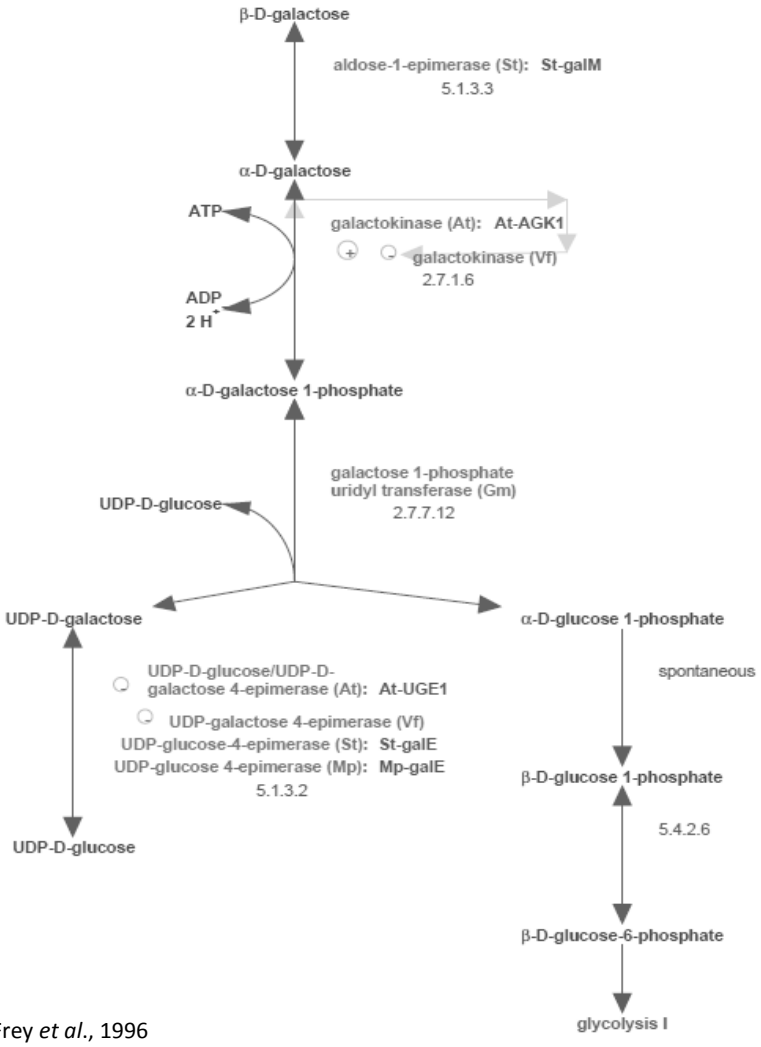


Figure 35: The Leloir pathway. Frey *et al.*, 1996

galactose (the *mgl*BAC operon) (Fig. 36 A and B) are controlled by a repressor, GalR, and an isorepressor, GalS (Weickert and Adhya, 1993). Both operons are induced by D-galactose or by its nonmetabolizable analog, D-fucose. For GalR, inducer binding results in a loss of repressor affinity for DNA (Majumdar and Adhya, 1987). The same mechanism is presumed to mediate *mgl* induction, which is under

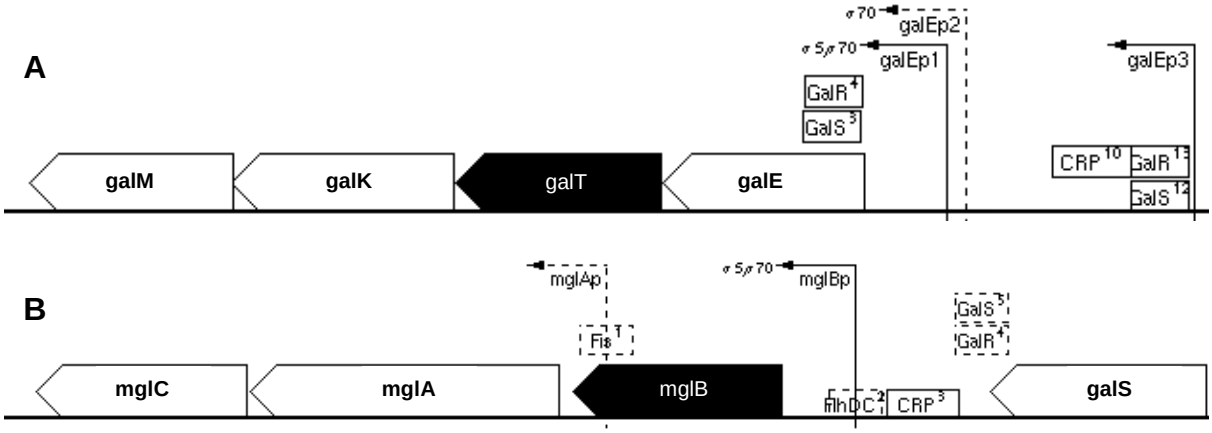


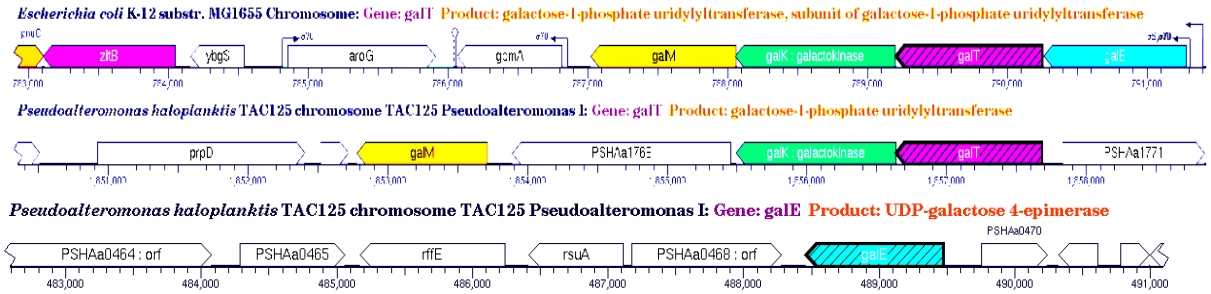
Figure 36: *gal*ETKM (A) ang *mgl*BAC (B) gene local context. GalR and GalS boxes indicate repressor binding sites and green box indicates cyclic AMP receptor protein activator binding site. Arrows and loops indicate promoters and terminators sites respectively. EcoCyc: *Nucl. Acid. Res*. 37:D464-70 2009

Table 5: Cross-species comparison of *gal*R orthologs (MetaCyc)

Organism	Gene	Gene Product
E. coli K-12 substr. MG1655	*gal*R	GalR transcriptional dual regulator
P. haloplanktis TAC125	PSHAa1771	putative GalR repressor of contiguous operon, likely to bind a galactoside

the control of GalS (Weickert and Adhya, 1992a). GalR and GalS have overlapping regulatory specificities, as indicated by ultrainduction of the gal operon. Both belong to the GalR-LacI family of regulators (Weickert and Adhya, 1992b). While *gal*S is negatively autoregulated and positively regulated by cyclic AMP (cAMP) receptor protein (CRP), *gal*R is constitutively expressed.
In *P. haloplanktis* TAC125 genome either the *mgl* operon and the *gal*S regulator are not present. On the other hand a *gal*R orthologue was found showing 42,7% identity to *Ecgal*R (Tab. 5). Moreover, *P. haloplanktis* TAC125 *gal* operon shows a different organisation compared to *E. coli* (Fig. 37). The *gal*E gene (PSHAa0469) is not included in the cluster and a new gene appears between *gal*K and *gal*M, PSHAa1768. It is predicted to encode a putative sodium/hexose cotransport protein but it shows only 20% identity with *Ecgal*P.

Figure 37: Multi-genome alignment of *gal*T and *gal*E local gene context. Genes colour indicates orthologous groups.



When the intergenic distances between *P. haloplanktis* TAC125 *gal* genes are considered, it appears that only *gal*T and *gal*K are included in *gal* operon. Those two genes are indeed overlapped (-1bp distance) showing the typical structure of *P. haloplanktis* TAC125 translationally coupled genes intergenic regions. A promoter scan was performed on *gal*M and PSHAa1768 upstream regions (Bprom, Softberry) and it revealed the presence of consensus sequences for the constitutive transcription of the analysed genes (data not shown). Although the same analysis carried on *gal*T upstream region did not reveal the presence of canonical consensus sequences for GalR binding, we considered worth to investigate on the transcriptional regulation of *P. haloplanktis* TAC125 *gal*TK operon in the presence of D-galactose.

Transcriptional analysis of gluconate permease PSHAb0479

The relative amount of gluconate permease PSHAb0479 mRNA in *P. haloplanktis* TAC125 cells was evaluated during growth in defined medium containing L-glutamate as carbon source in the presence/absence of 0,5% w/v D-gluconic acid. Real Time PCR analysis on cDNA synthesised from total RNA extracted after about 24h of shaken flask cultivation at 15°C revealed that the addition of D-gluconic acid in the culture medium induces up to 20 folds increase of PSHAb0479 transcription (Fig. 38). This finding confirms the presence of a functional D-gluconate dependent

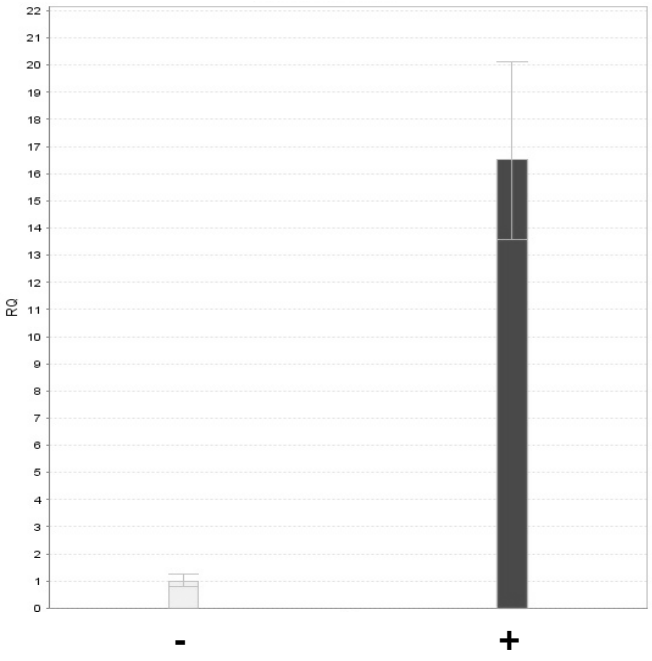


Figure 38: Real Time PCR relative quantification of *P. haloplanktis* TAC125 gluconate permease PSHAb0479 mRNA in the presence (+) or absence (-) of D-gluconic acid. RQ: relative quantification

regulation mechanism for the transcription of genes involved in the uptake of gluconate in *P. haloplanktis* TAC125. Moreover, the psychrophilic gluconate permease promoter represents an interesting tool for the construction of new regulated expression vectors for recombinant proteins production at low temperatures. Indeed, sodium gluconate is a user friendly compound, highly soluble and quite inexpensive. Furthermore, D-gluconate is also a good substrate for *P. haloplanktis* TAC125 growth and therefore the new promoter could be alternatively used for constitutive expression of recombinant proteins in media containing D-gluconate as carbon source.

Transcriptional analysis of galTK operon

The effect of D-galactose on *P. haloplanktis* TAC125 *gal*TK operon transcription was evaluated by Real Time PCR relative quantification of *gal*T mRNA after 24h growth in defined medium containing L-glutamate as carbon source at 15°C in the presence/absence of 0,5% w/v D-galactose. The analysis was performed on cDNA synthesised from total RNA. Surprisingly the analysis revealed that the amount of *gal*T mRNA in the cell doesn't significantly change when galactose is added in the culture medium (Fig. 39). The ability of the psychrophilic bacterium to grow in media containing D-galactose as sole carbon source indicates that the transcription of

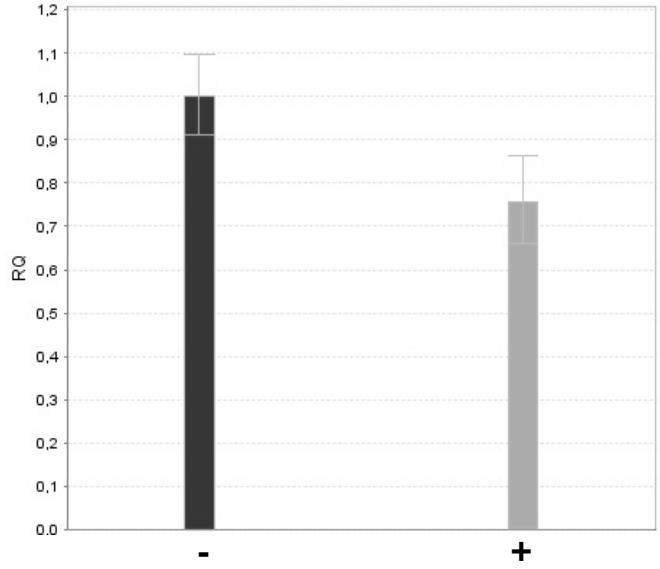


Figure 39: Real Time PCR relative quantification of *P. haloplanktis* TAC125 *galT* (PSHAa1770) mRNA in the presence (+) or absence (-) of D-galactose. RQ: relative quantification

*gal*TK operon in *P. haloplanktis* TAC125 is constitutively derepressed. The lack of GalS isorepressor and the different distribution of *gal* genes in *P. haloplanktis* TAC125 genome suggest that the transcriptional regulation of genes involved in galactose metabolism has been counter selected by the evolution of the psychrophilic bacterium. Moreover the in silico analysis of *P. haloplanktis* TAC125 *gal*R promoter (Bprom, Softberry) underlined the presence of a conserved CRP binding site. The lack of cAMP-CAP complex in *P. haloplanktis* TAC125 can be the reason of insufficient

expression of GalR repressor which causes constitutive transcription of the genes responsible for galactose catabolism. However the transcriptional efficiency of *gal*TK promoter can still be further investigated for constitutive expression of recombinant genes in *P*. *haloplanktis* TAC125.

Alginate dependent transcription of PSHAa1748 alginate lyase

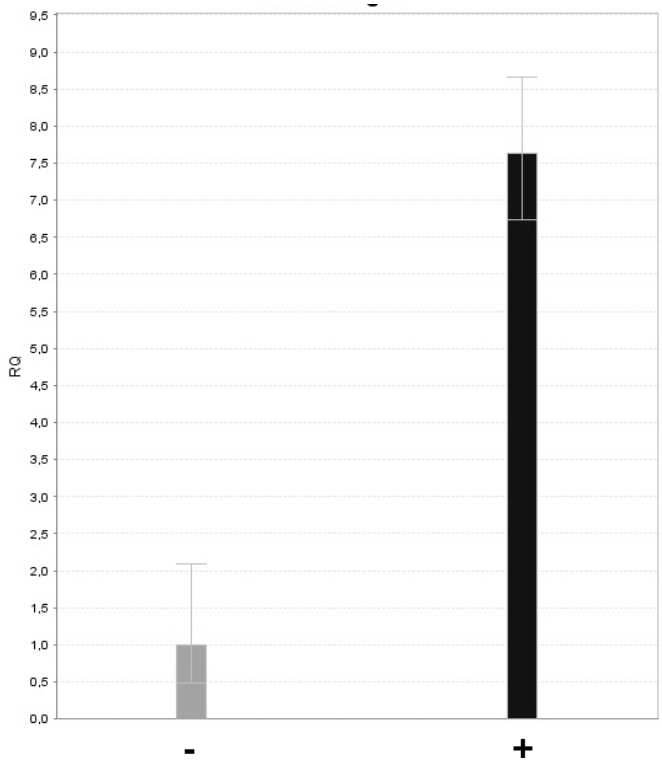


Figure 40: Real Time PCR relative quantification of *P. haloplanktis* TAC125 alginate lyase PSHAa1748 mRNA in the presence (+) or absence (-) of alginic acid. RQ: relative quantification

An *in silico* analysis of *P*. *haloplanktis* TAC125 genome revealed the presence of three putative alginate lyase coding genes. Previous experiments demonstrated that two genes are always expressed while the PSHAa1748 mRNA was only detected by non quantitative RT-PCR when alginate was added to the culture medium (unpublished results). The induction efficiency of alginate on *P*. *haloplanktis* TAC125 alginate lyase PSHAa1748 promoter was then investigated by Real Time PCR relative quantification. The analysis was carried on PSHAa1748 cDNA synthesised from total RNA extracted after about 24h of shaken flask cultivation at 15°C in L-glutamate deefined medium in the presence/absence of 0,3% w/v alginic acid. As shown in Figure 40, the addition of alginic acid leads to about 10 folds increase in PSHAa1748 gene expression. This makes the PSHAa1748 promoter a promising candidate for the construction of a new regulated psychrophilic expression system. However, although alginate is a quite inexpensive compound, it is very poorly soluble in water and highly viscous limiting its use at high concentrations. Therefore, alginic acid utilisation as inducer for recombinant gene expression in *P*. *haloplanktis* TAC125 should be carefully optimised to ensure good induction efficiency even at low concentrations.

Temperature downshift induction: analysis of psychrophilic Trigger Factor differential expression

The peculiar ability of *P*. *haloplanktis* TAC125 to grow in a wide range of temperatures (0-25°C) suggests that a molecular adaptation to temperature changes should occur even at transcriptional level. The identification of *P*. *haloplanktis* TAC125 promoters whose activity is specifically induced at low temperatures could represents an attractive feature for the construction of an effective cold-inducible expression system for recombinant protein production in *P*. *haloplanktis* TAC125. Previous experiments focused on the understanding of cell adaptation to the life at low temperatures by proteomics did not underline the presence of specific sets of proteins (such as Cold Shock Proteins, Csp) whose expression is up- or down-regulated at low temperatures (unpublished data). More recently, a differential

proteomic analysis of *P*. *haloplanktis* TAC125 grown at 4 and 18°C revealed that a particular protein, identified as the Trigger Factor (TF), results to be up to 50 folds over-expressed in response to temperature downshift (D'Amico S., personal communication).
TF is a peptidyl-prolyl cis/trans isomerase that acts as a molecular chaperone by maintaining the newly synthesized protein in an open conformation. In prokaryotes, the ribosome-associated Trigger Factor is the first chaperone that newly synthesized polypeptides encounter when they emerge from the ribosomal exit tunnel (Hoffmann *et al.,* 2006). In *E*. *coli*, Trigger Factor is induced upon cold shock and enhances its viability at low temperatures (Kandror and Goldberg, 1997).
We analysed differential expression of *P*. *haloplanktis* TAC125 *tig* gene, encoding the psychrophilic Trigger Factor, at different temperature (4 and 15°C) in order to identify a new potential cold-inducible psychrophilic promoter to be used for recombinant gene expression at low temperatures.
The quantification of *P*. *haloplanktis* TAC125 TF relative expression at different temperatures was carried out by Real Time PCR experiments on cDNA synthesised from total RNA extracted after about 24h of shaken flask cultivation in L-glutamate defined medium at 15°C and 4°C. Unfortunately, the analysis revealed that the amount of *tig* mRNA does not significantly change between the two tested temperatures (Fig. 41). The obtained result indicates that the observed up-regulation of TF protein expression at low temperatures probably occur at a translational or post-translational level and therefore other targets have to be searched out for the identification new psychrophilic regulated promoters induced by temperature downshift.

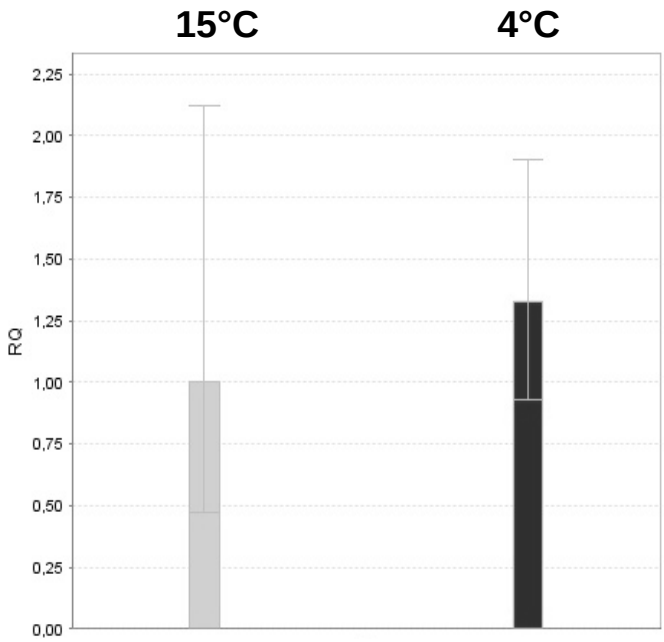


Figure 41: Real Time PCR relative quantification of *P. haloplanktis* TAC125 *Trigger Factor* PSHAa2063 mRNA at different temperatures. RQ: relative quantification

Section 2: Extracellular protein secretion in *P. haloplanktis* TAC125
Protein secretion into the extra-cellular environment is one of most desirable strategy to allow a rapid and not expensive recovery of recombinant proteins. Secretion to the culture medium has several advantages over intracellular recombinant protein production. These advantages include simplified downstream processing, enhanced biological activity, higher product stability and solubility, and N-terminal authenticity of the expressed peptide (Cornelis, 2000; Makrides, 1996; Mergulhaõ *et al*., 2004). If the product is secreted to the culture medium, cell disruption is not required for recovery. As bacteria usually do not secrete amounts of proteins higher then they have in the intracellular space, recovery of a recombinant gene product can be greatly simplified by a secretion strategy that minimises contamination from host proteins.

The realisation of a "cold" recombinant secretion system in the Antarctic Gram-negative bacterium *P. haloplanktis* TAC125 has been previously reported (Cusano *et al*., 2006b). This system efficiently conjugates the obvious advantages of extracellular protein targeting with the positive effect of low temperature on the recombinant product solubility. Indeed, low expression temperature can facilitate the correct folding of "difficult" products (Sahdev *et al*., 2008) and the use of *P. haloplanktis* TAC125 as expression system allowed the efficient production of some "intractable" proteins in soluble and active form at temperature as low as 4°C (Parrilli *et al*., 2008). The cold-adapted secretion system makes use of the psychrophilic α-amylase from *P. haloplanktis* TAB23 (Feller *et al*., 1992) as secretion carrier. Three chimerical proteins, made of the psychrophilic α-amylase fused to an intracellular protein, were translocated in the extracellular medium with a secretion yield always above 80% (Cusano *et al*., 2006b). The system also allowed the correct disulphide bond formation of chimera components, secreting a fully active passenger (Cusano *et al*., 2006b).
A deep understanding of the molecular mechanism driving extracellular protein secretion in the psychrophilic bacterium can pave the way to strain engineering strategies aimed at the construction of a *P. haloplanktis* TAC125 stain with improved secretion ability and reduced extracellular proteases content which can affect the quality of recombinant products (Cusano *et al*., 2006b). By an *in silico* analysis of *P. haloplanktis* TAC125 genome it is possible to indentify the presence of only one canonical secretion system, a Type II secretion machinery (T2SS), homologous to the General Secretory Pathway (GSP) already described in many other Gram-negative bacteria (Sandkvist, 2001). Moreover, experimental evidences suggested that in *P. haloplanktis* TAC125 the secretion of the cold α-amylase depends on a still uncharacterised pathway.
The last part of this work has been focused on the study of *P. haloplanktis* TAC125 secretion systems. Starting from the molecular and phenotypic characterisation of the canonical T2SS, we developed a *P. haloplanktis* TAC125 mutant strain with reduced extracellular proteolytic activity. Moreover the existence of a functional still uncharacterised Psychrophilic Secretion System (PSS) responsible for the cold α-amylase extracellular localisation was demonstrated and the first key component of this secretion machinery, the protein PssA, was identified.

Results of this section are reported into the following published articles:

General Secretory Pathway from marine Antarctic Pseudoalteromonas haloplanktis TAC125
E. Parrilli, **M. Giuliani**, M.L. Tutino. ***Marine Genomics*** 2008, 1(3-4): 123-128

Cell engineering of Pseudoalteromonas haloplanktis TAC125: construction of a mutant strain with reduced exo-proteolytic activity
E. Parrilli, A.M. Cusano, **M. Giuliani** and M.L. Tutino. ***Microbial Cell Factories*** 2006, 5 (Suppl 1):P36

*Ph*PssA is required for alpha-amylase secretion in Antarctic *Pseudoalteromonas haloplanktis*
E. Parrilli, **M. Giuliani**, C. Pezzella, A. Danchin, G. Marino, M.L. Tutino. ***Microbiology*** 2010, DOI 10.1099/mic.0.032342-0- In press

Marine Genomics 1 (2009) 123–128

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General Secretory Pathway from marine Antarctic *Pseudoalteromonas haloplanktis* TAC125

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ABSTRACT

In Gram-negative bacteria Type II secretion system (T2SS) is one of six protein secretion machineries that permit the export of proteins from within the bacterial cell to the extra-cellular milieu and/or into target host cells. The ability to secrete proteins in the extra-cellular medium is a key aspect of the physiology of Gram-negative bacteria. Indeed, secreted proteins provide the means, among others, by which microorganisms interact and modify the surrounding environment. This is a crucial aspect for microorganisms capable to survive in hostile extreme habitats, such as Antarctica. The sequencing and the annotation of marine Antarctic *Pseudoalteromonas haloplanktis* TAC125 (*P. haloplanktis* TAC125) genome paved the way to investigate on the molecular mechanisms of Type II dependent protein secretion in this bacterium. The present work reports a detailed *in silico* description and a functional analysis of *P. haloplanktis* TAC125 T2SS pathway, which features were compared with other already characterized T2SS machineries. The cold adapted Type II secretion cluster resulted to be characterized by the notably presence of a downstream and physically associated gene (called *gspXX*) which was found only in two other Alteromonadales T2SS gene clusters. A transcriptional analysis of some *P. haloplanktis* TAC125 T2SS genes demonstrated that the psychrophilic cluster was constitutively transcribed during the cell growth and that *gspXX* gene is co-transcribed with another gene of the cluster. These data support the inclusion of *gspXX* into the cold-adapted Type II secretion system cluster and point towards the existence of a novel and previously not described component of the T2SS machinery. This evidence opens new questions concerning the role played by GspXX protein in the architecture/function of the *P. haloplanktis* TAC125 T2SS machinery.

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1. Introduction

The external layer of the cell envelope of Gram-negative bacteria is an essential barrier against undesirable compounds and a place of intense trafficking of proteins and signals. This layer allows the exchange of various small nutrient molecules between the cells and the environment. Nevertheless, it is a compartment of traffic for large proteins such as enzymes and toxins.

Numerous studies dealing with the molecular mechanism of protein secretion have revealed that Gram-negative bacteria evolved different strategies to achieve this process. Among them, the Type II protein secretion system (T2SS), also known as the General Secretory Pathway (GSP), is part of a two steps process in which proteins are first translocated across the inner membrane by the Sec (de Keyzer et al., 2003) or TAT (Voulhoux et al., 2001) pathway and then, are transported from the periplasm to the exterior by an outer membrane secretin (Filloux, 2004; Cianciotto, 2005; Sandkvist, 2001).

Depending on the species, between 12 and 15 genes (often called *gsp* genes) have now been identified as essential for Type II secretion, and the homologous genes and gene products have been designated, for most species, by the letters A through O and S. Although the T2SS serves as an outer membrane translocase, essential components of this system are found in four distinct sub cellular locations. Together, these proteins form a trans-envelope complex that spans the entire length of periplasm and penetrates both the outer and the cytoplasmic membranes.

GspD (also known as secretin) is embedded in outer membrane where it works as gated secretion pore (Marciano et al., 1999; Filloux, 2004). A pilus-like structure comprising one major (GspG) and four minor (GspH, -I, -J, -K) pseudopilins (Nunn and Lory, 1993; Bleves et al., 1998; Sauvonnet et al., 2000) extends between cytoplasmic and outer membrane (Hu et al., 2002). The presumably retractable assembly of pseudopilins might facilitate protein secretion by acting as a piston (Sandkvist, 2001).

Four cytoplasmic membrane proteins GspL, GspM, GspF and GspC form a hierarchically structured complex (Tsai et al., 2002; Filloux,

Abbreviations: T2SS, Type II secretion system; GSP, General Secretory Pathway; TAT, twin arginine translocation; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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2004). Being able to mediate multiple protein–protein interactions, this inner membrane (IM) complex may function as an energy- or signal-transmitting module to couple the various molecular events required for exoprotein secretion. The function of the GspC protein is unknown. Its principal feature is the presence of a PDZ (Pallen and Ponting, 1997) domain in the C-terminal region of the protein. PDZ domains mediate a variety of protein–protein interactions. It is widely thought that PDZ domain-containing proteins mediate the organization of multi-molecular complexes at sites of membrane specialization. Direct interactions have been demonstrated between GspC and GspD (Lee et al., 2000), as well as between GspC and the major pseudopilin GspG (Lee et al., 2005). Classically, within the Type II secretion, GspM was shown to be crucial for the stability of GspL (Michel et al., 1998). The stabilization process is reciprocal because the abundance of GspM in the cell depends on GspL, indicating that these two Gsp components interact with each other. In addition, GspL is known to interact with the cytosolic secretion ATPase GspE (Sandkvist et al., 1995). GspE is essential for the T2SS functionality (Sandkvist et al., 1995) since likely it works as the energy-generating component for the T2SS.

Beyond these core components, there are several other proteins that are not conserved among the majority of genera, including GspN, GspA, GspB, GspO and GspS. Possible functions have been suggested for some of them. For instance, GspN protein is, like GspC, a bitopic IM protein and the *Xantomonas campestris* GspN was proposed to take part to the formation of the GspL–GspM complex (Lee et al., 2001; Tsai et al., 2002). GspO is a specialized peptidase localized in the inner membrane, likely involved in the N-terminal processing of the pseudopilins, while GspS might assist the placement of the secretin GspD in the outer membrane (Peabody et al., 2003; Filloux, 2004).

GspA and GspB form a complex within the cytoplasmic membrane, the role of this complex remains elusive looking at the panel of putative proposed functions, which include energy transduction, stabilization or increased expression of the Gsp complex and piloting the secretin to the OM (Filloux, 2004).

Historically, the study of T2SS started from bacteria with pathogenic capabilities; however, recent studies have shown that GSP functionality may also confer an ecologic advantage to bacteria growing in specific environments. Presently, there are genetic and functional data to indicate the relevance of T2SS in bacteria that are strictly environmental non-pathogens. Indeed, many non-pathogenic organisms secrete proteins, such as cellulases and other degradative enzymes, that help them adapt to their lifestyle.

For example, the T2SS pathway plays a fundamental role in the lifestyle of the eukaryote-associated bacterium *Pseudoalteromonas tunicata* D2, as the production of secondary metabolites with inhibitory activity against bacteria and other organisms is directly correlated with the existence of a functional GSP system in this bacterium (Egan et al., 2002). Recently, it has also been shown that T2SS might promote the persistence of the *Legionella pneumophila* in aquatic habitats by facilitating both intracellular growth in fresh water amoebae and extra-cellular growth at 'low' temperatures (Soderberg et al., 2004). Recent reports on several *Shewanella* species have significantly contributed to increase our knowledge on the different functions undertaken by the T2SS system, further shifting the focus of the T2SS research from pathogenicity to ecology. *Shewanella putrefaciens* is able to reduce Fe(III) and Mn(IV) as part of the anaerobic respiratory process due to a metal reductase complex that uses the GSP pathway to be transported across the outer membrane (DiChristina et al., 2002). In different *Shewanella* species, the GSP pathway is directly associated with the transport of enzymes that carry out important biogeochemical functions in the marine environment. For example, *Shewanella oneidensis* strain MR-1 produces conductive pilus-like appendages (nanowires) used to transfer electrons between cells and metal-oxide surfaces under oxygen limitation (Gorby et al., 2006).

The evidence that the Type II secretion pathway is directly involved in exporting enzymes with different ecological functions suggests that the GSP pathway has a significant role in the ecology of environmental bacteria. As often observed in Pseudoalteromonadales, the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 (*P. haloplanktis* TAC125) (Médigue et al., 2005) has a very good secretory ability, likely related to the basic nutritional consequences of protein secretion if the enzymes to be exported are hydrolases, i.e. enzymes able to broaden up the nutritive substrate profile. The sequencing and the annotation of *P. haloplanktis* TAC125 genome (Médigue et al., 2005) paved the way to investigate on the molecular mechanisms of protein secretion in this Antarctic bacterium. The annotation of *P. haloplanktis* TAC125 genome highlighted the presence of a gene cluster coding for a Type II secretion machinery which functionality have been recently reported (Parrilli et al., 2008). The present work is aimed at a detailed *in silico* description and a functional analysis of *P. haloplanktis* TAC125 GSP pathway, which features have been compared with other already characterized T2SS machineries.

2. Materials and methods

2.1. Strains and plasmids

P. haloplanktis TAC125 (Médigue et al., 2005) was isolated from Antarctic sea water. *Escherichia coli* DH5α (Hanahan, 1983) was used as host for the gene cloning. *E. coli* strain S17-1(λ*pir*) was used as donor in intergeneric conjugation experiments (Tascon et al., 1993).

2.2. Growth conditions and analytical procedures

P. haloplanktis TAC125 was grown in aerobic conditions at 15 °C in TYP broth (16 g/l yeast extract, 16 g/l bacto tryptone, 10 g/l marine mix) at pH 7.5, supplemented with ampicillin 200 μg/ml, or chloramphenicol 25 μg/ml if transformed when required. Antarctic bacteria transformation was achieved by intergeneric conjugation as previously reported (Duilio et al., 2004).

E. coli cells were routinely grown in Terrific broth (Sambrook and Russell, 2001) at 37 °C. When required, antibiotics were added at the following concentrations in liquid cultures: 100 μg/ml of ampicillin, or cloramphenicol at 50 μg/ml final concentration. Genetic manipulations were carried out as previously described (Sambrook and Russell, 2001).

P. haloplanktis TAC125 DNA genomic purification was performed by ChargeSwitch gDNA Mini Bacteria Kit (Invitrogen).

Table 1
Plasmids and oligonucleotides primers used in this work.

Plasmids		
pUCC	Vector deriving from the pUCLoriT/oriR plasmid, containing the T/R box, the transcription termination signal from *Ph*TAC125 *aspC* gene and chloramphenicol resistance gene	(Tutino et al., 2002)
pUCRP	Vector deriving from the pUC18 plasmid, containing the T/R box and the transcription termination signal from *Ph*TAC125 *aspC* gene and PSHAb0363 promoter region	(Papa et al., 2007)
pUCRD*gspE*	Plasmid containing the gspE gene under the control of PSHAb0363 promoter	(This work)
Oligonucleotides		
Oligo *gspESalI-NdeIfw*	5′-TCAGCTGCATATGACTAAGGCGATTG-3′	
Oligo *gspEXbaI-PmeIrev*	5′-AATCTAGAGTTTAAACAAACCGCCAAG-3′	
Oligo 1	5′-TTGCATGCATGCGCATCATCCGG-3′	
Oligo 2	5′-AAGAGCTCCTTCACTGAGCATCG-3′	
Oligo 3	5′-TTTCTAGAATCCTATTGTGTCACCAC-3′	
Oligo 4	5′-AAGAGCTCAATCGTCCGTTTACCG-3′	
Oligo 5	5′-TTGAGCTCGCAGATGTGAGCTTTGG-3′	
Oligo 6	5′-AAGAATTCTTTTAGGGTTGCATCAGC-3′	
Oligo 7	5′-TTGCATGCGTTTTAATGCAATTACCC-3	
Oligo 8	5′-AAGAGCTCTAATTTGATGGTATAGCG-3	

Protein samples were analyzed by Polyacrylamide Gel Electrophoresis (SDS-PAGE) (12% acrylamide, w/v) according to standard methods (Sambrook and Russell, 2001).

2.3. Construction of vector pUCRDgspE

pUCRD*gspE* was constructed starting from pUCC vector, a plasmid deriving from the pUCLOriT/R plasmid (Tutino et al., 2002), containing the T/R box, the transcription termination signal from *Ph*TAC125 *aspC* gene and chloramphenicol resistance gene. In this vector was cloned the *gspE* gene under the control of L-malate inducible promoter. DNA fragment containing promoter region of PSHAb0363 gene(P363) was extracted from pUCRP (Papa et al., 2007) vector by a double *Hind*III and *Nde*I digestion. The *gspE* gene was amplified to introduce *Nde*I and *Pme*I restriction sites (primers oligo *gspESal*I-*Nde*Ifw and oligo *gspEXba*I-*Pme*Irev see Table 1) and the PCR product was subjected to a double *Nde*I and *Pme*I digestion. Promoter region and *gspE* gene were introduced in pUCC vector digested using *Hind*III and *Sma*I restriction enzymes.

2.4. RNA isolation and RT-PCR analysis

P. haloplanktis TAC125 cells were grown at 15 °C in TYP medium and total RNA was extracted according to Tosco et al. (2003). RNA was reverse transcribed using SuperScript II RNase H-Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. cDNAs were amplified using as primers specific oligonucleotides (Table 1) designed on *P. haloplanktis* TAC125 genome sequence, and Taq polymerase (Promega, Madison, WI, USA) according to the manufacturer's instructions. The reaction mixture was amplified (denaturation at 95 °C for 45 s; annealing at 58 °C for 45 s; extension at 72 °C for 1 min, 35 cycles).

2.5. Zymographic assay

P. haloplanktis TAC125 wild type and *gspE* mutant strains were grown in standard conditions and culture samples were collected. Samples were centrifuged at 10000 ×*g* for 5 min at 4 °C and the upper phase was collected for further analysis. The collected culture media were tenfold concentrated by Centricon (AMICON, exclusion size 5 kDa), and 12 µl were loaded onto a non reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing gelatin (1.5 mg ml^{-1}). After electrophoresis, gel was soaked twice with 2.5% Triton X-100 (v/v) solution for a total of 60 min to remove SDS. The gel was then incubated in a developing buffer (50 mM Tris–HCl, pH 7.5, containing 5 mM $CaCl_2$) for 18 h at 15 °C, rinsed with water, and stained with Coomassie blue R250. Areas of gelatin digestion corresponding to proteolytic activies were visualized as unstained regions in the gel.

3. Results and discussion

3.1. In silico analysis of gsp cluster from P. haloplanktis TAC125

P. haloplanktis TAC125 General Secretory Pathway is located on its larger chromosome and it is made up of twelve genes organized into a cluster including *gspC-N* (Fig. 1). Respect to Type II secretion pathways present in other Gram-negative bacteria, the psychrophilic GSP contains all genes codifying functions necessary for the secretion system activity i.e.: the gene encoding the secretion ATPase GspE and pseudopilin genes *gspG*, *-H*, *-I*, *-J*, *-K*. *P. haloplanktis* TAC125 genome also contains the genes encoding the cytoplasmatic platform components (*gspC*, *-F*, *-L*, *-M*) and the secretin (*gspD*), but it is devoid of genes encoding GspA, -B, -O, -S proteins.

As for the absence of *gspO* gene in Type II secretion cluster, in those species where the O gene is not physically associated to the rest of the Type II secretion genes, it is often found clustered with a subset of genes required for Type IV pilus biogenesis. In these species the prepilin peptidase exhibits a dual function, i.e. it is required for processing and methylation of both the Type IV prepilin subunits as well as the prepilin-like components encoded by the Type II secretion genes *gspG*, *-H*, *-I*, *-J*, and *-K* (Strom et al., 1993; Pugsley and Dupuy, 1992). It is therefore likely that in *P. haloplanktis* the role of the prepilin peptidase is played by the protein codified by PSHAa0380 gene which is located in a conserved *Ph*TAC125 genome region coding for a canonical Type IV pilus biogenesis system (Médigue et al., 2005).

A databank search looking at the distribution of the *gspN* gene in T2SS containing genomes highlighted that this gene is present only in a reduced number of GSP clusters, thus confirming the likely dispensability of its encoded protein, which function still remains not understood. Interestingly, as shown in Fig. 1, *Ph*TAC125 contains a *gspN* gene and 21 bp downstream of its stop codon, an open reading frame is found, in the same direction of the *gsp* cluster. This coding sequence, annotated as PSHAa0243 gene, is characterized by a canonical SD sequence (5′-AAAGAG) centered at −8 from the ATG start codon and encodes a hypothetical protein of 195 aa (hereafter called GspXX). The position of this gene is suggestive of a potential involvement of its encoded product in the cold-adapted GSP function, and it makes atypical the *P. haloplanktis* TAC125 *gsp* cluster. Other two Alteromonadales, *P. tunicata* D2 (NZ_AAOH00000000) and *Alteromonadales* bacterium TW-7 (NZ_AAVS00000000), present a similar Type II gene cluster organization (Fig. 1), that includes a gene homologue to PSHAa0243 (Evans et al., 2008). The GspXX protein contains highly conserved regions, one of which is the YgfB-YecA domain (IPRO11978), so called because it was found for the first time in the *E. coli* proteins YgfB and YecA. This domain, which function is still unknown, characterizes several other proteins and

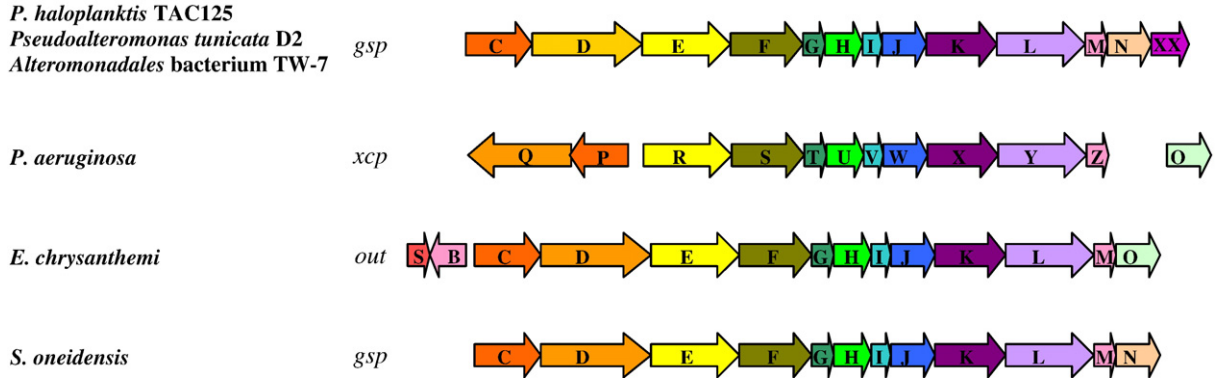

Fig. 1. Schematic representation of *Pseudoalteromonas haloplanktis* TAC125, *P. tunicata* D2, *Alteromonadales* bacterium TW-7, *Pseudomonas aeruginosa*, *Erwinia chrysanthemi*, and *Schewanella oneidensis* Type II secretion clusters. Genes (from A to O and S) are represented by arrowheads to indicate their orientation, and homologous functions are filled by the same color. The *P. aeruginosa* and *E. chrysanthemi* T2SS gene clusters have a different designation, and have been called *xcp* and *out* gene clusters, respectively. Spaces are introduced to indicate that genes are not adjacent. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Identity percentages between *P. haloplanktis* TAC125 GSP proteins and their homologues in selected bacteria.

	Pseudomonas aeruginosa	*Erwinia chrysanthemi*	*Shewanella oneidensis*	*Pseudoalteromonas tunicata* D2	*Alteromonadales* bacterium TW-7
GspC	–	28	37	51	76
GspD	35	48	55	75	89
GspE	64	63	72	80	90
GspF	47	55.	58	76	89
GspG	50	58	57	75	94
GspH	36	22	35	59	82
GspI	36	39	35	62	80
GspJ	30	34	31	64	87
GspK	28	34	37	52	81
GspL	25	25	33	49	75
GspM	20	27	33	46	80
GspN	–	23	26	41	85
GspXX	–	–	–	34	76

consists of two tightly associated 3-helical bundles with different twists (Galkin et al., 2004).

Fig. 1 also presented the genetic organization of T2SS gene cluster in *S. oneidensis*, *Pseudomonas aeruginosa* and *Erwinia chrysanthemi*. These micro-organisms have been selected due to the close phylogenetic relationship with *P. haloplanktis* TAC125 (*S. oneidensis*) or because their T2SS systems have been extensively characterized.

Each *P. haloplanktis* TAC125 *gsp* gene product was pair wise compared to the homologous protein (if present) from the other selected micro-organisms, and the resulting identity percentages are reported in Table 2. As a whole, the identity percentages reflect the phylogenetic relatedness between *P. haloplanktis* TAC125 and the compared bacteria, where Tw-7 is the closest one. Some Gsp proteins (i.e. GspC, -L and -M) resulted to be less conserved, while GspE, -F, and -G turned out to be proportionally more invariant. These observations are in good agreement with those previously reported in literature (Sandkvist et al., 1995).

3.2. *P. haloplanktis TAC125 genome encodes a functional GSP protein secretion system*

In a recent paper (Parrilli et al., 2008), we described the construction of a *P. haloplanktis* TAC125 genomic mutant strain in which GSP-dependent secretion was completely abolished. This mutant was obtained by insertional mutagenesis of *gspE* gene that encodes a specialized ATPase whose inactivation in other Gram-negative bacteria resulted in the total loss of T2SS functionality (Sandkvist et al., 1995). When grown at 4 °C, the *P. haloplanktis* TAC125 *gspE* mutant strain resulted to secrete a reduced number of proteases with respect to the wild type strain (Parrilli et al., 2008), thus demonstrating the cold-adapted GSP functionality at 4 °C.

In order to asses the GSP functionality in different environmental conditions, the *P. haloplanktis* TAC125 *gspE* mutant strain grown at 15 °C in rich medium, and concentrated culture supernatants of *P. haloplanktis* TAC125 wild type and *gspE* mutant were analyzed by SDS-PAGE (Fig. 2). The analysis of the extra-cellular protein content demonstrated that also in these growth conditions the *gspE* mutant actually secretes lower amounts of proteins with respect to the wild type strain (Fig. 2). Extracellular protein secretion is however not completely abolished in the *gspE* mutant strain, thus supporting the functionality of other secretion pathways different from T2SS in *P. haloplanktis* TAC125.

The extra-cellular protease secretion in *P. haloplanktis* TAC125 *gspE* mutant strain was investigated by gelatin zymography of concentrated culture supernatants. As shown in Fig. 3, the *gspE* mutant sample contains a notably reduced number of proteolytic activities as compared to the protease content of wild type sample.

To exclude that the inability of *P. haloplanktis* TAC125 *gspE* mutant cells to secrete proteases was due to a polar effect on expression of *gspE* neighboring genes, a complementation experiment was carried out. The complementing plasmid, pUCRD*gspE*, was constructed to restore the GspE function in *P. haloplanktis* TAC125-*gspE* cells. This plasmid contains the DNA sequence encoding *gspE* gene under the control of a regulated promoter (Papa et al., 2007), which function is induced by L-malate.

The mutant strain was transformed with pUCRD*gspE* plasmid and the recombinant cells were grown at 15 °C in rich medium in the presence of 0.1% malate. The extra-cellular protease secretion in *P. haloplanktis* TAC125 *gspE* (pUCRD*gspE*) complemented strain was investigated by gelatin zymography of concentrated culture supernatants. As shown in Fig. 3, the secretion of the psychrophilic proteases is partially restored in *P. haloplanktis* TAC125 *gspE* complemented cells. The partial recovery of protease secretion is likely a consequence of the altered stoichiometry of Type II protein complex components.

3.3. *Transcriptional analysis of GSP cluster from P. haloplanktis TAC125*

As previously discussed, GSP system is one of the most widespread protein secretion systems in Gram-negative bacteria (Cianciotto, 2005). This observation may reflect an early appearance of this system during bacteria evolution, associated to its efficiency in protein translocation.

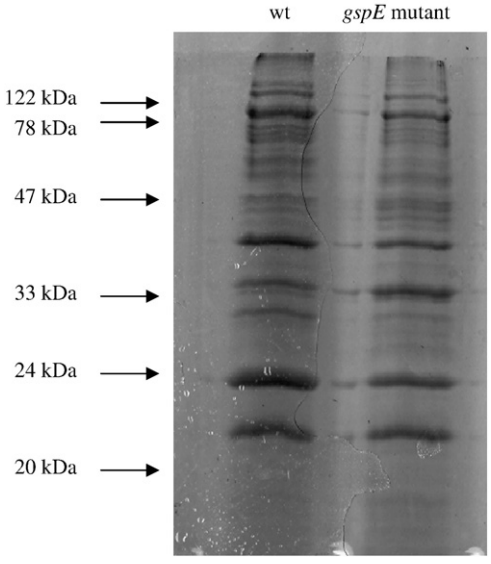

Fig. 2. 12% SDS-PAGE analysis of tenfold concentrated culture supernatants of *P. haloplanktis* TAC125 wild type (wt) and *gspE* mutant cells (*gspE* mutant) grown in rich medium at 15 °C.

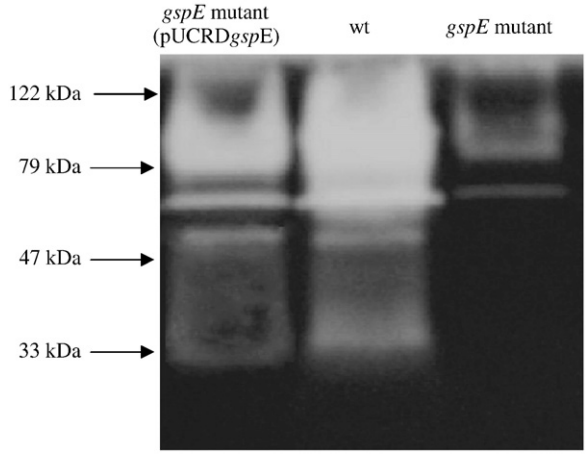

Fig. 3. Zymography of ten fold concentrated supernatants withdrawn from *P. haloplanktis* TAC125 wild type, *P. haloplanktis* TAC125 *gspE* mutant, and *P. haloplanktis* TAC125 *gspE* mutant-(pUCRD*gspE*) cultures at middle exponential phase. In this experiment the zymographic developing time was 18 h, a condition that assures the detection of all proteases contained in the sample.

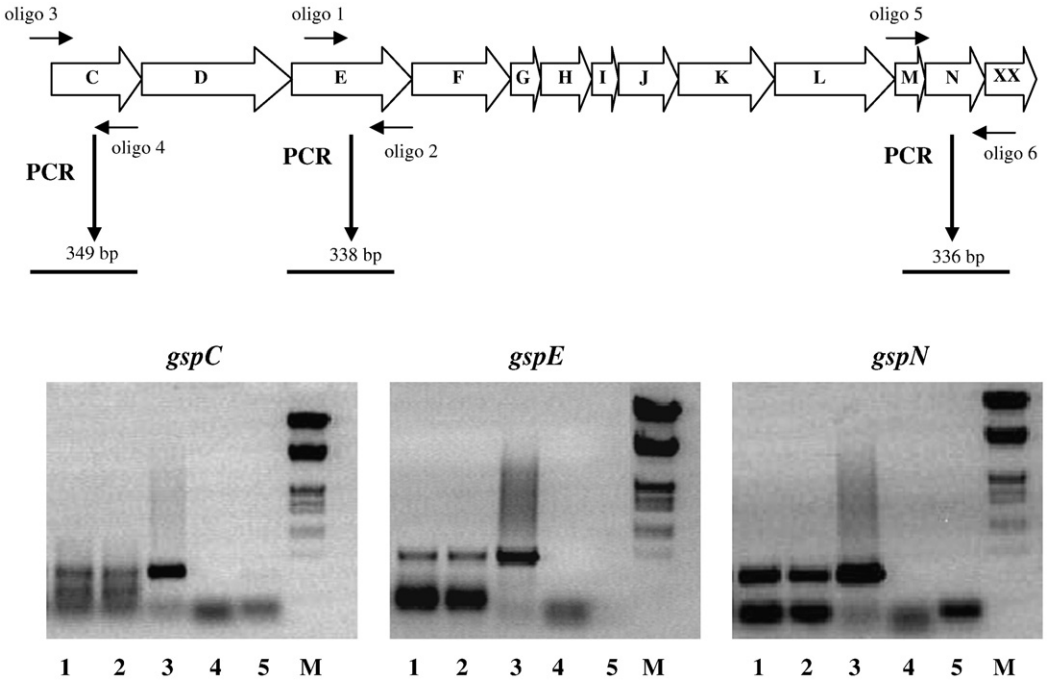

Fig. 4. Non quantitative Reverse Transcriptase RT-PCR analysis of *gspC*, *gspE*, and *gspN* genes in *P. haloplanktis* TAC125 cells. 1, RT-PCR analysis of sample withdrawn at 15 h; 2, RT-PCR analysis of sample withdrawn at 47 h; 3, positive control, PCR reaction carried out on *P. haloplanktis* TAC125 genomic DNA; 4, negative control, PCR reaction carried on *P. haloplanktis* TAC125 DNA-free total RNA; 5, PCR negative control; M, broad range DNA ladder. The Fig. contains schematic representations of the expected PCR products, where black arrows indicate the annealing position of oligonucleotide primers (see Table 1) used for PCR experiments.

Although extremely conserved in structure/genetic organization, T2SS functionality can be subjected to a wide array of regulatory networks, often associated to the specific role to be exerted by the secreted passenger(s). Therefore, reports can be found in literature either on constitutively expressed T2SS machineries or on GSP secretion systems which function is actually regulated in a growth-phase-dependent manner as a result of quorum sensing or auto induction mechanisms (Sandkvist, 2001).

In order to asses if in *P. haloplanktis* TAC125 the *gsp* genes expression is growth-phase-dependent, a Reverse Transcriptase (RT)-

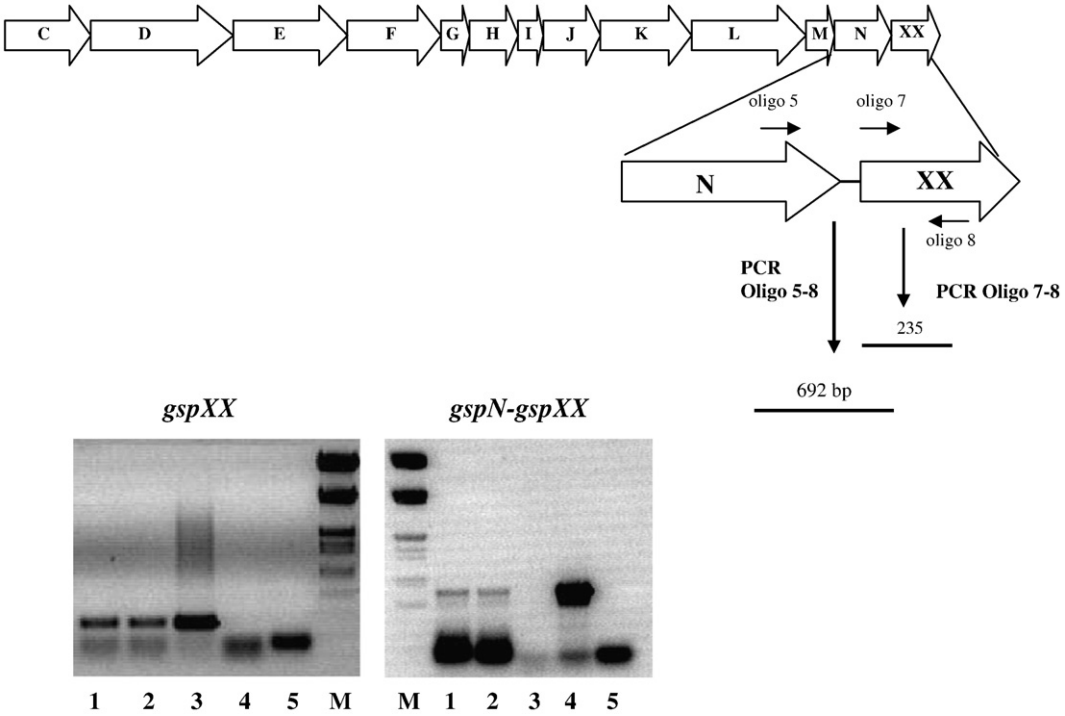

Fig. 5. Non quantitative Reverse Transcriptase RT-PCR analysis of *gspXX* gene. Panel *gspXX*: 1, RT-PCR analysis of sample withdrawn at 15 h; 2 RT-PCR analysis of sample withdrawn at 47 h; 3, positive control, PCR reaction carried out on *P haloplanktis* TAC125 genomic DNA; 4, negative control, PCR reaction carried on *P haloplanktis* TAC125 DNA-free total RNA; 5 PCR negative control; M, broad range DNA ladder. Panel *gspXX–gspN*: non quantitative Reverse Transcriptase RT-PCR analysis of the region between *gspN* and *gspXX* genes. Lanes 1 to M, see legend of Panel *gspXX*. The figure contains schematic representations of expected PCR products where black arrows indicate the annealing position of oligonucleotides primers (see Table 1) used for PCR experiments.

PCR analysis was performed. Total RNA was extracted from cell samples withdrawn at exponential (15 h) and stationary (47 h) growth phases and was used to evaluate the transcription of *gspC*, *gspE*, and *gspN*. As shown in Fig. 4 (lanes 1 and 2), all the tested *gsp* genes resulted to be constitutively expressed in *P. haloplanktis* TAC125 cells. These results suggest that GSP functionality may be required over the development of psychrophilic bacterial culture.

The same approach was used to investigate the expression of *gspXX* gene. The Reverse Transcriptase (RT)-PCR analysis shown in Fig. 5, panel *gspXX*, demonstrates that the gene is actually expressed in the tested conditions, and that its expression is growth-phase-independent.

As previously observed, the relative position of *gspXX* and *gspN* genes suggests that they could be co-transcribed. A transcriptional analysis performed using oligonucleotides designed to amplify the region between the two genes (Fig. 5, panel *gspN–gspXX*) was carried out. The presence of a PCR product of the expected size and sequence demonstrated that *gspXX* and *gspN* genes are co-transcribed in these experimental conditions. This evidence supports the inclusion of *gspXX* into the cold-adapted *gsp* cluster, and opens new questions concerning the role played by GspXX protein in the architecture/function of the psychrophilic T2SS machinery.

4. Conclusion

The present paper reports a detailed *in silico* description of *P. haloplanktis* TAC125 T2SS gene cluster, the first one isolated from an Antarctic marine bacterium. This cluster is characterized by the notably presence of a downstream and physically associated gene (called *gspXX*) which was found only in two other Alteromonadales *gsp* gene clusters. The cold-adapted GSP machinery was demonstrated to be responsible for the extra-cellular addressing of a large number of *P. haloplanktis* TAC125 proteins, proteases amongst others. Transcriptional regulation of T2SS gene expression was also investigated, and it turned out that the tested psychrophilic *gsp* genes were constitutively transcribed during the cell growth. Furthermore, the *gspXX* transcription was verified, and the inclusion of this gene into the cold-adapted *gsp* cluster was demonstrated by the co-transcription of the *gspN–gspXX* genes. These data pointed towards the existence of a novel and previously not described component of the GSP machinery, which role should be investigated to possibly relate its presence with the adaptation to marine psychrophilic lifestyle.

Acknowledgements

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References

Bleves, S., Voulhoux, R., Michel, G., Lazdunski, A., Tommassen, J., Filloux, A., 1998. The secretion apparatus of *Pseudomonas aeruginosa*: identification of a fifth pseudopilin, XcpX (GspK family). Mol. Microbiol. 27, 31–40.

Cianciotto, N., 2005. Type II secretion: a protein secretion system for all seasons. Trends Microbiol. 13, 581–588.

de Keyzer, J., van der Does, C., Driessen, A.J., 2003. The bacterial translocase: a dynamic protein channel complex. Cell. Mol. Life. Sci. 60, 2034–2052.

DiChristina, T.J., Moore, C.M., Haller, C.A., 2002. Dissimilatory Fe(III) and Mn(IV) reduction by *Shewanella putrefaciens* requires ferE, a homolog of the *pulE* (*gspE*) type II protein secretion gene. J. Bacteriol. 184, 142–151.

Duilio, A., Tutino, M.L., Marino, G., 2004. Recombinant protein production in Antarctic Gram-negative bacteria. Methods Mol. Biol. 267, 225–237.

Egan, S., James, S., Holmstrom, C., Kjelleberg, S., 2002. Correlation between pigmentation and antifouling compounds produced by *Pseudoalteromonas tunicata*. Environ. Microbiol. 4, 433–442.

Evan, F.F., Egan, S., Kjelleberg, S., 2008. Ecology of type II secretion in marine gammaproteobacteria. Environ. Microbiol. 10, 1101–1107.

Filloux, A., 2004. The underlying mechanisms of Type-II protein secretion. Biochim. Biophys. Acta 1694, 163–179.

Galkin, A., Sarikaya, E., Lehmann, C., Howard, A., Herzberg, O., 2004. X-ray structure of HI0817 from *Haemophilus influenzae*: protein of unknown function with a novel fold. Proteins 57, 874–877.

Gorby, Y.A., Yanina, S., McLean, J.S., Rosso, K.M., Moyles, D., Dohnalkova, A., 2006. Electrically conductive bacterial nanowires produced by *Shewanella oneidensis* strain MR-1 and other microorganisms. Proc. Natl. Acad. Sci. U. S. A. 103, 11358–11363.

Hanahan, D., 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166, 557–580.

Hu, N.T., Leu, W.M., Lee, M.S., Chen, A., Chen, S.C., Song, Y.L., Chen, L.Y., 2002. XpsG, the major pseudopilin in *Xanthomonas campestris* pv. *campestris*, forms a pilus-like structure between cytoplasmic and outer membranes. Biochem. J. 365, 205–211.

Lee, H.M., Tyan, S.W., Leu, W.M., Chen, L.Y., Chen, D.C., Hu, N.T., 2001. Involvement of the XpsN protein in formation of the XpsL–xpsM complex in *Xanthomonas campestris* pv. *campestris* type II secretion apparatus. J. Bacteriol. 183, 528–535.

Lee, H.M., Wang, K.C., Liu, Y.L., Yew, H.Y., Chen, L.Y., Leu, W.M., Chen, D.C., Hu, N.T., 2000. Association of the cytoplasmic membrane protein XpsN with the outer membrane protein XpsD in the type II protein secretion apparatus of *Xanthomonas campestris* pv. *campestris*. J. Bacteriol. 182, 1549–1557.

Lee, M.S., Chen, L.Y., Leu, W.M., Shiau, R.J., Hu, N.T., 2005. Associations of the major pseudopilin XpsG with XpsN (GspC) and secretin XpsD of *Xanthomonas campestris* pv. *campestris* type II secretion apparatus revealed by cross-linking analysis. J. Biol. Chem. 280, 4585–4591.

Marciano, D.K., Russel, M., Simon, S.M., 1999. An aqueous channel for filamentous phage export. Science 284, 1516–1519.

Médigue, C., Krin, E., Pascal, G., Barbe, V., Bernsel, A., Bertin, P.N., Cheung, F., Cruveiller, S., D'Amico, S., Duilio, A., Fang, G., Feller, G., Ho, C., Mangenot, S., Marino, G., Nilsson, J., Parrilli, E., Rocha, E.P.C., Rouy, Z., Sekowska, A., Tutino, M.L., Vallenet, D., von Heijne, G., Danchin, A., 2005. Coping with cold: the genome of the versatile marine Antarctica bacterium *Pseudoalteromonas haloplanktis* TAC125. Genome Res. 15, 1325–1335.

Michel, G., Bleves, S., Ball, G., Lazdunski, A., Filloux, A., 1998. Mutual stabilization of the XcpZ and XcpY components of the secretory apparatus in *Pseudomonas aeruginosa*. Microbiology 144, 3379–3386.

Nunn, D.N., Lory, S., 1993. Cleavage, methylation, and localization of the *Pseudomonas aeruginosa* export proteins XcpT, -U, -V, and -W. J. Bacteriol. 175, 4375–4382.

Pallen, M.J., Ponting, C.P., 1997. PDZ domains in bacterial proteins. Mol. Microbiol. 26, 411–413.

Papa, R., Rippa, V., Sannia, G., Marino, G., Duilio, A., 2007. An effective cold inducible expression system developed in *Pseudoalteromonas haloplanktis* TAC125. J. Biotechnol. 127, 199–210.

Parrilli, E., De Vizio, D., Cirulli, C., Tutino, M.L., 2008. Development of an improved *Pseudoalteromonas haloplanktis* TAC125 strain for recombinant protein secretion at low temperature. Microbial Cell Factories, vol. 7, p. 2.

Peabody, C.R., Chung, Y.J., Yen, M.R., Vidal-Ingigliardi, D., Pugsley, A.P., Saier, M.H., 2003. type II protein secretion and its relationship to bacterial type IV pili and archaeal flagella. Microbiology 149, 3051–3072.

Pugsley, A.P., Dupuy, B., 1992. An enzyme with type IV prepilin peptidase activity is required to process components of the general extra-cellular protein secretion pathway of *Klebsiella oxytoca*. Mol. Microbiol. 6, 751–760.

Sambrook, J., Russell, D.W., 2001. Molecular Cloning in A Laboratory Manual, 3rd Ed. Edited by Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sandkvist, M., 2001. Biology of type II secretion. Mol. Microbiol. 40, 271–283.

Sandkvist, M., Bagdasarian, M., Howard, S.P., DiRita, V.J., 1995. Interaction between the autokinase EpsE and EpsL in the cytoplasmic membrane is required for extracellular secretion in Vibrio cholerae. EMBO J. 14, 1664–1673.

Sauvonnet, N., Vignon, G., Pugsley, A.P., Gounon, P., 2000. Pilus formation and protein secretion by the same machinery in *Escherichia coli*. EMBO J. 19, 2221–2228.

Soderberg, M.A., Rossier, O., Cianciotto, N.P., 2004. The type II protein secretion system of *Legionella pneumophila* promotes growth at low temperatures. J. Bacteriol. 186, 3712–3720.

Strom, M.S., Nunn, D.N., Lory, S., 1993. A single bifunctional enzyme,PilD, catalyzes cleavage and N-methylation of proteins belonging to the type IV pilin family. Proc. Natl. Acad. Sci. U. S. A. 90, 2404–2408.

Tascon, R.I., Rodriguez-Ferri, E.F., Gutierrez-Martin, C.B., Rodriguez-Barbosa, I., Berche, P., Va zquez-Boland, J.A., 1993. Transposon mutagenesis in *Actinobacillus pleuropneumoniae* with a Tn10 derivative. J. Bacteriol. 175, 5717–5722.

Tosco, A., Birolo, L., Madonna, S., Lolli, G., Sannia, G., Marino, G., 2003. GroEL from the psychrophilic bacterium *Pseudoalteromonas haloplanktis* TAC 125: molecular characterization and gene cloning. Extremophiles 7, 17–28.

Tsai, R.T., Leu, W.M., Chen, L.Y., Hu, N.T., 2002. A reversibly dissociable ternary complex formed by XpsL, XpsM and XpsN of the *Xanthomonas campestris* pv. *campestris* type II secretion apparatus. Biochem. J. 367, 865–871.

Tutino, M.L., Parrilli, E., Giaquinto, L., Duilio, A., Sannia, G., Feller, G., Marino, G., 2002. Secretion of alpha-amylase from *Pseudoalteromonas haloplanktis* TAB23: two different pathways in different hosts. J. Bacteriol. 184, 5814–5817.

Voulhoux, R., Ball, G., Ize, B., Vasil, M.L., Lazdunski, A., Wu, L.F., Filloux, A., 2001. Involvement of the twin-arginine translocation system in protein secretion via the type II pathway. EMBO J. 20, 6735–6741.

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Poster Presentation

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Cell engineering of *Pseudoalteromonas haloplanktis* TAC125: construction of a mutant strain with reduced exo-proteolytic activity

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Background

We have already shown that using cold-adapted bacteria as host vectors, some "intractable" proteins can be efficiently produced at temperature as low as 4°C [1,2]. Furthermore, we set up a "cold" gene-expression system implemented for the secretion of recombinant proteins in the Antarctic Gram-negative bacterium *Pseudoalteromonas haloplanktis* TAC125 (*Ph*TAC125). Such a system could effectively conjugate the positive effect of low temperature on the recombinant product solubility with the obvious advantages linked to extra-cellular protein targeting. This novel system makes use of the psychrophilic α-amylase from *Ph*TAB23 [3] as secretion carrier. Several chimerical proteins were produced and used to test the versatility and efficiency of the novel secretion system. All the chimerical proteins were efficiently produced and secreted (Cusano AM, Ph. D thesis 2005 Università di Napoli "Federico II"). However, bacteria belonging to *Pseudoalteromonas* genus are reported to secrete a wide range of exo-proteins, especially proteases. This feature could hamper both applicability and efficiency of the cold-adapted secretion system, due to the possible recombinant product degradation.

The *Ph*TAC125 genome sequence [4] was recently determined. The *in silico* genome analysis highlighted the presence of a putative Type II secretion system (T2SS), while the extra-cellular targeting of the cold α-amylase depends on a still uncharacterized secretion pathway [4].

Results

Construction of the Ph*TAC125 suicide vector*

Figure 1 shows the Vs suicide vector constructed to generate *Ph*TAC125 genomic mutants. It is characterised by the presence of: i) the pJB3-derived *oriT* (1), a DNA fragment

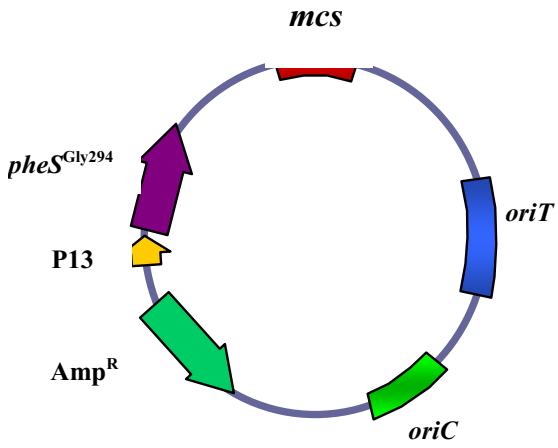

Figure 1
Schematic representation of the *Ph*TAC125 Vs suicide vector. mcs, Multiple cloning site; *oriT*, origin of conjugative transfer [1]; *oriC* and Amp^R, origin of replication and beta-lactamase encoding gene from the pUC18 vector; P13, synthetic psychrophilic promoter; $pheS^{Gly294}$, *E. coli* gene encoding a mutated version of the α subunit of Phe-tRNA synthase.

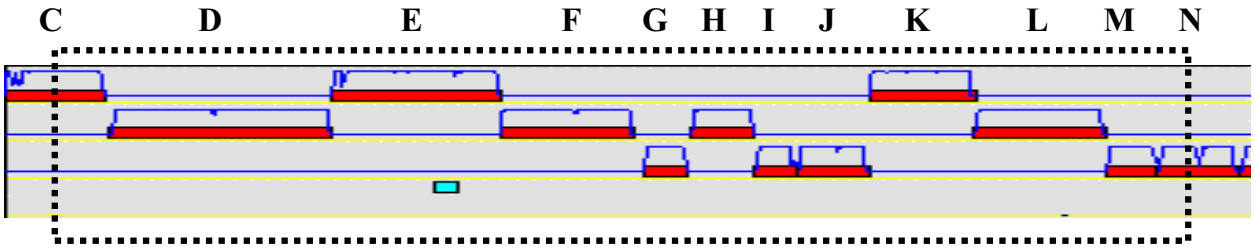

Figure 2
Genetic organization of the *Ph*TAC125 *gsp* cluster and *gspCN* deletion. The *Ph*TAC125 *gsp*$^-$ mutant was generated by deleting a genomic region corresponding to that displayed into the dotted rectangle.

responsible for the initiation of the conjugative transfer between an *Escherichia coli λpir* strain (donor) and the psychrophilic cells (acceptor); ii) the *E.coli blaM* gene, encoding a mesophilic β-lactamase which is used as selection gene to isolate the first site-specific integration event; iii) *pheS*^{Gly294}, which encodes a mutated version of the *E. coli* α subunit of Phe-tRNA synthase [5], which renders bacteria sensitive to *p*-chlorophenylalanine. This phenylalanine analog is used as counterselective agent for the isolation of those strains in which a second recombination event occurred. To assure a proper level of *pheS*^{Gly294} expression, its transcription was subjected to the control of a psychrophilic synthetic promoter (P13).

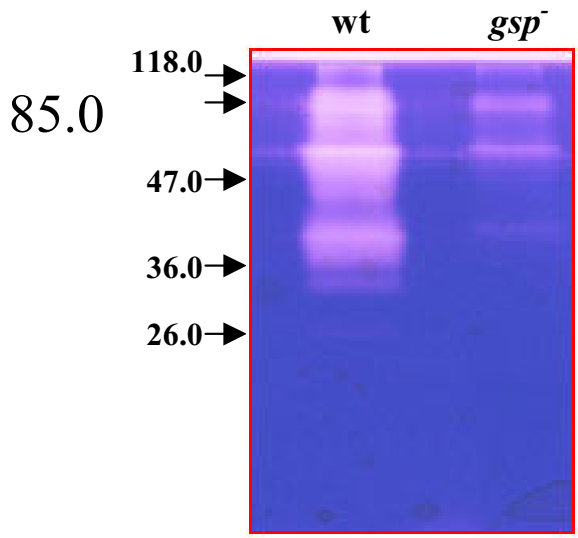

Figure 3
Gelatin zymography of *Ph*TAC125 wt and *gsp*$^-$ supernatants. Psychrophilic cells were grown in TYP medium at 4°C till late exponential phase. Culture supernatants were recovered by culture centrifugation, 10 times concentrated and loaded onto a 10% SDS-PAGE containing bovine gelatin. After the electrophoresis run, the gel was washed to remove the Na-SDS and incubated in the development solution overnight at 15°C. Finally the gel is stained with Comassie blue and destained. Molecular weight markers were marked in kDa.

Construction of a Ph*TAC125* gsp$^-$ *[Δ*gspCN*] strain*

To inactivate the T2SS pathway in *Ph*TAC125 (Figure 2), a deletion strategy was applied. Two genomic fragments were PCR amplified by using specific oligonucleotides as primers. They correspond to the 5' 360 bp portion of *gspC* and 3' 300 bp portion of *gspN* respectively. The fragments were suitably digested and cloned into the Vs vector. The resulting vector (VsCN) was mobilized by intergeneric conjugation [1] into *Ph*TAC125, and the cells were plated at 4°C on TYP solid medium containing 30 μg/ml carbenicillin to select those in which a single recombination event occurred. Second recombination event was induced by repeated plating of mutant psychrophilic cells at 4°C on minimum solid medium containing 20 mM *p*-ClPhe. The occurrence of the correct deletion was checked by sequencing the specific PCR fragments.

Phenotypic characterization of Ph*TAC125* gsp$^-$ *strain*

The global exo-proteolytic activity of the *Ph*TAC125 *gsp*$^-$ strain was analyzed by *in gel* zymography and compared to that of the wild type strain. As shown in figure 3, culture supernatant of *gsp*$^-$ strain contains a reduced number of exo-proteases.

Conclusion

We report here a cell engineering approach to the construction of a *Ph* TAC125 strain with reduced exo-protease activity. By applying a gene-placements strategy, we obtained a mutant strain in which the gene cluster encoding the T2SS was almost totally deleted. While the growth behavior and some physiological features of the *gsp*$^-$ mutant are indistinguishable from the wild type ones, the deleted strain displays a remarkable reduction in the pro-

tease content in the culture supernatant. This aspect makes the *Ph*TAC125*gsp*⁻ mutant a promising host for the recombinant secretion into the host extra-cellular medium of proteins with biotechnological potential.

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References

1. Duilio A, Tutino ML, Marino G: **Recombinant protein production in Antarctic Gram negativebacteria.** *Methods Mol Biol* 2004, **267:**225-237.
2. Duilio A, Marino G, Mele A, Sannia G, Tutino ML: **Ufficio Italiano Brevetti e Marchi n. RM2003/A000155.** .
3. Tutino ML, Duilio A, Parrilli E, Remaut E, Sannia G, Marino G: **A novel replication element from an Antarctic plasmid as a tool for the expression of proteins at low temperature.** *Extremophiles* 2001, **5:**257-264.
4. Médigue C, Krin E, Pascal G, Barbe V, *et al.*: **Coping with cold: the genome of the versatile marine Antarctica bacterium *Pseudoalteromonas haloplanktis* TAC125.** *Genome Research* 2005, **15:**1325-35.
5. Kast P: **pKSS – a second-generation general purpose cloning vector for efficient positive selection of recombinant clones.** *Gene* 1994, **138:**109-14.

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PssA is required for α-amylase secretion in Antarctic *Pseudoalteromonas haloplanktis*

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Extracellular protein secretion is an essential feature in bacterial physiology. The ability to efficiently secrete diverse hydrolytic enzymes represents a key nutritional strategy for all bacteria, including micro-organisms living in extreme and hostile habitats, such as cold environments. However, little is known about protein secretion mechanisms in psychrophilic bacteria. In this study, the recombinant secretion of a cold-adapted α-amylase in the Antarctic Gram-negative *Pseudoalteromonas haloplanktis* TAC125 was investigated. By a combination of several molecular techniques, the function of the *pssA* gene was related to α-amylase secretion in this psychrophilic bacterium. Deletion of the *pssA* gene completely abolished amylase secretion without affecting the extracellular targeting of other substrates mediated by canonical secretion systems. The *pssA* gene product, PssA, is a multidomain lipoprotein, predicted to be localized in the bacterial outer membrane, and displaying three TPR (tetratricopeptide repeat) domains and two LysM modules. Based on functional annotation of these domains, combined with the experimental results reported herein, we suggest a role for PssA as a molecular adaptor, in charge of recruiting other cellular components required for specific α-amylase secretion. To the best of our knowledge, no proteins exhibiting the same domain organization have previously been linked to protein secretion.

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INTRODUCTION

Protein export systems are present in all living organisms. Bacteria have evolved several complex systems for protein export. Didermic bacteria face a special challenge in this regard, since secreted proteins must cross the inner membrane, the periplasmic space, and the outer membrane. Secretion of proteins takes place via a variety of mechanisms, from simple one-component systems to complex multi-component pathways (Desvaux *et al.*, 2009; Johnson *et al.*, 2006).

This wide diversity in the structure of protein secretion systems can be interpreted in the light of the central role played by extracellular protein secretion in bacterial physiology. Secreted proteins provide the means by which micro-organisms interact with and modify their surrounding environment, also a crucial aspect necessary for survival in hostile extreme habitats, such as Antarctica. Although several cold-adapted exoenzymes have been characterized, the small amount of information available on the mechanisms responsible for their extracellular addressing has come from our laboratory (Cusano *et al.*, 2006a, b; Parrilli *et al.*, 2008b, 2009; Tutino *et al.*, 2001). The development of a cold-adapted expression system (Parrilli *et al.*, 2008a) paved the way to investigate the cellular machineries devoted to protein secretion in Antarctic bacterial hosts. The α-amylase from *Pseudoalteromonas haloplanktis* TAB23 (Feller *et al.*, 1992) was chosen as a model enzyme. This enzyme is synthesized as a preproenzyme, composed of a signal peptide, the mature enzyme (49 kDa), and a C-terminal propeptide (21 kDa), which constitutes a structurally independent domain that neither exhibits any foldase function nor affects the amylase catalytic activity (Feller *et al.*, 1998). The export of the α-amylase precursor through the inner membrane likely occurs via the Sec pathway (Cao & Saier, 2003; Feller *et al.*, 1998). Once in the periplasmic space, α-amylase undergoes folding into its catalytically competent form (Feller *et al.*,

Abbreviations: T2SS, type II secretion system; TPR, tetratricopeptide repeat.

Supplementary material is available with the online version of this paper.

1998). Subsequently, secretion machinery drives its extracellular translocation. The proenzyme is found in the *P. haloplanktis* TAB23 culture supernatant as a precursor until the late exponential growth phase, when the action of an extracellular protease removes the C-terminal domain (Feller *et al.*, 1998). Although the C-terminal propeptide sequence is not homologous to that of β-autotransporters (Henderson *et al.*, 2004), in a previous paper (Feller *et al.*, 1998) it was suggested that it may function as a protein secretion helper when amylase is produced in *Escherichia coli*. However, different results were obtained when the secretion of recombinant α-amylase and its truncated version, devoid of the C-terminal domain, was investigated either in the enzyme source strain, P. haloplanktis TAB23, or in another Antarctic bacterium, *P. haloplanktis* TAC125 (Tutino *et al.*, 2002). It was demonstrated that the C-terminal propeptide is not mandatory for α-amylase secretion and that the presence of the C-terminal domain does not interfere either with the secretion kinetics or with the maximal secretion and production yield (Tutino *et al.*, 2002).

In the light of the above results, we decided to study the secretion of the truncated α-amylase (hereafter, 'α-amylase' indicates the protein without C-terminal domain). This approach was chosen to simplify data analysis and interpretation, since it allows ruling out the occurrence of C-terminal-domain-mediated effects on protein secretion, if any. α-Amylase secretion was studied in *P. haloplanktis* TAC125. Several reasons prompted us to use this bacterium as recombinant host: (i) it is the first Antarctic micro-organism whose genome has been sequenced and annotated (Médigue *et al.*, 2005); (ii) in this bacterium, recombinant α-amylase secretion occurs in a way similar to that in the source strain (Tutino *et al.*, 2001, 2002); (iii) *P. haloplanktis* TAC125 is devoid of endogenous α-amylase activity (Tutino *et al.*, 2001); and (iv) the cold-adapted α-amylase was successfully used as secretion carrier in a cold gene-expression system for secretion of heterologous proteins in *P. haloplanktis* TAC125 (Cusano *et al.*, 2006b; Parrilli *et al.*, 2008b).

The *in silico* analysis of the *P. haloplanktis* TAC125 genome (Médigue *et al.*, 2005) demonstrated that the bacterium possesses, besides specialized machineries for secretion of type IV pili and curli components, only one other secretion system: a type II secretion system (T2SS) (Parrilli *et al.*, 2009) homologous to already described T2SSs in many other Gram-negative bacteria (Johnson *et al.*, 2006). This secretion system is not involved in recombinant α-amylase secretion in *P. haloplanktis* TAC125 (Parrilli *et al.*, 2008b). Indeed, a *P. haloplanktis* TAC125 mutant strain in which the T2SS was knocked out was able to specifically secrete the cold-adapted α-amylase like the wild-type strain (Parrilli *et al.*, 2008b), thus suggesting the occurrence of an as yet uncharacterized secretion pathway.

In this paper we report the identification of a cold-adapted protein required for α-amylase secretion in *P. haloplanktis* TAC125.

METHODS

Bacterial strains and growth condition. *P. haloplanktis* TAC125 (Médigue *et al.*, 2005) was isolated from Antarctic sea water. *E. coli* DH5α (Hanahan, 1983) was used as host for the gene cloning. *E. coli* EPI100 [F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74* *recA1 endA1 araD139* Δ(*ara*, *leu*)7697 *galU galK* λ^- *rpsL nupG*] was used as host for the cosmid library screening and for *in vitro* complementation.

P. haloplanktis TAC125 was grown in aerobic conditions at 15 °C in TYP broth at pH 7.5, supplemented with ampicillin (200 μg ml^{-1}) or chloramphenicol (50 μg ml^{-1}) if transformed. *E. coli* was routinely grown in Luria–Bertani broth (Sambrook & Russell, 2001) at 20 °C or at 37 °C supplemented with ampicillin (100 μg ml^{-1}), chloramphenicol (50 μg ml^{-1}) or kanamycin (50 μg ml^{-1}) if transformed. Transformation of *P. haloplanktis* was achieved by intergeneric conjugation (Parrilli *et al.*, 2008a).

Construction and screening of cosmid library. Construction of a cosmid library of *P. haloplanktis* TAC125 genomic DNA was performed as described in the pWEB cosmid cloning kit EPICENTRE brochure. The number of cosmid clones required to ensure that any given *P. haloplanktis* TAC125 DNA sequence is contained within the library is 420; the calculated total number of independent clones contained within the constructed *Ph*TAC125 cosmid library was 616. The cosmid clones were transformed by electroporation with pBBR*amy*ΔCt and plated on selective LB plates containing 100 μg ampicillin ml^{-1}, 50 μg chloramphenicol ml^{-1} and 1 % soluble starch. The plates were incubated at 20 °C and colonies able to secrete amylase were detected by the amylase activity test (Filloux *et al.*, 1985). The selected clones were grown in liquid culture at 20 °C in LB medium with 100 μg ampicillin ml^{-1} and 50 μg chloramphenicol ml^{-1}. The amylase activity was tested on the culture medium of each clone. The amylase secretion yield of clones displaying higher amylase activity was evaluated by assaying amylase and β-lactamase activity in the extracellular medium and corresponding intracellular extracts.

General techniques for plasmid construction. Standard methods were employed for DNA manipulation and isolation, amplification by PCR, and DNA sequencing (Sambrook & Russell, 2001; Ausubel *et al.*, 1994).

Construction of pBBR*amy*ΔCt vector. To produce the *P. haloplanktis* TAB23 α-amylase lacking its C-terminal propeptide in *E. coli* EPI100 cells an expression vector, pBBR*amy*ΔCt, was synthesized. The DNA fragment containing the *amy*ΔCt gene under control of the *lacZ* promoter was excised by *Tfi*I/*Xba*I double digestion of pαH12wt* (Feller *et al.*, 1998) followed by enzymic reaction to fill protruding ends. The DNA fragment was inserted into *Acc*I-digested pBBR122 (Antoine & Locht, 1992) after fill-in of protruding ends.

Construction of cosmid subclones. ***Subclone 1.*** 12-26 cosmid DNA was digested with *Bgl*II and derived DNA fragments were loaded in a 0.7 % agarose gel and separated by electrophoresis. The 9201 bp DNA fragment was purified from the gel and cloned into pUC18 vector (Norrander *et al.*, 1983) previously digested with *Bgl*II.

Subcone 2. To obtain a subclone containing the cosmid region between the PSHAb0127 and PSHAb0133 genes, 12-26 cosmid DNA was digested with *Bst*1107I and the protruding ends obtained were subjected to intramolecular ligation.

Subclone 3. 12-26 cosmid DNA was digested with *Ava*I and the DNA fragments obtained were loaded in a 0.7 % agarose gel and separated by electrophoresis. The 9268 bp DNA fragment was purified from the gel and cloned into pGEM vector previously digested with *Ava*I.

Subclone 4. PSHAb0139 and PSHAb0140 were amplified from *P. haloplanktis* TAC125 genomic DNA in two separate PCRs. Primers used to amplify the first fragment of 1102 bp were designed to introduce a *Sph*I restriction site and to cover the natural *Pvu*II restriction site (oligo 140PvuII forward and oligo 140SphI reverse; see Supplementary Table S1, available with the online version of this paper). Primers used to amplify the second fragment of 1646 bp were designed to introduce an *Eco*RI restriction site and to cover the natural *Pvu*II restriction site (oligo 139EcoRI forward and oligo 140PvuII reverse; see Supplementary Table S1). The two fragments were digested with *Pvu*II/*Sph*I and *Eco*RI/*Pvu*II respectively and ligated into pUC18 (*Sph*I/*Eco*RI).

Subclone 5. The 12-26 cosmid DNA was digested with *Bam*HI and the DNA fragments obtained were loaded in a 0.7 % agarose gel and separated by electrophoresis. The 13580 bp DNA fragment was purified from the gel and cloned into pUC18 vector previously digested with *Bam*HI.

Construction of pFCamyΔCt-*pssA* vector. To complement *pssA* function in *P. haloplanktis* TAC125-Δ*pssA* mutant cells the expression vector pFC*amy*ΔCt-*pssA* was constructed. The 2504 bp region including the *pssA* gene and its upstream region was excised by *Fsp*I digestion of subclone 4 and then ligated into the α-amylase psychrophilic expression vector pFC*amy* (Cusano *et al.*, 2006b) digested by *Bgl*II after enzymic fill-in of protruding ends.

Amplification of the *P. haloplanktis* TAB23 *pssA* gene homologue. Two internal primers 43F and 43R (Supplementary Table S1), designed to amplify a 595 bp fragment of the *pssA* gene, were used to perform a PCR amplification on the *P. haloplanktis* TAB23 genome. The sequence of the amplified fragment was determined and aligned in the BLAST nucleotide database.

Protein electrophoresis and immunoblotting. Protein samples were analysed by SDS-PAGE (12 %, w/v, acrylamide) according to standard methods (Ausubel *et al.*, 1994). For immunoblotting, the proteins were transferred to a PVDF membrane (Millipore). For immunodetection of proteins, *P. haloplanktis* TAB23 anti-α-amylase (Feller *et al.*, 1998) or anti-β-lactamase antisera were diluted in blocking buffer (phosphate-buffered saline, 5 % skimmed milk). Peroxidase-conjugated anti-rabbit IgG (Sigma-Aldrich) was used as secondary antibody. Proteins were detected by chemiluminescence (Pierce).

Zymographic assay. *P. haloplanktis* TAC125 wild-type and *P. haloplanktis* TAC125-Δ*pssA* mutant strains were grown at 15 °C in standard conditions and the zymographic assay was performed as previously reported (Parrilli *et al.*, 2008b).

Enzymic assays. α-Amylase activity was assayed by using the Boehringer-Roche kit AMYL under the conditions previously reported (Feller *et al.*, 1998). β-Lactamase activity was assayed according to O'Callaghan *et al.* (1972).

RESULTS

Isolation of a cosmid clone containing the *P. haloplanktis* TAC125 genomic fragment responsible for the recombinant α-amylase secretion

To identify the genes encoding the secretion machinery responsible for the extracellular addressing of the cold-adapted α-amylase in *P. haloplanktis* TAC125, an *in vivo* heterologous complementation assay was set up.

Since *E. coli* cannot secrete the cold-adapted α-amylase devoid of its C-terminal propeptide (Feller *et al.*, 1998), this bacterium was used as a host for screening the *P. haloplanktis* TAC125 genomic DNA cosmid library, looking for cold-adapted gene product(s) allowing secretion of the α-amylase in the mesophilic host. The use of a cosmid library was appropriate because secretion pathway genes are usually organized in large gene clusters.

The cosmid library of randomly generated *P. haloplanktis* TAC125 genomic DNA fragments (~40 kb long) was obtained using pWEB cosmid cloning kit and the recombinant cosmids were transferred into recipient cells already containing an expression vector for production of the α-amylase (pBBR*amy*ΔCt). The pBBR*amy*ΔCt plasmid was synthesized by cloning the *amy*ΔCt gene in the pBBR122 vector. This broad-host-range vector was chosen since its replication is compatible with the replication of the pWEB vector, while it carries two selection genes, which confer resistance to chloramphenicol and kanamycin, respectively. *E. coli* cells co-transformed with pBBR*amy*ΔCt and pWEB cosmid library clones were plated at 20 °C on a selective solid medium containing starch. In these conditions, an amylolytic halo will only surround colonies that can secrete an amylase activity in the extracellular medium.

About 600 clones were screened on plates, evaluating the size of the colonies versus the haloes, and 60 clones displaying wider amylolytic haloes were grown in liquid culture at 20 °C. Amylase activity was tested in the culture medium of each clone. Nine clones displaying higher amylase activity were subjected to further analysis. The amylase and β-lactamase activities were assayed in the extracellular medium and corresponding intracellular extracts of the nine clones (data not shown) to determine the amylase secretion yield (expressed as the percentage of total enzymic activity detected in the extracellular medium). The localization of the periplasmic β-lactamase was monitored to rule out that the extracellular targeting of the recombinant cold-adapted α-amylase was due to host outer membrane leakage. One clone (*E. coli* clone 12-26) passed this last screening step, being able to secrete the α-amylase into the culture medium with a secretion yield of about 35 %, under conditions where we did not detect significant amounts of β-lactamase (Table 1). Therefore, the production of the heterologous cold-adapted proteins encoded by the 12-26 cosmid insert did not alter the stability of *E. coli* cell envelope, but conferred amylase secretion ability to the *E. coli* cells (Table 1).

The sequencing of the *P. haloplanktis* TAC125 genomic DNA insert contained in the 12-26 cosmid locates this fragment as the region between bp positions 152464 and 189746 of *P. haloplanktis* TAC125 chromosome b, containing the CDSs from PSHAb0127 and PSHAb0152 (see Supplementary Fig. S1A, Supplementary Table S2, and [12]

Table 1. Extracellular α-amylase and β-lactamase activities in *E. coli* EPI100/pBBR*amy*ΔCt cells transformed with the 12-26 cosmid (*E. coli*/pBBR*amy*ΔCt + 12-26) at different growth phases

Enzyme activities are expressed as percentages of the total activity recovered in cells and culture supernatants. As control, the same values referring to *E. coli* cells co-transformed with pBBR*amy*ΔCt and a non-secreting cosmid clone (13-64 cosmid) are reported. Each experiment was carried out in triplicate; means ± SD are shown.

Growth phase	*E. coli*/pBBR*amy*ΔCt + 12-26		*E. coli*/pBBR*amy*ΔCt + 13-64		*E. coli*/pBBR*amy*ΔCt + pUC18	
	α-Amy	β-Lac	α-Amy	β-Lac	α-Amy	β-Lac
Early	39 % (1.4 ± 0.1 IU)	5 % (15.8 ± 4 IU)	9 % (0.28 ± 0.1 IU)	4 % (22.4 ± 5 IU)	9 % (0.33 ± 0.3 IU)	5 % (294 ± 8 IU)
Exponential	25 % (3.3 ± 0.1 IU)	16 % (62.7 ± 5 IU)	8 % (0.33 ± 0.1 IU)	5 % (20.6 ± 2 IU)	12 % (0.69 ± 0.1 IU)	10 % (448 ± 5 IU)
Stationary	34 % (7.3 ± 0.4 IU)	15 % (48.3 ± 3 IU)	7 % (0.71 ± 0.3 IU)	5 % (19.1 ± 3 IU)	13 % (1.52 ± 0.3 IU)	15 % (862 ± 4 IU)

http://www.genoscope.cns.fr/agc/mage/psychroscope and http://bioinfo.hku.hk/PsychroList/).

The selected DNA portion does not code either for a T2SS component or for any proteins of the already characterized secretion pathways. It encodes a few proteins of known function and several proteins of unknown function (Supplementary Table S2).

Construction of 12-26 cosmid subclones and test for their ability to promote α-amylase secretion

To find out which of the genes were involved in α-amylase secretion, several subclones of the 12-26 cosmid insert were constructed. Different portions of the *P. haloplanktis* TAC125 genomic DNA region between CDS PSHAb0127 and PSHAb0152 were cloned using different strategies. These subclones are illustrated in Supplementary Fig. S1B.

Subclone 1 includes a 9201 bp fragment that contains a portion of CDS PSHAb0127, full PSHAb0128 and PSHAb0129 genes, and a large section of CDS PSHAb0130. The 9268 bp DNA fragment cloned in subclone 2 includes the region between CDS PSHAb129 and CDS PSHAb134 in addition to a portion of CDS PSHAb135. In subclone 3, the region between the PSHAb0127 and PSHAb0133 genes is present. Subclone 4 contains a genomic DNA fragment that includes CDS PSHAb0139 and CDS PSHAb0140. In Subclone 5, a 13 580 bp DNA fragment includes the region between PSHAb0141 and PSHAb0152. Attempts to clone the DNA region comprising PSHAb0135 to PSHAb0138 failed, although several cloning strategies were applied.

E. coli/pBBR*amy*ΔCt cells were transformed with each subclone, and recombinant cells were grown in liquid culture at 20 °C. Amylase activity was tested in the culture medium of each clone. The α-amylase was detected only in the extracellular medium of *E. coli* cells transformed with subclone 4. Extracellular medium and corresponding cellular extract of *E. coli*/pBBR*amy*ΔCt + Subclone 4 recombinant cells were analysed by Western blotting using anti-*Ph*TAB23 α-amylase serum (Fig. 1a) and anti-β-lactamase serum (Fig. 1b). The α-amylase was present in both extracellular and intracellular extract of *E. coli*/pBBR*amy*ΔCt + Subclone 4 cells, whereas the periplasmic β-lactamase remained confined to the intracellular extract of recombinant *E. coli* cells. These results indicate that subclone 4 confers to *E. coli* cells the ability to secrete the α-amylase.

Subclone 4 includes a DNA fragment that contains two CDSs, PSHAb0139 and PSHAb0140 (hereafter called *pssA*) (Supplementary Fig. S1B). *In silico* analysis revealed that PSHAb0139 codes for a putative transcriptional regulator, and this gene displays 28.27 % identity with the homologous *E. coli* gene *yfgA* (Supplementary Table S2). The protein encoded by CDS PSHAb0140 (hereafter called PssA) is a 577 aa lipoprotein and the sequence analysis of its leader peptide (LipoP prediction: http://www.cbs.dtu.dk/services/LipoP; Juncker *et al.*, 2003) predicted that it is transported to and inserted into the inner face of the outer membrane via the Lol lipoprotein sorting pathway (Tokuda & Matsuyama, 2004). Moreover, *in silico* analysis of the PssA amino acid sequence with the InterPro software (http://www.ebi.ac.uk/InterProScan; Zdobnov & Apweiler, 2001) predicted the presence of three tetratricopeptide repeat (TPR) domains (IPR001440), encompassing amino acid positions 40 and 160 of the protein, and of two LysM peptidoglycan-binding domains (IPR002482) at the C-terminal side. No significant similarities were found between the central domain of the protein and proteins present in international protein databases.

PssA protein, labelled as putative lytic cell-wall-binding lipoprotein (Supplementary Table S2), has no homologue in the *E. coli* proteome. As shown in Supplementary Fig. S1C, the gene cluster surrounding *pssA* is conserved in beta- and gamma-proteobacteria. Furthermore, in the genomes of many Gram-negative bacteria the CDSs ranging between PSHAb0134 and PSHAb0142 are in synteny (filled rectangles in Supplementary Fig. S1C), with the sole exception of *pssA*.

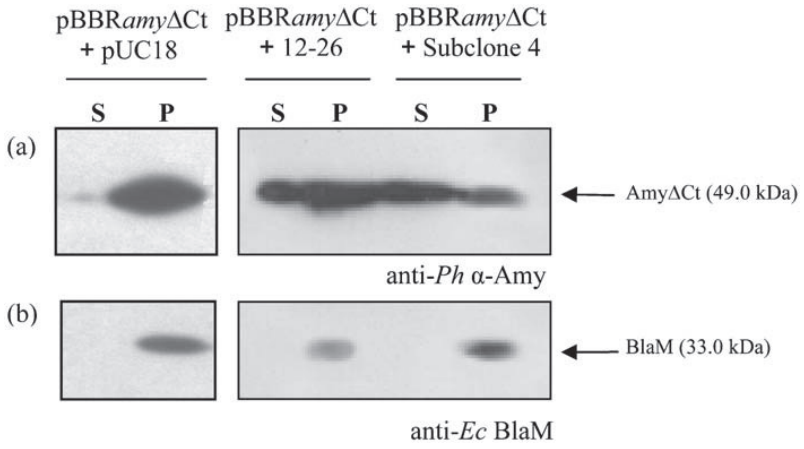

Fig. 1. Analysis of α-amylase localization in *E. coli*/pBBR*amy*ΔCt cells transformed with subclone 4. Samples from *E. coli*/pBBR*amy*ΔCt transformed with pUC18 or 12-26 cosmid were used as negative and positive controls respectively. (a) Western blot analysis with anti-*P. haloplanktis* α-amylase polyclonal antiserum of extracellular samples (S) and corresponding cellular extract (P). (b) Western blot analysis with anti-*E. coli* β-lactamase polyclonal antiserum of extracellular supernatant (S) and corresponding cellular extract (P).

Identification of *pssA* gene orthologues in *P. haloplanktis* TAB23 and other Gram-negative bacteria

A PCR experiment (data not shown) carried out on *P. haloplanktis* TAB23 genomic DNA using internal primers designed on the *pssA* sequence led to the specific amplification of a 595 bp DNA fragment. The sequence of the amplified fragment showed 99 % identity with the *pssA* sequence, indicating the presence of a PssA-coding gene homologue in the α-amylase source strain.

The PssA sequence was used as template for a homology search in protein databases, and gene products having a similar domain organization to PssA were found in three marine bacteria only: *Colwellia psychrerythraea* (Methé *et al.*, 2005), *Pseudoalteromonas tunicata* D2, (GenBank AAOH00000000) and an unclassified *Alteromonadales* strain named TW-7 (GenBank AAVS00000000).

Construction and phenotype analysis of the *P. haloplanktis* TAC125-Δ*pssA* mutant

To assess the role of PssA protein in α-amylase secretion, a *P. haloplanktis* TAC125 mutant was constructed in which the *pssA* gene was knocked out.

Functional inactivation of the *P. haloplanktis* TAC125 *pssA* gene was achieved by two-step gene deletion mutagenesis. The mutation was obtained by using a suicide vector (pVS), suitably constructed for *P. haloplanktis* TAC125 (Parrilli *et al.*, 2006, 2008b). The Δ*pssA* mutated gene is characterized by a 607 bp deletion that results in a sequence frame shift.

Total RNA was extracted from the *P. haloplanktis* TAC125-Δ*pssA* deletion mutant and subjected to reverse transcriptase RT-PCR analysis to evaluate transcription of the *pssA* and PSHAb0139 genes. The deletion mutagenesis carried out in *P. haloplanktis* TAC125-Δ*pssA* abolished *pssA* gene transcription while it did not affect PSHAb0139 gene expression (data not shown). The growth behaviour of *P. haloplanktis* TAC125-Δ*pssA* in standard conditions remained comparable to that of the wild-type strain (data not shown), suggesting that inactivation of the *pssA* gene does not affect cell viability.

To evaluate whether inactivation of the *pssA* gene may alter the general secretion ability of the bacterium in a non-specific way, for instance by interfering with a process that could indirectly impair all protein secretion mechanisms, the secretion of extracellular proteases by *P. haloplanktis* TAC125-Δ*pssA* was evaluated. This group of extracellular enzymes was analysed since it was previously demonstrated (Parrilli *et al.*, 2008b, 2009) that the majority of extracellular proteases in *P. haloplanktis* TAC125 are secreted by the type II secretory pathway. Extracellular protease secretion in *P. haloplanktis* TAC125-Δ*pssA* was investigated by gelatin zymography of culture supernatant. As shown in Fig. 2, the extracellular medium of the *P. haloplanktis* TAC125-Δ*pssA* mutant contains a number of proteolytic activities similar to those detected in the wild-type.

PssA is necessary for α-amylase secretion in *P. haloplanktis* TAC125

Secretion of cold-adapted α-amylase by *P. haloplanktis* TAC125-Δ*pssA* was investigated. The mutant strain was transformed with pFC*amy*ΔCt, a vector previously constructed for the recombinant secretion of α-amylase in *P. haloplanktis* (Cusano *et al.*, 2006b). The recombinant mutant strain was grown at 15 °C and the α-amylase secretion was evaluated by Western blot analysis of intracellular and extracellular protein samples withdrawn at different growth phases. As shown in Fig. 3(a), the α-amylase was present only in the intracellular extracts, demonstrating that the inactivation of the *pssA* gene completely abolishes the secretion of this enzyme in *P. haloplanktis* TAC125-Δ*pssA*.

To exclude the possibility that the inability of *P. haloplanktis* TAC125-Δ*pssA* cells to secrete the recombinant α-amylase was due to a polar effect on expression of *pssA* neighbouring genes, a complementation experiment

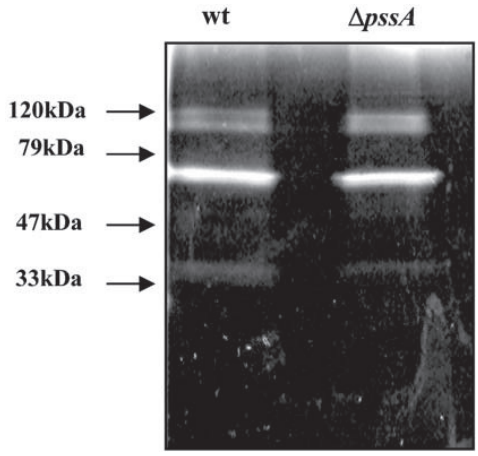

Fig. 2. Protease zymography of *P. haloplanktis* TAC125 wild-type (wt) and *P. haloplanktis* TAC125-Δ*pssA* mutant (Δ*pssA*) culture supernatants. Samples were withdrawn at the mid-exponential phase and concentrated tenfold. The zymographic developing time was 18 h, a condition that ensures the detection of all proteases contained in the sample.

was carried out. The complementing plasmid, pFC*amy*ΔCt-*pssA*, was constructed to restore PssA function in *P. haloplanktis* TAC125-Δ*pssA* cells. This plasmid contains both the *amy*ΔCt gene and the DNA sequence encoding *pssA* and its upstream region (150 bp long), in which the presence of a putative promoter sequence was predicted (SoftBerry BPROM software: http://linux1.softberry.com/berry.phtml). The mutant strain was transformed with pFC*amy*ΔCt-*pssA* and the recombinant cells were grown in standard conditions. Intracellular and extracellular protein samples were withdrawn at different growth phases, and subjected to Western blotting analysis with anti-*P. haloplanktis* α-amylase (Fig. 3b) and anti-*E. coli* β-lactamase sera (Fig. 3c). As shown in Fig. 3(b, c), the α-amylase was detected in the extracellular samples, while the β-lactamase was always fully associated with the intracellular extracts, thus demonstrating that the secretion of the α-amylase is partially restored in *P. haloplanktis* TAC125-Δ*pssA*/pFC*amy-pssA* complemented cells.

DISCUSSION

Over the last several years our group has been involved in unravelling the molecular mechanism involved in the secretion of the cold-adapted α-amylase from the marine Antarctic bacterium *P. haloplanktis* TAB23. This α-amylase is still one of the most cold-tolerant proteins intensively studied to clarify the structure/function relationships which underlie enzyme cold-adaptation (D'Amico *et al.*, 2006). This cold-adapted enzyme is likely translocated by a Sec system in the periplasmic space, where it attains its catalytically competent folded state (Feller *et al.*, 1998), and then an as yet unidentified secretion machinery catalyses its extracellular translocation.

The development of genetic tools for manipulation of Antarctica bacteria (Parrilli *et al.*, 2008a) allowed us to demonstrate that the C-terminal propeptide is not mandatory for α-amylase recombinant secretion either in the source strain or in the related strain *P. haloplanktis* TAC125 (Tutino *et al.*, 2002). Indeed, the propeptide can be replaced by other protein domains without affecting the secretion of the chimeric proteins (Cusano *et al.*, 2006b; Parrilli *et al.*, 2008b). Therefore it can be assumed that the mature α-amylase (i.e. devoid of the C-terminal propeptide) contains some molecular signals responsible for its specific secretion. To investigate the nature and structure of these secretion signals, mature α-amylase was subjected to a deletion mutagenesis analysis (Cusano *et al.*, 2006a). Features necessary for exoenzyme secretion were localized in a α-amylase domain, and a likely 3D recognition between α-amylase and the cognate cold-adapted secretion machinery was suggested (Cusano *et al.*, 2006a).

These results may point towards the involvement of a T2SS secretion pathway (Johnson *et al.*, 2006), which is the only canonical secretion system identified *in silico* in the *P. haloplanktis* TAC125 genome. However our recent results demonstrated that the extracellular targeting of the cold-adapted α-amylase does not depend on the T2SS in *P. haloplanktis* TAC125 (Parrilli *et al.*, 2008b).

To identify cellular components involved in the secretion of α-amylase, a *P. haloplanktis* TAC125 genomic DNA cosmid library was constructed and screened in *E. coli*. By this *in vivo* complementation experiment a *P. haloplanktis* TAC125 genomic fragment was selected, which did not encode any of the components of previously characterized secretion pathways. Data reported in this paper allowed us to identify a protein (PssA) required for the secretion of α-amylase in *P. haloplanktis* TAC125. The presence of a PssA homologue was demonstrated only in *P. haloplanktis* TAB23, i.e. the α-amylase source strain, and in three marine bacteria.

In silico analysis suggests that PssA is a lipoprotein that may be anchored into the inner face of the outer membrane. The PssA protein is predicted to contain three TPR domains; the TPR is an imperfect 34 aa repeat that is found as a protein–protein interaction module that mediates the assembly of multiprotein complexes (Blatch & Lässle, 1999; D'Andrea & Regan, 2003; Das *et al.*, 1998).

Recently, two lipoproteins equipped with TPR domains have been related to the functionality of the bacterial type IV pilus biogenesis system, which closely resemble the T2SS (Peabody *et al.*, 2003), and reported data (Carbonnelle *et al.*, 2005; Nudleman *et al.*, 2005) indicate that TPR-containing lipoproteins have the potential to interact with and/or form an outer-membrane multiprotein complex.

The PssA protein contains also two LysM domains in its C-terminal region. This protein module was originally

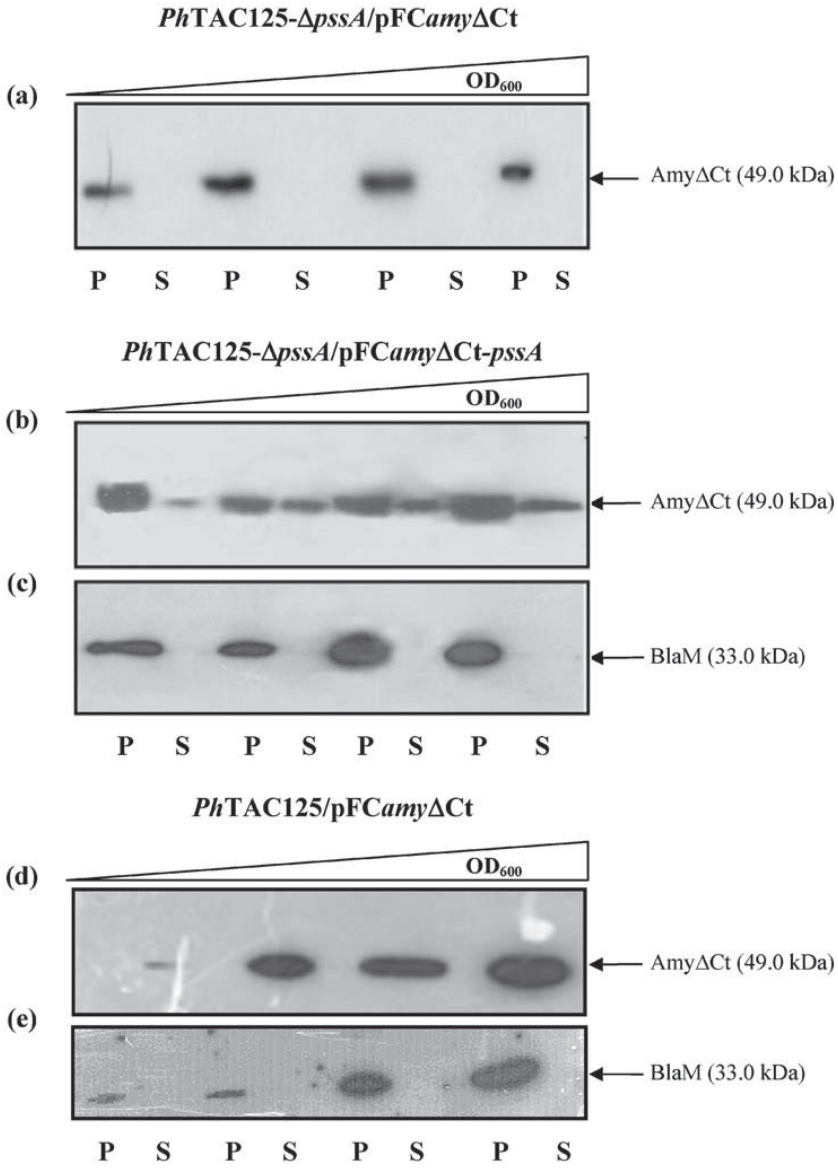

Fig. 3. Western blot analysis of extracellular samples (S) and corresponding cellular extracts (P) collected at different growth phases of *P. haloplanktis* TAC125-Δ*pssA*/pFC*amy*ΔCt (a), *P. haloplanktis* TAC125-Δ*pssA*/pFC*amy*ΔCt-*pssA* (b, c) and *P. haloplanktis* TAC125/pFC*amy*ΔCt (positive controls; d, e). In (a), (b) and (d), Western blot analysis was carried out by using anti-*P. haloplanktis* α-amylase polyclonal antiserum, while in (c) and (e) anti-*E. coli* β-lactamase polyclonal antiserum was used.

identified in enzymes that degrade bacterial cell walls but it is also present in many other bacterial proteins (Birkeland, 1994). The available data suggest that the LysM domain is a general peptidoglycan-binding module (Bateman & Bycroft, 2000).

In summary, PssA is a putative multidomain lipoprotein predicted to be localized in the inner leaflet of the outer membrane and displaying three TPR domains and two LysM modules. To the best of our knowledge, no proteins exhibiting the same domain organization have previously been related to protein secretion systems. Based on the functional annotation of TPR and LysM domains, combined with experimental evidence reported in the present work, a model for the role of PssA protein is suggested (Fig. 4) in which PssA interacts with other proteins and/or with itself while bound to the peptidoglycan, allowing the specific secretion of cold-adapted α-amylase. According to the proposed model, the lack of PssA completely abolishes α-amylase secretion. Symmetrically, PssA overproduction may alter the secretion complex stoichiometry, affecting amylase secretion efficiency. A similar effect was reported when some proteins of a multicomponent secretion system were overproduced (Ball *et al.*, 1999). This may account for the partial recovery of amylase secretion in the complemented strain (Fig. 3b), where *pssA* was supplied in multiple copies.

Surprisingly, the production of the PssA protein permits recombinant *E. coli*/pBBR*amy*ΔCt + Subclone 4 cells to secrete the cold-adapted α-amylase. The most plausible explanation of this result is that PssA can recruit some *E. coli* proteins, forming a protein complex able to recognize and secrete the α-amylase. Data presented in this paper do not allow speculation on structural similarities and/or

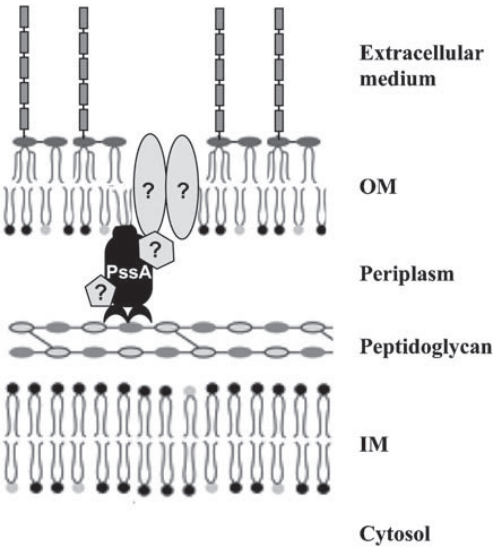

Fig. 4. Model for the role of PssA protein. The protein is proposed to work as a molecular adaptor, able to interact with other proteins (outer-membrane and/or periplasmic ones) while bound to the peptidoglycan, allowing the specific secretion of the cold-adapted α-amylase. OM, outer membrane; IM, inner membrane.

differences between the protein complexes responsible for amylase secretion in *P. haloplanktis* TAC125 and recombinant *E. coli*/pBBR*amy*ΔCt + Subclone 4 cells. This aspect is currently under investigation and will be the subject of a following paper.

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REFERENCES

Antoine, R. & Locht, C. (1992). Isolation and molecular characterization of a novel broad-host-range plasmid from *Bordetella bronchiseptica* with sequence similarities to plasmids from gram-positive organisms. *Mol Microbiol* **6**, 1785–1799.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1994). *Current Protocols in Molecular Biology*. New York: Wiley.

Ball, G., Chapon-Hervé, V., Bleves, S., Michel, G. & Bally, M. (1999). Assembly of XcpR in the cytoplasmic membrane is required for extracellular protein secretion in *Pseudomonas aeruginosa*. *J Bacteriol* **181**, 382–388.

Bateman, A. & Bycroft, M. (2000). The structure of a LysM domain from *E. coli* membrane-bound lytic murein transglycosylase D (MltD). *J Mol Biol* **299**, 1113–1119.

Birkeland, N. K. (1994). Cloning, molecular characterization and expression of the genes encoding the lytic functions of lactococcal bacteriophage phi LC3:a dual lysis system of modular design. *Can J Microbiol* **40**, 658–665.

Blatch, G. L. & Lässle, M. (1999). The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. *Bioessays* **21**, 932–939.

Cao, T. B. & Saier, M. H., Jr (2003). The general protein secretory pathway: phylogenetic analyses leading to evolutionary conclusions. *Biochim Biophys Acta* **1609**, 115–125.

Carbonnelle, E., Hélaine, S., Prouvensier, L., Nassif, X. & Pelicic, V. (2005). Type IV pilus biogenesis in *Neisseria meningitidis*: PilW is involved in a step occurring after pilus assembly, essential for fibre stability and function. *Mol Microbiol* **55**, 54–64.

Cusano, A. M., Parrilli, E., Duilio, A., Sannia, G., Marino, G. & Tutino, M. L. (2006a). Secretion of psychrophilic alpha-amylase deletion mutants in *Pseudoalteromonas haloplanktis* TAC125. *FEMS Microbiol Lett* **258**, 67–71.

Cusano, A. M., Parrilli, E., Marino, G. & Tutino, M. L. (2006b). A novel genetic system for recombinant protein secretion in the Antarctic *Pseudoalteromonas haloplanktis* TAC125. *Microb Cell Fact* **5**, 40.

D'Amico, S., Collins, T., Marx, J. C., Feller, G. & Gerday, C. (2006). Psychrophilic microorganisms: challenges for life. *EMBO Rep* **7**, 385–389.

D'Andrea, L. D. & Regan, L. (2003). TPR proteins: the versatile helix. *Trends Biochem Sci* **28**, 655–662.

Das, A. K., Cohen, P. W. & Barford, D. (1998). The structure of the tetratricopeptide repeats of protein phosphatase 5: implications for TPR-mediated protein-protein interactions. *EMBO J* **17**, 1192–1199.

Desvaux, M., Hébraud, M., Talon, R. & Henderson, I. R. (2009). Secretion and subcellular localizations of bacterial proteins: a semantic awareness issue. *Trends Microbiol* **17**, 139–145.

Feller, G., Lonhienne, C., Deroanne, C., Libioulle, J., Van Beeumen, J. & Gerday, C. (1992). Purification, characterization, and nucleotide sequence of the thermolabile alpha-amylase from the Antarctic psychrotroph *Alteromonas haloplanctis* A23. *J Biol Chem* **267**, 5217–5221.

Feller, G., D'Amico, S., Benotmane, A. M., Joly, F., Van Beeumen, J. & Gerday, C. (1998). Characterization of the C-terminal propeptide involved in bacterial wall spanning of alpha-amylase from the psychrophile *Alteromonas haloplanctis*. *J Biol Chem* **273**, 12109–12115.

Filloux, A., Joyet, P., Murgier, M. & Lazdunski, A. (1985). Cloning and expression of a *Bacillus licheniformis* alpha-amylase gene in *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **30**, 203–207.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.

Henderson, I.R., Navarro-Garcia, F., Desvaux, M., Fernandez, R.C. & Ala'Aldeen, D. (2004). Type V protein secretion pathway: the autotransporter story. *Microbiol Mol Biol Rev* **68**, 692–744.

Johnson, T. L., Abendroth, J., Hol, W. G. & Sandkvist, M. (2006). Type II secretion: from structure to function. *FEMS Microbiol Lett* **255**, 175–186.

Joris, B., Englebert, S., Chu, C. P., Kariyama, R., Daneo-Moore, L., Shockman, G. D. & Ghuysen, J. M. (1992). Modular design of the *Enterococcus hirae* muramidase-2 and *Streptococcus faecalis* autolysin. *FEMS Microbiol Lett* **70**257–264.

Juncker, A. S., Willenbrock, H., von Heijne, G., Nielsen, H., Brunak, S. & Krogh, A. (2003). Prediction of lipoprotein signal peptides in Gram-negative bacteria. *Protein Sci* **12**, 1652–1662.

17

Kast, P. (1994). pKSS-a second-generation general purpose cloning vector for efficient positive selection of recombinant clones. *Gene* **138**, 109–114.

Médigue, C., Krin, E., Pascal, G., Barbe, V., Bernsel, A., Bertin, P. N., Cheung, F., Cruveiller, S., D'Amico, S. & other authors (2005). Coping with cold: the genome of the versatile marine Antarctica bacterium *Pseudoalteromonas haloplanktis* TAC125. *Genome Res* **15**, 1325–1335.

Methé, B. A., Nelson, K. E., Deming, J. W., Momen, B., Melamud, E., Zhang, X., Moult, J., Madupu, R., Nelson, W. C. & other authors (2005). The psychrophilic lifestyle as revealed by the genome sequence of *Colwellia psychrerythraea* 34H through genomic and proteomic analyses. *Proc Natl Acad Sci U S A* **102**, 10913–10918.

Mol, O. & Oudega, B. (1996). Molecular and structural aspects of fimbriae biosynthesis and assembly in *Escherichia coli*. *FEMS Microbiol Rev* **19**25–52.

Norrander, J., Kempe, T. & Messing, J. (1983). Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. *Gene* **26**, 101–106.

Nudleman, E., Wall, D. & Kaiser, D. (2005). Cell-to-cell transfer of bacterial outer membrane lipoproteins. *Science* **309**, 125–127.

O'Callaghan, C. H., Morris, A., Kirby, S. M. & Shingler, A. H. (1972). Novel method for detection of β-lactamase by using a chromogenic cephalosporin substrate. *Antimicrob Agents Chemother* **1**, 283–288.

Papa, R., Rippa, V., Sannia, G., Marino, G. & Duilio, A. (2007). An effective cold inducible expression system developed in *Pseudoalteromonas haloplanktis* TAC125. *J Biotechnol* **127**, 199–210.

Parrilli, E., Cusano, A. M., Giuliani, M. & Tutino, M. L. (2006). Cell engineering of *Pseudoalteromonas haloplanktis* TAC125: construction of a mutant strain with reduced exo-proteolytic activity. *Microb Cell Fact* **5**36.

Parrilli, E., Duilio, A. & Tutino, M. L. (2008a). Heterologous protein expression in psychrophilic hosts. In *Psychrophiles: from Biodiversity to Biotechnology*, pp. 365–379. Edited by R. Margesin, F. Schinner, J. C. Marx & C. Gerday. Berlin & Heidelberg: Springer.

Parrilli, E., De Vizio, D., Cirulli, C. & Tutino, M. L. (2008b). Development of an improved *Pseudoalteromonas haloplanktis* TAC125 strain for recombinant protein secretion at low temperature. *Microb Cell Fact* **7**, 2.

Parrilli, E., Giuliani, M. & Tutino, M. L. (2009). General secretory pathway from marine Antarctic *Pseudoalteromonas haloplanktis* TAC125. *Marine Genomics* **1**, 123–128.

Peabody, C. R., Chung, Y.-J., Yen, M.-R., Vidal-Ingigliardi, D., Pugsley, A. P. & Saier, M. H., Jr (2003). Type II protein secretion and its relationship to bacterial type IV pili and archaeal flagella. *Microbiology* **149**, 3051–3072.

Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Thanassi, D. G. & Hultgren, S. J. (2000). Assembly of complex organelles: pilus biogenesis in gram-negative bacteria as a model system. *Methods* **20**111–126.

Tokuda, H. & Matsuyama, S. (2004). Sorting of lipoproteins to the outer membrane in *E. coli*. *Biochim Biophys Acta* **1694**, IN1–9.

Tutino, M. L., Duilio, A., Parrilli, E., Remaut, E., Sannia, G. & Marino, G. (2001). A novel replication element from an Antarctic plasmid as a tool for the expression of proteins at low temperature. *Extremophiles* **5**, 257–264.

Tutino, M. L., Parrilli, E., Giaquinto, L., Duilio, A., Sannia, G., Feller, G. & Marino, G. (2002). Secretion of α-amylase from *Pseudoalteromonas haloplanktis* TAB23: two different pathways in different hosts. *J Bacteriol* **184**, 5814–5817.

Yen, M. R., Peabody, C. R., Partovi, S. M., Zhai, Y., Tseng, Y. H. & Saier, M. H., Jr (2002). Protein-translocating outer membrane porins of gram-negative bacteria. *Biochim Biophys Acta* **1562**, 6–31.

Zdobnov, E. M. & Apweiler, R. (2001). InterProScan – an integration platform for the signature recognition methods in InterPro *Bioinformatics* **17**, 847–848.

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Conclusions

The use of psychrophilic bacteria as hosts for recombinant proteins production is a promising strategy for improving the quality of recombinant products in terms of solubility and correct folding. By the use of the psychrophilic genetic expression systems available so far, many examples are reported of soluble production "difficult" proteins from different origins in the Antarctic bacterium *P. haloplanktis* TAC125. However, to achieve the goal of the utilisation the cold expression system for recombinant proteins production at industrial scale, the development of new genetic tools for recombinant genes expression at low temperatures is required in order to improve the performances of the psychrophilic expression system and to make it competitive with the well established microbial cell factories such as *E. coli*.

The identification of new regulated psychrophilic promoters, for example, can be a useful strategy to improve protein production yields. Indeed, physical separation between bacterial growth phase and recombinant protein expression by the use of a regulated promoter and an efficient induction strategy can improve the productivity of the entire system. By a rational strategy a set of potentially regulated genes were identified in *P. haloplanktis* TAC125 genome. Transcriptional analysis of target genes was performed in a cheap minimal medium in the presence of inexpensive compounds of physical stimuli as inducers. The analysis led to the identification of the gluconate permease PSHAb0479 which is up-regulated in the presence of D-gluconic acid in the culture medium resulting in about 20 folds increase in corresponding mRNA levels. Moreover the alginate lyase PSHAa1748 resulted to be up to 10 folds over-expressed when alginic acid is added to the culture. The two identified promoters represent promising candidates for the construction of new regulated psychrophilic expression systems for recombinant proteins production at low temperatures.

Another useful strategy to improve the yields of soluble products and to facilitate the downstream processes is the recombinant protein addressing into extracellular medium. The reduced extracellular proteins content can indeed significantly simplify the purification process and the lower local concentration of recombinant products can further overcome the aggregation phenomena and inclusion bodies formation. An efficient system for recombinant proteins extracellular secretion in *P. haloplanktis* TAC125 was already developed making use of a psychrophilic α-amylase as secretion carrier. However only a few information are available so far about protein secretion mechanisms in psychrophilic bacteria. An *in silico* analysis of *P. haloplanktis* TAC125 genome reveals the presence of only one canonical secretion system, the T2SS GSP. Molecular and phenotypic characterisation of *P. haloplanktis* TAC125 GSP secretion system was performed and it revealed that GSP is responsible of the secretion of most of *P. haloplanktis* TAC125 extracellular proteases. This observation led to the construction of an engineered *P. haloplanktis* TAC125 strain where the GSP system was inactivated resulting in lower extracellular proteases content. Moreover, GSP system inactivation did not completely abolished proteins extracellular secretion in *P. haloplanktis* TAC125 indicating the presence of at least another still uncharacterised system also responsible for the secretion of the cold α-amylase carrier protein. In order to improve the efficiency of the system used for extracellular secretion of recombinant proteins in *P. haloplanktis* TAC125 the mechanism involved in psychophilic α-amylase secretion was investigated. By a combination of several molecular techniques, the function of *pss*A gene was related to α-amylase secretion in the psychrophilic bacterium. *pss*A gene deletion completely abolished the amylase secretion without affecting the extracellular targeting of other

substrates mediated by canonical secretion systems. The *pss*A gene product, PssA, is a multidomain lipoprotein, predicted to be localised into the bacterial outer membrane, and displaying three TPR (Tetratrico Peptide Repeat) domains and two LysM modules. Relying on functional annotation of these domains combined with the experimental results obtained, we have suggested a role for PssA as a molecular adaptor in charge of recruiting other cellular components required for specific α-amylase secretion.

Data collected have provided important information which can be used for the construction of engineered *P. haloplanktis* TAC125 strains with improved ability in recombinant proteins extracellular secretion.

MATERIALS AND METHODS

Strains and plasmids

Pseudoalteromonas haloplanktis TAC125 was isolated from Antarctic sea water (Medigue *et al*., 2005). *Escherichia coli* DH5α (Hanahan, 1983) was used as host for the gene cloning. *E. coli* strain S17-1(λpir) was used as donor in interspecific conjugation experiments (Tascon *et al*., 1993).

Growth conditions

P. haloplanktis TAC125 was grown at 15°C in aerobic conditions in TYP broth (16 g L^{-1} yeast extract, 16 g L^{-1} bacto tryptone, 10 g L^{-1} marine mix) at pH 7.5, supplemented with ampicillin 100 µg/ml when required. *P. haloplanktis* TAC125 cultivation in synthetic media was performed in SCHATZ mineral medium (Papa *et al*., 2007) supplemented with different amino acidic carbon sources listed in table 4 and with the addition of 100 µg/ml ampicillin and 0,4%w/v L-malate when required. Antibody fragments production was performed at 15°C in SCHATZ mineral medium supplemented with 0,5% w/v L-leucine, 0,5% w/v L-isoleucine and 1% w/v L-valine (LIV medium), 100 µg/ml ampicillin with additional 0,4%w/v L-malate as inducer. Experimental conditions for antibody fragments production processes are as follows.
P. haloplanktis TAC125 batch and continuous cultivations in bioreactor were performed in LIV medium at 15°C in a STR 3L fermenter (Applikon) connected to an ADI 1030 Bio Controller (Applikon) with a working volume of 1L. The culture was mantained in fully aerobic conditions (DOT ≥ 30%) by an airflow of 20 L h^{-1} and a stirring rate of 500 rpm. The culture pH was maintained at 7.00 by automatic addition of H_2SO_4 5% v/v. The cell biomass from a preinoculum, performed in shaken flask with the same medium and temperature used for the successive experiment in batch, was used to inoculate batch cultures. Chemostat cultivation was performed as described on SCHATZ medium supplemented with 1xLIV or 0,5% w/v L-leucine at a dilution rate of D = 0.05 h^{-1} and a working volume of 1.0L.
Antarctic bacteria transformation was achieved by intergeneric conjugation as previously reported (Duilio *et al*., 2004b).
E. coli cells were routinely grown in LB broth (Sambrook and Russell, 2001) at 37°C. When required, 100 µg/ml of ampicillin was added in liquid and solid cultures.
Cell growth was monitored by measuring the optical density (OD) at 600 nm using a UVIKON 922 spectrophotometer (Kontron).
Genetic manipulations were carried out following standard procedures (Sambrook and Russell, 2001).
All media component were purchased from Sigma-Aldrich.

Plasmids construction

DNA manipulation and analysis were performed according to Sambrook and Russell, 2001. Plasmidic DNA extraction and fragments purification was carried out with the QUIAEX II kit from Qiagen. Restriction enzymes, T4 DNA ligase, alkaline phosphatase, *Phusion* DNA polymerase were supplied by Boehringer-Roche, Promega, Fermentas or Finnzyme. Plasmids and oligonucleotide primers used in this work are listed in Table 6.

ScFvOx expression vectors construction – The *scFvOx-c-myc* gene was PCR amplified according to standard methods (Sambrook and Russell, 2001) from the source vector p*ScPelB,* kindly provided by Dr B. Soehling, University of Halle (Germany) in order to introduce 5' *Sma*I-*Sal*I and 3' *Eco*RI restriction sites by using primers Sc-SS-fw and Sc-tagE respectively (Tab 6). The amplified fragment was

cloned into pGEMT*easy* (Promega) vector and its nucleotide sequence was checked by sequencing to rule out the occurrence of mutations during synthesis.
For the construction of vectors pPM13*psD-scFvOx-c-myc* and pPM13*psA-scFvOx-c-myc* used for the screening of signal peptide for periplasmic secretion, the *scFvOx-c-myc* gene was double digested with *Sal*I/*Eco*RI and inserted into the corresponding sites of the psychrophilic periplasmic vectors pPM13*psD* and pPM13*psA* (unpublished results). For the construction of pFF*amy-scFvOx-c-myc* vector for ScFvOx extracellular secretion the *scFvOx-c-myc* gene was double digested with *Sma*I/*Eco*RI and ligated into pFF*amy** vector (Cusano *et al*., 2006) previously digested with the same restriction enzymes. For pUCRP-*scFv* expression vector construction the *psD-scFvOx-c-myc* gene was PCR amplified according to standard methods (Sambrook and Russell, 2001) from the previously constructed pPM13*psD-scFvOx-c-myc* vector in order to introduce the 5' *Nde*I and 3' *Xho*I restriction sites and to remove the stop codon by using the primers PsD-N-fw and the c-Myc-X-rv respectively (Tab. 6). The amplified fragment was cloned into pGEMT*easy* (Promega) vector and its nucleotide sequences was checked by sequencing to rule out the occurrence of mutations during synthesis. The gene fragment was then digested with *Nde*I/*Xho*I and cloned into a modified pUCRP vector (unpublished results) in-frame to a C-terminal 6xHis tag (Fig. 14).

Fab 3H6 operon and expression vectors construction – Fab 3H6 *lc* and *hc* genes were fused to the sequence coding the psychrophilic leader peptide PsD separately as follows.The Fab *lc* gene was PCR amplified by standard methods from pGAPZalphaA-*lc* source vector, kindly provided by Prof. D. Mattanovich, University of Natural Resources and Applied Life Sciences, Vienna (BOKU). Lc-S-fw and Lc-BE-rv primers (Tab. 6) were used to insert a 5' *Sal*I and 3' *Bst*98I-*Eco*RI restriction sites respectively. Moreover, the reverse primer was designed in order to introduce a Shine Dalgarno sequence (Fig. 10) into the 3' *lc* gene by silent mutagenesis. The amplified fragment was cloned into pGEMT*easy* (Promega) vector and its nucleotide sequence was checked by sequencing to rule out the occurrence of mutations during synthesis. The fragment was then digested with *Sal*I/*Eco*RI and cloned into the corresponding sites of a previously digested pPM13*psD* vector thus obtaining a pPM13*psD-lc* plasmid. In parallel, Fab *hc* gene was PCR amplified from pGAPZalphaA-*lc* source vector, kindly provided by Prof. D. Mattanovich, University of Natural Resources and Applied Life Sciences, Vienna (BOKU), in order to add a 5' *Acc*65I and 3' *Xho*I/*Eco*RI restriction sites by using primers Hc-A-fw and Hc-XE-rv respectively (Tab. 6). The fragment encoding the PsD signal peptide was also PCR amplified from pPM13*psD* vector in order to insert 5' *Bam*HI/*Bst*98I and 3' *Acc*65I restriction sites by using PsD-BB-fw and PsD-A-rv primers respectively (Tab. 6). The amplified *hc* and *psD* gene fragments were digested with *Bam*HI/*Acc*65I and *Acc*65I/*Eco*RI and cloned into pUC18 plasmid (New England Biolabs) previously digested with *Bam*HI/*Eco*RI vector and their nucleotide sequences was checked by sequencing.
For Fab 3H6 operon construction (Fig. 10) the resulting gene fusion *psD-hc* was excised from pUC18*psD-hc* by *Bst*98I/*Xho*I double digestion. In parallel, the fusion gene *psD-lc* was excised from the previously constructed pPM13*psD-lc* vector by *Nde*I/*Bst*98I double digestion. The two obtained gene fragments: *psD-lc* (*Nde*I/*Bst*98I) and *psD-hc* (*Bst*98I/*Xho*I) were finally cloned into a modified pPM13 vector (unpublished results) previously digested with *Nde*I/*Xho*I in-frame to a C-terminal 6xHis tag coding sequence thus obtaining the pPM13-*fab* expression vector.

For pUCRP-*fab* expression vector construction the *fab* operon was excised from the previously constructed pPM13-*fab* plasmid by *Nde*I/*Eco*RI double digestion and cloned into the same restriction sites of pUCRP expression vector (Papa *et al*., 2007).

VHH D6.1 expression vector construction – The *vhh* D6.1 gene was PCR amplified according to standard methods (Sambrook and Russell, 2001) from pHEN-D6.1 source vector kindly provided by Dr. A. De Marco, IFOM-IEO campus Milan, in order to insert a 5' *Sal*I and a 3' *Not*I restriction sites by using primers VH-S-fw and VH-N-rv respectively (Tab. 6). The amplified fragment was cloned into pGEMT*easy* (Promega) vector and its nucleotide sequence was checked by sequencing to rule out the occurrence of mutations. The *vhh* D6.1 gene was then digested with *Sal*I/*Not*I and cloned into the previously constructed pUCRP-*scFv* expression vector by replacing the *scFvOx* gene excised by a *Sal*I/*Not*I double digestion. The resulting expression vector, pUCRP-*vhh* contains the *vhh* D6.1 gene in-frame to N-terminal PsD and C-terminal c-Myc and 6xHis tag coding sequences (Fig. 14)

Table 6: Plasmids and oligonucleotides

Plasmid	Description	References
pPM13	Vector deriving from the pUC18 plasmid, containing the T/R box, the psychophilic promoter 13 and the transcription termination signal from *Ph*TAC125 *aspC* gene	Duilio et al., 2004
pPM13*psD*	pPM13 containing PhDsbA signal peptide coding sequence	Unpublished results
pPM13*psA*	pPM13 containing Phα- amylase signal peptide coding sequence	Unpublished results
pPM13-his	pPM13 containing C-terminal 6xHis tag coding sequence	Unpublished results
pPM13*psD-scFv*	Expression vector for constitutive periplasmic production of ScFvOx-c-Myc antibody fragment	This work
pFF*amy**	Expression vector for N-terminal amylase fusion for extracellular secretion	Cusano *et al*., 2006
pFF*amy-scFv*	Expression vector for constitutive production and extracellular secretion of ScFvOx	This work
pUCRP	Psychrophilic expression vector containing PSHAb0363 promoter region	Papa *et al*., 2007
pUCRP*β-gal*	Expression vector for L-malate inducible production of psychrophlic β-galactosidase from *P. haloplanktis* TAE79	Papa *et al*., 2007
pUCRP-his	pUCRP containing C-terminal 6xHis tag coding sequence	Unpublished results
pUCRP-*scfv*	Expression vector for periplasmic L-malate inducible production of ScFvOx-c-Myc-6xHis antibody fragment	This work
pPM13*psD-lc*	Expression vector for periplasmic production of Fab 3H6 light chain	This work
pUC18*psD-hc*	pUC18 cloning vector containing the Fab 3H6 hc gene fused to psD signal peptide coding sequence	This work
pPM13-*fab*	Expression vector for constitutive Fab 3H6 periplasmic production	This work
pUCRP-*fab*	Expression vector for periplasmic L-malate inducible production of Fab 3H6	This work
pUCRP-*vhh*	Expression vector for periplasmic L-malate inducible production of VHHD6.1-c-Myc-6xHis	This work

Oligonucleotides	Sequence 5' - 3'	Restriction site
Sc-SS-fw	CAGCCCCGGGTCGACATGGCCG'	*Sal*I-*Sma*I
Sc-tagE	GCTTGTCGAATTCCTATGCGGCCC	*Eco*RI
PsD-N-fw	CGGCGCATATGCTTAAAAAATTAAAACTGAG	*Nde*I
c-Myc-X-rv	ATATCTCGAGGGCCCTTGCGGCCCCATTC	*Xho*I
Lc-S-fw	ATAATGTCGACGAAACAACTGTGACCCAGTC	*Sal*I
Lc-BE-rv	CGAATTCTTAAGCATTAACACTCTCCTCTGTTGAAG	*Eco*RI/*Bst*98I
Hc-A-fw	TAGGTACCGTCCAACTGCAGCAGTCTG	*Acc*65I
Hc-XE-rv	AGAATTCTACTCGAGACAAGATTTGGGCTCAAC	*Eco*RI/*Xho*I
PsD-BB-fw	GGAGGATCCATGCTTAAGAAATTAAAACTGAG	*Bam*HI/*Bst*98I
PsD-A-fw	CAGGTACCTTCAAAGTTTGCTGCCAAGTG	*Acc*65I
VH-S-fw	ATCGTGTCGACATGGCTGAGGTGC	*Sal*I
VH-N-rv	ATATATGCGGCCGCAATGGAGACGGTG	*Not*I
Fab-RT-fw	TTCAACAGAGGAGAGTGTTAATGC	none
qRT-PCR primers	**Sequence 5' - 3'**	**Target sequence**
0363-fw:	TTGGCATAATGCTCAGTTTTCATT	PSHAb0363
0363-rv:	ACCGTAAATAAGTGTAGCGGCAAA	PSHAb0363
GP-fw	CGATGATAGCCGGGATTTTC	PSHAb0479
GP-rv	CGCCCATTCCTTTTTGGATA	PSHAb0479
GalT-fw	GGGTGCAGGTATTCGAAAACA	PSHAa1770
GalT-rv	GTAATTGCTGCTGCGACCAA	PSHAa1770
Alg-fw	GGTGAAGGGTGACGAGTCGTT	PSHAa1748
Alg-rv	CGCGTTTACACGGCGAATA	PSHAa1748
TF-fw	TGGCGAAGTAATGCAACGTAAT	PSHAa2063
TF-rv	CTTCAAGTGCTTTTGGAGCAAA	PSHAa2063

Analytical procedures

Biomass determination - For biomass determination suitable sample volumes were washed in demineralised water, collected and dried on pre-weighed filter discs and dried at 110°C until constant weight. The dry cell weight was correlated with OD at 600nm throughout the following equation:

Dry cell weight (g L^{-1}) = 0,74 x OD_{600nm}

Cell lysis – For recombinant β-galactosidase production analysis aliquots of bacterial pellet corresponding to 1 mL culture volume were resuspended in 1mL Lysis buffer (Na phosphate buffer 0,1M pH 7,8, EDTA 2 mM, DTT 1 mM, Triton X-100 1% v/v, lisozyme 5 mg mL^{-1}, PMSF 1 mM) and incubated at 15°C for 30'. The suspension was then centrifuged at 10000rpm for 15 mins at 4°C and the supernatant used for activity assays. For analysis of recombinant antibody fragments production and purification, aliquots of bacterial pellet corresponding to 50 mL culture volume were resuspended in Na phosphate buffer 50 mM pH 8, NaCl 300 mM, PMSF 1 mM and subjected to 5 cycles of French Press (Sinstem, Limited Basic Z Model) at 1,8 kbar. The resulting suspension was subjected to ultracentrifugation (Beckman 50.2Ti) at 45000rpm for 2h at 4°C and the supernatant used for further analysis.

Periplasmic proteins preparation- Bacterial pellets were resuspended in 1/20 of culture volume of borate buffer ($Na_2B_4O_7$ 200 mM, NaCl 130 mM, EDTA 5 mM, pH 8) and incubated 18h at 4°C. The suspension was centrifuged at 8000 rpm for 15min at 4°C and the supernatant used for further analysis.

SDS-PAGE and Western blotting analysis – Protein samples (5 μg protein extracts and 500ng pure proteins) were analyzed by Polyacrylamide Gel Electrophoresis (Sodium Dodecyl Sulphate-PAGE) (10% acrylamide, w/v) according to standard methods (Sambrook and Russell, 2001). For Western blotting analysis, 1 μg protein extracts were subjected to standard polyacrylamide gel electrophoresis (SDS-PAGE), under non-reducing conditions in the case of Fab 3H6, and transferred to a polyvinylidene difluoride membrane (PVDF) (Immobilon PSQ, Millipore).

Proteins immunodetection - After blocking the membrane 1h at RT in blocking (buffer phosphate buffer saline (PBS), 5% w/v skimmed milk, 0,05% v/v Triton X-100) immunodetection was performed as follows: for Fab 3H6 anti-light chain detection, anti-human-κ-light chain mAb-alkaline phosphatase conjugate (Sigma) was diluted 1:2000 in blocking buffer and incubated 2h at RT. Blots were developed using the colorimetric AP-substrate kit (Biorad). For Fab 3H6 HC detection anti-His tag mAb (Sigma-Aldrich) was diluted 1:1000 in blocking buffer and incubated for 16h at 4°C. Peroxidase conjugate anti-mouse IgG (Calbiochem) was used as secondary antibody. Proteins were detected by chemiluminescence (Pierce).
For ScFvOx and VHHD6.1 detection anti c-Myc mAb (Calbiochem) was diluted 1:5000 in blocking buffer and incubated for 1h at RT. Peroxidase conjugate anti-mouse IgG (Calbiochem) was used as secondary antibody. Proteins were detected by chemiluminescence (Pierce).
For cold α-amylase detection *P. haloplanktis* TAB23 anti-α-amylase antiserum was diluted 1:4000 in blocking buffer and incubated 1h at RT. Peroxidase conjugate anti-rabbit IgG (Sigma-Aldrich) was used as secondary antibody. Proteins were detected by chemiluminescence's (Pierce).

ELISA quantification – For Fab 3H6 quantification a sandwich ELISA was performed as described in previous studies (Gach *et al*., 2007). Pure Fab 3H6 used as standard was purchased from Rockland.
Concentrations of active ScFvOx were determined by ELISA assay as previously described by Lange and co-workers (Lange *et al*., 2005). Refolded purified ScFvOx IB produced in *E. coli* (Lange *et al*., 2005) was used as standard for the assay.

Enzymatic assays - Protein concentration was determined with the Bio-Rad protein assay (Bradford, 1976), using bovine serum albumine as standard.
Recombinant cold-active β-galactosidase was assayed spectrophotometrically at 25°C as previously reported (Hoyoux *et al*., 2001).
Alkaline phoshatase activity assay was performed according to Jones *et al*., 1989.
Enymatic kinetic were registered with a DU7500 spectrophotometer (Beckman).

Amino acids and total ammonium quantification – Residual amino acids concentation during *P. haloplanktis* TAC125 cultivation was evaluated by AccQ·Tag ™ Amino Acid Analysis method as described by Cohen and Michaud, 1993. AccQ·Fluor™ Reagent Kit was purchased from Waters. Sample derivatisation has been performed accordind to manufacturer's instructions. For calibration standard 50 pmol of each amino acid derivative (L-Leu, L-Ile, L-Val) were used separately. For calibration curve, different dilutions of L-Leu, L-Ile and L-Val derivative were used separately at

concentrations ranging from 0,0125 to 3,1250 mM. For total ammonium quantification a calibration curve ranging from 0,32 to 10,00 mM NH_4Cl derivative was used. A Waters Alliance 2695 HPLC system (Waters) was utilised for the HPLC analyses. AccQ·Tag ™ amino acid column Nova-Pak C18, 4 µm (150×3.9 mm) from Waters was used. The column was thermostated at 37 °C and 10 µL was the injection volume (concentration of amino acids 5–200 pmol). A gradient mobile phase was used for chromatography. The mobile phase consisted of eluent A (140 mM NaOAc, 1,107 mg L^{-1} EDTA, 2,35 mL L^{-1} triethylamine pH 5,05 with H_3PO_4) and eluent B (60% v/v acetonitrile, HPLC grade). The best gradient separation program was the following:
Time 0 min: A—100%, B—0%
Time 0.5 min: A—98%, B—2%
Time 15 min: A—93%, B—7%
Time 19 min: A—90%, B—10%
Time 29 min: A—67%, B—33%
Time 37 min: A—0%, B—100%
Time 38 min: A—100%, B—0%
Time 50 min: A—100%, B—0%

L-malate quantification – Residual L-malate concentration during *P. haloplanktis* TAC125 cultivation was analysed by HPLC (Hewlett Packard 1050) analysis using an ionic exchange column Aminex HPX-87H (Bio Rad). The mobile phase was 15 mM sulphuric acid. For the calibration curve L-malate concentrations ranging from 0,93 up to 29,85 mM were used.

RNA preparation and RT - Total RNA was isolated from pellets corresponding to 500µl of *P. haloplanktis* TAC125 cell culture (RNasy Mini kit, Qiagen) and subjected to in-column DNase treatment (Rnase-Free Dnase Set, Qiagen). Quality of the RNA isolation was checked by gel electrophoresis followed by quantification in spectrophotometer according to Sambrook and Russell, 2001. Only samples showing a A_{260nm}/A_{280nm} ratio ≥ 0,8 were used for further experiments.
Reverse transcription (RT) reactions were performed using SuperScript II RNase H^- Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions using 100 pmol of specific primers (Tab. 6) on approximately 5 µg of total purified RNA. In addition, RT negative control reactions were performed where water was added instead of template.

Analysis of mRNA by RT-PCR - For the evaluation of Fab 3H6 operon mRNA stability PCR amplifications (Sambrook and Russell 2001) were performed using 2 µl of RT reaction sample as a template, *Taq* DNA polymerase (Promega) using Fab-RT-fw and Hc-XE-rv specific primers (Tab. 6) An additional PCR reaction on DNA-free total RNA was carried as negative control. PCR products were visualised by agarose gel electrophoresis on a 1% agarose gel containing 1 µg mL^{-1} ethidium bromide.

Analysis of mRNA by qRT-PCR - The qRT-PCR method was used to determine the relative amount of specific transcriptional products in the presence/absence of different compounds or physical stimuli. The qRT-PCR was performed with cDNAs prepared from 2 separate cultures per treatment. A total of 12 data were obtained per point derived from two cDNA separate preparations from each culture. Each of the four cDNA samples obtained was amplified in triplicate experiments.
Real-time PCR was carried out using a StepOne™ Real Time PCR System (Applied Biosystems) and the amplification of the target sequences was detected using SYBR

Green technology. The housekeeping gene *ihf*B was chosen as an internal control to correct for variations of mRNA amounts and cDNA synthesis efficiency.
The primers for specific amplification were designed by Primer Express® Software Version 3.0 (Applied Biosystems) and are listed in Table 6. qRT-PCR amplification mixtures (20 µl) contained 2 µl template cDNA, 2x SYBR® Green I Master Mix (10 µl) (Applied Biosystems) and 300 nM forward and reverse primer. A non-template control reaction mixture was included for each gene. The PCR cycling programme was as follows: holding stage, 1 cycle of 95°C for 10 min; cycling stage, 40 cycles at 95°C for 15 s, 60°C for 60 s; melting curve stage 1 cycle at 95°C for 15 s, 60°C for 60 s, 95°C for 15 s with a temperature increment of +0,3°C. Specificity of the reaction was checked by analysis of the melting curve of the final amplified product. Experiments and data analysis were performed using StepOne™ Software v2.0 by ΔΔCt method (Applied Biosystems User Bulletin, 1997).

ScFvOx and VHHD6.1 affinity purification

The 6xHis tagged recombinant antibody fragments ScFvOx and VHHD6.1 were affinity purified on Ni^{2+}-NTA resin in batch conditions. 200 µl of HIS-Select Nickel Affinity Gel resin (Sigma-Aldrich), pre-equilibrated with binding buffer (Na-phosphate buffer 50 mM pH 8, NaCl 300 mM) were incubated with about 25 mg of crude protein extracts for 16h at 4°C while shaking. Five washing steps were performed with washing buffer (Na-phosphate 50 mM pH 8, NaCl 300 mM, imidazole 30 mM). Protein elution was carried with 50 µl of elution buffer (Na-phosphate 50 mM pH 8, NaCl 300 mM, imidazole 250 mM). A second elution step was performed with 50 µl of the same elution buffer supplemented with 500 mM imidazole.

REFERENCES

Arbabi-Ghahroudi M, Tanha J, MacKenzie R: Prokaryotic expression of antibodies. *Canc Metast Rev.* (2005), 24: 501–19

Baneyx F and Mujacic M: Recombinant protein folding and misfolding in *Escherichia coli.Nature Biotechnol.* (2004), 22(11): 1399-408

Blatny JM, Brautaset T, Winther-Larsen HC, Haugan K, Valla S: Construction and use of a versatile set of broad-host-range cloning and expression vectors based on the RK2 replicon. *Appl Environ Microbiol*. (1997), 63: 370-9

Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. (1976), 72: 248-54

Bird RE, Hardman KD, Jacobsen JW, Johnson S, Kaufman BM, Lee SM, Lee T, Pope SH, Riordan GS, Whitlow M: Single-chain antigen-binding proteins. *Science* (1988), 242: 423-26

Bryson S, Julien JP, Isenman DE, Kunert R, Katinger H, Pai EF: Crystal structure of the complex between the F′ab fragment of the cross-neutralizing anti-HIV-1 antibody 2F5 and the F_{ab} fragment of its anti-idiotypic antibody 3H6. *J Mol Biol.* (2008) 382: 910-9

Canadian Corporate News Monoclonal Antibodies Report: 2008 Update (2008)

Caspi R, Foerster H, Fulcher CA, Kaipa P, Krummenacker M, Latendresse M, Paley S, Rhee SY, Shearer AG, Tissier C, Walk TC, Zhang P, Karp PD: The MetaCyc Database of metabolic pathways and enzymes and the BioCyc collection of Pathway/Genome Databases. *Nucl Acids Res.* (2008), 36:D623-31

Cohen SA and Michaud DF: Synthesis of a fluorescent derivatizing reagent, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, and its application for the analysis of hydrolysate amino acids via high-performance liquid chromatography, *Anal Biochem.* (1993), 211: 279-87

Cornelis P: Expressing genes in different *Escherichia coli* compartments. *Curr Opin Biotechnol.* (2000), 11: 450-4

Cusano AM, Parrilli E, Duilio A, Sannia G, Marino G, Tutino ML: Secretion of psychrophilic alpha-amylase deletion mutants in *Pseudoalteromonas haloplanktis* TAC125. *FEMS Microbiol Lett*. (2006a), 258: 67-71

Cusano AM, Parrilli E, Marino G, Tutino ML: A novel genetic system for recombinant protein secretion in the Antarctic *Pseudoalteromonas haloplanktis* TAC125. *Microb Cell Fact.* (2006b) 5: 40

Duilio A, Madonna S, Tutino ML, Pirozzi M, Sannia G, Marino G: Promoters from a cold-adapted bacterium: definition of a consensus motif and molecular characterization of UP regulative elements. *Extremophiles* (2004), 8**:** 125-32

Evans D, Das R: Monoclonal antibody therapies: evolving into a $ 30 billion market. Datamonitor, London, UK (2005)

Farid SS: Process economics of industrial monoclonal antibody manufacture. *J Chrom B* (2007), 848: 8-18

Feller G, Lonhienne C, Deroanne C, Libioulle J, Van Beeumen J, Gerday C. Purification, characterization, and nucleotide sequence of the thermolabile alpha-amylase from the Antarctic psychrotroph *Alteromonas haloplanctis* A23. *J Biol Chem*. (1992), 267: 5217-21

Feller G, D'Amico S, Benotmane AM, Joly F, Van Beeumen J, Gerday C: Characterization of the C-terminal propeptide involved in bacterial wall spanning of alpha-amylase from the psychrophile *Alteromonas haloplanctis*. *J Biol Chem.* (1998) 273: 12109-15

Ferrer-Miralles N, Domingo-Espín J, Corchero JL, Vázquez E, Villaverde A: Microbial factories for recombinant pharmaceuticals. *Microb Cell Fact.* (2009), 8:17

Fiedler U and Conrad U: High-level production and long-term storage of engineered antibodies in transgenic tobacco seeds. *Bio/Technol.* (1995), 13: 1090-3

Frenken LG, Hessing JG, Van den Hondel CA, Verrips CT: Recent advances in the large-scale production of antibody fragments using lower eukaryotic microorganisms. *Res Immunol.* (1998), 149: 589-99

Frey PA: The Leloir pathway: a mechanistic imperative for three enzymes to change the stereochemical configuration of a single carbon in galactose. *FASEB J.* (1996), 10(4): 461-70

Gacha JS, Maurer M, Hahna R, Gasser B, Mattanovich D, Katinger H, Kunert R: High level expression of a promising anti-idiotypic antibody fragment vaccine against HIV-1 in *Pichia pastoris. J Biotechnol.* (2007), 128: 735-46

Gasser B, Saloheimo M, Rinas U, Dragosits M, Rodríguez-Carmona E, Baumann K, Giuliani M, Parrilli E, Branduardi P, Lang C, Porro D, Ferrer P, Tutino ML, Mattanovich D, Villaverde A: Protein folding and conformational stress in microbial cells producing recombinant proteins: a host comparative overview. *Microb Cell Fact.* (2008), 7:11

Got PA, Scherrmann JM: Stereoselectivity of antibodies for the bioanalysis of chiral drugs. *Pharm Res.* (1997), 14: 1516-23

Graham BM, Porter AJ, Harris WJ: Cloning, expression and characterization of a single-chain antibody fragment to the herbicide paraquat. *J Chem Technol Biotechnol.* (1995), 63: 279-89

Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hamers C, Songa EB, Bendahman N and Hamers R: Naturally occurring antibodies devoid of light chains. *Nature* (1993), 363: 446-8

Hanahan D: Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol*. (1983), 166: 557-80

Haney SA, Platko JV, Oxender DL, Calvo JM: Lrp, a leucine-responsive protein, regulates branched-chain amino acid transport genes in *Escherichia coli*. *J Bacteriol*. (1992), 174(1): 108-15

Haydon DJ and Guest JR: A new family of bacterial regulatory proteins. *FEMS Microbiol Lett.* (1991), 63: 291-5

Hoffmann A, Merz F, Rutkowska A, Zachmann-Brand B, Deuerling E, Bukau B: Trigger factor forms a protective shield for nascent polypeptides at the ribosome. *J Biol Chem.* (2006) 281: 6539-45

Hollinger P and Hudson PJ: Engineered antibody fragments and the rise of single domains. *Nat Biotechnol.* (2005) 23: 1126-36

Hotta A, Kamihira M, Itoh K, Morshed M, Kawabe M, Ono KI, Matsumoto H, Nishijima KI, Iijima S: Production of anti-CD2 chimeric antibody by recombinant animal cells. *J Biosci Bioeng.* (2004), 98(4): 298-303

Hoyoux A, Jennes I, Dubois P, Genicot S, Dubail F, Francois JM, Baise E, Feller G, Gerday C: Coldadapted beta-galactosidase from the Antarctic psychrophile *Pseudoalteromonas haloplanktis*. *Appl Environ Microbiol*. (2001), 67: 1529-35

Istúriz T, Palmero E, Vitelli-Flores J: Mutations affecting gluconate catabolism in *Escherichia coli*. Genetic mapping of the locus for the thermosensitive gluconokinase. *J Gen Microbiol.* (1986), 132: 3209-12

Izu H, Adachi O, Yamada M: Gene organization and transcriptional regulation of the gntRKU operon involved in gluconate uptake and catabolism of *Escherichia coli. J Mol Biol.* (1997), 267(4): 778-93

Jones JV, Mansour M, James H, Sadi D, Carr RI: A substrate amplification system for enzyme-linked immunoassays. Demonstration of its applicability for measuring anti-DNA antibodies. *J Immunol Methods* (1989) 118: 79-84

Joosten V, Lokman C, van den Hondel C, Punt PJ: The production of antibody fragments and antibody fusion proteins by yeasts and filamentous fungi. *Microb Cell Fact.* (2003), 2:1

Kandror O and Goldberg AL: Trigger factor is induced upon cold shock and enhances viability of *Escherichia coli* at low temperatures. *Proc Natl Acad Sci USA* (1997), 94(10): 4978-81

Klemm P, Tong S, Nielsen H, Conway T: The *gntP* gene of *Escherichia coli* involved in gluconate uptake. *J Bacteriol.* (1996), 178: 61-7

Köhler G and Milstein C: Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* (1975), 256 : 495-7

Kolaj O, Spada S, Robin S, Wall JG: Use of folding modulators to improve heterologous protein production in *Escherichia coli. Microb Cell Fact.* (2009),8:9

Kunert RE, Weik R, Ferko B, Stiegler G, Katinger H: Anti-idiotypic antibody Ab2/3H6 mimics the epitope of the neutralizing anti-HIV-1 monoclonal antibody 2F5. *AIDS* (2002), 16(4): 667-8

Landgraf JR, Boxer JA, Calvo JM: *Escherichia coli* Lrp (leucine-responsive regulatory protein) does not directly regulate expression of the *leu* operon promoter. *J Bacteriol*. (1999), 181(20): 6547-51

Lange C, Patil G, Rudolph R: Ionic liquids as refolding additives: N′-alkyl and N′-(ω-hydroxyalkyl) N-methylimidazolium chlorides. *Protein Sci*. (2005), 14(10): 2693-701

Madonna S, Papa R, Birolo L, Autore F, Doti N, Marino G, Quemeneur E, Sannia G, Tutino ML, Duilio A: The thiol-disulphide oxidoreductase system in the cold-adapted bacterium *Pseudoalteromonas haloplanktis* TAC 125: discovery of a novel disulfide oxidoreductase enzyme. *Extremopiles* (2006) 10: 41-51

Majumdar A, and Adhya S: Probing the structure of Gal operator-repressor complexes. *J Biol Chem.* (1987), 262: 13258-62

Makrides SC: Strategies for achieving high-level expression of genes in *Escherichia coli*. *Microbiol Rev.* (1996), 60: 512-38

Médigue C, Krin E, Pascal G, Barbe V, Bernsel A, Bertin PN, Cheung F, Cruveiller S, D'Amico S, Duilio A, Fang G, Feller G, Ho C, Mangenot S, Marino G, Nilsson J, Parrilli E, Rocha EP, Rouy Z, Sekowska A, Tutino ML, Vallenet D, von Heijne G, Danchin A: Coping with cold: the genome of the versatile marine Antarctica bacterium *Pseudoalteromonas haloplanktis* TAC125. *Genome Res.* (2005), 15(10): 1325-35

Mergulhaõ F, Monteiro G, Cabral J, Taipa M: Design of bacterial vector systems for the production of recombinant proteins in *Escherichia coli*. *J Microb Biotechnol.* (2004), 14: 1-14

Monegal A, Ami D, Martinelli C, Huang E, Aliprandi M, Capasso P, Francavilla C, Ossolengo G, de Marco A: Immunological applications of single-domain llama recombinant antibodies isolated from a naïve library. *Prot Eng Des Selec.* (2009) 22(4): 273-80

Moran MA, Buchan A, González JM, Heidelberg JF, Whitman WB, Kiene RP, Henriksen JR, King GM, Belas R, Fuqua C, Brinkac L, Lewis M, Johri S, Weaver B, Pai G, Eisen JA, Rahe E, Sheldon WM, Ye W, Miller TR, Carlton J, Rasko DA, Paulsen IT, Ren Q, Daugherty SC, Deboy RT, Dodson RJ, Durkin AS, Madupu R, Nelson WC, Sullivan SA, Rosovitz MJ, Haft DH, Selengut J, Ward N: Genome sequence of *Silicibacter pomeroyi* reveals adaptations to the marine environment. *Nature* (2004), 432(7019): 910-3

Muyldermans S and Lauwereys M: Unique single-domain antigen binding fragments derived from naturally occurring camel heavy-chain antibodies. *J Mol Recognit.* (1999), 12:131-140

Oppenheim D and Yanofsky C: Translational coupling during expression of the tryptophan operon in *Escherichia coli*. *Genetics* (1980), 95: 785-95

Oxender DL and Quay SC: Regulation of leucine transport and binding proteins in *Escherichia coli. J Cell Physiol.* (1976), 89(4): 517-21

Papa R, Glagla S, Danchin A, Schweder T, Marino G, Duilio A: Proteomic identification of a two-component regulatory system in *Pseudoalteromonas haloplanktis* TAC125. *Extremophiles*. (2006) 10(6): 483-91

Papa R, Rippa V, Sannia G, Marino G, Duilio A: An effective cold inducible expression system developed in *Pseudoalteromonas haloplanktis* TAC125. *J Biotechnol.* (2007), 127(2): 199-210

Park JS, Han KY, Lee JH, Song JA, Ahn KY, Seo HS, Jun Sim SJ, Kim SW, Lee J: Solubility enhancement of aggregation-prone heterologous proteins by fusion expression using stress-responsive *Escherichia coli* protein, RpoS. *BMC Biotechnol.* (2008), 8: 15

Parrilli E, Duilio A, Tutino ML: Heterologous protein expression in psychrophilic hosts. In *Psychrophiles: from Biodiversity to Biotechnology*. Edited by: RMea. Berlin Heidelberg: Springer-Verlag; (2008): 365-79

Parrilli E, De Vizio D, Cirulli C, Tutino ML: Development of an improved *Pseudoalteromonas haloplanktis* TAC125 strain for recombinant protein secretion at low temperature. *Microb Cell Fact.* (2008), 7: 2

Patil G, Rudolph R, Lange C: *In vitro*-refolding of a single-chain Fv fragment in the presence of heteroaromatic thiols. *J Biotechnol.* (2008), 134: 218-21

Penrose WR, Nichoalds GE, Piperino JR, Oxender DL: Purification and Properties of a Leucine-binding Protein from *Escherichia coli*. *J Biol Chem*. (1968), 243(22):5921-8

Peterson E, Owens SM, Henry RL: Monoclonal Antibody Form and Function: Manufacturing the Right Antibodies for Treating Drug Abuse. *AAPS J.* (2006), 8(2): E383-E390

Quay SC and Oxender DL: Regulation of Branched-Chain Amino Acid Transport in *Escherichia coli*. *J Bacteriol*. (1976), 127(3): 1225-38

Quay SC, Dick TE, Oxender DL: Role of transport systems in amino acid metabolism: leucine toxicity and the branched-chain amino acid transport systems. *J Bacteriol.* (1977), 129(3): 1257-65

Rahmanian M, Claus DR, Oxender DL: Multiplicity of Leucine Transport Systems in *Escherichia coli* K-12. *J Bacteriol*. (1973), 116(3): 1258-66

Rosano GL and Ceccarelli EA: Rare codon content affects the solubility of recombinant proteins in a codon bias-adjusted *Escherichia coli* strain. *Microb Cell Fact*. (2009), 8: 41

Sahdev S, Khattar SK, Saini KS: Production of active eukaryotic proteins through bacterial expression systems: a review of the existing biotechnology strategies. *Mol Cell Biochem.* (2008) 307: 249-64

Sambrook J, Russell DW: Molecular Cloning in A Laboratory Manual. 3. *Cold Spring Harbor Laboratory*, Cold Spring Harbor, NY; (2001)

Sandkvist M: Biology of type II secretion. *Mol Microbiol*. (2001), 40: 271-83

Schierle CF, Berkmen M, Huber D, Kumamoto C, Boyd D, Beckwith J: The DsbA Signal Sequence Directs Efficient, Cotranslational Export of Passenger Proteins to the *Escherichia coli* Periplasm via the Signal Recognition Particle Pathway. *J Bacteriol.* (2003), 185(19): 5706-13

Schrama D, Reisfeld RA, Jürgen CB: Antibody targeted drugs as cancer therapeutics. *Nat Rev Drug Discov.* (2006), 5: 147-59

Scotti C and Gherardi E: Structural basis of affinity maturation of the TEPC15/Vκ 45.1 anti-2-phenyl-5-oxazolone antibodies. *J Mol Biol.* (2006), 359: 1161-9

Spooner RA, Murray S, Rowlinson-Busza G, Deonarain MP, Chu A, Epenetos AA: Genetically engineered antibodies for diagnostic pathology. *Hum Pathol.* (1994), 25: 606-14

Tascon RI, Rodriguez-Ferri EF, Gutierrez-Martin CB, Rodriguez-Barbosa I, Berche P, Va zquez-Boland JA. Transposon mutagenesis in *Actinobacillus pleuropneumoniae* with a Tn10 derivative. *J Bacteriol*. (1993), 175: 5717-22

Tutino ML, Duilio A, Fontanella B, Moretti MA, Sannia G, Marino G: Plasmids from Antarctic bacteria. In: R. Margesin and F. Schinner, Editors *Cold-adapted organisms: ecology, physiology, enzymology and molecular biology*. *Spriger, Berlin*. (1999) pp.335-48

Tutino ML, Duilio A, Parrilli E, Remaut E, Sannia G, Marino G: A novel replication element from an Antarctic plasmid as a tool for the expression of proteins at low temperature. *Extremophiles*. (2001), 5: 257-64

Tutino ML, Parrilli E, Giaquinto L, Duilio A, Sannia G, Feller G, Marino G: Secretion of α-amylase from *Pseudoalteromonas haloplanktis* TAB23: two different pathways in different hosts. *J Bacteriol*. (2002), 184: 5814-17

Ullers RS, Houben ENG, Raine A, ten Hagen-Jongman CM, Ehrenberg M, Brunner J, Oudega B, Harms N, Luirink J: Interplay of signal recognition particle and trigger factor at L23 near the nascent chain exit site on the *Escherichia coli* ribosome. *J Cell Biol.* (2003), 161: 679-84

Vallejo LF and Rinas U: Strategy for recovery of active protein through refolding of bacterial inclusion body proteins. *Microb Cell Fact.* (2004), 3, 2-12

Vigentini I, Merico A, Tutino ML, Compagno C, Marino G: Optimization of recombinant Human Nerve Growth Factor production in the psychrophilic *Pseudoalteromonas haloplanktis*. *J Biotechnol.* (2006), 127:141-50

Wade H, Scanlan TS: The structural and functional basis of antibody catalysis. *Annu Rev Biophys Biomol Struct.* (1997), 26 :461-93

Weickert M, and Adhya S: Isorepressor of the *gal* regulon in *Escherichia coli*. *J Mol Biol*. (1992a) 226: 69-83

Weickert M, and Adhya S: A family of bacterial regulators homologous to Gal and Lac repressors. *J Biol Chem.* (1992b), 267: 15869-74

Weickert M, and Adhya S: Control of Transcription of Gal Repressor and Isorepressor Genes in *Escherichia coli. J Bacteriol.* (1993), 175: 251-8

Wickner W and Leonard MR: *Escherichia coli* Preprotein Translocase. *J Biol Chem.* (1996), 271(47): 29514-16

Wu SC, Ye R, Wu XC, Ng SC, Wong SL: Enhanced Secretory Production of a Single-Chain Antibody Fragment from *Bacillus subtilis* by Coproduction of Molecular Chaperones. *J Bacteriol.* (1998), 180(11): 2830-35

Yokota T, Milenic DE, Whitlow M, Schlom J: Rapid tumor penetration of a single-chain F_v and comparison with other immunoglobulin forms. *Cancer Res.* (1992), 52: 3402-8

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- E. Parrilli, **M. Giuliani**, M.L. Tutino: "General Secretory Pathway from marine Antarctic *Pseudoalteromonas haloplanktis* TAC125" *Marine Genomics* 2008, **1**(3-4): 123-128
- E. Parrilli, A.M. Cusano, **M. Giuliani** and M.L. Tutino: Cell engineering of *Pseudoalteromonas haloplanktis* TAC125: construction of a mutant strain with reduced exo-proteolytic activity" *Microbial Cell Factories* 2006, **5** (Suppl 1):P36
- E. Parrilli, **M. Giuliani**, C. Pezzella, A. Danchin, G. Marino, M.L. Tutino: "*Ph*PssA is required for alpha-amylase secretion in Antarctic *Pseudoalteromonas haloplanktis*" *Microbiology* 2010, DOI 10.1099/mic.0.032342-0- In press

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- M.L. Tutino, E. Parrilli, C. De Santi, **M. Giuliani**, G. Marino, D.de Pascale: "Cold-adapted esterases and lipases: a biodiversity still under-exploited" *Current Chemical Biology* 2010 - In press
- E. Parrilli, **M. Giuliani**, G. Marino, M.L. Tutino: "Influence of production process design on inclusion bodies protein: the case of an Antarctic flavohemoglobin" *Microbial Cell Factories* 2009 – Submitted
- E. Parrilli, **M. Giuliani**, D. Giordano, R. Russo, G. Marino, C. Verde, M.L. Tutino: "The role of a 2-on-2 haemoglobin in oxidative and nitrosative stress resistance of Antarctic *Pseudoalteromonas haloplanktis* TAC125" *Research in Microbiology* 2009 - Submitted
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Oral communications:

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- **M. Giuliani**, E. Parrilli, M.L. Tutino: "Antibody fragments production in a cold-adapted cell factory" **Cortona Procarioti 2008** , Cortona (AR), Italy, March 14-15th, 2008

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International Courses:

- **OSSIBE 4**, the 4th Oulu Summer School in Bioprocess Engineering "Protein production- what can go wrong and haw can you improve it fast?" Oulu, Finland, June 11-15th 2007
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Other publications

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Review

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Protein folding and conformational stress in microbial cells producing recombinant proteins: a host comparative overview

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Abstract

Different species of microorganisms including yeasts, filamentous fungi and bacteria have been used in the past 25 years for the controlled production of foreign proteins of scientific, pharmacological or industrial interest. A major obstacle for protein production processes and a limit to overall success has been the abundance of misfolded polypeptides, which fail to reach their native conformation. The presence of misfolded or folding-reluctant protein species causes considerable stress in host cells. The characterization of such adverse conditions and the elicited cell responses have permitted to better understand the physiology and molecular biology of conformational stress. Therefore, microbial cell factories for recombinant protein production are depicted here as a source of knowledge that has considerably helped to picture the extremely rich landscape of in vivo protein folding, and the main cellular players of this complex process are described for the most important cell factories used for biotechnological purposes.

Review

One of the main bottlenecks in recombinant protein production is the inability of the foreign polypeptides to reach their native conformation in heterologous host cells, which usually results into their prevalence in the insoluble cell fraction. The unusually high and non-phys-

iological rates of recombinant protein production and the occurrence of significant amounts of misfolded protein species drive the cells to a global conformational stress condition. This situation is characterized by a series of individual physiological responses provoked in order to minimize any toxicity of misfolded protein species and to restore cellular folding homeostasis. The generalized use of microbial cell factories for biological synthesis of proteins and the growing interest in the physiological aspects of conformational stress have converted recombinant cells into schools of protein folding, from which scientists are learning about the cell-protein relationships during the complex process of in vivo protein folding.

The purpose of this review is to summarize the major concepts of the cell biology of protein folding. For that, eukaryotic cells, illustrated by yeasts and filamentous fungi are dissected regarding the mechanics and composition of their folding machinery, misfolding stress responses and strategies to cope with conformational stress. The complexity of the folding, trafficking and secretion machineries of these cell factories is presented versus the relatively simple folding scheme in bacterial cells such as *Escherichia coli* that are also common hosts for recombinant protein production. Despite the existing obvious differences, evolutionary conserved physiological traits regarding folding stress can be identified when comparing eukaryotic and prokaryotic hosts. Furthermore, practical implications of all these findings to improve protein production processes are discussed in their biotechnological context.

Protein folding and conformational stress in eukaryotic cells

Yeasts and filamentous fungi are among the most frequently used eukaryotic cell systems for recombinant protein production, in part due to the performance of post-translational modifications that bacteria cannot perform, that are, in most cases, required for proper protein activity. In eukaryotic cells, endoplasmatic reticulum (ER) resident proteins are responsible for correct protein folding. The list of such folding-assistant proteins includes calnexin, chaperones of the hsp70 and hsp90 families (e.g. BiP/Grp78, Grp94), the protein disulfide isomerases (Pdi) which catalyze the formation of disulfide bonds and the peptidyl-prolyl-isomerases. Some of the post-translational modifications such as N-glycosylation are initiated in the ER lumen. Both natural and recombinant proteins are only exported to the Golgi by vesicular transport when their correct conformation has been assured by a glucose-dependent surveillance mechanism of the ER. Unless there is a differing signal, proteins intended for secretion are directed from the Golgi to the outside of the plasma membrane by specific transport vesicles [1,2]. A schematic overview of the protein folding processes is presented in Figure 1, while the responses to secretion stress are summarized in Figure 2.

The protein folding process and subsequent secretion is a rather complex process involving many interacting participants. Due to this interdependence, genetically increasing the rate of one step can lead to rate-limitation of another one, which can then become the bottleneck of the expression system. Moreover, in most cases the rate limiting step in the eukaryotic secretion pathway has been identified to be the exit of proteins from the ER [3]. Linked to this control point is a mechanism called ER-associated protein degradation (ERAD), which is responsible for the retention of misfolded or unmodified non-functional proteins in the ER and their subsequent removal. Protein degradation is executed by linking the misfolded protein to ubiquitin after it has been re-translocated into the cytosol through the same ER translocon pore where it had been imported. The ubiquitin-marked protein is then recognized and degraded by the 26S proteasome in the cytosol (recently reviewed by [4,5].

Two quality control systems in the ER ensure that only correctly folded, modified and assembled proteins travel further along the secretory pathway. The UDP-glucose:glycoprotein glucosyltransferase (UGT) is a central player of glycoprotein quality control in the ER (reviewed among others by [6]). After addition of the core glycan (GlcNac2-Man9-Glc3) to specific asparagine residues of the nascent polypeptide, the three terminal glucose residues have to be clipped off before the protein can exit the ER. Non-native polypeptides are tagged for reassociation with the ER-lectin calnexin by readdition of the terminal glucose onto the N-glycan mediated by UGT. This enzyme specifically recognizes and binds to molten globule-like folding intermediates, thereby acting as sensor of the protein folding status. Re-glucosylation of erroneous glycoproteins prevents their release from the calnexin cycle and subsequent secretion. Upon persistent misfolding, N-glycosylated polypeptides are slowly released from calnexin and enter a second level of retention-based ER quality control by aggregating with the BiP chaperone complex [7]. This correlates with the loss in the ability to emend misfolding. The BiP complex is involved in co-translational translocation of the nascent polypeptide into the ER lumen and preferentially binds to hydrophobic patches. Prolonged binding to either calnexin or the BiP complex targets the polypeptides to the ERAD, however, the exact mechanisms remain elusive (reviewed by [6]). The fact that accumulation of proteins in the ER is able to influence the synthesis of foldases and chaperones such as BiP and Pdi by transcriptional activation in the nucleus lead to the conclusion early on that there must be an intracellular signalling pathway from the ER to the nucleus,

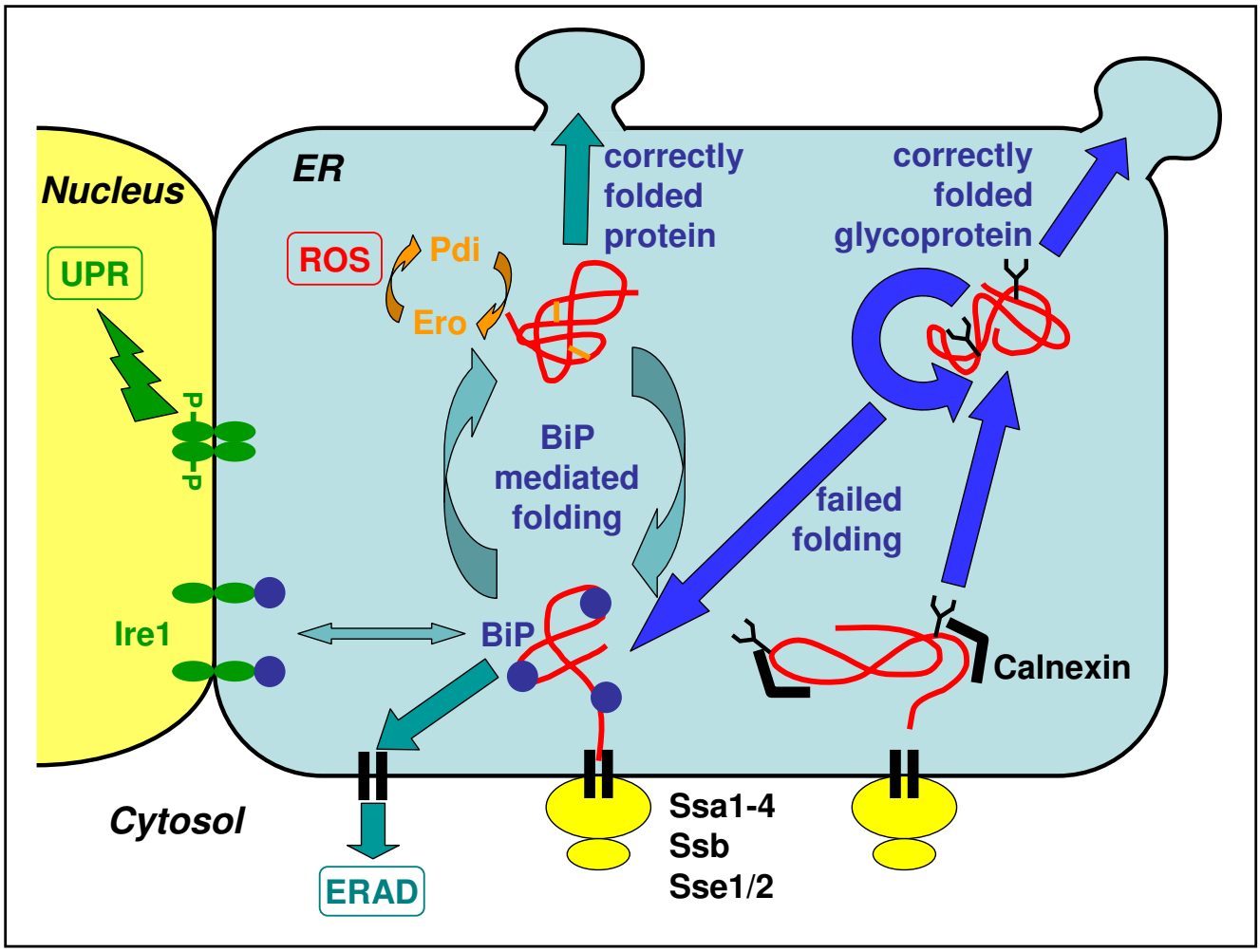

Figure 1

Schematic representation of protein folding, quality control, degradation and secretion in yeast (as an example for lower eukaryotic cells). Secretory proteins are transported into the ER through the Sec61 translocon complex of the ER membrane either co-translationally or post-translationally. In the latter case, cytosolic chaperones (Ssa1-4, Ssb, Sse1/2) support solubility and prevent aggregation of the polypeptide chains. After translocation to the ER, nascent polypeptides are bound by BiP and mediated to mature folding in an ATP-dependent cyclic process of release of and binding to BiP. The formation of correct disulfide bonds is mediated in a cycle of Pdi and Ero activity, which may lead to the formation of reactive oxygen species (ROS). Correctly folded protein is released to transport vesicles, while prolonged BiP binding, indicating misfolding, leads to retrograde translocation to the cytosol and proteasomal degradation (ERAD). Nascent glycoproteins are bound by calnexin and mediated to correct folding and processing of the N-glycans. Failed folding leads to binding by the BiP complex and targeting to ERAD, while correctly folded and processed glycoproteins are released to transport vesicles. Prolonged binding of BiP to partially misfolded proteins leads to the induction of the unfolded protein response (UPR), mediated by Ire1 (see also figure 2).

called the unfolded protein response (UPR) (for reviews see [8,9]).

After having passed ER quality control successfully, proteins intended for secretion have to be transported to the Golgi network. Specialized cargo vesicles that selectively incorporate these proteins bud from the ER and are targeted to the Golgi membrane by the activity of the coat protein complex II (COPII). In the Golgi network proteins undergo additional post-translational modifications and are subjected to sorting mechanisms that finally target them to their final destination. Possible trafficking routes include direction to the plasma membrane, to the endosomal compartments, to the vacuole, as well as retrograde transport to the ER (review by [10]). Secretory proteins are then delivered to the cell surface by specialized post-Golgi

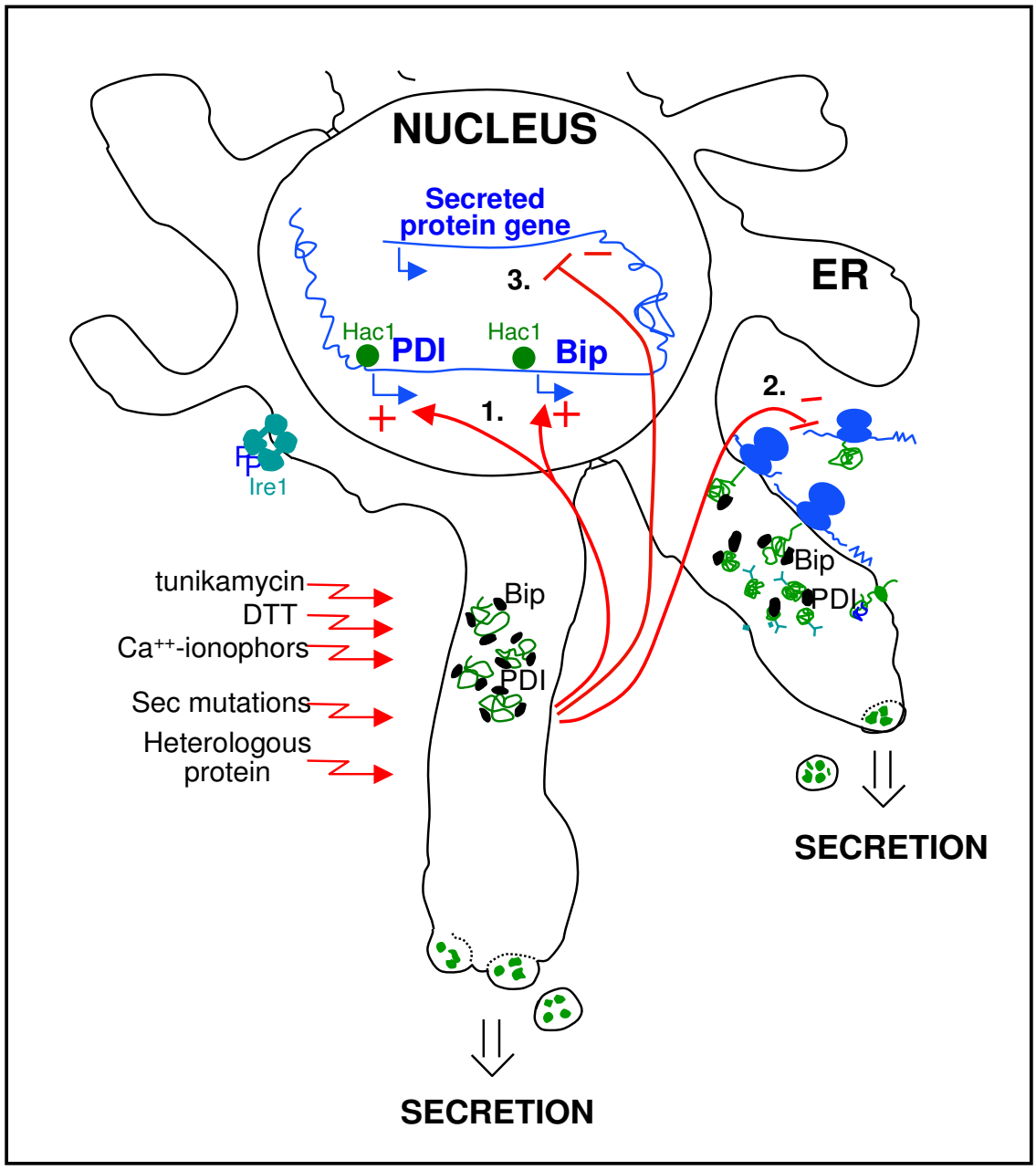

Figure 2
Schematic representation of secretion stress responses in eukaryotes Secretory proteins are translocated to the ER either during their translation or post-translationally. Folding of these proteins in the ER can be disturbed by environmental factors or it can be inhibited experimentally by agents inhibiting protein folding like dithiothreitol (DTT) and Ca-ionophores or agents inhibiting glycosylation like tunicamycin. It has been observed that foreign proteins often do not fold well and cause conformational stress. Several responses of the cell to impaired protein folding in the ER have been discovered: 1.) Unfolded protein response (UPR). Genes encoding folding helpers like the chaperone Bip and the foldase protein disulfide isomerase Pdi, and a large number of other genes involved in other functions of the secretory pathway are induced. The proteins Ire1 and Hac1 involved in this signal transduction pathway are shown in the figure. 2.) Translation attenuation. The translation initiation factor eIF2 alpha is phosphorylated, and subsequently translation initiation is inhibited. This reduces the influx of proteins into the ER. This response is only known from mammalian cells. 3.) Repression under secretion stress (RESS). The mRNA levels of genes encoding secreted proteins are down-regulated during ER stress. This response has been discovered in filamentous fungi, but there is evidence for its occurrence in plants.

secretory vesicles that dock to and fuse with the plasma membrane. The process called exocytosis includes targeting of the secretory vesicles to the appropriate membrane mediated by the Exocyst, a multiprotein complex, and by interaction of the v-SNAREs (vesicle, in yeast: Snc1/2 proteins) and t-SNAREs (target membrane; Sso1/2p and Sec9p) and release of the cargo proteins outside the cell after fusion of the secretory vesicle with the plasma membrane.

Impact of the environment on folding and folding stress

During the recent years, it has become evident that a variety of metabolic and environmental stresses may have a strong impact on recombinant protein production. Both types of stress factors occurring during industrial production processes in yeasts, along with potential metabolic and cell engineering approaches to overcome production constraints, were reviewed in Mattanovich et al. [11]. Among environmental factors that affect protein folding and secretion, especially temperature, low pH, high osmolarity and oxidative stress may play an important role.

While many studies have been performed on optimizing fermentation conditions for maximum specific productivity in yeasts, data correlating increased product yields to improved protein folding and secretion mechanisms are still missing. Similar reports regarding the impact of cultivation conditions on protein production in filamentous fungi remain scarce and usually limited to case studies [12-14]. Wang et al. [15] reviewed the impacts of bioprocess strategies on recombinant protein production in filamentous fungi, and concluded that the major effect of the environmental changes correlates to varying morphological forms, which exhibit different secretory capacities.

Temperature

Temperature has a profound impact on cell metabolism and abundance/regulation of folding-related genes/proteins (hsp70 family, ER-membrane proteins, etc.). Lowering the cultivation temperature from 30 to 20–25°C has been reported to increase product titers in yeasts in several cases [16-19]. While it may be speculated that a lower growth temperature is leading to lower specific growth rates, thus enabling folding of the recombinant proteins at a lower rate, it was shown recently in chemostat cultures that actually gene regulatory events take place. In continuous cultures of *Pichia pastoris* expressing a human antibody Fab fragment specific productivity of the heterologous protein was significantly increased during the chemostat process at lower temperature (1.4-fold on average). Several genes related to protein targeting to the ER and folding (*SSA4*, *SEC53*, *KAR2*, *ERO1*) and core metabolism genes were found among the genes down-regulated at 20°C, as were the product genes [20]. Transcription of genes involved in the regulation of vesicular transport, exocytosis, ER-associated protein degradation as well as markers for response to oxidative and hyperosmotic stress was enhanced in comparison to 25°C steady state. The reduction in transcriptional activity of the core metabolism is a likely explanation for the reduced mRNA levels of the product genes (LC and HC), which were under control of the glycolytic GAP (glyceraldehyde 3-phosphate dehydrogenase) promoter. The authors hypothesized that at lower temperature a reduced amount of folding stress is imposed on the cells, consequently leading to a higher rate of correctly folded product. Although lower temperature has been shown to improve protein secretion rates, this still depends on the nature of the heterologous protein. Production of a hyperthermophilic enzyme was improved by cultivation at higher temperature (40°C) in *Saccharomyces cerevisiae*, thereby reducing ER folding stress [21].

Additionally to regulatory events, many positive effects of temperature shifts on protein production might be linked to cell wall composition (porosity) and cell cycle. Indeed, increased levels of chitin and cell wall linking beta-glucans have been determined in yeast cells grown at 37°C compared to 22°C in batch cultures [22].

Generally, it turns out that cultivation at an optimized temperature is one of the crucial parameters for improved specific productivity, as it is likely to direct carbon fluxes towards heterologous protein production, and maintains the cells in the more secretion competent phases of the cell cycle.

Oxygenation

Redox processes play a major role in heterologous protein production, both related to the oxidation of the product to form disulfide bonds, and to oxidative stress of the host cell during cultivation. Cultivation of methylotrophic yeasts like *P. pastoris* on methanol leads to significant oxidative stress, which may be relieved by the addition of antioxidants like ascorbic acid [23]. Similarly, the expression of antioxidant enzymes like superoxide dismutase was reported to relieve oxidative stress [24].

Apart from environmental stressors, oxidative stress can be imposed on the host cells by intrinsic factors such as leakage in the respiratory pathway, beta-oxidation of lipids, or accumulation of misfolded protein in the ER. There is strong evidence that oxidative stress is connected to growth temperature. While in most cases lower growth temperature results in lower oxidative stress, Gasser et al. [20] showed that the genes coding for the key regulatory enzymes of both the cell redox homeostasis (thioredoxin reductase *TRR1*, thioredoxin peroxidase *TSA1*, glutathione oxidoreductase *GLR1*) and osmoregulation (mitogen-activated protein (MAP) kinase *HOG1*) were

induced at the lower temperature where higher secretion rates occur. Generally, the secretory pathway compartments maintain a higher oxidized status compared to the cytosol in order to enable disulfide-bond formation. Finally the electrons generated during the oxidative folding cycles are transferred to molecular oxygen and may lead to the formation of reactive oxygen species [25].

Interestingly, it was shown recently that very low oxygen supply enhances the secretion rate of heterologous proteins in *P. pastoris* significantly, which led to the development of a hypoxic fed batch strategy with over 2-fold increased productivity [26].

Osmolarity

So far no clear connection between medium osmolarity and protein folding has been established. Previous data indicate that the response is extremely transient [27]; and even less is known of the effect of osmolarity on heterologous protein production. Mager and Siderius [28] describe temporary cell growth arrest (either at G1 or G2/M) upon hyperosmotic stress conditions accompanied by the induction of the high osmolarity glycerol (HOG) kinase pathway in *S. cerevisiae*. Intracellular glycerol levels are increased in order to adjust osmo-balance through the modification of cell wall integrity. Unlike in animal cells where an osmotic shock leads to increased exocytosis [29], and hyperosmotic GS-NS0 mammalian cells that exhibit an increased specific production rate (albeit decreased growth rate) as compared with iso-osmotic cultures [30], osmo-regulated secretion behaviour in fungi remains unproven. In methanol grown *P. pastoris* cells, salt stress prior to induction was shown to have a positive effect on single chain antibody scFv titers [19], while Lin et al. [18] reported a negative effect of salt supplementation on the secretion of an Fc fusion protein.

pH

Osmolarity and pH seem to trigger highly interrelated responses. From an industrial point of view the main desired effect of low pH is to reduce the activity of host proteases which can lead to severe protein degradation (reviewed among others by [31]), but no uniform picture has been assigned to the correlation of pH and protease activity in the culture broth. Both in yeasts and filamentous fungi changing the pH of the culture medium can significantly improve protein yields, however, this effect is most probably not directly associated with improved protein folding mechanisms. On the other hand, lower extracellular pH requires higher energy to maintain intracellular pH values constant/physiological, thereby delaying cell growth and enforcing the cell wall barrier [22,32,33]. Subsequently this more rigid cell wall may diminish secretion efficiency of the pH stressed cells. Lin at al. [18] tested different pH values (ranging from 3.0 to 7.2) during fed batch production of a Fc fusion protein in *P. pastoris* and reported detection of the heterologous protein only at the highest pH of 7.2, however, the authors conclude that the pH optimum is strongly protein and strain dependent.

Folding stress and heterologous protein production

The ER-resident chaperone BiP (binding protein, in yeast encoded by *KAR2*) belongs to Hsp70 family of heat shock proteins and it is present in the lumen of the endoplasmatic reticulum of all eukaryotes. The yeast homologue is sometimes referred to as Grp78. Binding to BiP prevents the nascent part of secretory or transmembrane proteins from misfolding, until synthesis of the protein is finished. It has been suggested that BiP is not only involved in the translocation of the nascent polypeptides across the ER membrane into the ER lumen, but that it is a key element of an ER-resident quality control mechanism that prevents unfolded proteins from leaving the ER [34]. Other functions associated to BiP are the solubilisation of folding precursors, stabilization of unassembled protein subunits and redirecting misfolded polypeptide chains to the cytosol for ubiquitin-labeling and subsequent degradation by the proteasome (ERAD, ER-associated protein degradation, [35]). Besides a basal constitutive expression level, BiP transcription is induced by the presence of mutant and misfolded proteins in the ER lumen and by stress effects that result in the accumulation of unfolded proteins [36], presumably including the high level expression of heterologous proteins. A saturation of the secretory pathway seems possible, as extractable levels of free folding assistants BiP and Pdi1 decrease when heterologous proteins are overexpressed in *S. cerevisiae* [37]. Kauffman et al. [38] observed an induction of BiP during the expression of a scFv fragment in this yeast species, and Hohenblum et al. [39] have reported increased levels of BiP upon expression of recombinant human trypsinogen in *P. pastoris*. Likewise, biPA and pdiA transcript levels were increased due to heterologous protein overexpression, as well as upon high level secretion of homologous enzymes in filamentous fungi [40-42].

ER-associated protein degradation is a complex process in which misfolded proteins in the ER are redirected to the translocon for retranslocation to the cytosol, where they are subjected to proteasomal degradation. Additionally, excess subunits of multimeric proteins that are unable to assemble are degraded through the ERAD mechanism. According to Plemper et al. [43], the malfolded proteins are retro-translocated through the Sec61-complex translocon pore, through which they had entered the lumen of the ER before, accompanied by ubiquitination at the cytosolic side of the ER membrane. The labeling of substrates destined for degradation by the cytosolic 26S proteasome requires an Ub (ubiquitin) activating enzyme, an

Ub conjugating enzyme and an Ub ligase besides ubiquitin itself. In *P. pastoris* three essential components of the ERAD pathway have been shown to be up-regulated upon production of an antibody Fab fragment in correlation to higher protein secretion rates: *HRD1*, coding for an Ub protein ligase, that is able to recruit Ub conjugating enzymes (such as the gene product of *UBC1*) next to the Sec61 translocon pore complex [20].

Prolonged ER retention of misfolded proteins entails repetitive rounds of oxidative protein folding attempts by foldases such as Pdi and consequently results in the generation of reactive oxygen species (ROS). Alleviation of the ER stress is accomplished by the upregulation of the UPR and subsequent induction of the ERAD, however, prolonged UPR induction can also contribute to the stress situation by the accumulation of ROS. In this context, both oxidative stress and ERAD occur in addition to UPR induction when hydrophobic cutinase accumulates in the ER of *S. cerevisiae* [44], while hirudin production in *P. pastoris* lead to increased levels of ROS [23]. Recently it has been shown that overstraining or failure of the ERAD components leads to persistent ER stress conditions and subsequent cell death in both yeasts and higher eukaryotic cells [45,46].

The unfolded protein response pathway is activated by a unique mechanism not known in any other signal transduction pathway (for a recent review see [47]). The sensor protein Ire1p [48] resides in the ER membrane and possesses both kinase and endonuclease activities. When unfolded proteins accumulate in the ER, Ire1p undergoes autophosphorylation and oligomerisation, and catalyses the cleavage of the mRNA encoding the UPR transcription factor, called Hac1/hacA in yeasts and filamentous fungi [49,50] or Xbp1 in mammalian cells [51]. In this way Ire1p initiates an unconventional intron splicing event that has been shown in *S. cerevisiae* to be completed by tRNA ligase [52]. Splicing of yeast *HAC1* mRNA removes a translation block mediated by the intron [53] and enables formation of the activator protein. For mammalian Xbp1 it has been shown that the unspliced mRNA produces an unstable protein that represses the UPR target genes, whereas the spliced mRNA is translated to a potent, stable activator protein [51]. In the filamentous fungi *Trichoderma reesei, Aspergillus nidulans* [50] and *Aspergillus niger* [54], the hac1/hacA mRNA is truncated at the 5' flanking region during UPR induction, in addition to the unconventional intron splicing. This truncation removes upstream open reading frames from the mRNAs, most probably increasing translation initiation at the start codon of the *HAC1*/*HACA* open reading frame. Kincaid and Cooper [46] identified a novel function of Ire1p in the degradation of mRNAs encoding selected secretory proteins thus avoiding potential overload of the ER and the translocon complex *a priori*.

ER-associated stress responses such as UPR and ERAD were reported to be induced upon overexpression of several heterologous proteins, *e.g.*, human tissue plasminogen activator (t-PA) in *T. reesei* [55] and *A. niger* [56], and bovine chymosin in *A. nidulans* [57]. Similarily, overexpression of Fab fragments [20] and *Rhizopus oryzae* lipase [58] revealed UPR induction in *P. pastoris*.

In another layer of ER stress regulation, mammalian cells can attenuate translation initiation during unfolded protein accumulation into the ER, in order to reduce the influx of proteins to the ER. This regulation pathway is initiated by the ER membrane kinase PERK that has some similarity with Ire1 [59]. PERK phosphorylates the translation initiation factor eIF2alpha, resulting in drastic reduction in translation. This mechanism is not known in yeasts or filamentous fungi, and PERK orthologues can not be found in the genomes of the lower eukaryotes. Interestingly, the filamentous fungi *T. reesei* [60] and *A. niger* [61] have an alternative mechanism for controlling the protein influx to the ER. In conditions of ER stress the mRNAs encoding secreted proteins are rapidly down-regulated. This mechanism called RESS (repression under secretion stress) was shown to be dependent on the promoters of the genes encoding secreted proteins, and thus it probably functions at the level of transcription [60]. It has been observed that in *Arabidopsis thaliana* a large number of genes encoding secreted proteins are down-regulated when cells are exposed to ER stress [62], implying the possibility that RESS might also exist in plants.

Overcoming folding stress for improved protein production

Although promising expectations emerged that increased BiP levels would result in increased folding capacity in the ER, and thus improved secretion rates, the findings were rather inconsistent and unpredictable. Some studies emphasize that overproduction of BiP stimulates protein secretion in *S. cerevisiae* (5-fold increase in secretion of human erythropoietin [63], 26-fold increase in bovine prochymosin [64], 2.5-fold increase in the titer of antithrombotic hirudin due to 2.5 times higher biomass yields [65]). While the secretion level of plant thaumatin in *Aspergillus awamori* was increased up to 2.5-fold compared to a wild type strain due to bipA overexpression [66], the secretory behaviour of the same protein was not affected by overexpression of *KAR2* in *S. cerevisiae* [64]. According to Wittrup and coworkers, a reduction of BiP levels leads to decreased secretion of foreign proteins, however, no effect was observed upon a 5-fold overexpression of BiP on secretion levels of three different recombinant proteins in *S. cerevisiae* [67], and neither for cutinase in *A. awamori* [68]. Other reports even suggest a

negative impact of BiP overexpression, as extracellular levels of *A. niger* glucose oxidase (GOX) decreased 10-fold upon BiP overexpression in *Hansenula polymorpha* [69]. As prolonged binding to BiP seems to direct proteins rather to degradation than to the secretory pathway, it becomes more obvious why the overexpression of this chaperone alone does not result in higher levels of secreted foreign proteins, but can negatively influence expression levels, as reported by Kauffman *et al*. [38] and van der Heide *et al*. [69]. Interestingly, *Pyrococcus furiosus* beta-glucosidase secretion in *S. cerevisiae* is diminished with increased BiP levels, but benefited from higher protein disulfide isomerase (Pdi) levels, although the protein did not contain any disulfide bonds [70], pointing at the chaperone activity of Pdi, as discussed below.

Conesa et al. [71] examined the impact of overexpression of two ER quality control factors, BiP and calnexin, on the secretion of glycosylated *Phanerochaete chrysosporium* manganese peroxidase (MnP) in *A. niger*, as the expression levels of these genes were induced upon recombinant protein production. While BiP overproduction diminished manganese peroxidase secretion levels severely, overexpression of calnexin resulted in a four- to fivefold increase in the extracellular MnP levels. Higher levels of calnexin also showed beneficial effects in mammalian and baculo virus expression systems [72,73]. Recently, the co-overexpression of calnexin was shown to stimulate the secretion of three glycoproteins and one unglycosylated product (HSA) in *H. polymorpha* (2–3 fold on average; [74]). On the other hand, secretion of human serum albumin (HSA) remained unaffected by raising calnexin levels in *Schizosaccharomyces pombe* [75], while in *S. cerevisiae* deletion of the calnexin gene *CNE1* was reported to enhance secretion of both antitrypsin [76] and unstable lysozymes [77,78].

Protein disulfide isomerase (Pdi) is a multifunctional protein resident in the ER lumen that is responsible for the correct formation of disulfide bonds during oxidative folding and the isomerisation of uncorrectly folded disulfides. Apart from this foldase activity, Pdi also acts as a chaperone. An additional *PDI* gene copy in *S. cerevisiae* successfully improved secretion of human growth factor by 10-fold, of *S. pombe* acid phosphatase by 4-fold [63] and of human lysozyme by around 30–60% [79]. Human lysozyme as well as HSA production could also be enhanced by the same strategy in *Kluyveromyces lactis* (1.8 fold and 15 fold, respectively; [80,81]). Both *S. cerevisiae PDI1* and the *P. pastoris* own homolog were proven to be functional in *P. pastoris* by facilitating secretion of the human parathyroid hormone (hPTH, [82]), human anti HIV1 2F5 Fab [83], and *Necator americanus* secretory protein Na-ASP1 [84], the latter reporting a correlation between the secretory enhancement and the *PDI* copy number. Generally, no clear picture emerged from the co-overexpression of the two folding helpers, BiP and Pdi. Whereas synergistic action of BiP and Pdi was suggested regarding the improvement of the secretion of various single chain fragments (scFv) in *S. cerevisiae* [85], a 2-fold increase in secretion of the A33scFv in *P. pastoris* was only achieved by additional copies of *KAR2*, but not *PDI*, and not by the combination of both [86], in analogy to the antagonistic effect observed in CHO cells [87]. Coexpression of *KAR2*, *PDI1* or *SSO2* exhibited no effect on secretion of gamma-Interferon (IFNgamma) in *H. polymorpha* [88]. Moreover, coexpression of *cypB*, which encodes a foldase of the ER secretory pathway [89], did no increase production of tissue plasminogen activator (t-PA) in *A. niger*, although t-PA production elicited an UPR response detectable through elevated transcript levels of *bip*, *pdi* and *cypB* [90]. Thus, it seems that the effect of coexpression of chaperone and foldase genes strongly depends on the properties of the target protein and, moreover, it seems that fine-tuned overexpression of these genes are required to generate a functional secretory network to improve foreign protein overproduction. For example, in *A. niger*, overexpression of *bip* to a certain threshold was beneficial for plant sweet protein thaumatin production, but above this threshold level thaumatin production decreased [66]. Similarly, defined levels of Pdi were required for optimum thaumatin secretion in *A. niger* [91].

The flavoenzyme Ero1 is required for oxidation of protein dithiols in the ER. It is oxidized by molecular oxygen and acts as a specific oxidant of protein disulfide isomerase (Pdi). Disulfides generated de novo within Ero1 are transferred to Pdi and then to substrate proteins by dithiol-disulfide exchange reactions [92]. Duplication of either *KlPDI1* or *KlERO1* genes led to a similar increase in HSA yield in *K. lactis*, while duplication of both genes accelerated the secretion of HSA and improved cell growth rate and yield. Increasing the dosage of *KlERO1* did not affect the production of human interleukin 1beta, a protein that has no disulfide bridges [93].

Finally, another approach to stimulate the secretory pathway concertedly is to overexpress the unfolded protein response (UPR) activating transcription factor Hac1. Transcriptional analyses in *S. cerevisiae* revealed that up to 330 genes are regulated by Hac1, most of them belonging to the functional groups of secretion or the biogenesis of secretory organelles (e.g. ER-resident chaperones, foldases, components of the translocon). Interestingly, genes encoding proteins involved in protein degradation, vesicular trafficking, lipid biogenesis and vacuolar sorting are also induced by Hac1 [94]. In this context, Higashio and Kohno [95] describe the stimulation of ER-to-Golgi transport through the UPR by inducing COPII vesicle formation. The homologs of *S. cerevisiae HAC1* in *T. reesei*

(hac1) and *A. nidulans* (hacA) have been identified [50] and the effects of UPR induction by constitutive overexpression of these genes have been evaluated. The heterologous overexpression of *T. reesei* hac1 in *S. cerevisiae* yielded a 2.4-fold improvement in *Bacillus* α-amylase secretion, and a slight increase in the yeast endogenous invertase as well as in total protein secretion. *S. cerevisiae HAC1* overexpression was shown to enhance secretion of the endogenous invertase (2-fold), and recombinant α-amylase (70% increase), but did not effect secretion of *T. reesei* EGI, a protein supposed to accumulate in the ER. Disruption of *HAC1* in *S. cerevisiae* led to a reduced secretion of the two recombinant proteins (α-amylase -75%, EGI -50%), but not of the endogenous invertase [96]. Similar results could also be seen in *A. awamori*, where overproduction of *A. awamori* hacA ameliorated secretion of *Trametes versicolor* laccase and bovine preprochymosin 7-fold and 2.8 fold, respectively [97], and in *P. pastoris*, where heterologous expression of *S. cerevisiae HAC1* increased the secretion rate of a Fab antibody fragment [83].

Novel strategies: genome wide-screening

All these approaches are limited to the existing knowledge base. Novel processes might be identified and targeted to improve secretion (including non-UPR regulated genes) through different approaches. In this regard, high throughput flow cytometry and cell sorting are valuable tools to isolate overproducing clones [98]. One approach is to screen overexpression libraries for improved secretion of heterologous protein, which is anchored to the cell surface via agglutinin (Aga2p) and detected by immunofluorescent staining. Shusta et al. [99] showed that the levels of surface-displayed single chain T-cell receptors correlated strongly with the soluble expression of the respective proteins. A 3-fold higher secreting clone could be isolated out of a library potentially as large as 10^8 in a couple of weeks [100]. Screening of a yeast cDNA library in *S. cerevisiae* surface display strains identified cell wall proteins, translational components and the folding assistant Ero1 as beneficial for the secretion of various antibody fragments [101]. However, one potential drawback of this high throughput method is that the display efficiency of the protein of interest can be dominated by its fusion partner Aga2p, as BiP and *PDI* overexpression had no effect on surface display levels of the scFvs although they increased soluble expression levels [85].

Furthermore, genome-wide analytical tools like DNA microarrays are regarded as data mining source for physiological effects, stress regulation and host engineering. Sauer et al. [102] have analysed the differential transcriptome of a *P. pastoris* strain overexpressing human trypsinogen versus a non-expressing strain. 13 out of the 524 significantly regulated genes were selected, and their *S. cerevisiae* homologs were overexpressed in a *P. pastoris* strain producing a human antibody Fab fragment [103]. Five previously characterized secretion helpers (*PDI1*, *ERO1*, *SSO2*, *KAR2*/BiP and *HAC1*), as well as 6 novel, hitherto unidentified, factors, more precisely Bfr2 and Bmh2 involved in protein transport, the chaperones Ssa4 and Sse1, the vacuolar ATPase subunit Cup5 and Kin2, a protein kinase connected to exocytosis, proved their benefits for practical application in lab scale production processes by increasing both specific production rates as well as volumetric productivity of an antibody fragment up to 2.5-fold in fed batch fermentations of *P. pastoris* [103].

Protein folding and conformational stress in prokaryotic cells

Since early recombinant DNA times, bacteria (especially *E. coli*) have been the most widely used microorganisms for recombinant protein production due to genetic simplicity, fast growth rate, high cell density production and availability of a spectrum of genetic systems, among others. For production processes being efficient, foreign genes are expressed from plasmids and under the control of inducible promoters, what results into non physiological and unusually high transcription rates. Strong production of recombinant proteins can lead to a stressful situation for the host cell, with the extent of the bacterial stress response being determined by the specific properties of the recombinant protein, and by the rates of transcription and translation [104]. This fact has a clear and profoundly negative impact on productivity and probably protein quality. In addition, recombinant proteins fail, very often, to reach their native conformation when produced in bacteria [105]. This is caused by a coincidence of diverse events impairing protein folding including bottlenecks in transcription and translation, undertitration of chaperones and proteases, improper codon usage and inability of disulfide-bond formation [106,107]. Misfolded protein species usually deposit as amorphous masses of insoluble material called inclusion bodies [108], recorded as by-products of bacterial protein production processes. Inclusion bodies are mainly formed by the deposition of unfolded or partially misfolded protein species that interact through hydrophobic patches unusually exposed to the solvent and with high amino acid sequence homology [109,110]. The specificity in protein aggregation makes inclusion bodies highly pure in composition and therefore enriched in the recombinant protein itself. However, also truncated versions of the recombinant product, other plasmid-encoded proteins, but also defined host cell proteins can get entrapped within bacterial inclusion bodies [111-116]. Moreover, the presence of folding assistant proteins in inclusion bodies [117-119] confirm that specific interactions lead to entrappment of cellular proteins in these aggregates. The high purity of inclusion bodies makes them a convenient source of easily extractable pro-

tein that must be refolded in vitro by denaturing procedures, a fact that has been largely exploited for biotechnology purposes [120]. The potential routes of a newly synthesized protein in the bacterial cytosol are illustrated in Figure 3.

Although inclusion bodies are mainly found in the cytoplasm, they occur also in the bacterial periplasm if proteins have been engineered to present a leader peptide for secretion [121]. In fact, a control quality system mostly separated from that acting in the cytoplasm assist folding of secreted proteins in the periplasmic space of gram negative bacteria. This is regulated through the combined activity of two partially overlapping systems, regulated by the alternate σ factor σ^E and by the Cpx envelope stress signalling system, that intricately combine the activity of chaperones and proteases [122,123]. However, the simultaneous activation of stress signals in both bacterial compartments upon the production of misfolding prone proteins strongly suggest a close physiological and genetic connection between cytoplasmic and extracytoplasmic systems [124]. The quality control and conformational stress in the periplasmic space has been extensively revised elsewhere [121,125].

Different to the unfolded protein response (UPR) described in eukaryotic cells, the physiological reaction to conformational stress in the bacterial cytoplasm has not

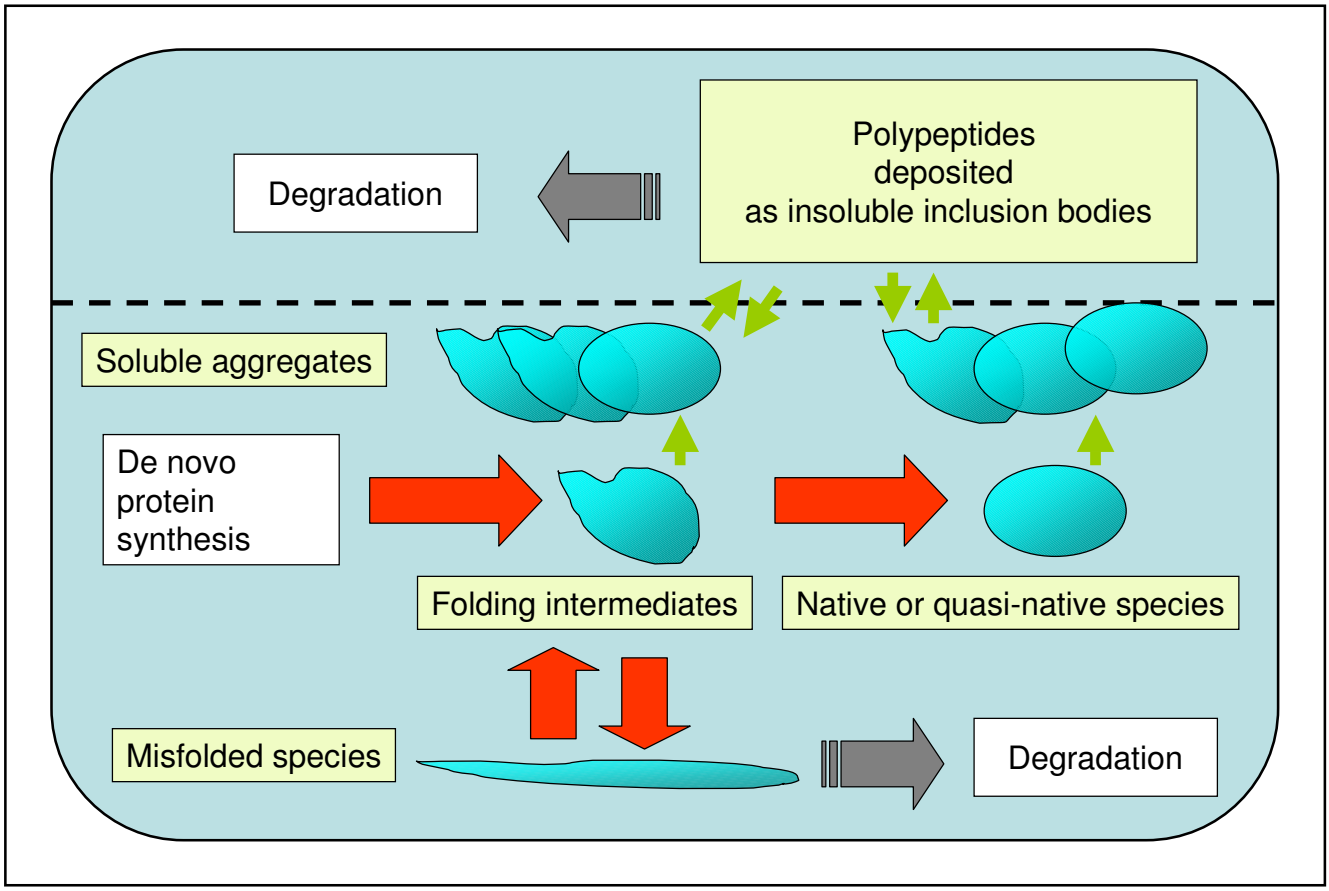

Figure 3
Schematic representation of protein folding and aggregation in recombinant *E. coli*. After de novo synthesis, a fraction of recombinant proteins (especially heterologous proteins with conformationally complex disulfide bridges) do not reach their native conformation and aggregate as insoluble deposits named inclusion bodies. Protein aggregates already exist in the soluble cell fraction, and can involve native or quasi-native protein species. The main cytoplasmic chaperones involved in the protein folding process (red arrows) include the trigger factor, DnaK, DnaJ, GrpE, GroEL and GroES. Both soluble aggregates and individual protein species can enter the virtual insoluble cell fraction (indicated by a dashed line) and deposit as inclusion bodies, in a fully reversible process (green arrows). Protein release from inclusion bodies is mainly controlled by DnaK, ClpB and IbpA,B. Proteases (Ion, ClpP and others) attack both soluble and insoluble species with folding defects. In particular, proteases degrade inclusion body proteins in situ, or through a more complex process intimately related to the protein release process, and therefore, strongly dependent on DnaK.

received any similar precise name. Transcriptome analysis of recombinant *E. coli* has resulted in a catalogue of genes up-regulated during protein production [126,127]. Among them several heat shock genes have been identified (including those encoding the proteases Lon, ClpP, HslV and HslU, and the chaperones IbpA, IbpB, DnaK, DnaJ, ClpB, HtpG, MopA and MopB among others) but also other ones not directly involved in protein quality (such as YagU, YojH, YbeD and others) and whose precise role remains to be identified. This fact indicates that the conformational stress imposed by protein production is more complex and physiologically distinguishable from that caused by thermal denaturation, namely the heat shock response [128,129], and includes several overlapping stress responses [104] Well characterized stress events have been observed during recombinant protein production such as SOS DNA repair [130] and stringent responses [131], although it is still be solved whether such reactions are directly associated to the prevalence of unfolded or misfolded protein species and the eventual connection with the σ^{32}-regulated heat shock response. The expression of some of these stress genes is being used as a convenient marker of conformational stress in recombinant cells [132].

The bacterial conformational stress itself has been poorly characterized from its physiological side. Instead, many efforts have been addressed to a rather practical issue such as minimizing aggregation, what in turn has resulted in a better comprehension of in vivo protein folding processes. Since solubility has been considered for a long time being synonymous with protein quality, increasing the relative yield of soluble protein has been targeted by physicochemical approaches. From already classical studies, it is well known that high temperatures impair protein folding and favour aggregation of the recombinant proteins as inclusion bodies [133,134]. Therefore, reducing the growth temperature has been a general strategy used to minimize inclusion body formation [135-137] that, like other strategies, has rendered moderately positive, but unpredictable and product-dependent results [107]. Fusion of folding-reluctant species to highly soluble homologous or thermostable proteins has in some cases, resulted in moderate enhancement of the passenger protein solubility [106,138,139].

Chaperones and protein degradation

Folding failures of recombinant proteins produced in *E. coli* is generally attributed to a limitation in the cell concentration of folding assistant elements, which cannot process the newly synthesized aggregation prone polypeptides. This assumption is physiologically supported by the overexpression of chaperone genes, in particular of chaperone genes from the heat-shock protein family, in response to recombinant protein overproduction [126,127,133]. Thus, coproduction of the main heat shock chaperones (specially GroEL and DnaK) together with the target protein has been largely explored as a way to minimize aggregation and to enhance the solubility of the recombinant protein product (reviewed in [140-143]). In many cases, solubility has been significantly enhanced by coexpression of individual chaperone genes, while in others an even negative effect on product stability and host viability has been observed. Selection of the suitable chaperone(s) is still a trial-and-error process. However, more recent results indicate that complete chaperones gene sets rather than individual chaperone genes with synergistic and/or cooperative activities (such as DnaK-DnaJ-GrpE and GroEL-GroES sets) will lead to a more predictable improvement of target protein solubility. [144-147].

Interestingly, when producing enzymes or fluorescent proteins in DnaK$^-$ cells, the biological activities and therefore the conformational quality of aggregated polypeptides is much more close to that of soluble versions, compared to wild type cells [148-150]. Furthermore, the overexpression of the *dnaK* gene along with a model GFP recombinant protein dramatically reduces the specific fluorescence of a GFP fusion in both soluble and insoluble versions [151]. This indicates that DnaK directly or indirectly impairs the folding state of the aggregated proteins. In this regard, the production of GFP variants in absence of DnaK results in highly fluorescent inclusion bodies [152]. In these cells, both the protein yield and quality were dramatically enhanced although the solubility is lower than in the wild type, as expected. This occurs by the inhibition of GFP proteolysis mediated by the proteases Lon and ClpP, which participate in the in vivo disintegration of inclusion bodies in absence of protein synthesis [153,154]. Probably, such proteases act coordinately in a disaggregation complex [155-157] in which DnaK, ClpB and IbpAB remove aggregated polypeptides for proteolytic digestion. Therefore, although solubility can be indeed enhanced by high levels of DnaK, GroEL and other chaperones it occurs at expenses of quality and yield, probably by generally stimulating proteolysis [116]. In fact, solubility and conformational quality are not only non coincident parameters [158] but highly divergent protein features [152].

Disulfide-bond formation in recombinant E. coli

Usually, the cytoplasmic space of *E. coli* is a reducing environment. Therefore, disulfide-bonds within proteins are not formed, a fact that represents an additional obstacle for proper folding of many recombinant proteins. There are two approaches to produce disulfide-bonded proteins in *E. coli* expression, namely *in vitro* refolding of inclusion body proteins under appropriate redox conditions [120] or manipulating *in vivo* conditions by either converting

the cytoplasm into an oxidizing environment or secreting the protein into the periplasmic space or even further into the culture medium (less reducing environments). Correct disulfide bond formation in the periplasm of *E. coli* is a catalyzed process, where the oxidation of cysteine pairs occurs through the transfer of disulfides from the highly oxidizing DsbA/DsbB proteins to the proof-reading proteins DsbC/DsbD which are able to rearrange non-native disulfides to their native configuration [159]. In particular, overexpression of DsbC has been shown to increase the yield of correctly disulfide-bonded proteins in the periplasm of *E. coli* [160-162]. The co-expression of eukaryotic protein disulfide isomerases in *E. coli* can also favour the formation of disulfide bonds in the periplasmic space [163,164].

Disulfide bond formation in the cytoplasm of *E. coli* can occur when the genes encoding thioredoxin reductase (*trxB*) and glutathione oxido-reductase (*gor*) are inactivated [165,166]. A double-mutant strain containing appropriate mutations, known as Origami, has been used, for example, to generate active variants of tissue-type plasminogen activator [165] and functional antibody fragments in the *E. coli* cytoplasm [167,168]. In some cases, recovery of functional cytoplasmic disulfide-bonded proteins can be further enhanced by coexpressing signal sequence deficient periplasmic chaperones and/or disulfide-bond isomerases such as DsbC [165,167,169,170]. Unfortunately, *trxB gor* mutants exhibit impaired growth characteristics [112,165], but, at least for antibody fragments it has been shown that expression yields of correctly disulfide-bonded proteins in the cytoplasm can be similar to those obtained by secretion into the periplasmic space [171].

Protein folding and secretion in non-conventional bacterial expression systems

Although *E. coli* is still the most commonly used prokaryotic organism for heterologous protein production, other bacterial hosts are becoming more and more attractive.

Gram-positive Bacilli strains are also frequently employed at industrial level. In contrast to *E. coli*, their outer envelope has no lipopolysaccharides, also called "endotoxins" since they exert a pyrogenic activity in humans or other mammals. Therefore, many pharmaceutically relevant products have been obtained in several strains [172]. In addition, the Bacilli strains are attractive hosts because they have a naturally high secretion capacity, as they export proteins directly into the extracellular medium. Amongst Bacilli species, the protein secretion pathway in *B. subtilis* have been deeply investigated at molecular level and a comprehensive literature survey is reported in [173]. Several bottlenecks in the expression and secretion of heterologous proteins have been highlighted [174]. The secretory pathway of proteins can be divided into three functional stages: the early stage, involving the synthesis of secretory pre-proteins, their interaction with chaperones and binding to the secretory translocase complex; the second stage, consisting in translocation across the cytoplasmic membrane; and the last stage, including removal of the N-terminal signal peptide, protein refolding and passage through the cell wall. A pivotal role in the whole secretion process is played by molecular chaperones [175]. *B. subtilis* has two types of molecular chaperones, intracellular and extra-cytoplasmic molecular chaperones. GroES, GroEL, DnaK, DnaJ and GrpE are intracellular molecular chaperones. Besides being involved in and largely responsible for protein folding and minimizing aggregation, these chaperones maintain pre-proteins (the products to be secreted) in translocation-competent conformations [176]. PrsA is the only known extracytoplasmic folding factor in *B. subtilis*. PrsA is a lipoprotein that consists of a 33 kDa lysine-rich protein part and the N-terminal cysteine with a thiol-linked diacylglycerol anchoring the protein to the outer leaflet of the cytoplasmic membrane [177]. Subsequent folding of a secreted mature protein into a stable and active conformation usually requires PrsA protein. In *prsA* mutants, the secretion and stability of some model proteins is decreased, if not abolished, while overproduction of PrsA enhances the secretion of exoproteins engineered to be expressed at a high level [178].

There is, however, a physiologic limit to the overloading of *B. subtilis* secretory pathway. The massive production of homologous and heterologous exoproteins has been reported to induce a phenomenon called "protein secretion stress response" [179]. The CssRS two-component regulatory system is able to detect the presence of partially folded or unfolded exo-protein intermediates and activates the transcription of several genes, among which a key role is played by *htrAB*. These genes encode two membrane localised serine proteases involved in the proteolysis of aberrant products [180].

Several gene expression systems using non-conventional prokaryotic organisms as host cells have been developed over the last decades. Each bacterial host was generally implemented to overcome defined problems/bottlenecks observed during the recombinant production of specific protein classes in conventional systems, such as *E. coli* and *B. subtilis*. The use of such non-conventional systems is still very limited and largely suffers from the lack of molecular details concerning host physiology and any other phenomenon related to massive recombinant protein production. Notwithstanding, some of them may represent useful model systems to further investigate on the optimization of recombinant protein folding and quality.

In this context, some interest has been raised by the implementation of an Antarctic Gram negative bacterium as production host. *Pseudoalteromonas haloplanktis* TAC125 was isolated from a sea water sample collected in the vicinity of the Dumont d'Urville Antarctic station, in Terre Adélie. It is characterised by fast growth rates, combined with the ability to reach very high cell densities even in uncontrolled laboratory growth conditions and to be easily transformed by intergeneric conjugation [181]. There features made *P. haloplanktis* TAC125 an attracting host for the development of an efficient gene-expression system for the recombinant protein production at low temperatures of thermal-labile and aggregation-prone proteins [182]. Furthermore, it was the first Antarctic Gram-negative bacterium which genome was fully determined and carefully annotated [183].

Since high temperatures have a general negative impact on protein folding due to the strong temperature dependence of hydrophobic interactions that mainly drive the aggregation reaction [184], and favour conformational stress, the production of recombinant proteins at low temperatures represents an exciting model to study the dynamics of protein folding and misfolding and to improve the quality of the products. The growth of *E. coli* below 37°C has been often explored to minimize aggregation but without consistent, protein-irrespective results. Also, the use of suboptimal growth temperatures might negatively affect the biology of the host cell and the performance of the process in undesirable and non predictable ways. Recombinant protein production in psychrophilic bacteria, i.e. at temperature as low as 4°C, may minimize undesired hydrophobic interactions during protein folding, desirably resulting in enhancing the yield of soluble and correctly folded products while operating close to the optimal growth range. Furthermore, with respect to mesophilic cells growing at suboptimal temperatures, psychrophiles contain a full set of folding factors already adapted to assist optimally, when required, protein folding at freezing temperatures.

The efficiency of the cold-adapted expression system was tested by producing several aggregation-prone products in *P. haloplanktis* TAC125, such as a yeast α-glucosidase [182], the mature human nerve growth factor [182], and a cold adapted lipase [185]. All the recombinant products resulted to be fully soluble and biologically competent.

Concluding remarks

In vivo protein folding is a very complex issue that involves many cellular proteins and physiological responses. During recombinant protein production, conformational stress conditions elicited by the synthesis of aggregation prone polypeptides profoundly alter the physiology of the host cell, triggering mechanisms addressed to manage potentially toxic misfolding protein species and to recover the cell folding homeostasis. The use of different microorganims as factories for recombinant protein production, including yeast, filamentous fungi and bacteria has resulted in dramatic gains of information about the biology of such stress responses, and has provided valuable information to better understand the mechanics of in vivo protein folding and aggregation.

However, so far it has not been possible to create the "perfect folding environment". Especially with respect to industrial protein production processes, the direct impact of altered process conditions on recombinant protein folding remains unclear. Ongoing research in the authors' labs is targeted to elucidate the physiological responses of different eukaryotic and prokaryotic microbial hosts on a genome wide level in order to interrelate environmental stresses to protein folding/aggregation mechanisms and eliminate bottlenecks.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

All authors contributed equally to this manuscript, and read and approved the final version.

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References

1. Gething MJ, Sambrook J: **Protein folding in the cell.** *Nature* 1992, **355(6355):**33-45.
2. Stryer , Lubert : **Biochemie.** *Spektrum der Wissenschaft VerlagsGmbH* 1995.
3. Shuster JR: **Gene expression in yeast: protein secretion.** *Curr Opin Biotechnol* 1991, **2(5):**685-690.
4. Brodsky JL, Scott CM: **Tipping the delicate balance: defining how proteasome maturation affects the degradation of a substrate for autophagy and endoplasmic reticulum associated degradation (ERAD).** *Autophagy* 2007, **3(6):**623-625.
5. Meusser B, Hirsch C, Jarosch E, Sommer T: **ERAD: the long road to destruction.** *Nat Cell Biol* 2005, **7(8):**766-772.
6. Kleizen B, Braakman I: **Protein folding and quality control in the endoplasmic reticulum.** *Curr Opin Cell Biol* 2004, **16(4):**343-349.
7. Molinari M, Galli C, Vanoni O, Arnold SM, Kaufman RJ: **Persistent glycoprotein misfolding activates the glucosidase II/UGT1-**

driven calnexin cycle to delay aggregation and loss of folding competence. *Mol Cell* 2005, **20(4):**503-512.
8. Ma Y, Hendershot LM: **The unfolding tale of the unfolded protein response.** *Cell* 2001, **107(7):**827-830.
9. Patil C, Walter P: **Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals.** *Curr Opin Cell Biol* 2001, **13(3):**349-355.
10. Nakano A: **Yeast Golgi apparatus – dynamics and sorting.** *Cell Mol Life Sci* 2004, **61(2):**186-191.
11. Mattanovich D, Gasser B, Hohenblum H, Sauer M: **Stress in recombinant protein producing yeasts.** *J Biotechnol* 2004, **113(1–3):**121-135.
12. Garcia-Kirchner O, Segura-Granados M, Rodriguez-Pascual P: **Effect of media composition and growth conditions on production of beta-glucosidase by Aspergillus niger C-6.** *Appl Biochem Biotechnol* 2005, **121–124:**347-359.
13. Liu CQ, Chen QH, Cheng QJ, Wang JL, He GQ: **Effect of cultivating conditions on alpha-galactosidase production by a novel Aspergillus foetidus ZU-G1 strain in solid-state fermentation.** *J Zhejiang Univ Sci B* 2007, **8(5):**371-376.
14. Wang L, Ridgway D, Gu T, Moo-Young M: **Effects of process parameters on heterologous protein production in Aspergilus niger fermentation.** *Journal of Chemical Technology and Biotechnology* 2003, **78(12):**1259-1266.
15. Wang L, Ridgway D, Gu T, Moo-Young M: **Bioprocessing strategies to improve heterologous protein production in filamentous fungal fermentations.** *Biotechnol Adv* 2005, **23(2):**115-129.
16. Hackel BJ, Huang D, Bubolz JC, Wang XX, Shusta EV: **Production of soluble and active transferrin receptor-targeting single-chain antibody using Saccharomyces cerevisiae.** *Pharm Res* 2006, **23(4):**790-797.
17. Li Z, Xiong F, Lin Q, d'Anjou M, Daugulis AJ, Yang DS, Hew CL: **Low-temperature increases the yield of biologically active herring antifreeze protein in Pichia pastoris.** *Protein Expr Purif* 2001, **21(3):**438-445.
18. Lin H, Kim T, Xiong F, Yang X: **Enhancing the production of Fc fusion protein in fed-batch fermentation of Pichia pastoris by design of experiments.** *Biotechnol Prog* 2007, **23(3):**621-625.
19. Shi X, Karkut T, Chamankhah M, Alting-Mees M, Hemmingsen SM, Hegedus D: **Optimal conditions for the expression of a single-chain antibody (scFv) gene in Pichia pastoris.** *Protein Expr Purif* 2003, **28(2):**321-330.
20. Gasser B, Maurer M, Rautio J, Sauer M, Bhattacharyya A, Saloheimo M, Penttilä M, Mattanovich D: **Monitoring of transcriptional regulation in Pichia pastoris under protein production conditions.** *BMC Genomics* 2007, **8:**179.
21. Smith JD, Richardson NE, Robinson AS: **Elevated expression temperature in a mesophilic host results in increased secretion of a hyperthermophilic enzyme and decreased cell stress.** *Biochim Biophys Acta* 2005, **1752(1):**18-25.
22. Aguilar-Uscanga B, Francois JM: **A study of the yeast cell wall composition and structure in response to growth conditions and mode of cultivation.** *Lett Appl Microbiol* 2003, **37(3):**268-274.
23. Xiao A, Zhou X, Zhou L, Zhang Y: **Improvement of cell viability and hirudin production by ascorbic acid in Pichia pastoris fermentation.** *Appl Microbiol Biotechnol* 2006, **72(4):**837-844.
24. Li JR, Yu P: **Expression of Cu, Zn-superoxide dismutase gene from Saccharomyces cerevisiae in Pichia pastoris and its resistance to oxidative stress.** *Appl Biochem Biotechnol* 2007, **136(1):**127-139.
25. Tu BP, Weissman JS: **Oxidative protein folding in eukaryotes: mechanisms and consequences.** *J Cell Biol* 2004, **164(3):**341-346.
26. Baumann K, Maurer M, Dragosits M, Cos O, Ferrer P, Mattanovich D: **Hypoxic fed batch cultivation of Pichia pastoris increases specific and volumetric productivity of recombinant proteins.** *Biotechnol Bioeng* 2008, **100(1):**177-83.
27. Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO: **Genomic expression programs in the response of yeast cells to environmental changes.** *Mol Biol Cell* 2000, **11(12):**4241-4257.
28. Mager WH, Siderius M: **Novel insights into the osmotic stress response of yeast.** *FEMS Yeast Res* 2002, **2(3):**251-257.
29. Ollivier H, Pichavant-Rafini K, Puill-Stephan E, Calves P, Nonnotte L, Nonnotte G: **Effects of hyposmotic stress on exocytosis in isolated turbot, Scophthalmus maximus, hepatocytes.** *J Comp Physiol [B]* 2006, **176(7):**643-652.
30. Wu MH, Dimopoulos G, Mantalaris A, Varley J: **The effect of hyperosmotic pressure on antibody production and gene expression in the GS-NS0 cell line.** *Biotechnol Appl Biochem* 2004, **40(Pt 1):**41-46.
31. Jahic M, Veide A, Charoenrat T, Teeri T, Enfors SO: **Process technology for production and recovery of heterologous proteins with Pichia pastoris.** *Biotechnol Prog* 2006, **22(6):**1465-1473.
32. Fernandes AR, Mira NP, Vargas RC, Canelhas I, Sa-Correia I: **Saccharomyces cerevisiae adaptation to weak acids involves the transcription factor Haa1p and Haa1p-regulated genes.** *Biochem Biophys Res Commun* 2005, **337(1):**95-103.
33. Kapteyn JC, ter Riet B, Vink E, Blad S, De Nobel H, Van Den Ende H, Klis FM: **Low external pH induces HOG1-dependent changes in the organization of the Saccharomyces cerevisiae cell wall.** *Mol Microbiol* 2001, **39(2):**469-479.
34. Dorner A, Kaufman R: **The levels of endoplasmic reticulum proteins and ATP affect folding and secretion of selective proteins.** *Biologicals* 1994, **22(2):**103-112.
35. Nishikawa S, Fewell S, Kato Y, Brodsky J, Endo T: **Molecular chaperones in the yeast endoplasmic reticulum maintain the solubility of proteins for retrotranslocation and degradation.** *J Cell Biol* 2001, **153(5):**1061-1070.
36. Kozutsumi Y, Segal M, Normington K, Gething M, Sambrook J: **The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins.** *Nature* 1988, **332(6163):**462-464.
37. Robinson A, Wittrup K: **Constitutive overexpression of secreted heterologous proteins decreases extractable BiP and protein disulfide isomerase levels in Saccharomyces cerevisiae.** *Biotechnol Prog* 1995, **11(2):**171-177.
38. Kauffman K, Pridgen E, Doyle Fr, Dhurjati P, Robinson A: **Decreased protein expression and intermittent recoveries in BiP levels result from cellular stress during heterologous protein expression in Saccharomyces cerevisiae.** *Biotechnol Prog* 2002, **18(5):**942-950.
39. Hohenblum H, Gasser B, Maurer M, Borth N, Mattanovich D: **Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant Pichia pastoris.** *Biotechnol Bioeng* 2004, **85(4):**367-375.
40. Nyyssönen E, Keränen S: **Multiple roles of the cellulase CBHI in enhancing production of fusion antibodies by the filamentous fungus Trichoderma reesei.** *Curr Genet* 1995, **28(1):**71-79.
41. Pakula T, Salonen K, Uusitalo J, Penttilä M: **The effect of specific growth rate on protein synthesis and secretion in the filamentous fungus Trichoderma reesei.** *Microbiology* 2005, **151(Pt 1):**135-143.
42. Punt P, van Gemeren I, Drint-Kuijvenhoven J, Hessing J, van Muijlwijk-Harteveld G, Beijersbergen A, Verrips C, van den Hondel C: **Analysis of the role of the gene bipA, encoding the major endoplasmic reticulum chaperone protein in the secretion of homologous and heterologous proteins in black Aspergilli.** *Appl Microbiol Biotechnol* 1998, **50(4):**447-454.
43. Plemper RK, Bordallo J, Deak PM, Taxis C, Hitt R, Wolf DH: **Genetic interactions of Hrd3p and Der3p/Hrd1p with Sec61p suggest a retro-translocation complex mediating protein transport for ER degradation.** *J Cell Sci* 1999, **112(Pt 22):**4123-4134.
44. Sagt C, Müller W, van der Heide L, Boonstra J, Verkleij A, Verrips C: **Impaired cutinase secretion in Saccharomyces cerevisiae induces irregular endoplasmic reticulum (ER) membrane proliferation, oxidative stress, and ER-associated degradation.** *Appl Environ Microbiol* 2002, **68(5):**2155-2160.
45. Haynes CM, Titus EA, Cooper AA: **Degradation of misfolded proteins prevents ER-derived oxidative stress and cell death.** *Mol Cell* 2004, **15(5):**767-776.
46. Kincaid MM, Cooper AA: **ERADicate ER stress or die trying.** *Antioxid Redox Signal* 2007, **9(12):**2373-2387.
47. Ron D, Walter P: **Signal integration in the endoplasmic reticulum unfolded protein response.** *Nat Rev Mol Cell Biol* 2007, **8(7):**519-529.
48. Cox JS, Shamu CE, Walter P: **Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase.** *Cell* 1993, **73(6):**1197-1206.
49. Cox JS, Walter P: **A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response.** *Cell* 1996, **87(3):**391-404.

50. Saloheimo M, Valkonen M, Penttila M: **Activation mechanisms of the HAC1-mediated unfolded protein response in filamentous fungi.** *Mol Microbiol* 2003, **47(4):**1149-1161.
51. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K: **XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor.** *Cell* 2001, **107(7):**881-891.
52. Sidrauski C, Cox JS, Walter P: **tRNA ligase is required for regulated mRNA splicing in the unfolded protein response.** *Cell* 1996, **87(3):**405-413.
53. Ruegsegger U, Leber JH, Walter P: **Block of HAC1 mRNA translation by long-range base pairing is released by cytoplasmic splicing upon induction of the unfolded protein response.** *Cell* 2001, **107(1):**103-114.
54. Mulder HJ, Saloheimo M, Penttila M, Madrid SM: **The transcription factor HACA mediates the unfolded protein response in Aspergillus niger, and up-regulates its own transcription.** *Mol Genet Genomics* 2004, **271(2):**130-140.
55. Arvas M, Pakula T, Lanthaler K, Saloheimo M, Valkonen M, Suortti T, Robson G, Penttila M: **Common features and interesting differences in transcriptional responses to secretion stress in the fungi Trichoderma reesei and Saccharomyces cerevisiae.** *BMC Genomics* 2006, **7:**32.
56. Guillemette T, van Peij N, Goosen T, Lanthaler K, Robson G, van den Hondel C, Stam H, Archer D: **Genomic analysis of the secretion stress response in the enzyme-producing cell factory Aspergillus niger.** *BMC Genomics* 2007, **8:**158.
57. Sims AH, Gent ME, Lanthaler K, Dunn-Coleman NS, Oliver SG, Robson GD: **Transcriptome analysis of recombinant protein secretion by Aspergillus nidulans and the unfolded-protein response in vivo.** *Appl Environ Microbiol* 2005, **71(5):**2737-2747.
58. Resina D, Bollók M, Khatri N, Valero F, Neubauer P, Ferrer P: **Transcriptional response of P. pastoris in fed-batch cultivations to Rhizopus oryzae lipase production reveals UPR induction.** *Microb Cell Fact* 2007, **6:**21.
59. Harding HP, Zhang Y, Ron D: **Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase.** *Nature* 1999, **397(6716):**271-274.
60. Pakula TM, Laxell M, Huuskonen A, Uusitalo J, Saloheimo M, Penttila M: **The effects of drugs inhibiting protein secretion in the filamentous fungus Trichoderma reesei. Evidence for down-regulation of genes that encode secreted proteins in the stressed cells.** *J Biol Chem* 2003, **278(45):**45011-45020.
61. Al-Sheikh H, Watson AJ, Lacey GA, Punt PJ, MacKenzie DA, Jeenes DJ, Pakula T, Penttila M, Alcocer MJ, Archer DB: **Endoplasmic reticulum stress leads to the selective transcriptional down-regulation of the glucoamylase gene in Aspergillus niger.** *Mol Microbiol* 2004, **53(6):**1731-1742.
62. Martinez IM, Chrispeels MJ: **Genomic analysis of the unfolded protein response in Arabidopsis shows its connection to important cellular processes.** *Plant Cell* 2003, **15(2):**561-576.
63. Robinson A, Hines V, Wittrup K: **Protein disulfide isomerase overexpression increases secretion of foreign proteins in Saccharomyces cerevisiae.** *Biotechnology (NY)* 1994, **12(4):**381-384.
64. Harmsen M, Bruyne M, Raué H, Maat J: **Overexpression of binding protein and disruption of the PMR1 gene synergistically stimulate secretion of bovine prochymosin but not plant thaumatin in yeast.** *Appl Microbiol Biotechnol* 1996, **46(4):**365-370.
65. Kim M, Han K, Kang H, Rhee S, Seo J: **Coexpression of BiP increased antithrombotic hirudin production in recombinant Saccharomyces cerevisiae.** *J Biotechnol* 2003, **101(1):**81-87.
66. Lombraña M, Moralejo F, Pinto R, Martín J: **Modulation of Aspergillus awamori thaumatin secretion by modification of bipA gene expression.** *Appl Environ Microbiol* 2004, **70(9):**5145-5152.
67. Robinson A, Bockhaus J, Voegler A, Wittrup K: **Reduction of BiP levels decreases heterologous protein secretion in Saccharomyces cerevisiae.** *J Biol Chem* 1996, **271(17):**10017-10022.
68. van Gemeren I, Beijersbergen A, van den Hondel C, Verrips C: **Expression and secretion of defined cutinase variants by Aspergillus awamori.** *Appl Environ Microbiol* 1998, **64(8):**2794-2799.
69. van der Heide M, Hollenberg C, van der Klei I, Veenhuis M: **Overproduction of BiP negatively affects the secretion of Aspergillus niger glucose oxidase by the yeast Hansenula polymorpha.** *Appl Microbiol Biotechnol* 2002, **58(4):**487-494.
70. Smith J, Tang B, Robinson A: **Protein disulfide isomerase, but not binding protein, overexpression enhances secretion of a non-disulfide-bonded protein in yeast.** *Biotechnol Bioeng* 2004, **85(3):**340-350.
71. Conesa A, Jeenes D, Archer DB, van den Hondel CA, Punt PJ: **Calnexin overexpression increases manganese peroxidase production in Aspergillus niger.** *Appl Environ Microbiol* 2002, **68(2):**846-851.
72. Chung JY, Lim SW, Hong YJ, Hwang SO, Lee GM: **Effect of doxycycline-regulated calnexin and calreticulin expression on specific thrombopoietin productivity of recombinant Chinese hamster ovary cells.** *Biotechnol Bioeng* 2004, **85(5):**539-546.
73. Higgins MK, Demir M, Tate CG: **Calnexin co-expression and the use of weaker promoters increase the expression of correctly assembled Shaker potassium channel in insect cells.** *Biochim Biophys Acta* 2003, **1610(1):**124-132.
74. Klabunde J, Kleebank S, Piontek M, Hollenberg CP, Hellwig S, Degelmann A: **Increase of calnexin gene dosage boosts the secretion of heterologous proteins by Hansenula polymorpha.** *FEMS Yeast Res* 2007, **7(7):**1168-1180.
75. Marechal A, Tanguay PL, Callejo M, Guerin R, Boileau G, Rokeach LA: **Cell viability and secretion of active proteins in Schizosaccharomyces pombe do not require the chaperone function of calnexin.** *Biochem J* 2004, **380(Pt 2):**441-448.
76. Parlati F, Dominguez M, Bergeron JJ, Thomas DY: **Saccharomyces cerevisiae CNE1 encodes an endoplasmic reticulum (ER) membrane protein with sequence similarity to calnexin and calreticulin and functions as a constituent of the ER quality control apparatus.** *J Biol Chem* 1995, **270(1):**244-253.
77. Arima H, Kinoshita T, Ibrahim HR, Azakami H, Kato A: **Enhanced secretion of hydrophobic peptide fused lysozyme by the introduction of N-glycosylation signal and the disruption of calnexin gene in Saccharomyces cerevisiae.** *FEBS Lett* 1998, **440(1–2):**89-92.
78. Song Y, Sata J, Saito A, Usui M, Azakami H, Kato A: **Effects of calnexin deletion in Saccharomyces cerevisiae on the secretion of glycosylated lysozymes.** *J Biochem (Tokyo)* 2001, **130(6):**757-764.
79. Hayano T, Hirose M, Kikuchi M: **Protein disulfide isomerase mutant lacking its isomerase activity accelerates protein folding in the cell.** *FEBS Lett* 1995, **377(3):**505-511.
80. Bao W, Fukuhara H: **Secretion of human proteins from yeast: stimulation by duplication of polyubiquitin and protein disulfide isomerase genes in Kluyveromyces lactis.** *Gene* 2001, **272(1–2):**103-110.
81. Iwata T, Tanaka R, Suetsugu M, Ishibashi M, Tokunaga H, Kikuchi M, Tokunaga M: **Efficient secretion of human lysozyme from the yeast, Kluyveromyces lactis.** *Biotechnol Lett* 2004, **26(23):**1803-1808.
82. Vad R, Nafstad E, Dahl L, Gabrielsen O: **Engineering of a Pichia pastoris expression system for secretion of high amounts of intact human parathyroid hormone.** *J Biotechnol* 2005, **116(3):**251-260.
83. Gasser B, Maurer M, Gach J, Kunert R, Mattanovich D: **Engineering of Pichia pastoris for improved production of antibody fragments.** *Biotechnol Bioeng* 2006, **94(2):**353-361.
84. Inan M, Aryasomayajula D, Sinha J, Meagher M: **Enhancement of protein secretion in Pichia pastoris by overexpression of protein disulfide isomerase.** *Biotechnol Bioeng* 2006, **93(4):**771-778.
85. Shusta E, Raines R, Plückthun A, Wittrup K: **Increasing the secretory capacity of Saccharomyces cerevisiae for production of single-chain antibody fragments.** *Nat Biotechnol* 1998, **16(8):**773-777.
86. Damasceno L, Anderson K, Ritter G, Cregg J, Old L, Batt C: **Co-overexpression of chaperones for enhanced secretion of a single-chain antibody fragment in Pichia pastoris.** *Appl Microbiol Biotechnol* 2006.
87. Borth N, Mattanovich D, Kunert R, Katinger H: **Effect of increased expression of protein disulfide isomerase and heavy chain binding protein on antibody secretion in a recombinant CHO cell line.** *Biotechnol Prog* 2005, **21(1):**106-111.
88. Klabunde J, Hollenberg C, Gellissen G: **Impact of overexpressed secretory-pathway genes on the secretion of IFNgamma in**

Hansenula polymorpha applying an rDNA-cointegration approach for assessment. *FEMS Yeast Res* 2005.
89. Derkx P, Madrid S: **The foldase CYPB is a component of the secretory pathway of Aspergillus niger and contains the endoplasmic reticulum retention signal HEEL.** *Mol Genet Genomics* 2001, **266(4):**537-545.
90. Wiebe M, Karandikar A, Robson G, Trinci A, Candia J, Trappe S, Wallis G, Rinas U, Derkx P, Madrid S, *et al.*: **Production of tissue plasminogen activator (t-PA) in Aspergillus niger.** *Biotechnol Bioeng* 2001, **76(2):**164-174.
91. Moralejo F, Watson A, Jeenes D, Archer D, Martín J: **A defined level of protein disulfide isomerase expression is required for optimal secretion of thaumatin by Aspegillus awamori.** *Mol Genet Genomics* 2001, **266(2):**246-253.
92. Gross E, Kastner D, Kaiser C, Fass D: **Structure of Ero1p, source of disulfide bonds for oxidative protein folding in the cell.** *Cell* 2004, **117(5):**601-610.
93. Lodi T, Neglia B, Donnini C: **Secretion of human serum albumin by Kluyveromyces lactis overexpressing KlPDI1 and KlERO1.** *Appl Environ Microbiol* 2005, **71(8):**4359-4363.
94. Travers K, Patil C, Wodicka L, Lockhart D, Weissman J, Walter P: **Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation.** *Cell* 2000, **101(3):**249-258.
95. Higashio H, Kohno K: **A genetic link between the unfolded protein response and vesicle formation from the endoplasmic reticulum.** *Biochem Biophys Res Commun* 2002, **296(3):**568-574.
96. Valkonen M, Penttilä M, Saloheimo M: **Effects of inactivation and constitutive expression of the unfolded- protein response pathway on protein production in the yeast Saccharomyces cerevisiae.** *Appl Environ Microbiol* 2003, **69(4):**2065-2072.
97. Valkonen M, Ward M, Wang H, Penttilä M, Saloheimo M: **Improvement of foreign-protein production in Aspergillus niger var. awamori by constitutive induction of the unfolded-protein response.** *Appl Environ Microbiol* 2003, **69(12):**6979-6986.
98. Mattanovich D, Borth N: **Applications of cell sorting in biotechnology.** *Microb Cell Fact* 2006, **5:**12.
99. Shusta EV, Kieke MC, Parke E, Kranz DM, Wittrup KD: **Yeast polypeptide fusion surface display levels predict thermal stability and soluble secretion efficiency.** *J Mol Biol* 1999, **292(5):**949-956.
100. Rakestraw JA, Baskaran AR, Wittrup KD: **A flow cytometric assay for screening improved heterologous protein secretion in yeast.** *Biotechnol Prog* 2006, **22(4):**1200-1208.
101. Wentz AE, Shusta EV: **A novel high-throughput screen reveals yeast genes that increase secretion of heterologous proteins.** *Appl Environ Microbiol* 2007, **73(4):**1189-1198.
102. Sauer M, Branduardi P, Gasser B, Valli M, Maurer M, Porro D, Mattanovich D: **Differential gene expression in recombinant Pichia pastoris analysed by heterologous DNA microarray hybridisation.** *Microb Cell Fact* 2004, **3(1):**17.
103. Gasser B, Sauer M, Maurer M, Stadlmayr G, Mattanovich D: **Transcriptomics-based identification of novel factors enhancing heterologous protein secretion in yeasts.** *Appl Environ Microbiol* 2007, **73(20):**6499-6507.
104. Hoffmann F, Rinas U: **Stress induced by recombinant protein production in Escherichia coli.** *Adv Biochem Eng Biotechnol* 2004, **89:**73-92.
105. Baneyx F, Mujacic M: **Recombinant protein folding and misfolding in Escherichia coli.** *Nat Biotechnol* 2004, **22(11):**1399-1408.
106. Sorensen HP, Mortensen KK: **Advanced genetic strategies for recombinant protein expression in Escherichia coli.** *J Biotechnol* 2005, **115(2):**113-128.
107. Sorensen HP, Mortensen KK: **Soluble expression of recombinant proteins in the cytoplasm of Escherichia coli.** *Microb Cell Fact* 2005, **4(1):**1.
108. Villaverde A, Carrio MM: **Protein aggregation in recombinant bacteria: biological role of inclusion bodies.** *Biotechnol Lett* 2003, **25(17):**1385-1395.
109. Carrio M, Gonzalez-Montalban N, Vera A, Villaverde A, Ventura S: **Amyloid-like properties of bacterial inclusion bodies.** *J Mol Biol* 2005, **347(5):**1025-1037.
110. Speed MA, Wang DI, King J: **Specific aggregation of partially folded polypeptide chains: the molecular basis of inclusion body composition.** *Nat Biotechnol* 1996, **14(10):**1283-1287.
111. Hart R, Rinas U, Bailey J: **Protein composition of Vitreoscilla hemoglobin inclusion bodies produced in Escherichia coli.** *J Biol Chem* 1990, **265(21):**12728-12733.
112. Neubauer A, Soini J, Bollok M, Zenker M, Sandqvist J, Myllyharju J, Neubauer P: **Fermentation process for tetrameric human collagen prolyl 4-hydroxylase in Escherichia coli: Improvement by gene optimisation of the PDI/beta subunit and repeated addition of the inducer anhydrotetracycline.** *J Biotechnol* 2007, **128:**308-321.
113. Rinas U, Bailey JE: **Protein compositional analysis of inclusion bodies produced in recombinant Escherichia coli.** *Appl Microbiol Biotechnol* 1992, **37(5):**609-614.
114. Rinas U, Boone TC, Bailey JE: **Characterization of inclusion bodies in recombinant Escherichia coli producing high levels of porcine somatotropin.** *J Biotechnol* 1993, **28(2–3):**313-320.
115. Rinas U, Bailey JE: **Overexpression of bacterial hemoglobin causes incorporation of pre-beta-lactamase into cytoplasmic inclusion bodies.** *Appl Environ Microbiol* 1993, **59(2):**561-566.
116. Rinas U, Hoffmann F, Betiku E, Estape D, Marten S: **Inclusion body anatomy and functioning of chaperone-mediated in vivo inclusion body disassembly during high-level recombinant protein production in Escherichia coli.** *J Biotechnol* 2007, **127(2):**244-257.
117. Allen SP, Polazzi JO, Gierse JK, Easton AM: **Two novel heat shock genes encoding proteins produced in response to heterologous protein expression in Escherichia coli.** *J Bacteriol* 1992, **174(21):**6938-6947.
118. Carrio MM, Villaverde A: **Construction and deconstruction of bacterial inclusion bodies.** *J Biotechnol* 2002, **96(1):**3-12.
119. Carrio MM, Villaverde A: **Localization of chaperones DnaK and GroEL in bacterial inclusion bodies.** *J Bacteriol* 2005, **187(10):**3599-3601.
120. Vallejo LF, Rinas U: **Strategies for the recovery of active proteins through refolding of bacterial inclusion body proteins.** *MicrobCell Fact* 2004, **3(1):**11.
121. Miot M, Betton JM: **Protein quality control in the bacterial periplasm.** *MicrobCell Fact* 2004, **3(1):**4.
122. Connolly L, De Las Penas A, Alba B, Gross C: **The response to extracytoplasmic stress in Escherichia coli is controlled by partially overlapping pathways.** *Genes Dev* 1997, **11(15):**2012-2021.
123. Hunke S, Betton JM: **Temperature effect on inclusion body formation and stress response in the periplasm of Escherichia coli.** *Mol Microbiol* 2003, **50(5):**1579-1589.
124. Zahrl D, Wagner M, Bischof K, Koraimann G: **Expression and assembly of a functional type IV secretion system elicit extracytoplasmic and cytoplasmic stress responses in Escherichia coli.** *J Bacteriol* 2006, **188(18):**6611-6621.
125. Duguay A, Silhavy T: **Quality control in the bacterial periplasm.** *Biochim Biophys Acta* 2004, **1694(1–3):**121-134.
126. Jurgen B, Lin HY, Riemschneider S, Scharf C, Neubauer P, Schmid R, Hecker M, Schweder T: **Monitoring of genes that respond to overproduction of an insoluble recombinant protein in Escherichia coli glucose-limited fed-batch fermentations.** *Biotechnol Bioeng* 2000, **70(2):**217-224.
127. Lesley SA, Graziano J, Cho CY, Knuth MW, Klock HE: **Gene expression response to misfolded protein as a screen for soluble recombinant protein.** *Protein Eng* 2002, **15(2):**153-160.
128. Arsene F, Tomoyasu T, Bukau B: **The heat shock response of Escherichia coli.** *Int J Food Microbiol* 2000, **55(1–3):**3-9.
129. Bukau B: **Regulation of the Escherichia coli heat-shock response.** *Mol Microbiol* 1993, **9(4):**671-680.
130. Aris A, Corchero JL, Benito A, Carbonell X, Viaplana E, Villaverde A: **The expression of recombinant genes from bacteriophage lambda strong promoters triggers the SOS response in Escherichia coli.** *Biotechnol Bioeng* 1998, **60(5):**551-559.
131. Harcum SW, Bentley WE: **Heat-shock and stringent responses have overlapping protease activity in Escherichia coli. Implications for heterologous protein yield.** *Appl Biochem Biotechnol* 1999, **80(1):**23-37.
132. Schultz T, Martinez L, de MA: **The evaluation of the factors that cause aggregation during recombinant expression in E. coli is simplified by the employment of an aggregation-sensitive reporter.** *MicrobCell Fact* 2006, **5:**28.
133. Hoffmann F, Rinas U: **Kinetics of heat-shock response and inclusion body formation during temperature-induced produc-**

tion of basic fibroblast growth factor in high-cell-density cultures of recombinant Escherichia coli. *Biotechnol Prog* 2000, **16(6):**1000-1007.
134. Strandberg L, Enfors SO: **Factors influencing inclusion body formation in the production of a fused protein in Escherichia coli.** *Appl Environ Microbiol* 1991, **57(6):**1669-1674.
135. Vasina JA, Baneyx F: **Recombinant protein expression at low temperatures under the transcriptional control of the major Escherichia coli cold shock promoter cspA.** *Appl Environ Microbiol* 1996, **62(4):**1444-1447.
136. Vasina JA, Baneyx F: **Expression of aggregation-prone recombinant proteins at low temperatures: a comparative study of the Escherichia coli cspA and tac promoter systems.** *Protein Expr Purif* 1997, **9(2):**211-218.
137. Vasina JA, Peterson MS, Baneyx F: **Scale-up and optimization of the low-temperature inducible cspA promoter system.** *Biotechnol Prog* 1998, **14(5):**714-721.
138. De Marco V, Stier G, Blandin S, de Marco A: **The solubility and stability of recombinant proteins are increased by their fusion to NusA.** *Biochem Biophys Res Commun* 2004, **322(3):**766-771.
139. Huang H, Liu J, de MA: **Induced fit of passenger proteins fused to Archaea maltose binding proteins.** *Biochem Biophys Res Commun* 2006, **344(1):**25-29.
140. Baneyx F, Palumbo JL: **Improving heterologous protein folding via molecular chaperone and foldase co-expression.** *Methods Mol Biol* 2003, **205:**171-197.
141. Hoffmann F, Rinas U: **Roles of heat-shock chaperones in the production of recombinant proteins in Escherichia coli.** *Adv Biochem Eng Biotechnol* 2004, **89:**143-161.
142. Schlieker C, Bukau B, Mogk A: **Prevention and reversion of protein aggregation by molecular chaperones in the E. coli cytosol: implications for their applicability in biotechnology.** *J Biotechnol* 2002, **96(1):**13-21.
143. Thomas JG, Ayling A, Baneyx F: **Molecular chaperones, folding catalysts, and the recovery of active recombinant proteins from E. coli. To fold or to refold.** *Appl Biochem Biotechnol* 1997, **66(3):**197-238.
144. de Marco A, de Marco V: **Bacteria co-transformed with recombinant proteins and chaperones cloned in independent plasmids are suitable for expression tuning.** *J Biotechnol* 2004, **109(1–2):**45-52.
145. de Marco A, Deuerling E, Mogk A, Tomoyasu T, Bukau B: **Chaperone-based procedure to increase yields of soluble recombinant proteins produced in E. coli.** *BMC Biotechnol* 2007, **7(1):**32.
146. de Marco A: **Protocol for preparing proteins with improved solubility by co-expressing with molecular chaperones in Escherichia coli.** *Nat Protoc* 2007, **2(10):**2632-2639.
147. Nishihara K, Kanemori M, Kitagawa M, Yanagi H, Yura T: **Chaperone coexpression plasmids: differential and synergistic roles of DnaK-DnaJ-GrpE and GroEL-GroES in assisting folding of an allergen of Japanese cedar pollen, Cryj2, in Escherichia coli.** *Appl Environ Microbiol* 1998, **64(5):**1694-1699.
148. Garcia-Fruitos E, Gonzalez-Montalban N, Morell M, Vera A, Ferraz RM, Aris A, Ventura S, Villaverde A: **Aggregation as bacterial inclusion bodies does not imply inactivation of enzymes and fluorescent proteins.** *MicrobCell Fact* 2005, **4:**27.
149. Garcia-Fruitos E, Carrio MM, Aris A, Villaverde A: **Folding of a misfolding-prone beta-galactosidase in absence of DnaK.** *Biotechnol Bioeng* 2005, **90(7):**869-875.
150. Gonzalez-Montalban N, Garcia-Fruitos E, Ventura S, Aris A, Villaverde A: **The chaperone DnaK controls the fractioning of functional protein between soluble and insoluble cell fractions in inclusion body-forming cells.** *MicrobCell Fact* 2006, **5:**26.
151. Martinez-Alonso M, Vera A, Villaverde A: **Role of the chaperone DnaK in protein solubility and conformational quality in inclusion body-forming Escherichia coli cells.** *FEMS Microbiol Lett* 2007, **273(2):**187-195.
152. Garcia-Fruitos E, Martinez-Alonso M, Gonzalez-Montalban N, Valli M, Mattanovich D, Villaverde A: **Divergent Genetic Control of Protein Solubility and Conformational Quality in Escherichia coli.** *J Mol Biol* 2007, **374:**195-205.
153. Carrio MM, Villaverde A: **Role of molecular chaperones in inclusion body formation.** *FEBS Lett* 2003, **537(1–3):**215-221.
154. Vera A, Aris A, Carrio M, Gonzalez-Montalban N, Villaverde A: **Lon and ClpP proteases participate in the physiological disintegration of bacterial inclusion bodies.** *J Biotechnol* 2005, **119(2):**163-171.
155. Mogk A, Schlieker C, Friedrich KL, Schonfeld HJ, Vierling E, Bukau B: **Refolding of substrates bound to small Hsps relies on a disaggregation reaction mediated most efficiently by ClpB/DnaK.** *J Biol Chem* 2003, **278(33):**31033-31042.
156. Mogk A, Deuerling E, Vorderwulbecke S, Vierling E, Bukau B: **Small heat shock proteins, ClpB and the DnaK system form a functional triade in reversing protein aggregation.** *Mol Microbiol* 2003, **50(2):**585-595.
157. Mogk A, Bukau B: **Molecular chaperones: structure of a protein disaggregase.** *Curr Biol* 2004, **14(2):**R78-R80.
158. Gonzalez-Montalban N, Garcia-Fruitos E, Villaverde A: **Recombinant protein solubility-does more mean better?** *Nat Biotechnol* 2007, **25(7):**718-720.
159. Nakamoto H, Bardwell JC: **Catalysis of disulfide bond formation and isomerization in the Escherichia coli periplasm.** *Biochim Biophys Acta* 2004, **1694:**111-119.
160. Hu X, O'Dwyer R, Wall JG: **Cloning, expression and characterisation of a single-chain Fv antibody fragment against domoic acid in Escherichia coli.** *J Biotechnol* 2005, **120:**38-45.
161. Maskos K, Huber-Wunderlich M, Glockshuber R: **DsbA and DsbC-catalyzed oxidative folding of proteins with complex disulfide bridge patterns in vitro and in vivo.** *J Mol Biol* 2003, **325:**495-513.
162. Qiu J, Swartz JR, Georgiou G: **Expression of active human tissue-type plasminogen activator in Escherichia coli.** *Appl Environ Microbiol* 1998, **64:**4891-4896.
163. Ostermeier M, De Sutter K, Georgiou G: **Eukaryotic protein disulfide isomerase complements Escherichia coli dsbA mutants and increases the yield of a heterologous secreted protein with disulfide bonds.** *J Biol Chem* 1996, **271:**10616-10622.
164. Zhan X, Schwaller M, Gilbert HF, Georgiou G: **Facilitating the formation of disulfide bonds in the Escherichia coli periplasm via coexpression of yeast protein disulfide isomerase.** *Biotechnol Prog* 1999, **15:**1033-1038.
165. Bessette PH, Aslund F, Beckwith J, Georgiou G: **Efficient folding of proteins with multiple disulfide bonds in the Escherichia coli cytoplasm.** *Proc Natl Acad Sci USA* 1999, **96:**13703-13708.
166. Stewart EJ, Aslund F, Beckwith J: **Disulfide bond formation in the Escherichia coli cytoplasm: an in vivo role reversal for the thioredoxins.** *EMBO J* 1998, **17:**5543-5550.
167. Jurado P, Ritz D, Beckwith J, de LV, Fernandez LA: **Production of functional single-chain Fv antibodies in the cytoplasm of Escherichia coli.** *J Mol Biol* 2002, **320:**1-10.
168. Santala V, Lamminmaki U: **Production of a biotinylated single-chain antibody fragment in the cytoplasm of Escherichia coli.** *J Immunol Methods* 2004, **284:**165-175.
169. Heo MA, Kim SH, Kim SY, Kim YJ, Chung J, Oh MK, Lee SG: **Functional expression of single-chain variable fragment antibody against c-Met in the cytoplasm of Escherichia coli.** *Protein Expr Purif* 2006, **47:**203-209.
170. Levy R, Weiss R, Chen G, Iverson BL, Georgiou G: **Production of correctly folded Fab antibody fragment in the cytoplasm of Escherichia coli trxB gor mutants via the coexpression of molecular chaperones.** *Protein Expr Purif* 2001, **23(2):**338-347.
171. Fernandez LA: **Prokaryotic expression of antibodies and affibodies.** *Curr Opin Biotechnol* 2004, **15:**364-373.
172. Terpe K: **Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems.** *Appl Microbiol Biotechnol* 2006, **72(2):**211-222.
173. van Wely K, Swaving J, Freudl R, Driessen A: **Translocation of proteins across the cell envelope of Gram-positive bacteria.** *FEMS Microbiol Rev* 2001, **25(4):**437-454.
174. Li W, Zhou X, Lu P: **Bottlenecks in the expression and secretion of heterologous proteins in Bacillus subtilis.** *Res Microbiol* 2004, **155(8):**605-610.
175. Bolhuis A, Tjalsma H, Smith HE, de Jong A, Meima R, Venema G, Bron S, van Dijl JM: **Evaluation of Bottlenecks in the Late Stages of Protein Secretion in Bacillus subtilis.** *Appl Environ Microbiol* 1999, **65(7):**2934-2941.
176. Yuan G, Wong S: **Isolation and characterization of Bacillus subtilis groE regulatory mutants: evidence for orf39 in the dnaK operon as a repressor gene in regulating the expression of both groE and dnaK.** *J Bacteriol* 1995, **177(22):**6462-6468.

177. Leskelä S, Wahlström E, Kontinen V, Sarvas M: **Lipid modification of prelipoproteins is dispensable for growth but essential for efficient protein secretion in Bacillus subtilis: characterization of the Lgt gene.** *Mol Microbiol* 1999, **31(4):**1075-1085.
178. Kontinen V, Sarvas M: **The PrsA lipoprotein is essential for protein secretion in Bacillus subtilis and sets a limit for high-level secretion.** *Mol Microbiol* 1993, **8(4):**727-737.
179. Hyyryläinen H, Bolhuis A, Darmon E, Muukkonen L, Koski P, Vitikainen M, Sarvas M, Prágai Z, Bron S, van Dijl J, *et al.*: **A novel two-component regulatory system in Bacillus subtilis for the survival of severe secretion stress.** *Mol Microbiol* 2001, **41(5):**1159-1172.
180. Westers H, Westers L, Darmon E, van Dijl J, Quax W, Zanen G: **The CssRS two-component regulatory system controls a general secretion stress response in Bacillus subtilis.** *FEBS J* 2006, **273(16):**3816-3827.
181. Duilio A, Tutino M, Marino G: **Recombinant protein production in Antarctic Gram-negative bacteria.** *Methods Mol Biol* 2004, **267:**225-237.
182. Parrilli E, Duilio A, Tutino ML: **Heterologous protein expression in psychrophilic hosts.** In *Psychrophiles: from Biodiversity to Biotechnology* Edited by: RMea. Berlin Heidelberg: Springer-Verlag; 2008:365-379.
183. Médigue C, Krin E, Pascal G, Barbe V, Bernsel A, Bertin P, Cheung F, Cruveiller S, D'Amico S, Duilio A, *et al.*: **Coping with cold: the genome of the versatile marine Antarctica bacterium Pseudoalteromonas haloplanktis TAC125.** *Genome Res* 2005, **15(10):**1325-1335.
184. Kiefhaber T, Rudolph R, Kohler H, Buchner J: **Protein aggregation in vitro and in vivo: a quantitative model of the kinetic competition between folding and aggregation.** *Biotechnology (NY)* 1991, **9(9):**825-829.
185. de Pascale D, Cusano AM, Autore F, dP Parrilli E, Marino G, Tutino ML: **The cold-active Lip1 lipase from the Antarctic bacterium Pseudoalteromonas haloplanktis TAC125 is a member of a new bacterial lipolytic enzyme family.** *Extremophiles* 2008.

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Cold-Adapted Esterases and Lipases: A Biodiversity Still Under-Exploited

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Abstract: Micro-organisms that thrive at low temperatures produce cold-adapted enzymes which generally display high catalytic efficiency making these biocatalysts particularly interesting either for investigating stability/flexibility relationships, or for their quite wide applications.

Psychrophilic lipases and esterases have attracted attention because of their increasing use in the organic synthesis of chiral intermediates due to their low optimum temperature and high activity in cold conditions, which are favourable properties for the production of relatively frail compounds. In addition, these enzymes have an advantage under low water conditions due to their inherent greater flexibility, wherein the activity of mesophilic and thermophilic enzymes is severely impaired by an excess of rigidity.

In this review we present an up to date overview on some psychrophilic esterases and lipases from microbial sources. The different experimental strategies available for the search of psychrophilic biocatalysts and their application to discover novel cold-adapted lipolytic enzymes will be outlined. Some structural features that justify the unusually high enzymatic activity at low temperature will be discussed, in view of the recent achievements concerning the use of cold-adapted lipases and esterases in the synthesis of fine chemicals.

Keywords: Psychrophilic micro-organisms, cold-active enzymes, α/β hydrolase fold, ester synthesis.

INTRODUCTION

Life at low temperatures is abundant, diverse and widespread, with organisms from all three domains of life being represented. These cold-adapted, or psychrophilic, micro-organisms have been found to not only endure, but to flourish under the harsh conditions of permanently low-temperatures. In fact, for some, this environment is not only optimal, but mandatory for sustained cell proliferation, with moderate to high temperatures (e.g., >12°C) being deleterious [1]. Clearly, adaptations at level of structural and physiological organisation have occurred, which allow these psychrophilic micro-organisms to overcome key obstacles inherent to life at low temperatures [2].

An up-to-date survey of the adaptive features characteristic to psychrophilic organisms can be found in a recent book [3]. It is out of doubt that one of the strongest effect of low temperatures on biological systems is the slowdown of reaction rates, a simple observation that can result as the sum of several phenomena, such as an alterations in the strength of enzyme-substrate interactions, an increased viscosity of the solvent and an altered solubility of proteins, salts and gases and ultimately leading to protein cold-denaturation [4-5].

As a result of these diverse adverse effects, most biological reactions display an approximately 16- to 80-fold drop in activity on reducing the temperature from 37°C to 0°C. In contrast, psychrophilic micro-organisms have been found to maintain relatively high metabolic fluxes at low temperatures, indicating that adaptation of the enzymatic repertoire has taken place to allow for appropriate reaction rates.

Focusing only on microbial enzymes isolated from organisms inhabiting permanently cold environments, about 200 biocatalysts have been characterized so far (February 2009) [3]. The majority of them acquired specific structural features correlating with enzyme cold adaptation, but it is now widely accepted that no structural feature is present in all cold-adapted enzymes, and no structural features always correlate with cold adaptation [6]. It is frequently observed that proteins from psychrophiles are thermo labile and more flexible than their counterparts from thermophiles. Many cold-adapted proteins have regions of local flexibility, particularly around the active site. The high local flexibility is translated into a reduction in $\Delta H\#$ (change in activation enthalpy between the ES ground state and the corresponding activated complex), high k_{cat}, and, in the majority of cases, high K_m. Therefore, the often associated high activity and low stability of cold-adapted enzymes underlie a general principle of activity-stability trade-off [6].

The two above mentioned properties of cold adapted biocatalysts make these enzymes of great interest in many industrial applications, where a high catalytic efficiency in low temperatures processes is required and the easy inactivation of the added catalysts by moderate heating is an added value [7].

Esterases and lipases are the most applied biocatalysts in industrial applications. The reasons of this success are numerous and related to the wide diversity in the substrates recognised, combined to the exquisite chemoselectivity, regioselectivity and stereoselectivity frequently displayed by this class of enzymes. Furthermore, they are readily available in large quantities because many of them can be produced in high yields from microbial organisms and the crystal structures of many lipolytic enzymes have been solved, making possible the design of rational engineering strategies. Finally, they do not usually require cofactors nor do they catalyse side reactions.

Despite the lack of significant sequence similarity between lipases and esterases of different families, they do share a common three-dimensional fold, known as the α/β hydrolase fold. This fold is also found in proteins of other functional families, including Ser proteases, haloperoxidases, haloalkane-dehalogenases and chlorine-esterase, which highlights the value of the fold as a template for various enzymes.

It was discovered that lipases and esterases carry out their function as carboxyl ester hydrolases by virtue of a catalytic triad, which is a mirror image of that found in serine proteases such as chymotrypsin. The triad is composed of a serine, a carboxylic acid residue (Asp or Glu) and a histidine, in this order in the sequence,

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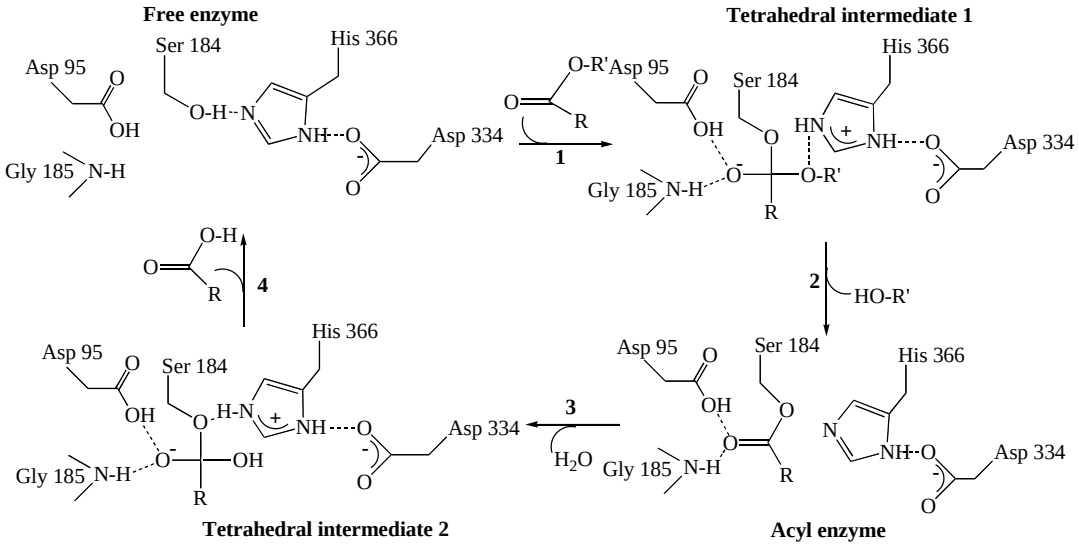

Fig. (1). Proposed CAL B reaction mechanism. In step 1 of the reaction, an ester substrate enters the active site and is subjected to nucleophilic attack by Ser177, activated by proton abstraction by the His346/Asp280 pair. The resulting tetrahedral intermediate is stabilised by interactions with the backbone nitrogen of Gly, and the protonated side chain of Asp. In step 2, His346 releases its proton to the alcohol product as the tetrahedral intermediate collapses. The alcohol leaves the site, and a water molecule enters (step 3). After being activated by the His/Asp pair, the water molecule attacks the acyl enzyme to generate a second tetrahedral intermediate, which then collapses to release the carboxylate product (step 4). Taken from [8].

and the mechanism of action proceeds through four consecutive steps. Fig. (**1**) summarizes this process as described for the *Candida antarctica* lipase B [8]. When substrates are hydrolysed, the carboxyl carbon of the leaving fatty acid is hydrogen-bound to the serine. The histidine (stabilized by the acidic residue) can donate a hydrogen to the leaving alcohol, after which a water molecule is added to the carbonyl group and the serine regains its hydroxyl group. As in the case of proteases there is an anion intermediate, stabilized in a so-called oxyanion hole by two or three hydrogen bonds [9].

Due to these structural/functional similarities, it is still difficult to predict *a priori* if a lipolytic enzyme is an esterase or a lipase. Although the presence of a so called "lid" in lipases has been sometimes claimed to discriminate between the two types of enzymes, the number of true lipases not displaying this structural feature is growing. Therefore, the most reliable tool to classify the lipolytic enzymes is the most classical one: testing the substrate specificity. Water-insoluble long-chain acylglycerols (≥C10) are very good substrates for lipases only, while esters with short-chain fatty acids (≤C10), at least partially soluble in water, are hydrolysed by esterases.

Is there a need for cold-adapted esterases and lipases? The interest on cold-adapted lipolytic enzymes is related to several aspects: they are interesting objects of study to highlight their physiological and/or structural adaptive mechanisms; many industrial and commercial applications would benefit from the use of psychrophilic enzymes; lipases are potentially useful in the production of secondary chemical compounds, of "domestic" compounds such as soap, and in the degradation of organic matter, i.e. "bioremediation". For instance, the most commercially important field of application for hydrolytic lipases is their addition to detergents, which are used mainly in household and industrial laundry and in household dishwashers. Godfrey and West (1996) reported that about 1000 tons of lipases are sold every year in the area of detergents. The use of cold-active lipase in the formulation of detergents is of great advantage in cold washing that mean to reduce the energy consumption and wear and tear of textile fibres [10].

In the next sections, we summarized recent results on some psychrophilic esterases and lipases from microbial sources. The different experimental strategies available for the search of psychrophilic biocatalysts and their application to discover novel cold-adapted lipolytic enzymes have been summarised. A brief discussion on several structural features that justify the unusually high enzymatic activity at low temperature of these enzymes have been reported, in view of the recent achievements concerning the use of cold-adapted lipases and esterases in the synthesis of fine chemicals.

1. STRATEGIES FOR THE SEARCH OF NOVEL COLD-ADAPTED LIPOLYTIC ENZYMES

The isolation of novel enzymes with specific properties is the result of several experimental strategies that could be distinguished in: classical, genomic and metagenomic approach. In the following paragraphs each strategy will be outlined together with some selected examples of cold-active lipolytic enzymes isolation. It is worth mentioning that the three approaches differ significantly in the expected size of the enzymatic repertoire potentially accessible to the screening procedures. In fact classical and genomic approaches require the microbial source isolation and its cultivation in laboratory conditions. This prerequisite makes it impossible to analyse the content of all the uncultivable microbes, a large fraction that can reach up to 99.8% of the micro-organisms present in some environments. To overcome this overwhelming limit, the metagenomic approach has been developed. This strategy allows the study of collective genomes of all micro-organisms thriving in a given habitat without the need of their culturing.

1.1. The Classical Approach

Classical approach consists in the isolation of the enzymatic activity of interest from a given bacterial strain by screening it with classical molecular techniques. As previously mentioned, cold-adapted microbial strains can be isolated from a wide array of cold environments either natural (i.e. polar and alpine ecosystems) or artificial (refrigerators or cold-rooms, for instance). These environments may be also characterized by other dominant parameters, such as unusually high values of pH or salinity, and these properties may be used as pre-selection criterion when a lypolitic enzyme with peculiar properties is searched. A rapid and cheap method to discriminate which strains possess lipolytic activity is used as first step in the screening programme. A convenient assay is the cultivation of the strains on agar plates containing 1% tributyrin. The strain

showing the highest lipolytic activity is then picked out and a suitable library of its genomic DNA is constructed. The chromosomal DNA is extracted and enzymatically digested in order to obtain fragments with sizes between 1 to 5 kb. The purified gene fragments are then ligated with commercial cloning vectors and the library is constructed by transforming the recombinant plasmids into competent *Escherichia coli* cells. A second screening step on agar plates is required for the identification of the library clones containing lipase encoding genes. Clones showing lipase activity by forming a clear halo on the agar plates containing tributyrin and supplemented with the appropriate antibiotic are finally selected. The new identified cold-active lipase is then over-expressed in the recombinant host and purified for further characterization.

At the moment, several microbial sources of cold-active lipases are available. As shown in Table **1**, the most of cold-active lipases/esterases producing micro-organisms are cold-adapted bacteria, mostly isolated from Antarctic and polar regions. Other powerful sources of cold-active lipolytic enzymes are bacterial genera isolated from deep-sea water and sediments or refrigerated milk and food samples. The investigations on cold-active lipases are also extended to mycotic sources (Table **1**). Even though only a few lipolytic fungi were reported to produce cold-active lipases, an extensive research has been carried out on the cold-active lipase B of *Candida antarctica* (CAL-B), which is widely used in industrial applications.

Over the last few years many examples of cold-active bacterial lipolytic enzymes isolated by classical approach were reported. The first example of isolation of cold-active lipolytic enzymes from a psychrophilic bacterium was from the Antarctic strain *Psychrobacter sp*. TAA144 (previously called *Moraxella* TAA144) [11]. Recently, the isolation of a cold-active lipase from the Antarctic deep-sea psychrotrophic bacterium *Psychrobacter sp*. 7195 and its characterization has been described [12]. By screening a genomic DNA library, an open reading frame of 954 bp coding for a lipase gene, *lipA1*, was identified, cloned, and expressed in *Escherichia coli* BL21 (DE3) cells. The purified recombinant LipA1 enzyme showed highest activity at pH 9.0 and low stability at temperatures higher than 30°C, indicating that it is a typical cold-adapted alkaline lipase.

Another recent paper reports the isolation of cold-active LipP from *Moritella* sp.2-5-10-1 by classical approach [13]. The recombinant LipP enzyme, expressed in *E. coli* BL21 (DE3), showed a relatively low activity maybe due to incorrect protein folding and its tendency to aggregate into inclusion bodies. It is interesting to note that several papers reports the limits highlighted by *E. coli* to correctly produce and process recombinant lipases and esterases [14]. One of the main limitations experienced while producing proteins in conventional bacterial mesophilic systems is the need to operate at their optimal growth temperature (usually 37ºC) for the production process. Since temperature has a general negative impact on protein folding, the production of recombinant proteins at low temperatures represents an exciting model to improve the quality of the products. Recombinant protein production in psychrophilic bacteria, i.e. at temperature as low as 4°C, may minimize undesired hydrophobic interactions during protein folding, possibly enhancing the yield of soluble and correctly folded products. Therefore, overexpression of cold-active lipase could be achieved in non conventional hosts naturally adapted to the growth at very low temperatures [15].

1.2. The Genomic Approach

The isolation of novel enzymes can also be achieved by combining *in silico* genome analysis with *in vivo* functional characterization. Genomic approach requires the knowledge of the whole genome sequence of the microbial source. Nowadays, several genomes of psychrophilic micro-organisms are available and their number in steadily increasing (Table **2**). By using bioinformatic tools is possible to identify genes potentially encoding lipolytic enzymes and to predict their properties or some new features by comparing in a multiple alignment protein coding regions highly conserved at the nucleotide level. After the *in silico* selection, the genes of interest are PCR amplified from the microbial source and then over-expressed in an appropriate recombinant host for further recombinant protein purification and characterization.

To illustrate this strategy, the Gram-negative Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 (*P. haloplanktis* TAC125), reports two examples of cold-active lipolytic enzyme characterization. The genome of the Antarctic organism contains several genes annotated as putative esterase/lipase encoding sequences. Recently, a gene coding for a putative esterase activity corresponding to the CDS PSHAa1385 has been functionally characterized [16]. The 843 bp gene was cloned, recombinantly expressed in *E. coli* and the encoded product was identified as a cytoplasmic, dimeric feruloyl esterase. Another *P. haloplanktis* TAC125 gene, PSHAa0051, selected on the bases of the peculiar features of the encoded product, which was annotated as "putative secreted esterase", was also recombinantly expressed in the same cold adapted bacterium [15]. The recombinant protein, named Lip1, found associated to the bacterial outer membrane although containing none of the already described anchoring domains, was purified and functionally characterized [17].

Table 1. Micro-Organism Producing Cold-Active Lipases

Bacteria	Source	References
Moraxella sp. strain TA144	Antarctic habitat	[11]
Psychrobacter immobilis strain B10	Antarctic habitat	[74]
Pseudomonas sp. strain B11-1	Alaskan soil	[75]
Pseudomonas fluorescens	Refrigerated human placental extracts	[76]
Pseudomonas sp. strain KB700A	Subterranean environment	[77]
Psychrobacter sp. Ant 300	Antarctic habitat	[78]
Photobacterium lipolyticum M37	Marine habitat	[79]
Psychrobacter sp. 7195	Antarctic habitat	[12]
Moritella sp. 2-5-10-1	Antarctic habitat	[13]
Fungi		
Candida antarctica	Antarctic habitat	[80]
Candida antarctica	Antarctic habitat	[81]

Table 2. Sequenced Genomes of Cold-Adapted Bacteria

Species/strain	Source	References
Colwellia psychrerythraea 34H	Artic marine sediment	[82]
Desulfotalea psychrophila LSv54	Marine sediment, Svarlbard	[83]
Methanococcoides burtonii DSM6242	Ace lake Vestfold Hills Eastern Antarctica	[84]
Pseudoaltermonas haloplanktis TAC125	Antarctic sea-water	[85]
Psychromonas ingrahamii 37	Under-ice sea-water, Alaska	[86]
Photobacterium profundum SS9	Amphipod, Sulu Trench	[87]
Psychrobacter arcticus 273-4	Tundra permafrost, Siberia	[88]
Psychrobacter cryohaloentis K5	Cryopeg, Siberia	[88]

1.3. The Metagenomic Approach

The major limitation of the two described methodologies, classical gene cloning and genomic approach, is the need to cultivate the lipase-producing micro-organisms in laboratory conditions. In spite of the great variety of potential sources of novel enzymes, it is estimated that more than 99% of the prokaryotic organisms are so far uncultivable [18] and therefore not accessible for biotechnology and basic research. However, valuable resources from uncultivable microbial communities can be exploited by using metagenomic approach. This approach is based on the culture-independent retrieval of genomic DNA from microbial communities living in particular environments [19]. Metagenomic libraries construction involves the same methods as the cloning of genomic DNA of individual micro-organisms; that is, DNA fragmentation by restriction-enzyme digestion or mechanical shearing, insertion of DNA fragments into an appropriate vector system, and transformation of the recombinant vectors into a suitable host. Once established, metagenomic libraries can be accessed and tested by multiple screening methodologies to discover novel biocatalysts. The principal testing criteria include the functional screening [20-22], the identification of interesting genes based on sequence homology through PCR or random sequencing [19] and the large-scale shotgun sequencing [23, 24], the hybridization [25] or the detection of substrate induced gene expression [26].

Several cold-active lipases have been identified by the metagenomic approach during the last years.

A novel low temperature active lipase was isolated from a metagenomic library of Baltic Sea sediment bacteria [27]. A fosmid library with inserts of 24-39 kb was generated and screened for clones producing lipolytic activity. A 978 bp open reading frame was selected encoding a putative lipase/esterase, called h1Lip1. Further characterization of the recombinant enzyme confirmed that it was a lipase.

The same strategy was adopted to search another cold-active lipase from the sea sediment samples at Edison Seamount, South West Pacific [28]. The sequence analysis of the selected fosmid clone revealed the presence of an open reading frame (*EML1*) showing similarities to lipases. EML1 enzyme was then recombinantly produced in *E. coli* BL21, and purified for biochemical characterization. Based on the data of optimum conditions for the recombinant enzyme activity and the calculated activation energy, it was suggested that EML1 is cold-active, consistent with the origin of the sample.

The reported examples show that metagenomic approach, using extreme environment samples, can provide an opportunity to isolate enzymes perfectly adapted to extreme working conditions which display unique primary sequence. On the other hand, the method suffers the general limitations of the heterologous gene-expression screening in *E. coli* which have been discussed in full details elsewhere [29].

2. STRUCTURAL/FUNCTIONAL RELATIONSHIP IN COLD-ADAPTED LIPOLYTIC ENZYMES

2.1. General Structural Features of Lipases and Esterases

The superfamily of carboxyl ester hydrolases (which comprises also lipases and esterases) includes ubiquitous enzymes of great physiological function with a wide range of substrate specificities; they are member of the α/β hydrolase family.

Since its original discovery [30] the α/β hydrolase fold has grown into a large superfamily of proteins that appear to be related by divergent evolution. The fold has proved remarkably adaptable, allowing it to carry out a truly impressive range of functions. The members vary in subunit size and oligomeric structure so that it is difficult to give a concise definition of the fold – they are predominantly simple hydrolytic enzymes with a conserved α/β structural core. Enzymatic catalysis utilises a 'catalytic triad' of residues that is usually comprised of a serine (nucleophile), a histidine and an aspartic acid that occur after the same secondary structure elements in the α/β structure – as shown in Fig. (**2**). The term catalytic triad was first applied to the active site residues of a number of families of proteolytic enzymes. These families include the well-known eukaryotic serine proteases, cysteine proteases, and subtilisin-like enzymes. The catalytic triad is a particularly useful arrangement of amino acids and has probably arisen several times during the course of evolution – the overall structures of the serine proteases and the α/β hydrolase fold enzymes are quite different and their triads are clearly related by convergent evolution.

What are the features of a typical α/β hydrolase fold protein? This question has been raised and addressed on several occasions [31], and it will be only briefly dealt with here. The basic canonical fold was originally defined as having eight strands, the second strand being antiparallel to the others. The strand order is 12435678 with helices that connect all. The strands have a super-helical twist such that the first and last strands are orthogonal to each other. The most conserved feature of the fold and its key signature is the so-called 'nucleophilic elbow' – a γ turn with the nucleophile at its apex. The sharpness of this turn requires that it posses a pair of non-Ramachandran backbone dihedral angles and small side chains, often glycine, on the residues at positions two before and two after the nucleophile (Nu-2, Nu+2). The residue at Nu+3 also needs to be small to avoid a steric clash with strand 4. This conservation of amino acids is frequently used to identify the nucleophile. The geometry of the nucleophilic elbow allows it to make a close approach to the substrate and also allows it to form part of the "oxy-anion binding hole" that stabilizes the negatively charged transition state formed during hydrolysis. This oxy-anion hole is usually formed by two backbone nitrogen atoms; the first always from the residue immediately following the nucleophile, the second usually between strand 3 and helix A. In most cases the oxy-anion hole is evident in the resting state of the enzyme, but in some cases substrate binding is required for activation [32]. The Asp/Glu and His residues of the

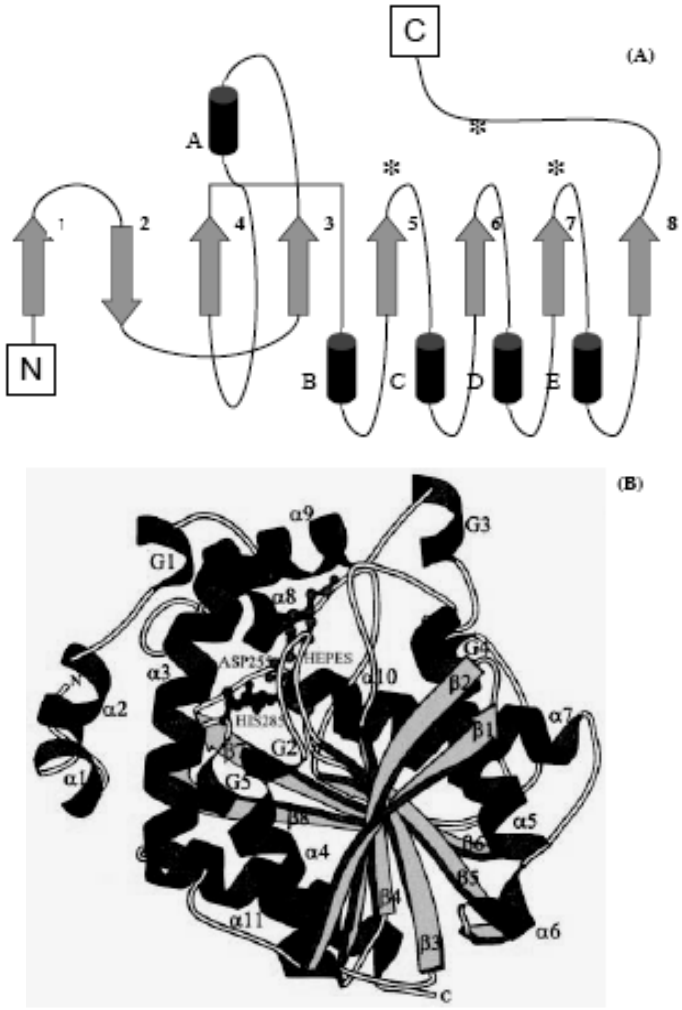

Fig. (2A,B): (A) Schematic representation of α/β idrolase canonical fold. * represent the catalytic residues. **(B)** The 3D overall fold of *Archeoglobus fulgidus* carboxylesterase: β-strands and helices belonging to the canonical α/β hydrolase fold are shown. The residues of the catalytic triad are shown in ball-and-stick representation: Ser160, His285 and Asp255. Taken from [89].

catalytic triad are usually located in a loop containing two reverse turns at the end of strands 7 and 8 respectively. Although the catalytic machinery of α/β hydrolase enzymes is very similar, the way by which they bind substrates varies from protein to protein. In most cases, substrate binding occurs in a "cap" domain that sits over the catalytic triad. The cap domains are built from one or more peptides that emanate from the C-terminal ends of strands 4, 6, 7 or 8. The differing sizes of the cap domains give rise to subunit molecular weights that vary considerably.

Although most of the α/β hydrolase fold enzymes can be easily classified as such, there are other members that have some, but not all of the fold characteristics. Perhaps the most profound difference in the family members is in their function. Most of the proteins are simple esterases, lipases or peptidases. The mechanisms of these proteins have been studied and in many cases appears to be very similar to that of the serine proteases.

Lipases and esterases are collectively known as lipolytic enzymes, which hydrolyze hydrophobic long- and short-chain carboxylic acid esters. Due to their respective solubility, esterase activity occurs in aqueous solution, while lipolytic reactions occur at the lipid–water interface, implying that the kinetics cannot be described by Michaelis–Menten equations, as these are valid only if the catalytic reaction takes place in one homogenous phase [33]. Lipolytic substrates usually form equilibrium between monomer, micellar and emulsified states, resulting in the need for a suitable model system to study lipase kinetics. The best-known phenomenon emerging from early kinetic studies of lipolytic reactions became known as 'interfacial activation', describing the fact that the activity of lipases is enhanced towards insoluble substrates that form an emulsion [9].

Lipases, in contrast to esterases, were therefore defined as carboxylesterases acting on emulsified substrates. The determination of their 3D structures seemed to provide an elegant explanation for interfacial activation: the active site of lipases was found to be covered by a surface loop, which was called the lid (or flap). Upon binding to the interface, this lid moves away, turning the 'closed' form of the enzyme into an 'open' form, with the active site now accessible to the solvent; at the same time, a large hydrophobic surface is exposed, which is thought to facilitate binding of the lipase to the interface. More recently, it turned out that the presence of a lid-like structure is not necessarily correlated with interfacial activation: lipases from *Pseudomonas aeruginosa* [34], *P. glumae* [35], and *Candida antarctica* CalA and CalB [36], and LipA lipase from *Serratia marcescens* [37], do not show interfacial activation but nevertheless have an amphiphilic lid covering their active sites. This observation led the conclusion that the presence of a lid domain and interfacial activation are unsuitable criteria to classify an enzyme as a lipase. Therefore, the current definition is rather simple: a lipase is a carboxylesterase that catalyses the hydrolysis of long-chain acylglycerols [33].

2.2. Lipolytic Enzymes Adaptation to Cold Conditions

Psychrophilic enzymes are of great interest in the scientific community, and are currently under study to characterize their physical and chemical properties in an attempt to understand the molecular basis of cold adaptation. Low temperatures have a negative effect on enzyme kinetics: any drop off in temperature results in an exponential decrease in reaction rate. For example, lowering the temperature by 10 C° causes a two-fold to four-fold decrease in enzyme activity [10, 38]. Therefore, enzymes from psychrophiles show high catalytic efficiency in the 0–20 °C temperature range, temperatures at which counterparts from mesophilic or thermophilic organisms do not allow adequate metabolic rates to support life or cellular growth. Such high activity balances the cold-induced inhibition of reaction rates. However, the structure of cold-adapted enzymes is also heat-labile. Indeed, low stability at moderate temperatures (usually >40 °C) is the other peculiar characteristic of psychrophilic enzymes [39-40]. This trend was revealed by calorimetric analysis of residual enzyme activities after incubation at increasing temperatures (it should be pointed out, however, that the loss of activity at moderate temperatures might not be always directly related to the loss of enzyme structure). It is generally believed that cold adaptation results from a combination of lack of selective pressure for thermostability and strong selection for high activity at low temperatures [41]. Psychrophilic enzymes are often characterized by high flexibility, which allows better interaction with substrates, and by lower activation energy requirements in comparison with their mesophilic and thermophilic counterparts. Hence, the presence of high flexibility could explain both thermolability and high catalytic efficiency at low temperatures [42]. The higher structural flexibility of psychrophilic enzymes, as compared to their mesophilic and thermophilic counterparts, could be the result of a combination of several features: weakening of intramolecular bonds (fewer hydrogen bonds and salt bridges as compared to mesophilic and thermophilic homologs have been shown); a decrease in compactness of the hydrophobic core; an increase in the number of hydrophobic side chains that are exposed to the solvent; longer and more hydrophilic loops; a reduced number of proline and arginine residues; and a higher number of glycine residues [43]. However, each protein family adopts its own strategy to increase its overall or local structural flexibility by using one or a combination of these structural modifications.

Earlier studies on the structural adaptation of extremophilic enzymes [44-47], were based on comparative analysis, also using homology modeling in cases where no experimental three-dimensional structures were available. These approaches could give valuable information on rules to be followed by protein engineers to produce modified enzymes with suitable features for biotechnological applications [48].

A clear example of this study was the model construction of the *Ph*TAC125 Lip1, using the crystal structure of the *Geobacillus stearothermophilus* carboxylesterase Est30 as template [17]. The most evident difference consisted in the presence of a large extra-loop (32 amino acids long), placed between a α-helix (αE) and the following β-sheet (β8) with respect to the *Geost* Est30 structure. As far the size of the active site pocket is concerned, *Ph*TAC125 Lip1 displayed a wider groove with respect to the *Geost* Est30 one and the *Ph*TAC125 Lip1 surface resulted to be more hydrophobic than that of *Geost* Est30. The above tendency to be more hydrophobic than the thermophilic counterpart is also observed in *Ph*TAC125 Lip1 active site pocket. *Geost* Est30 active site groove was characterised by the presence of a positively charged surface, which has no counterparts in the model of the psychrophilic enzyme.

Furthermore, because of their high catalytic efficiency at low temperatures, psychrophilic enzymes are investigated for their high potential economic benefit: in particular, they could be utilized in novel industrial processes requiring less energy.

3. USE OF THE COLD-ADAPTED ESTERASES AND LIPASES IN FINE CHEMICAL SYNTHESIS: ESTER SYNTHESIS

Novel catalytic synthetic methods in organic chemistry that have to satisfy increasingly stringent environmental constraints are in great demand by chemical industry. Moreover novel catalytic procedures are essential to produce new classes of organic compounds that are becoming targets of biomedical research Enzyme-catalysed chemical transformations are now widely recognized as a good alternative to traditional organic synthesis, and as convenient solutions to same intractable synthetic problems. In organic synthesis these biocatalysts can be used as the sole catalyst in reaction, in combination with other enzymes or with non biological reagents. In addition many enzymes can accept unnatural substrates and can be engineered to modify their stability substrate specificity and specific activity.

Lipase, due to their high catalytic efficiency, selectivity, and the "far than harsh" conditions in which they operate, are used to produce some of industrially important chemicals, traditionally manufactured from fats and oils by chemical processes, with greater rapidity and better specificity under mild conditions [49]. The increasing interest in bacterial lipases is based on their potential to catalyze the hydrolysis of fats (their natural function), trans-esterification, alcoholysis and esterification among other reactions. Indeed lipases, which cleave ester bonds of triacylglycerols with the subsequent release of free fatty acids, diacylglycerols, monoacylglycerols and glycerol, are also able to catalyze the reverse reactions (esterification, inter-esterification and trans-esterification) provided that the aqueous medium is replaced by an organic or a biphasic aqueous/organic medium. Indeed, various esters and lactones are among the substances synthesized using bacterial lipases [14], moreover the high selectivity of these enzymes is a key feature of these biocatalysts which are used in the resolution of racemic mixtures for preparation of optically pure compounds.

From the biotechnological standpoint there are numerous advantages of conducting enzymatic conversions in organic solvents as opposed to water: (i) high solubility of most organic compounds in nonaqueous media; (ii) ability to carry out new reactions impossible in water because of kinetic or thermodynamic restrictions; (iii) relative ease of product recovery from organic solvents as compared to water; and (iv) the insolubility of enzymes in organic media, which permits their easy recovery and reuse and thus eliminating the need for immobilization. It was demonstrated that some lipases act as a catalyst in the 99.98% organic medium; in addition, upon dehydration the enzymes acquire some remarkable new properties, e.g. they become more thermostable and more selective to trans-esterification and able to catalyze several processes including esterification, aminolysis, acyl exchange, thiotrans-esterification, and oximolysis; some of these reactions proceed to a substantial extent only in nonaqueous solvents [50].

Esterification is generally a water limited reaction because the equilibria catalyzed by hydrolytic enzymes is in favour of hydrolysis [51]. Water plays multiple roles on lipase-catalyzed esterifications performed in nonconventional media. It is widely known that water is necessary for the catalytic function of enzymes because it participates, directly or indirectly, in all non-covalent interactions that maintain the conformation of the catalytic site of enzymes. On the other hand, in esterification/hydrolysis reactions it is known that the water content affects the equilibrium conversion of the reactions as well as the distribution of products in the media [52]. Particularly for esterification, as the water content increases, lower equilibrium conversions are achieved. So, there is therefore a conflicting situation because under very low hydration conditions, enzyme efficiency is generally poor and hence reaction kinetics is too slow. Although the role of water in promoting enzyme action is not fully established, it is thought that when the associated water falls below a certain level, the enzymes become more rigid and then perhaps

less efficient [53]. Psychrophilic enzymes might therefore have a potential advantage for applications under low water conditions as a result of their inherent greater flexibility, which will be particularly useful in conditions wherein the activity of mesophilic and thermophilic enzymes is severely impaired by an excess of rigidity. This will allow the use of lower water content with the consequence of increasing yield [54].

Several ester synthesis reaction using cold-active lipases have been reported [14]. In particular, the lipase from *Candida antarctica* fraction B (CAL-B) [36] is a robust lipase in organic synthesis, showing high catalytic efficiency in several ester synthesis reactions [14].

3.1. Synthesis of Short-Chain Flavour Esters

Short-chain esters are compounds with wide applications mainly in food and cosmetic industries due to their characteristic fragrance and flavour. Moreover, some of these esters are also being used in the pharmaceutical industry. Most of the commercial esters can be directly obtained by extraction from plant materials, but the high cost and low quantities of product achieved make this technique inadequate for industrial applications. Then, the industrial production of this kind of compounds has been traditionally carried out by chemical synthesis. In the last decade, biotechnology has been considered for the production of esters used in food industry, since the obtained flavour can be labelled as 'natural' [55]. Thus, the enzymatic synthesis using lipases seems to be a competitive alternative to traditional chemical synthesis.

CAL-B was employed as a biocatalyst for esterification reactions leading to the synthesis of short-chain flavour esters in an organic solvent [56]. Lairos and co-workers demonstrated that *Candida antarctica* lipase CAL-B showed substrate specificity in the synthesis of esters in hexane involving reactions of short-chain acids having linear (acetic and butyric acids) and branched chain (isovaleric acid) structures, an unsaturated (tiglic acid) fatty acid, and phenylacetic acid with n-butanol and geraniol. Moreover they observed the alcohol specificity of the enzyme for esterification of acetic and butyric acids with four alcohols, such as n-butanol, isopentanol, 2-phenylethanol, and geraniol.

3.2. Regioselective and Enatioselective Ester Synthesis

Chiral compounds constitute a significant part of the fine chemical market, and biocatalysis has proven to be a valuable tool in the production of enantiomerically pure compounds. Therefore one of the main application of lipase lies clearly in the exploitation of the outstanding properties of biocatalysts with respect to chemoselectivity, regioselectivity and, particularly, stereoselectivity for the production of enantiomerically pure compounds.

C. antarctica lipase B is an excellent biocatalyst which provides some specific examples of stereoselective biotransformations. In a recent paper [57], the use of *C. antarctica* lipase B in the synthesis of monoester of pyridoxine (vitamin B6) was investigated. Pyridoxine (PN) is one of the three members of the vitamin B6 group, and it is the most important form of commercial vitamin B6, because its ester derivatives have broad applications in food industry, cosmetics and medical supplies [58]. Regioselective esterification of pyridoxine is a difficult task since the pyridine-ring possesses three hydroxyl groups (including one primary -OH and two secondary –OH) of similar reactivity; as a result, it is very difficult to discriminate among these three groups from a chemical point of view. Furthermore, the chemical methods leading to such regioselective analogues involve multi-step protection and deprotection procedures owing to its multiple hydroxyl groups. So the lipase enzymatic reactions were used to provide a helpful option to achieve regioisomer of pyridoxine ester. *C. antarctica* lipase B catalyzed esterification of pyridoxine [57] provides not only an effective selection of one out of the three hydroxyl groups thus leading to synthesis of regioselective monoester.

Ong and co-workers [59] proposed an alternative enzymatic route in the preparation of enantiopure (S)-ketoprofen using CAL-B biocatalyst making use of its remarkable properties e. g. regio, stereo, and substrate specificity. Ketoprofen, a non-steroidal anti-inflammatory drug (NSAID), is still marketed and administered as racemic mixture of "R" and "S" enantiomers, which are equivalent on an unit weight basis. However, (S)-ketoprofen and (R)-ketoprofen display significantly different pharmacological activities and benefits [60]. The paper investigates [59] the performance of free *Candida antarctica* lipase B (CALB) in a mixed solvent system for enantioselective esterification of (*R*)-ketoprofen, leaving the target product (*S*)-ketoprofen in unreacted form (Fig. **3**). The effects of enzyme loading, substrate concentration, alcohol to acid molar ratio, type of solvents and reaction temperature were studied and, in the optimum reaction conditions, a conversion of (*R*)-ketoprofen of 81% was obtained.

3.4. Polyester Synthesis

Usually, condensation poly-esterifications are performed by ester-interchange reactions or by direct esterification of hydroxyacids or diacid/diol combinations [61], using chemical catalysts. These reactions need harsh conditions (e.g., temperatures > 200°C) and metal catalysts are potentially problematic for certain product end uses. Indeed, these reaction conditions can limit product molecular weight and prevent the possibility of using building blocks that are not stable at such temperature-catalyst conditions.

The use of enzymes for polymer synthesis allows carrying out these reactions in the absence of heavy metals, at lower temperatures, and with increased selectivity. Biocompatibility, biodegradability, and environmental acceptability of biotechnologically produced polyesters are desired properties in agricultural and medical applications [62]. The discovery that lipases can also catalyze ester syntheses and trans-esterification reactions in organic solvent systems paved the way of enzyme catalyzed synthesis of biodegradable polyesters [63-65]. Indeed, lipases as catalysts can boast about (i) a promising substrate-conversion efficiencies for non-natural substrates, (ii) high enantio- and regioselectivity, (iii) catalyst recyclability.

Previous studies on lipase-catalyzed poly-esterifications have focused on reactions between diols and activated diacids. For example, Russell and co-workers [66] showed that by using CALB, the solventless co-polymerization of divinyl adipate and 1,4-butanediol gave the corresponding polyester with an average molecular weight (*M*w) of about 23200. However, activation of diacids is expensive and limits the potential technological impact of these methods. Important progress has been made in lipase-catalyzed co-polymerizations of acid and alcohol monomers using nonactivated

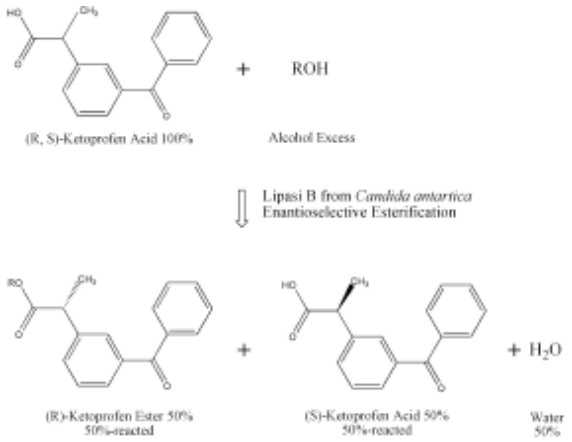

Fig. (3): Application of *Candida Antarctica* lipase B in the enantioselective esterification of (R,S)-ketoprofen. Taken from [59].

free acids [67-68]. The polymerization of aliphatic diols with isopthalic acid was described [69] using CALB as the catalyst. Uyama *et al*. [70] reported that *Candida antarctica* lipase catalyzed condensation reactions between sebacic acid and 1,5-pentanediol in bulk to give aliphatic polyesters with *M*n of 14 000. Binns *et al* [67] investigated reactions of adipic acid and 1,4-butanediol using CALB. The reactions were performed under solvent-free conditions and the product obtained was a polyesters with *M*w of about 2200. Mahapatro and co-workers reported the catalysis of condensation polymerizations between adipic acid and 1,8-octanediol by CALB [71]. Furthermore they extended the study to different substrates: the diols studied include 1,4-butanediol, 1,6-hexanediol, and 1,8-octanediol (HO-$(CH_2)n$OH, where n) 4, 6, and 8, respectively), the diacids studied include succinic, glutaric, adipic, and sebacic acids demonstrating that reactions with longer chain length diacids (sebacic and adipic acid) and diols (1,8-octane and 1,6-hexane diol) provided higher reactivity than systems with shorter chain-length diacids (succinic and glutaric) and 1,4-butanediol [72].

CALB was applied also for bulk poly-esterifications of linear aliphatic hydroxyacids like 6-hydroxyhexanoic acid, 10-hydroxydecanoic acid, 12-hydroxydodecanoic acid, and l6-hydroxyhexadecanoic acid [73]. The above references strongly established the possibility of performing polymerization reactions between diacids and diols in the presence and absence of solvents.

CONCLUSIONS AND PERSPECTIVES

In this review we reported an overview on cold adapted esterases and lipases from microbial sources, describing some structural features that justify the unusually high enzymatic activity at low temperature of these enzymes. Moreover our attention was focused on the different strategies available to discover novel cold-adapted lipolytic enzymes and on the recent use of cold-adapted lipases and esterases in the synthesis of fine chemicals.

Cold active lipases have proven to be promising enzymes to replace the conventional processes of the biotechnological industries. Moreover, these enzymes represent an extremely versatile group of enzymes that are capable of performing a variety of reactions thereby presenting a fascinating field for future research. However, several studies are necessary to overcome several bottlenecks concerning the high enzyme cost, its low activity and/or stability in some conditions and the low biodiversity of cold-adapted microbes explored so far. The development of novel recombinant DNA technologies such as, site-directed mutagenesis and metagenomics have a positive effect on the expression and production of greater amounts of recombinant proteins, which means more competitive prices, by introducing new or improved catalytic activities of these proteins at low temperature. Therefore, several efforts have to be made in order to attain economical overproduction of cold active lipase and to obtain modified lipase more stable and active.

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REFERENCES

[1] Xu Y, Nogi Y, Kato C, *et al*. *Moritella profonda* sp. nov. and *Moritella abyssi* sp. nov., two psychropiezophilic organisms isolated from deep Atlantic sediments. Int J Syst Evol Microbiol 2003; 53: 533–8.

[2] D'Amico S, Collins T, Marx JC, Feller G, Gerday C. Psychrophilic micro-organisms: challenger for life. EMBO Rep. 2006; 7: 385–9.

[3] Margesin R R, Schinner F, Marx J-C, Gerday C. In: Psychrophiles: from Biodiversity to Biotechnology (eds.), 2008. Springer-Verlag Berlin Heidelberg

[4] Makhatadze GI, Privalov PL. Hydration effects in protein unfolding. Biophys Chem 1994; 51: 291–9.

[5] Georlette D, Blaise V, Collins T, *et al.* Some like it cold: biocatalysis at low temperatures. FEMS Microbiol Rev 2004; 28: 25–42.

[6] Siddiqui KS, Cavicchioli R. Cold-adapted enzymes. Annu Rev Biochem. 2006; 75: 403-33.

[7] D'Amico S, Claverie P, Collins T. *et al.* Molecular basis of cold adaptation. Philos Trans R Soc Lond B Biol Sci 2002; 29: 917-25.

[8] Ericsson DJ, Kasrayan A, Johansson P, *et al.* X-ray structure of *Candida antarctica* lipase A shows a novel lid structure and a likely mode of interfacial activation. J Mol Biol 2008; 8: 109-19.

[9] Akoh CC, Lee GC, Liaw YC, Huang TH, Shaw JF. GDSL family of serine esterases/lipases. Prog Lipid Res 2004; 43: 534-52.

[10] Feller G, Gerday C. Psychrophilic enzymes: hot topics in cold adaptation. Nat Rev Microbiol 2003; 1: 200–8.

[11] Feller G. Thiry M, Arpigny JL, Gerday C. Cloning and expression in *Escherichia coli* of three lipase-encoding genes from the psychrotrophic Antarctic strain *Moraxella* TA144. Gene 1991; 102: 111-5.

[12] Zhang J, Lin S, Zeng R. Cloning, expression, and characterization of a cold-adapted lipase gene from an antarctic deep-sea psychrotrophic bacterium, *Psychrobacter* sp 7195. J Microbiol Biotechnol 2007; 17: 604–10.

[13] Yang X, Lin X, Fan T, Bian J, Huang X. Cloning and expression of lipP, a gene encoding a cold-adapted lipase from Moritella sp.2-5-10-1. Curr Microbiol 2008; 56: 194-8.

[14] Joseph B, Ramteke PW, Thomas G. Cold active microbial lipases: some hot issues and recent developments. Biotechnol Adv 2008; 26: 457-70.

[15] Parrilli E, Duilio A, Tutino ML. In: Heterologous protein expression in psychrophilic hosts. R. Margesin *et al.* (eds.) Psychrophiles: from Biodiversity to Biotechnology. Springer-Verlag Berlin Heidelberg 2008, 365-79.

[16] Aurilia V, Parracino A, Saviano M, Rossi M, D'Auria S. The psychrophilic bacterium *Pseudoalteromonas haloplanktis* TAC125 possesses a gene coding for a cold-adapted feruloyl esterase activity that shares homology with esterase enzymes from gamma- proteobacteria and yeast. Gene 2007; 397: 51-7.

[17] de Pascale D, Cusano AM, Autore F, *et al.* The cold-active Lip1 lipase from the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 is a member of a new bacterial lipolytic enzyme family. Extremophiles 2008; 12: 311-23.

[18] Rappe MS, Giovannoni SJ. The uncultured microbial majority. Annu Rev Microbiol 2003; 57: 369-394.

[19] Rondon MR, August PR, Bettermann AD, *et al.* Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured micro-organisms. Appl Environ Microbiol 2000; 66: 2541-7.

[20] Entcheva P, Liebl W, Johann A, Hartsch T, Streit W. Direct cloning from enrichment cultures, a reliable strategy for isolation of complete operons and genes from microbial consortia. Appl Environ Microbiol 2001; 67: 89-99.

[21] Gillespie DE, Brady SF, Bettermann AD, *et al.* Isolation of antibiotics turbomycin A and B from a metagenomic library of soil microbial DNA. Appl Environ Microbiol 2002; 68: 4301-6.

[22] Knietsch A, Waschkowitz T, Bowien S, Henne A, Daniel R. Construction and screening of metagenomic libraries derived from enrichment cultures: generation of a gene bank for genes conferring alcohol oxidoreductase activity on *Escherichia coli.* Appl Environ Microbiol 2003; 69: 1408-16.

[23] Venter JC, Remington K, Heidelberg JF, *et al.* Environmental genome shotgun sequencing of the Sargasso Sea. Science 2004; 304: 66-74.

[24] Tringe SG, von Mering C, Kobayashi A, *et al.* Comparative metagenomics of microbial communities. Science 2005; 308: 554-7.

[25] Stein JL, Marsh TL, Wu KY, Shizuya H, DeLong EF. Characterization of uncultivated prokaryotes: isolation and analysis of a 40-kilobase-pair genome fragment front a planktonic marine archaeon. J Bacteriol 1996; 178: 591-9.

[26] Uchiyama T, Abe T, Ikemura T, Watanabe K. Substrate induced gene-expression screening of environmental metagenome libraries for isolation of catabolic genes. Nat. Biotechnol 2005; 23: 88-93.

[27] Hårdeman F, Sjöling S. Metagenomic approach for the isolation of a novel low-temperature-active lipase from uncultured bacteria of marine sediment.FEMS Microbiol Ecol 2007; 59: 524-34.

[28] Jeon JH, Kim JT, Kim YJ, *et al*. Cloning and characterization of a new cold-active lipase from a deep-sea sediment metagenome. Appl Microbiol Biotechnol 2009; 81: 865-74.
[29] Daniel R. The Metagenomics of soil. Nat Rev Microbiol 2005; 3: 470–8.
[30] Ollis DL, Cheah E, Cygler M, *et al*. The alpha/beta hydrolase fold. Protein Engineering 1992; 5: 197-211.
[31] Nardini M, Dijkstra BW. Alpha/beta hydrolase fold enzymes: the family keeps growing. Curr Opin in Struct Biol 1999; 9: 732-7.
[32] Cambillau C, Vantilbeurgh H. Structure of Hydrolases - Lipases and Cellulases. Curr Opin in Struct Biol 1993; 3: 885-95.
[33] Jaeger KE, Reetz MT. Microbial lipases form versatile tools for biotechnology. Trends in Biotech 1998; 16: 396-403.
[34] Karadzic I, Masui A, Zivkovic LI, Fujiwara N. Purification and characterization of an alkaline lipase from *Pseudomonas aeruginosa* isolated from putrid mineral cutting oil as component of metalworking fluid. J Biosci Bioeng 2006; 102: 82-9.
[35] Noble ME, Cleasby A, Johnson LN, Egmond MR, Frenken LG. Analisys of the structure of *Pseudomonas glumae* lipase. Protein Eng 1994; 7: 559-62.
[36] Uppenberg J, Hansen MT, Patkar S, Jones TA. The sequence, crystal structure determination and refinement of two crystal forms of lipase B from *Candida Antarctica*. Structure 1994; 15:293-08. Erratum in: Structure 1994; 15: 453-4.
[37] Meier R, Drepper T, Svensson V, Jaeger KE, Baumann U. A calcium-gated lid and a large beta-roll sandwich are revealed by the crystal structure of extracellular lipase from *Serratia marcescens*. J Biol Chem 2007; 282: 31477-83.
[38] Lonhienne T, Gerday C, Feller G. Psychrophilic enzymes: revisiting the thermodynamic parameters of activation may explain local flexibility. Biochim Biophys Acta 2000; 1543: 1-10.
[39] D'Amico S, Marx JC, Gerday C, Feller G. Activity–stability relationships in extremophilic enzymes. J Biol Chem 2003; 278: 7891-6.
[40] Fields PA, Somero GN. Hot spots in cold adaptation: localized increases in conformational flexibility in lactate dehydrogenase A4 orthologs of Antarctic notothenioid fishes. Proc Natl Acad Sci USA 1998; 95: 11476-81.
[41] Papaleo E, Riccardi L, Villa C, Fantucci P, De Gioia L. Flexibility and enzymatic cold-adaptation: a comparative molecular dynamics investigation of the elastase family. Biochim Biophys Acta 2006; 1764: 1397-1406.
[42] Tronelli D, Maugini E, Bossa F, Pascarella S. Structural adaptation to low temperatures- analysis of the subunit interface of oligomeric psychrophilic enzymes. FEBS J 2007; 274: 4595-608.
[43] Gianese G, Bossa F, Pascarella S. Comparative structural analysis of psychrophilic and meso- and thermophilic enzymes. Proteins 2002; 47: 236-49.
[44] Szilagyi A, Zavodszky P. Structural differences between mesophilic, moderately thermophilic and extremely thermophilic protein subunits: results of a comprehensive survey. Structure Fold Des 2000; 8: 493-504.
[45] Russell RJM, Gerike U, Danson MJ, Hough DW, Taylor GL. Structural adaptations of the cold-active citrate synthase from an Antarctic bacterium. Structure 1998; 6: 351-61.
[46] Kim SY, Hwang KY, Kim SH, Sung HC, Han YS, Cho Y. Structural basis for cold adaptation. Sequence, biochemical properties, and crystal structure of malate dehydrogenase from a psychrophile *Aquaspirillium arcticum*. J Biol Chem 1999; 274: 11761-7.
[47] Aghajari N, Feller G, Gerday C, Haser R. Crystal structures of the psychrophilic alpha-amylase from *Alteromonas haloplanctis* in its native form and complexed with an inhibitor. Protein Sci 1998; 7: 564-72.
[48] Cavicchioli R, Siddiqui KS, Andrews D, Sowers KR. Low-temperature extremophiles and their applications. Curr Opin Biotechnol 2002; 13: 253-61.
[49] *Vulfson EN. Industrial applications of lipases. In: P Wooley, SB. Petersen* (*eds*) *Lipases, Cambridge, Great Britain*: *Cambridge. University Press. 1994;* 271
[50] Zaks A, Klibanov AM. Enzyme-catalyzed processes in organic solvents. Proc Natl Acad Sci 1985; 82: 3192-6.
[51] Borzeix F, Monot F, Vandecasteele JP. Strategies for enzymatic sterification in organic solvents: Comparison of microaqueous, biphasic, and micellar systems. Enzyme Microb Technol 1992; 14: 791-7.
[52] Yahya ARM, Anderson WA, Moo-Young M. Ester synthesis in lipase catalyzed reactions. Enzyme and Microbial Technology 1998; 23: 438-50.
[53] *Partridge J*, Halling PJ, Moore BD. *Practical route to high activity enzyme preparations for synthesis in organic media. Chem Commun 1998;* 32: *841-2*.
[54] Gerday C, Aittaleb M, Arpigny JL, *et al*. Psychrophilic enzymes: a thermodynamic challenge. Biochem Biophys Acta. 1997; 1342: 119-31.
[55] Gillies B, Yamazaki H, Armstrong DW. Production of flavour esters by immobilized lipase. Biotechnol Lett 1987; 10: 709-14.
[56] Larios A, García HS, Oliart RS, Alfaro GV. Synthesis of flavor and fragrance esters using *Candida antarctica* lipase. Appl Microbiol Biotechnol 2004; 65: 373-6.
[57] Zhang DH, Bai S, Sun Y. Food Lipase-catalyzed regioselective synthesis of monoester of pyridoxine (vitamin B6) in acetonitrile. Chemistry 2007; 102: 1012-9.
[58] Baldessari A, Mangone CP, Gros EG. Lipase-catalyzed acylation and deacylation reactions of pyridoxine, a member of vitamin-B6 group. Helv Chim Acta 1998; 81: 2407-13.
[59] Ong AL, Kamaruddin AH, Bhatia SW, Long WS, Lim ST, Kumari R. Performance of free *Candida antarctica* lipase B in the enantioselective esterification of (R)-ketoprofen. Enzyme Microb Technol 2006; 39: 924-9.
[60] Ossipov M H, Jerussi TP, Ren K, Sun H, Porreca F. Pain 2000; 87:193-9.
[61] Odian G. In: Principles of Polymerization. (2nd Ed. ed.), John Wiley & Sons, Inc., New York. 1981; 181-95.
[62] Evans JD, Sikdar SK. Biodegradable plastics: an idea whose time has come? Chemtech 1990; 20: 38-42.
[63] Wallace JS, Morrow CJ. Biocatalytic synthesis of polymers: synthesis of an optically active, epoxy-substituted polyester by lipase-catalyzed polymerization. J Polym Sci A, Polym Chem 1989; 27: 2553-67.
[64] Linko YY, Wang Z, Seppa¨la¨ J. Lipase-catalyzed synthesis of poly(1,4-butanediol succinate) in organic solvent. Biocatalysis 1994; 8: 269-82.
[65] Linko YY, Wang ZL, Seppa¨la¨ J. Lipase catalyzed linear aliphatic polyester synthesis in organic solvent. Enzyme Microb Technol 1995; 17: 506-11.
[66] Chaudhary A K, Lopez J, Beckman EJ, Russell AJ. Biocatalytic solvent-free polymerization to produce high molecular weight polyesters. Biotechnol Prog 1997; 13: 318-25.
[67] Binns F, Harffey P, Roberts S M, Taylor A. Studies of lipase-catalyzed polyesterification of an unactivated diacid/diol system. J Polym Sci PartA Polym Chem. 1998; 36: 2069-80.
[68] Linko YY, Wang ZL, Seppa¨la¨ J. Lipase-catalyzed synthesis of poly(1,4-butyl sebacate) from sebacic acid or its derivatives with 1,4-butanediol. J Biotechnol 1995; 40: 133-8.
[69] Linko YY, Lamsa M, Wu X, Uosukanum E, Seppala J, Linko P. Biodegradable products by lipase biocatalysis. J Biotechnol 1998; 66: 41-60.
[70] Uyama H, Inada K, Kobayashi S. Lipase-catalyzed synthesis of aliphatic polyesters by polycondensation of dicarboxylic acids and glycols in solvent-free system. Polym J 2000; 32: 440-3.
[71] Mahapatro A, Kumar A, Kalra B, Gross RA. Solvent free adipic acid/1,8-octanediol condensation polymerizations catalyzed by *Candida antarctica* lipase b. Macromolecules 2004; 37: 35-40.
[72] Mahapatro A, Kalra B, Kumar A, Gross RA. Lipase-catalyzed polycondensations: effect of substrates and solvent on chain formation, dispersity and end-group structure. Biomacromolecules 2003; 4: 544-51.
[73] Mahapatro A, Kumar A, Gross RA. Mild, solvent-free ω-hydroxy acid polycondensations catalyzed by *Candida antarctica* lipase B. Biomacromolecules 2004; 5: 62-8.
[74] Arpigny JL, Feller G, Gerday C. Cloning, sequence and structural features of lipase from Antarctic facultative psychrophilic *Psychrobacter immobilis* B10. Biochim Biophys Acta 1993; 1171: 331-3.
[75] Choo DW, Kurihara T, Suzuki T, Soda K, Esaki N. A cold-adapted lipase on an Alaskan psychrotroph, *Pseudomonas* sp. Strain B11-1:

gene cloning and enzyme purification and characterization. Apply Environ Microbiol 1998; 64: 486-91.

[76] Preuss J, Kaiser I, Gehring U. Molecular characterization of a phosphatidylcholine hydrolyzing phospholipase C. Eur J Biochem 2001; 268: 5081-91.

[77] Rashid N, Yuji S, Satoshi E, Haruyuki A, Tadayuki I. Low-temperature lipase from psychrotrophic *Pseudomonas* sp. Strain KB700A. Appl Environ Microbiol 2001; 67: 4064-9.

[78] Kulakova L, Galkina A, Nakayamab T, Nishinob T, Esakia N. Cold-active esterase from *Psychrobacter* sp. Ant300: gene cloning, characterization and the effects of Gly-Pro substitution near the active site on its catalytic activity and stability. Biochim Biophys Acta 2004; 1696: 59-65.

[79] Ryu HS, Kim HK, Choi WC, *et al*. New cold-adapted lipase from *Photobacterium lipolyticum* sp. Nov. that is closely related to filamentous fungal lipases. Appl Microbiol Biotechnol 2006; 70: 321-6.

[80] Rotticci-Mulder JC, Gustavsson M, Holmquist M, Hult K, Martinelle M. Expression in *Pichia pastoris* of *Candida Antarctica* lipase B and lipase B fused to cellulose-binding domain. Protein Expr and Purif 2001; 21: 386-92.

[81] Blank K, Morfill J, Gumpp H, Gaub HE. Functional expression of *Candida Antarctica* lipase B in *Escherichia coli*. J Biotechnol 2006; 125: 474-83.

[82] Methe BA, Nelson KE, Deming JW, *et al*. The psychrophilic lifestyle as revealed by the genome sequence of *Colwellia psychrerythraea* 34H through genomic and proteomic analyses. Proc Natl Acad Sci USA 2005; 102: 10913-8.

[83] Rabus R, Ruepp A, Frickey T, *et al*. The genome of *Desulfotalea psychrophila*, a sulfate-reducing bacterium from permanently cold Arctic sediments. Environ Microbiol 2004;6:887-02.

[84] Saunders NF, Thomas T, Curmi PM, *et al*. Mechanisms of thermal adaptation revealed from the genomes of the Antarctic Archaea *Methanogenium frigidum* and *Methanococcoides burtonii*. Genome Res 2003; 13: 1580-8.

[85] Médigue C, Krin E, Pascal G, *et al*. Coping with cold: the genome of the versatile marine Antarctica bacterium *Pseudoalteromonas haloplanktis* TAC125. Genome Res 2005; 15: 1325-35.

[86] Auman AJ, Breezee JL, Gosink JJ, Kampfer P, Staley JT. *Psychromonas ingrahamii* sp. nov., a novel gas vacuolate, psychrophilic bacterium isolated from Arcticpolar sea ice. Int. J Syst Evol Microbiol 2006; 56: 1001-7.

[87] Campanaro S, Vezzi A, Vitulo N, *et al*. Laterally transferred elements and high pressure adaptation in *Photobacterium profundum* strains. BMC Genomics 2005; 6: 122-137.

[88] Bakermans C, Ayala Del Rio HL, *et al*. *Psychrobacter cryohalolentis* sp. nov. and *Psycrhobacter arcticus* sp. nov., isolated from Siberian permafrost. Int J Syst Evol Microbiol 2006; 56: 1285-91.

[89] De Simone G, Menchise V, Manco G, *et al*. The crystal structure of a hyper-thermophilic carboxylesterase from the archaeon *Archaeoglobus fulgidus*. J Mol Biol 2001; 314: 507-18.

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Influence of production process design on inclusion bodies protein: the case of an Antarctic flavohemoglobin

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Abstract

Background
Protein over-production in *Escherichia coli* often results in formation of inclusion bodies (IBs). Some recent reports have shown that the aggregation into IBs does not necessarily mean that the target protein is inactivated and that IBs contain a high proportion of correctly folded protein. This proportion is variable depending on the protein itself, the genetic background of the producing cells and the expression temperature. In this paper we have evaluated the influence of the production process design on an inclusion bodies protein quality.

Results
The present paper describes the recombinant production in *Escherichia coli* of the flavohemoglobin from the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125. Flavohemoglobins are multidomain proteins requiring FAD and heme cofactors. The production was carried out in several different experimental setups differing in bioreactor geometry, oxygen supply and the presence of a nitrosating compound. Inall production processes, the recombinant protein accumulates in IBs, from which it was solubilized in non-denaturing conditions. Comparing structural properties of the solubilized flavohemoglobins, i.e. deriving from thedifferent process designs, our data demonstrated that the protein preparations differ significantly in the presence of cofactors (heme and FAD) and as far as their secondary and tertiary structure content is concerned.

Conclusions
Data reported in this paper demonstrate that other production process parameters, besides growth temperature, can influence the structure of a recombinant product even if it accumulates in IBs. To the best of our knowledge, this is the first reported example in which the structural properties of a protein solubilized from inclusion bodies have been correlated to the production process design.

Background
Protein over-production in *Escherichia coli* (*E. coli*) often results in formation of inclusion bodies (IBs). Aggregation most probably occurs as a consequence of interactions among the newly-formed folding intermediates which expose hydrophobic residues at their surface [1]. For a long time it was believed that IBs were compact, insoluble aggregates of misfolded proteins [2], remaining in the cell as biologically inactive deposits.
However, some recent reports have shown that the aggregation into IBs does not necessarily mean that the target protein is inactivated [3, 4]. Structural data collected from many model proteins revealed the presence of significant proportions of native-like secondary structure in IBs proteins [5, 6]. Consequently, it is not surprising that the analysis of the biological properties of IBs formed by enzymes demonstrated in some cases the occurrence of enzymatic activity inside the IBs [3, 7]. These evidences introduced the concept that IBs are

composed, at least partially, by functional polypeptides, whose deposition is necessarily driven by discrete aggregation determinants, that act irrespective of the global folding state of the protein [8]. It has been observed that IBs containing a high proportion of correctly folded protein can be easily solubilized under non-denaturing conditions [9] by using mild detergents or polar solvents, widely preserving the target protein folding.
The prevalence and extent of native structure and biological activity of IB proteins is variable depending on the protein itself, the genetic background of the producing cells and the expression temperature [6, 10].
Flavohemoglobins (flavoHbs) have been identified in a number of bacteria and yeasts [11]. These proteins are characterized by a modular structure, where a N-terminal hemoglobin domain, displaying a classical three-over-three α-helical sandwich motif around a single heme b [12], is linked to a C-terminal FAD-containing reductase domain which resembles ferredoxin reductase [13]. The flavoHbs C-terminal domain binds NAD(P)H and transfers electrons to the heme in the globin domain via FAD [14, 15].
It is generally believed that flavohemoglobins provide protection against NO and related reactive nitrogen species although the exact mechanisms of action is still under debate [16, 17, 18, 19, 20].
A flavoHb encoding gene (*PSHAa2880*) was identified by *in silico* genome analysis of the Antarctic Gram-negative marine eubacterium *Pseudoalteromonas haloplanktis* TAC125 (*P. haloplanktis* TAC125) [22]. In the present paper, the recombinant production of the psychrophilic flavoHb (hereinafter called *Ph*flavoHb) in *E. coli* cells was carried out in several different experimental setups in order to identify the best production condition . Indeed, previously reported results, on *E. coli* flavoHb, demonstrated that the heterologous over-production of flavoHb may lead to host cell damage [29] due to the action of flavoHb. as a potent generator of products of oxygen radical partial reduction (i.e., superoxide and peroxide) [27, 28]. Due to the expected toxicity of the recombinant product, flavoHb recombinant productions were carried out exploring several expression systems and/or microbial cell factories, with different results [29]. Amongst many other examples reported in literature, recombinant production of *E. coli* flavoHb in *E. coli* was obtained in absence of oxygen and in presence of nitrosating compound, an experimental setup in which the *hmp* gene expression is physiologic and the flavoHb activity is required [30]. Starting from the above information, in the present work, the recombinant production of the flavoHb from the Antarctic Gram-negative bacterium *P. haloplanktis* TAC125 [22] was performed in *E. coli* cells condition differing in presence of nitrosating compound and in O_2 viability.
All production processes resulted in the accumulation of the recombinant protein in IBs, from which it was solubilized in non-denaturing conditions. Comparing structural properties of the solubilized *Ph*flavoHbs, i.e. deriving from the different production processes, our data demonstrated that the protein preparations differ significantly in the presence of cofactors (heme and FAD) and in their secondary and tertiary structure, demonstrating the impact of the specific production process design on the quality of inclusion bodies protein.

Results

Recombinant production of *P. haloplanktis* TAC125 flavohemoglobin in *E. coli* cells resulted in full deposition of the protein in the inclusion bodies.

The *PSHAa2880* gene was PCR amplified to suitably introduce *NdeI* and *SalI* restriction sites, and cloned into pET22b vector corresponding sites, thus generating the recombinant pET22b-*2880* plasmid.
E. coli BL21(DE3) cells were transformed with the recombinant vector and, keeping in mind that proteins coming from psychrophilic micro-organisms are often characterized by a moderate to extreme thermal-lability [23], the production of the *Ph*flavoHb was carried out at 20°C. However, two different production process setups were explored. First, *E. coli* BL21(DE3)(pET22b-*2880*) recombinant cells were grown in a 7.5 L automatic fermenter, in which the recombinant mesophilic cells were grown aerobically at 20°C till the culture density reached the value of 0.6 OD at 600 nm. Induction was then performed by IPTG, in the

following conditions: i) addition of heme and FAD precursors (i.e. D-aminolevulinic acid, $FeCl_3$, and riboflavin); ii) addition of the nitrosating compound sodium nitroprusside (SNP), and iii) in microaerophilic conditions (dissolved oxygen tension always below 5% of saturation). Microareophilic conditions were achieved by stopping air supply during the next 16-18 hr of fermentation. The second experimental condition consisted in growing recombinant cells in shake flask at 20°C until the culture absorbance at 600 nm reached 0.6 OD, when the protein production was induced in the same conditions as in automatic fermenter but without SNP and in aerobic conditions.
Then, cells coming from the above production processes were analyzed looking for production and soluble/insoluble distribution of the recombinant protein by cell fractionation followed by SDS-PAGE analysis. Both production processes resulted in the total deposition of recombinant flavohemoglobin as cytosolic inclusion bodies (IBs), that were called flask-flavoHb IBs and ferm-flavoHb IBs if derived from cells grown in shake flasks or in fermenter, respectively.

Recombinant *P. haloplanktis* TAC125 flavohemoglobin is solubilized from inclusion bodies by non-denaturing solutions.
Treatment of *P. haloplanktis* TAC125 flavo-Hb IBs with different non-denaturing solvents such as low concentration of mild detergents or polar solvents was applied to the recovery of the recombinant protein in solution. Identical aliquots of flask-flavoHb IBs and ferm-flavoHb IBs were incubated overnight at 4°C with different non-denaturing solutions (i.e. buffered solutions containing 0.2% N-lauroyl sarcosine, or 5% DMSO, or 5% *n*-propanol, or 0.5% Triton X-100, or 1% Na-deoxycholate). Solubilized proteins were then separated from the insoluble matter by a centrifugation step and subjected to SDS-PAGE analysis. As shown in figure 1, both ferm-flavoHb IBs (panel A) and flask-flavoHb IBs (panel B) are partially solubilized in all tested conditions, although the solubilization yields (defined as the percentage of solubilized proteins relative to the total amount contained into the IBs sample) result to be quite different (Table 1).Percentage of solubilization of IBs in different solvents

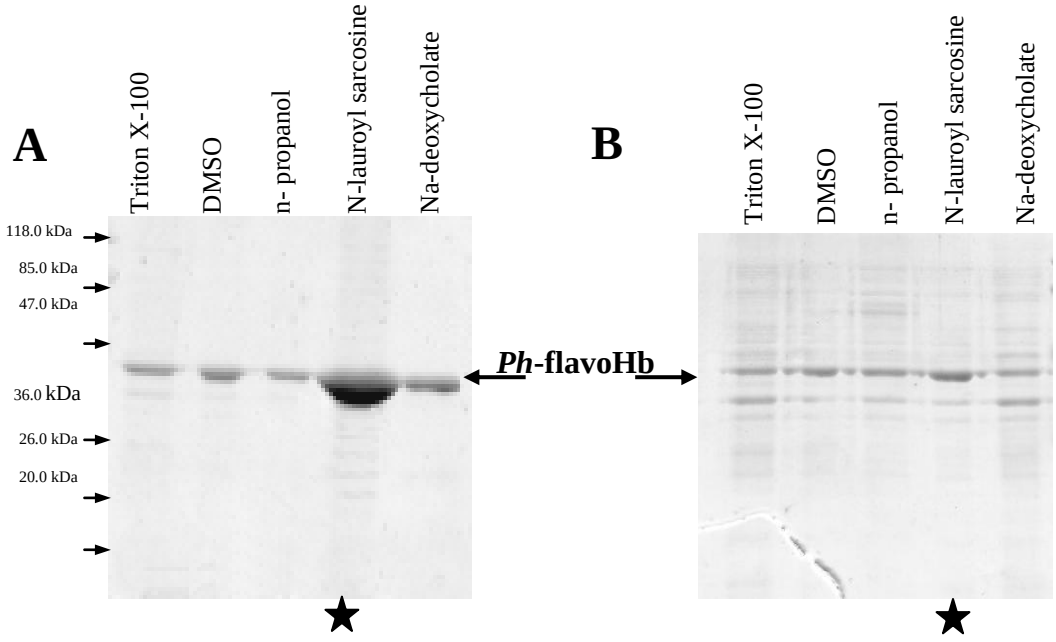

Figure 1 - Solubilization of flavoHb inclusion bodies produced in automatic fermenter (panel A) and shake flask (panel B)
Same amounts of IBs were re-suspended in 40 mM Tris/HCl, pH 8.0 buffer containing different non denaturing agents. Same volumes of solubilized proteins were analysed by SDS-PAGE. Black star indicates that the loaded sample corresponds to one tenth of the other samples.

	% solubilized protein	
Solubilization solvent	**ferm-flavoHb**	**flask-flavoHb**
5 % di n- propanol in 40 mM Tris/HCl, pH 8.0	6 ± 0.7	5 ± 0.1
00.5 % Triton X-100 in 40 mM Tris/HCl, pH 8.0	8 ± 0.6	10 ± 0.9
0.5 % DMSO in 40 mM Tris/HCl, pH 8.0	9 ± 0.9	5 ± 0.4
0.1 % Na-deoxycholate in 40 mM Tris/HCl, pH 8.0	22 ± 1.5	12 ± 0.3
0.2 % N-lauroyl sarcosine in 40 mM Tris/HCl, pH 8.0	95 ±1.0	57±0.8

Table 1 Percentage of solubilization of IBs in different solvents

was calculated using as 100% the protein concentration obtained dissolving IBs in urea 8 M and comparing this value with the protein concentration of samples obtained by treatment with different solvents. Evaluation of protein concentration was obtained by measuring the absorbance at 280 nm (Abs280). Indeed, best recovery in solution was obtained in N-lauroyl sarcosine either for ferm-flavoHb IBs or flask-flavoHb IBs (Table 1), but the corresponding solubilization yields exceed 95% in case of ferm-flavoHb IBs, while only about fifty percent of total proteins contained into IBs from flask culture went in solution. It is worth mentioning that in figure 1 the loaded amount of N-lauroyl sarcosine solubilized samples correspond to one tenth of the other samples.
The two solubilized IBs preparations also differ in their respective protein composition. As shown in figure 1, flask-flavoHb IBs seems to contain several proteins other than flavoHb (panel B), while IBs produced in fermenter contains almost only the psychrophilic recombinant protein (panel A).

Structural comparison of recombinant *P. haloplanktis* TAC125 ferm- and flask-flavoHb.
Flavohemoglobins extracted from either flask-flavoHb IBs (flask-flavoHb) or ferm-flavoHb IBs (ferm-flavoHb) were subjected to further analyses to investigate the presence of the two protein cofactors, i.e. heme and FAD.
Absorption spectra of heme-containing proteins are characterized by the presence of Soret signal, a peak centered at about 413 nm. Therefore, UV/VIS absorption spectra of each solubilized flavoHb were recorded, and their respective spectra regions between 380 and 450 nm are shown in figure 2. Only the ferm-flavoHb spectrum is characterized by the presence of a typical Soret signal, centered at about 413 nm (Figure 2). Then, the presence of the FAD cofactor in flask-flavoHb and ferm-flavoHb proteins was investigated by fluorescence measurements. In detail, emission spectra between 500 nm and 600 nm, exciting at 450 nm, were recorded and are shown in figure 3. An emission signal at 520 nm, which is indicative of the presence of the FAD cofactor, was only detected in the ferm-flavoHb fluorescence spectrum (Figure 3). To explore the secondary structure of flask-flavoHb and ferm-flavoHb proteins,

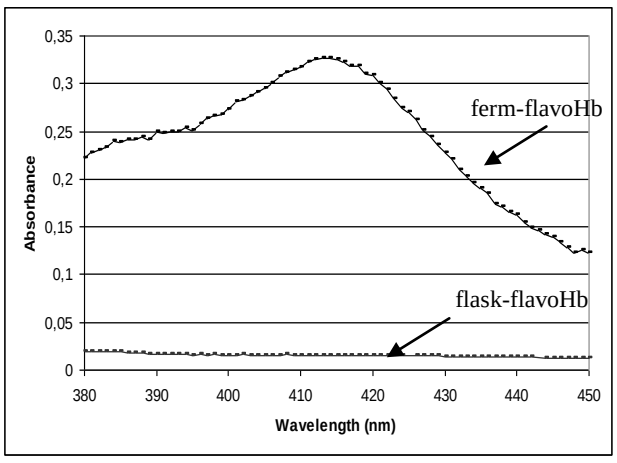

Figure 2 - Absorption spectra of ferm-flavoHb and flask-flavoHb
Absorption spectra of cold-adapted flavohemoglobin extracted from IBs produced in fermenter (ferm-flavoHb) and in flask (flask-flavoHb). The spectra were recorded in 0.2% N-lauroyl sarcosine, 40 mM Tris/HCl pH 8.0, and the proteins concentration was 2 µM.

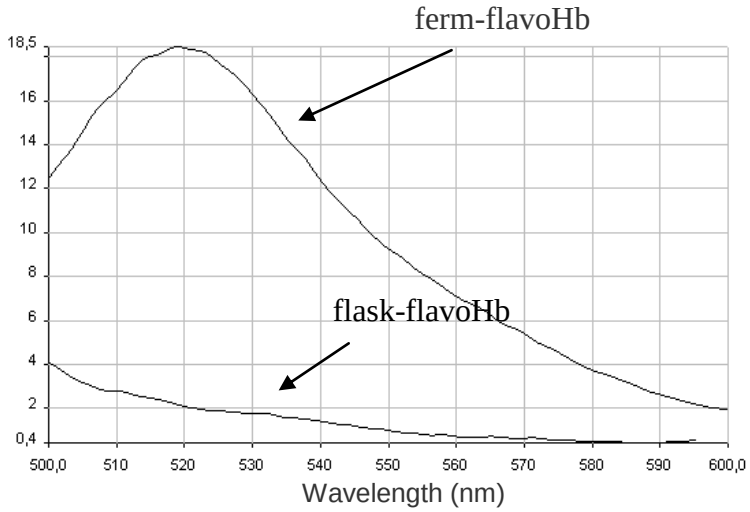

Figure 3 - Fluorescence spectra of ferm-flavoHb and flask-flavoHb
Fluorescence spectra of cold-adapted flavohemoglobin extracted from IBs produced in fermenter (ferm-flavoHb) and in flask (flask-flavoHb). The spectra were obtained exciting at 450 nm and recording emission between 500 nm and 600 nm. The proteins were in 0.2% N- lauroyl sarcosine, 40 mM Tris/HCl pH 8.0, and their concentration was 2 µg/µl

circular dichroism measurements were performed. As shown in figure 4, both proteins display secondary structure, although not identical since the two recorded CD spectra are clearly not superimposable. The collected CD data were used to calculate the percentage of α-helix, β-sheets and random coil for each protein (by using the software K2d, accessible through the site www.embl-heidelberg.de/~andrade/k2d [24, 25]). As shown in table 2, the two proteins differ significantly in their secondary structure content, and the ferm-flavoHb protein is predicted to have a higher content of either β-sheets or α-helix.

	Estimation of protein secondary structure from CD spectra by K2d	
	ferm-flavoHb	flask-flavoHb
α-helix	46 %	39 %
β-sheets	23 %	17 %
random coil	31 %	44 %

Table 2 - Percentage of calculated secondary structures of ferm- flavoHb and flask-flavoHb
Percentage of secondary structures was calculated from CD data spectra by K2d software (Merelo, Andrade). K2d offer an algorithm for the estimation of the percentages of protein secondary structure from UV circular dichroism spectra using a Kohonen neural network

The structural comparison between flask-flavoHb and ferm-flavoHb proteins was then extended to the study of fluorescence emission spectra of tryptophan residues (3 Trp residues are present in the *Ph*flavoHb sequence). Emission spectra in the range between 310 nm and 500 nm, exciting at 295 nm, were recorded and are presented in figure 5. Both proteins display an emission spectrum λ_{max} close to 338 nm, indicating that Trp residues are not solvent exposed. Both protein preparations were fully denatured by addition of guanidinium chloride (at a final concentration of 6 M) and the exposure of the tryptophan residues to a more polar environment was mirrored by the shift of the emission maximum to 366 nm (data not shown). Spectra presented in figure 5 differ in the intensity of λ_{max} signal, where flask-flavoHb protein has a fluorescence emission at 338 nm about three times higher than that of ferm-flavoHb. To investigate if the FAD cofactor, which is present only in ferm-flavoHb protein, is responsible for the observed quenching of the signal at 338 nm,

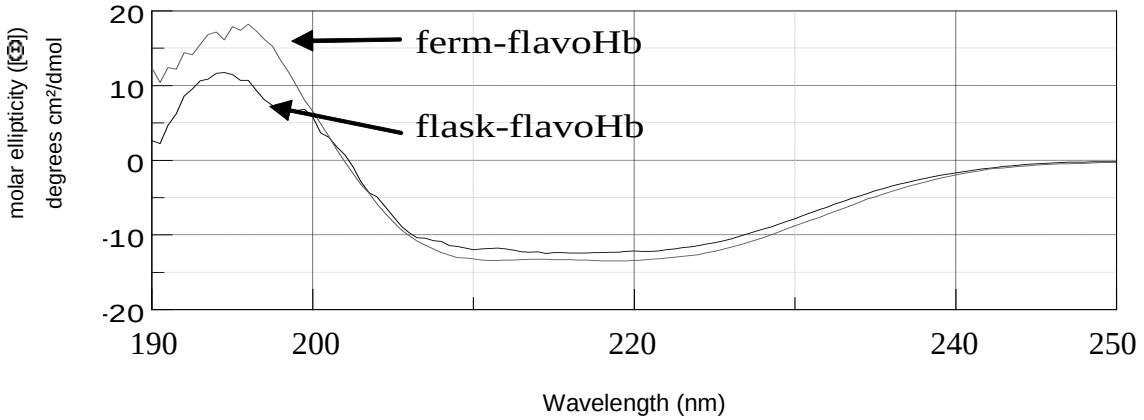

Figure 4 - CD analysis of ferm-flavoHb and flask-flavoHb
CD analysis of ferm-flavoHb (A) and flask-flavoHb (B). The CD spectra were recorded in phosphate buffer 40 mM pH 8.0 at 25°C, the proteins concentration was 100 ng/µl. In case of ferm-flavoHb secondary structure prediction, the program has given a maximum error of 0.080. The maximum error obtained for flask-flavoHb secondary structure prediction is 0.085. This means that the sum of the errors in the prediction of the alpha, beta and random percentage values divided by three is expected to be less then 0.085. In both cases the error values are below the threshold maximal error 0.227. Maximal errors above this value indicate that the result given by the network prediction is not reliable.

tryptophan fluorescence spectra of flask-flavoHb were recorded in the presence of two molar ratio of exogenous FAD. As shown in figure 6, the intensity of λ_{max} signal is not quenched by the addition FAD molecule, neither in the presence of the higher 1:1 FAD:protein molar ratio.

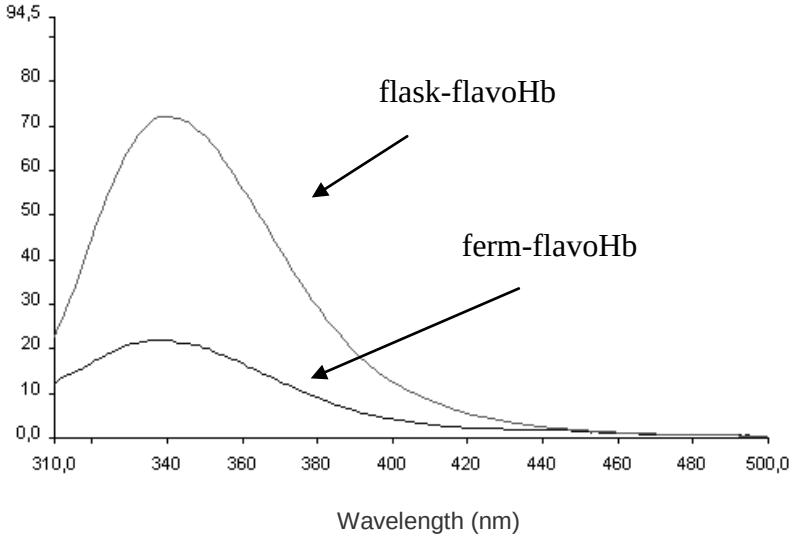

Figure 5 - Tryptophan fluorescence spectra of ferm-flavoHb and flask-flavoHb
Fluorescence spectra of ferm flavoHb (A) and flask-flavoHb (B) obtained recording an emission spectra between 200 nm and 500 nm exciting at 295 nm. Fluorescence spectra were recorded in 0. % N lauroyl sarcosine, 40 mM Tris/HCl pH 8.0, protein concentration was 2 µg/µl.

Analysis of different parameters influence on inclusion bodies protein

In order to understand what parameter is relevant in flavohemoglobin production, i. e. if the observed differences between flask-flavoHb ferm-flavoHb depend on the SNP presence or on oxygen availability, three different production process setups were explored. The production of the *Ph*flavoHb was carried out at 20°C in 7.5 L automatic fermenter, in microaerophilic conditions without SNP, in aerobic condition with and without SNP (in previously described induction conditions). Then, cells coming from the above production processes were analyzed and flavohemoglobin resulted to accumulate in inclusion bodies in all tested condition. *P. haloplanktis* TAC125 flavo-Hb IBs extracted from the different production processes were solubilized in presence of 0.2% N-lauroyl sarcosine (Figure 7). As shown in Figure 7, the solubilized IBs produced in fermenter contains almost only the

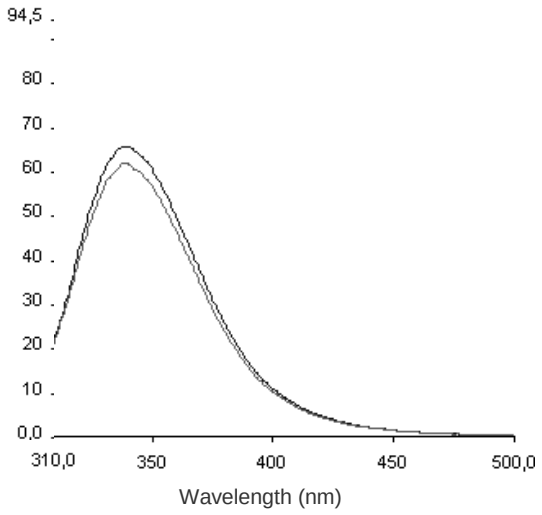

Figure 6 - Tryptophan fluorescence spectra of flask-flavoHb in presence of FAD

Tryptophan fluorescence spectra of flask-flavoHb in presence of FAD cofactor. FAD was added to flask-flavoHb protein at molar ratio of 1:10 and 1:1. Fluorescence spectra were recorded in 0.2% N-lauroyl sarcosine, 40 mM Tris/HCl pH 8.0, proteins concentration was 2 µg/µl.

psychrophilic recombinant protein indicating that the different protein composition of .solubilized IBs of flavoHb and fermHb (Figure 1) was due to the (fermentation device) system used to grow the cells, i.e flask or bioreactor. The analyses aimed to investigate the presence of the heme and FAD (data not shown) demonstrated that proteins obtained in microaerophilic conditions without SNP and in aerobic condition with and without SNP lack of both cofactors.

Discussion

Several recent reports have shown that IBs could contain proteins that posses a native-like secondary structure and an enzymatic activity [3, 5, 6, 7]. Moreover, it has been reported that IBs containing a high proportion of correctly folded protein can be easily solubilized under non-denaturing conditions [9]

In this paper we report a case in which using different process design we obtained always recombinant proteins in IBs easily solubilized under non-denaturing conditions t, although their common attitude to be solubilzed recombinant proteins derived from the different production prosses differ in presence of cofactors and in their secondary and tertiary structure. In detail, we produced a flavohemoglobins from the Antarctic Gram-negative bacterium *P. haloplanktis* TAC125 [22] in *E.coli* cells in fermenter in the presence of a nitrosating compound and in microaerobiosis. In parallel, *Ph*flavoHb was produced in *E. coli* recombinant cells in standard conditions, i.e. grown in shake flasks by an aerobic production scheme and in absence of SNP. Both processes, carried out at 20°C, resulted in the accumulation of the recombinant protein in cytoplasmic inclusion bodies. The treatment of ferm-flavoHb IBs and flask-flavoHb IBs with a N-lauroyl sarcosine solution highlighted unexpected differences in i) the solubilization yield, and ii) the composition of the solubilized matter. This evidence prompted us to carry out a structural comparison of the two recombinant *Ph*flavoHbs, in order to assess if the production setup could influence the main structural features of IBs proteins. Data reported in the present paper demonstrate that the two proteins differ significantly, mainly in the presence of FAD and heme cofactors. Indeed, when subjected to suitable spectroscopic analyses, proofs of the presence of heme and FAD were collected only in the case of ferm-flavoHb. Furthermore, CD spectra demonstrate

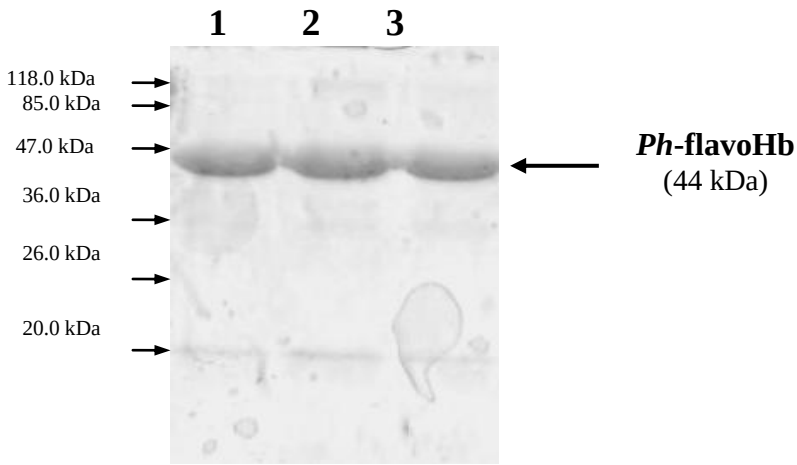

Figure 7 - FlavoHb inclusion bodies produced in automatic fermenter in different conditions

SDS-PAGE of flavoHb IBs extracted from culture obtained in microaerophilic conditions without SNP (1), in aerobic condition with (2)and without SNP(3). IBs were-suspended in 0.2% N-lauroyl sarcosine 40 mM Tris/HCl, pH 8.0.

that both proteins possess a secondary structure, but the ferm-flavoHb content of alpha-helix and beta-sheets is higher than that observed in the protein produced in flask.
Taking advantage from the presence of three tryptophan residues along the *Ph*flavoHb protein sequence, fluorescence emission spectra of tryptophan residues were recorded. Both spectra are characterized by a λ_{max} emission signal around 338 nm, a result indicative that the Trp residues are not exposed to the solvent. Indeed, in these experimental conditions, unfolded proteins usually present a shift of λ_{max} towards 350 nm. As expected, when the proteins were chemically denatured by guanidinium chloride a shift of the λ_{max} emission signal was observed (new maximum at 366 nm, data not shown). These data are highly suggestive that both proteins display a 3D structure, although likely not identical. In fact, fluorescence intensity at λ_{max} of flask-flavoHb is about three times higher than that of protein produced in fermenter. This different spectroscopic behavior is not justified by the likely quenching effect of FAD cofactor associated only to the ferm-flavoHb. Therefore, the observed difference in intensity of λ_{max} signal could be due to some amino acids residues close to tryptophan residues that work as fluorescence quencher in ferm-flavoHb. These results are suggestive of a different chemical surrounding around the tryptophan residues in flask-flavoHb and ferm-flavoHb proteins, indicating that the two proteins differ in 3D structure too.
Moreover reported results demonstrated that observed differences between flask-flavoHb ferm-flavoHb depend on a synergic effect of SNP presence and microaerophilic conditionsindee only in this condition we obtained a flavoHb endowed with FAD and heme cofactors
Lacking a structural/functional characterization of native *Ph*flavoHb, a final assessment on the quality of the recombinant proteins produced in this work cannot be formulated. However, the presence of FAD and heme cofactors, together with the collected indirect evidences of a different secondary and, eventually, tertiary structures, looks very promising of a better quality of flavoHb obtained in the presence of a nitrosating compound and in microaerobiosis. .

Conclusions

The present paper describes the recombinant production of a flavohemoglobin, a multidomain protein requiring FAD and heme cofactors for its activity. Two significantly different production process designs were exploredboth resulting in the full product accumulation in IBs. Data reported here demonstrate that other process parameters, besides growth temperature, influence the quality of a recombinant product even if it accumulates in IBs. To the best of our knowledge, this is the first reported example in which the quality of protein solubilized from inclusion bodies has been correlated to the production process design.

Methods

Bacterial strains, plasmid and culture condition

The *E. coli* BL21(DE3) (Novagen) strain was routinely used for cloning and expressing recombinant gene. Cells were grown in Luria-Bertani (LB) medium at 20°C. When required, Ampicillin (Sigma) was added at 100 µg/ml. Plasmid pET-22b (Novagen) was utilized for cloning and expression. Restriction and modifying enzymes were obtained from Promega. The oligonucleotides were custom synthesized from PRIMM.

Cloning of the PSHAa2880gene

The primer pairs for the *PSHAa2880* gene (Oligo 2880 fw 5' TTCATATGTTATCTGATAAAACTATTGAAA 3', Oligo 2880 rv 5' AAGTCGACTTATAGATCTTGATGCGG 3') were designed on the basis of the *P. haloplanktis* TAC125-genome sequence [31]. Sequences corresponding to the *Nde*I site and a *Sal*I site were introduced in the forward and reverse primers, respectively. The amplifications were performed in a mixture containing 80 ng of *P. haloplanktis* TAC125-genomic DNA as template, 50 pmol of each oligonucleotide primer, 1.8 mM MgCl2, 50 mM KCl, 20 mM Tris-HCl pH 8.3, 0.1% gelatine, 200 µM dNTP in a final volume of 50 µl. The mixtures were incubated at 95°C for 10 min, then 1.25 units of *Taq* DNA polymerase were added. Twenty cycles of amplification (consisting of 1 min at 95°C, 1.5 min

at 60°C and 1 min plus 5sec/cycle at 72°C) were carried out and followed by a cycle in which the extension reaction at 72°C was prolonged for 15 min in order to complete DNA synthesis. The amplified fragment was cloned and its nucleotide sequence checked to rule out the occurrence of any mutation during synthesis. The *Nde*I-*Sal*I-digested fragment of the *PSHAa2880* gene was then subcloned into the corresponding sites of the expression vector pET-22b, obtaining the plasmid pET22b-*2880.* The recombinant vector was used to transform *E. coli* BL21(DE3) cells, that were used for the following production processes. All DNA manipulation were performed as previously described [32]

Shake Flask Culture

For the over-expression of cold-adapted flavoHb in flask, a single colony of recombinant *E. coli* BL21(DE3) (pET22b-*2880*) was inoculated in LB medium supplemented with 4 g/L glucose and ampicillin (100 µg/ml) and allowed to grow at 20°C, in the rotary shaker, until absorbance at 600 nm reached ~0.6 OD. The culture was then induced with 1 mM isopropyl-β-D-thiogalactopyranoside and further incubated for another 16-18 h at 20°C in the presence of 50μM of D-aminolevulinic acid, 3 μM $FeCl_3$, 100 μM riboflavin. After production, the cell culture was aliquoted, centrifuged, and the supernatant was discarded. The bacterial pellet was stored for further analysis.

Laboratory Fermentation

A proper preinoculum of overnight grown recombinant *E. coli* BL21(DE3) (pET22b-*2880*) was diluted in 4.5 L of LB medium supplemented with 4 g/L glucose and ampicillin (100 µg/ml) in a 7.5-L Techfors *S* (Infors, HT Switzerland) automatic fermenter. Cells were grown aerobically at 20°C till the culture density reached the value of 0.6 OD at 600 nm. Induction was then performed by 1 mM IPTG, in the presence of 50μM mM D-aminolevulinic acid, 3 μM $FeCl_3$, 100 μM riboflavin and 0.4 mM sodium nitroprusside (SNP). Then, air supply was stopped during the next 16-18 hr of fermentation keeping a microaerophilic conditions (dissolved oxygen tension always below 5% of saturation).

In case of production of the *Ph*flavoHb in aerobic condition with and without SNP the preinoculum of overnight grown recombinant *E. coli* BL21(DE3) (pET22b-*2880*) was diluted in 2.5 L of medium and dissolved oxygen tension was maintained always above 30% of saturation.

After production, the cell culture was collected, centrifuged, and the supernatant was discarded. The bacterial pellet was stored for further analysis.

Protein inclusion bodies extraction

Biomass was harvested at the end of the production process by centrifugation, and the wet bacterial pellet was resuspended in 10 mM Tris/HCl, pH 8.0. Samples were kept on ice and disrupted by sonication using an Branson sonicator (Model B-15), using a program consisting of 20 cycles (30'' on, 60'' off, intensity 4.5). After disruption of the cells, samples were centrifuged at 5000 rpm for 30 min at a constant temperature of 4°C. The supernatant was discarded, and the inclusion bodies fraction was washed twice with chilled water, divided in several aliquots and incubated overnight for the solubilization at 4°C in 40 mM Tris/HCl, pH 8.0 buffer containing alternatively:

- 5% di n-propanol;
- 0.5% Triton X-100
- 5% DMSO
- 1% Na-deoxycholate.;
- 0.2% N-lauroyl sarcosine

As negative control the insoluble matter was treated with water. After the incubation, the suspensions were centrifuged at 4400x***g*** for 15 minutes at 4°C. The supernatants were analyzed by SDS-PAGE.

Protein concentration measurements

The Bradford method [33] was applied to determine protein concentration. In case of protein solubilized from IBs with different solvents a qualitative measure of protein content was determined by measuring the amount of light absorbed at 280 nm (Abs280).

Spectroscopic Measurements

UV–Vis absorption spectra were recorded in UNIKON 930spectrophotometer. Fluorescence measurements were carried out in a PERKIN ELMER LS 50B fluorospectrometer . Circular dichroism (CD) spectra were obtained in a Jasco spectropolarimeter (model J-715) equipped with a thermostatically controlled cell holder.

Authors' contributions

EP and MG performed the experiments and helped to draft the manuscript. EP and MLT drafted the manuscript and designed and coordinated the study. GM has been involved in manuscript preparation and critical reading. All authors read and approved the manuscript

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References

1. Schlieker C, Bukau B, Mogk, A: **Prevention and reversion of protein aggregation by molecular chaperones in the E. coli cytosol: implications for their applicability in biotechnology.** *J Biotechnol*. 2002, **96:**13-21.
2. Marston FA: **The purification of eukaryotic polypeptides synthesized in Escherichia coli.** *Biochem J.* 1986, **240:**1-12.
3. Garcia-Fruitos E, Gonzalez-Montalban N, Morell M, Vera A, Ferraz RM, Aris A, Ventura S, Villaverde A: **Aggregation as bacterial inclusion bodies does not imply inactivation of enzymes and fluorescent proteins.** *Microb Cell Fact*. 2005, **4:**27.
4. Ventura S, Villaverde A: **Protein quality in bacterial inclusion bodies.** *Trends Biotechnol.* 2006, **24:**179-185.
5. Ami D, Natalello A, Taylor G, Tonon G, Doglia SM: **Structural analysis of protein inclusion bodies by Fourier transform infrared microspectroscopy**. *Biochim Biophys Acta.* 2006, **1764:**793–799.
6. Gonzalez-Montalban N, Garcia-Fruitos E, Ventura S, Aris A, Villaverde A: **The chaperone DnaK controls the fractioning of functional protein between soluble and insoluble cell fractions in inclusion body-forming cells.** *Microb Cell Fact.* 2006, **5:**26.
7. Arie JP, Miot M, Sassoon N, Betton JM: **Formation of active inclusion bodies in the periplasm of Escherichia coli.** *Mol Microbiol.* 2006, **62:**427–437.
8. Gonzalez-Montalban N, Garcia-Fruitos E, Villaverde A: **Recombinant protein solubility-does more mean better?** *Nat Biotechnol.* 2007, **25:**718–720.
9. Jevsevar S, Gaberc-Porekar V, Fonda I, Podobnik B, Grdadolnik J, Menart V: **Production of nonclassical inclusion bodies from which correctly folded protein can be extracted.** *Biotechnol.Prog.* 2005, 21:632-639.
10. Garcia-Fruitos E, Carrio MM, Aris A, Villaverde A: **Folding of misfolding-prone beta-galactosidase in absence of DnaK.** *Biotechnol Bioeng.* 2005, **90:**869–875.
11. Ermler U, Siddiqui RA, Cramm R, Friedrich B: **Crystal structure of the flavohemoglobin from Alcaligenes eutrophus at 1.75 A resolution**. *EMBO J*. 1995, **14**:6067–6077.
12. Liu L, Zeng M, Hausladen A, Heitman J, Stamler JS: **Protection from nitrosative stress by yeast flavohemoglobin.** *Proc Natl Acad Sci.* 2000, **97:**4672–4676.
13. Tarricone C, Galizzi A, Coda A, Ascenzi P, Bolognesi M: **Unusual structure of the oxygen-binding site in the dimeric bacterial hemoglobin from Vitreoscilla sp.** *Structure.* 1997, **975:**497–507.
14. Poole RK, Ioannidis N, Orii Y: **Reactions of the Escherichia coli flavohaemoglobin (Hmp) with oxygen and reduced nicotinamide adenine dinucleotide: evidence for oxygen switching of flavin oxidoreduction and a mechanism for oxygen sensing.** *Proc Biol Sci.* 1994, **255:**251-258.
15. Andrews SC, Shipley D, Keen JN, Findlay JB, Harrison PM, Guest JR: **The haemoglobin-like protein (HMP) of Escherichia coli has ferrisiderophore reductase activity and its C-terminal domain shares homology with ferredoxin NADP$^+$ reductases.** *FEBS Lett.* 1992, **302**:247–252.
16. Gardner PR, Gardner AM, Martin LA, Salzman AL: **Nitric oxide dioxygenase: an enzymic function for flavohemoglobin** *Proc Natl Acad Sci.* 1998, **95:**10378–10383.
17. Hausladen A, Gow AJ, Stamler JS: **Nitrosative stress: metabolic pathway involving the flavohemoglobin.** *Pro. Nat. Acad Sci*. 1998, **95**:14100–14105.

18. Crawford MJ, Goldberg DE**: Regulation of the Salmonella typhimurium flavohemoglobin gene. A new pathway for bacterial gene expression in response to nitric oxide.** *J Biol Chem.* 1998, **273:**34028-32.
19. Membrillo-Hernández J, Coopamah MD, Anjum MF, Stevanin TM, Kelly A, Hughes MN, Poole RK: **The flavohemoglobin of Escherichia coli confers resistance to a nitrosating agent, a "Nitric oxide Releaser," and paraquat and is essential for transcriptional responses to oxidative stress.** *J Biol Chem.* 1999, 274:748–754.
20. Hausladen A, Gow A, Stamler JS: **Flavohemoglobin denitrosylase catalyzes the reaction of a nitroxyl equivalent with molecular oxygen.** *Proc Natl Acad Sci.* 2001, **98**:10108–10112.
21. Kim SO, Orii Y, Lloyd D, Hughes MN, Poole RK: **Anoxic function for the Escherichia coli flavohaemoglobin (Hmp): reversible binding of nitric oxide and reduction to nitrousoxide.** *FEBS Lett.* 1999, **445:**389–394.
22. Giordano D, Parrilli E, Dettaï A, Russo R, Barbiero G, Marino G, Lecointre G, di Prisco G, Tutino ML, Verde C: **The truncated hemoglobins in the Antarctic psychrophilic bacterium Pseudoalteromonas haloplanktis TAC125.** *Gene.* 2007, **398**:69-77.
23. Marx JC, Collins T, D'Amico S, Feller G, Gerday C: **Cold-adapted enzymes from marine Antarctic microorganisms.** *Mar Biotechnol.* 2007, **9:**293-304.
24. Andrade A, Chacón P, Merelo JJ, Morán F: **Evaluation of secondary structure of proteins from UV circular dichroism using an unsupervised learning neural network.** Prot. Eng. 1993, **6**:383-390.
25. Merelo JJ, Andrade MA, Prieto A, Morán F**: Proteinotopic Feature Maps.** *Neurocomputing***.** 1994. **6**:443-454.
26. Spiro S: **Regulators of bacterial responses to nitric oxide.** *FEMS Microbial rev.* 2007, **31:**193-211.
27. Wu G, Corker H, Orii Y, Poole RK: **Escherichia coli Hmp, an "oxygen-binding flavohaemoprotein", produces superoxide anion and self-destructs.** *Arch Microbiol.* 2004, **182:**193-203.
28. Membrillo-Hernández J, Ioannidis N, Poole RK: **The flavohaemoglobin (HMP) of Escherichia coli generates superoxide in vitro and causes oxidative stress in vivo.** *FEBS Lett.* 1996, **382:**141-4.
29. Lewis ME, Corker HA, Gollan B, Poole RK: **A survey of methods for the purification of microbial flavohemoglobins.** *Methods Enzymol.* 2008, **436:**169-86.
30. Gardner AM, Martin LA, Gardner PR, Dou Y, Olson JS: **Steady-state and transient kinetics of Escherichia coli nitric-oxide dioxygenase (flavohemoglobin). The B10 tyrosine hydroxyl is essential for dioxygen binding and catalysis**. *J Biol Chem.* 2000, **275:**12581-9.
31. Médigue C, Krin E, Pascal G, Barbe V, Bernsel A, Bertin PN, Cheung F, Cruveiller S, D'Amico S, Duilio A, Fang G, Feller G, Ho C, Mangenot S, Marino G, Nilsson J, Parrilli E, Rocha EP, Rouy Z, Sekowska A, Tutino ML, Vallenet D, von Heijne G, Danchin A: **Coping with cold: the genome of the versatile marine Antarctica bacterium Pseudoalteromonas haloplanktis TAC125**.*Genome Res.* 2005, **15:**1325-35.
32. Sambrook J, Russell DW: **Molecular Cloning** *in* A Laboratory Manual, 3rd Ed. 2001 Edited by Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
33. Bradford M: **A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding**. *Anal. Biochem.* 1976, **72:**248-254.

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The role of a 2-on-2 haemoglobin in oxidative and nitrosative stress resistance of Antarctic *Pseudoalteromonas haloplanktis* TAC125

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Abstract
The 2-on-2, previously named truncated haemoglobins, are monomeric low molecular weight oxygen-binding proteins that share the overall topology with vertebrate haemoglobins. Although several studies on 2-on-2 haemoglobins have been reported, their physiological and biochemical functions are not yet well defined, and various roles other than binding oxygen have been suggested. Three genes encoding 2-on-2 haemoglobins have been identified in *Pseudoalteromonas haloplanktis* TAC125 (*Ph*TAC125), a psychrophilic Antarctic marine bacterium, raising the question whether their function is related to its ability to cope with the high oxygen concentration in cold sea water. To investigate the function played by one of the three trHbs, *Ph*HbO, a *Ph*TAC125 genomic mutant strain was constructed, in which the PSHAa*0030* gene was knocked out. The mutant strain was grown under controlled conditions and several aspects of bacterium physiology were compared with those of wild-type cells when dissolved oxygen tension and growth temperature were changed. In details, we compared their respective growth behaviour, the transcription of same genes (the three 2-on-2 haemoglobins genes and of a gene encoding a flavohaemoglobin) and sensitivity to H_2O_2 and nitosating agents. Data presented in this paper point towards the involvement of *Ph*HbO in oxidative- and nitrosative-stress resistance, a key aspect of the molecular adaptation of *Ph* TAC125 to the Antarctic marine environment.

Key words*: Pseudoalteromonas haloplanktis* TAC125; cold-adapted bacterium; 2-on-2 haemoglobin; oxydative and nitrosative stress; peroxidase

Introduction
Life at low temperature imposes a wide array of challenges to marine bacteria. One of them is related to the increase of gas solubility in cold water. Indeed, at low temperatures the enhanced oxygen solubility significantly increases the production rate of toxic reactive O_2 species (ROS). From the genome analysis of *Colwellia psychroerythraea* [19] and *Desulfotalea psychrophila* [29], a common strategy to face this environmental challenge can be envisaged, consisting in developing enhanced antioxidant capacity owing to multiple genes that encode catalases and superoxide dismutases. By contrast, *in silico* analysis of the *Pseudoalteromonas haloplanktis* TAC125 (*Ph*TAC125) genome suggests that this Antarctic marine bacterium may cope with increased oxygen solubility by multiplying O_2-scavenging enzymes (such as dioxygenases) and deleting entire metabolic pathways which generate ROS as side products. The remarkable deletion of the ubiquitous molybdopterin-dependent metabolisms in the *Ph*TAC125 genome [18] can be regarded in this perspective. Furthermore, the micro-organism is remarkably resistant to H_2O_2 [18], and this ability was correlated

in silico to the presence of several enzymes involved in scavenging chemical groups affected by ROS (such as peroxiredoxins and peroxidases), and to the presence of one catalase-encoding gene (*katB*) and a possible paralog (PSHAa*1737*) [18].
In this context, our interest was focused on oxygen-binding proteins involved in oxygen scavenging and/or transport, eventually contributing to *Ph*TAC125 adaptation to high oxygen concentration in cold sea water.
Bacteria may produce three types of haemoglobins (Hbs), namely 2-on-2 or truncated Hbs (trHbs), monomeric Hbs and flavohaemoglobins (flavoHbs). 2-on-2 haemoglobins are monomeric low-molecular weight oxygen-binding haemoproteins and their fold is based on a "2 on 2" α-helical sandwich [35]. Three main trHb groups have been identified based on sequence clustering, i.e. group I (also named HbN), group II (also named HbO) and group III (also named HbP) [33, 34, 35]. All these trHbs are able to bind diatomic ligands such as O_2, CO, and NO, with different affinities [20] The high oxygen affinity displayed by most trHbs makes their role as O_2 transporters very unlikely [21, 35] Several other functions have been proposed, e.g. trHbs have been suggested to be involved in the response to oxidative and nitrosative stresses [5, 7] or to function as oxygen sensors [35].
The *Ph*TAC125 genome contains three genes encoding trHbs and one that codes for a flavoHb [9]. The presence of several 2-on-2 haemoglobins encoding genes raises the question whether their function can be related to *Ph* TAC125 ability to cope with the high oxygen concentration of cold sea water. This hypothesis and the availability of several genetic tools [25] suitably developed for *Ph*TAC125 prompted us to use this organism to investigate the functional role of 2-on-2 Hbs in Antarctic cell physiology. Indeed some microorganisms with interesting truncated globins (i.e. *Mycobacterium tuberculosis*, *Nostoc commune*, *Chlamydomonas eugamentos* and *Paramecium caudatum*) are not readily amenable to genetic manipulation; as a consequence, the experimental demonstrations of proposed physiological hypothesis are limited [28]. We have recently reported the recombinant production and purification of *Ph*HbO [9], one of the three *Ph*TAC125 trHbs. To investigate its physiological role, a genomic mutant strain was constructed, in which the *Ph*HbO encoding gene (PSHAa*0030*) was inactivated by insertional mutagenesis. The mutant strain was grown under controlled conditions and its growth behaviour was compared with that of wild-type cells, when dissolved oxygen tension (DOT) and growth temperature changed. This approach allowed proposing a likely role of *Ph*HbO in the adaptation of *Ph*TAC125 to the Antarctic marine environment.

Material and Methods

Bacterial strains and growth conditions. *Ph*TAC125 [18] was isolated from Antarctic sea water. *Escherichia coli* DH5α [11] was used as host for gene cloning and was routinely grown in Luria-Bertani broth [30] at 37°C with the appropriate antibiotic selection.
*Ph*TAC125 was grown in minimal medium [23] supplemented with 0.5% yeast extract and 0.2% galactose. 100 μg/ml ampicillin, 50 μg/ml chloramphenicol were added to liquid cultures when transformed. Antarctic bacterium was made recombinant by intergeneric conjugation as previously reported [25]. Batch cultivations were performed in a computer-controlled bioreactor (Sixfors Systhem, Infors) equipped with control units (pH, DOT, temperature, rpm) at 4°C and 15°C. pH was maintained at 7.0±0.05 by the addition of 1% H_2SO_4 or 5% NH_4OH. Under extreme aerobiosis condition, DOT was maintained always above 80% by modifying stirring speed and aeration rate. In microaerobiosis (DOT always below 5% saturation) air supply was stopped after inoculum. For each strain, the growth kinetics were followed in triplicate in at least two independent experiments.
Construction of plasmids. Standard methods were employed for DNA manipulation and isolation, amplification by PCR, and DNA sequencing [2, 30]. Restriction enzymes, T4 DNA ligase, alkaline phosphatase, T4 polynucleotide kinase Klenow fragment, *Taq* DNA polymerase were supplied from Boehringer-Roche, Amersham-Pharmacia Biotech, Promega, and New England Biolabs. DNA fragment purification was carried out with the QUIAEX II kit from Qiagen GmbH. The amplified fragments were cloned and checked by nucleotide sequencing to rule out the occurrence of mutations during synthesis.
Vector pVS*0030* and *Ph*TAC125-*30* mutant construction. The reaction of PCR [30] was employed to amplify a DNA fragment of PSHAa*0030* gene. *Ph*TAC125 genomic DNA was used as PCR

template and two primers were designed to amplify a 241bp-long region of the PHSAa*0030* gene and to introduce two SphI sites (Oligo S0030fw 5'-TTGCATGCATACTAGGTGGAGAAGC-3' and Oligo S0030rv 5'-AAGCATGCGCGCAAGTCTTGATC-3'). The amplified DNA fragment was digested by SphI, and inserted into the pVS plasmid [24] corresponding site. The pVS*0030* vector was mobilized by intergeneric conjugation into *Ph*TAC125 cells and insertion mutants were screened on plates at 4°C containing carbenicillin (30μg/ml) as selection agent.

Construction of vector pUC*0030*. pUC*0030* was constructed starting from pUCC vector, a plasmid deriving from the pUCLOriT/R plasmid [32], containing the T/R box, the transcription termination signal from the *Ph*TAC125 *aspC* gene and the chloramphenicol-resistance gene. The nucleotide region including the PSHAa*0030* gene and its upstream region was amplified to introduce HindIII and BamHI restriction sites using oligo 0030 fw (5'-TTAAGCTTTAGCTCCCTTACCGCC-3') and oligo 0030 rv (5'-AAGGATCCGTGCCAGCTTTAAGGC-3'). The PCR product was subjected to double HindIII and BamHI digestion, purified, and inserted into pUCC vector corresponding sites.

RNA preparation and RT-PCR. Total RNA was isolated (RNeasy Mini kit, Qiagen) from 500 μl aliquots of *Ph*TAC125-*30* mutant and *Ph*TAC125 wildtype cell cultures withdrawn at different growth times at 4°C, 15°C, microaerobiosis and extreme aerobiosis, and subjected to in-column DNase treatment (Rnase-Free Dnase Set, Qiagen). Reverse-transcription (RT) reactions using SuperScript II RNase H^- Reverse Transcriptase (Invitrogen) were performed using 100 pmol of specific primers. In detail, reverse transcriptase analyses were performed on approximately 5 μg of purified RNA using PHSAa*0030* specific primers 0030Sfw (5'-TTGCATGCATACTAGGTGGAGAAGC-3') and 0030Xrv (5'-AAGCATGCGCGCAAGTCTTGATC-3'), *Ph*TAC125 PHSAa*0458* specific primers 0458Erv (5'-CTTTGCTCGAGCATAGCATTAATTAG-3') and 0458Sfw (5'-AATGGGCATGCCGGCATAGAAAAAC-3'), PSHAa2880 specific primers 2880Srv (5'-GAGTATTTTCGCAAGCATGCAAATAC-3') and 2880Efw (5'-ATTTTGCTCGAGCACTACCCAATTG-3'), PSHAa2217 specific primers 2217Srv (5'-GCTTTTTGCATGCATAATAGCCAAGC-3') and 2217Efw (5'-TACCCAGCTCGAGGATGCATTTTATG-3'). PCR amplifications were performed using 2 μl of RT reaction sample as a template, *Taq* DNA polymerase (Promega) and specific target primer pairs. An additional PCR reaction on DNA-free total RNA was performed as negative control.

Disk diffusion assays. The sensitivity of the *Ph*TAC125-*0030* mutant and *Ph*TAC125 wild-type cells to 100 mM GSNO, 100 mM SNP, 256 mM H_2O_2, and 100 mM spermidine NONOate was assayed at 15°C in a plate diffusion assay [3].

Peroxidase assay. *E. coli* BL21(DE3) containing the plasmid pET-*HbO* was grown in LB medium supplemented with kanamycin (50 μg/ml) and *Ph*HbO production and purification was carried out as previously described [9].

The peroxidase activity of the purified protein was assaed by UV–VIS spectrophotometry (UNIKON 930) using ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) and H_2O_2 as substrates. The concentration of *Ph*HbO in the reaction was estimated by the method of Bradford. Formation of the ABTS cation radical was followed at 415 nm, using ε 415 nm = 36 mM^{-1} cm^{-1}. The pH values were 5.0, 7.0, and 8.0. using 100 mM citrate–phosphate (pH 3.0–7.0) and 100 mM Tris–HCl (pH 8.0–10.0). Assays were performed at 25 °C, 15°C and 4°C in a 1-cm cell in the presence of 20 mM ABTS and 5 mM H_2O_2. The specific activity was measured as the mean value of triplicate samples.

Results

Construction of the *Ph*TAC125-*30* mutant by insertional mutagenesis and growth of mutant cells under controlled conditions.

To assess the function of *Ph*HbO, a *Ph*TAC125 mutant strain (hereafter called *Ph*TAC125-*30* mutant) was constructed, in which the PSHAa*0030* gene was knocked-out. A PCR-amplified PSHAa*0030* gene fragment was cloned into the pVS suicide vector optimized for *Ph*TAC125 insertional mutagenesis [24]. The resulting vector, pVS*0030,* was mobilized in *Ph*TAC125 cells and insertion mutants were screened on plates containing carbenicillin as selection agent. Occurrence of the genome insertion was demonstrated by PCR analysis (data not shown) carried out on genomic DNA from a selected clone.

The growth profiles of the *Ph*TAC125-*30* mutant and wild-type cells were recorded at 4°C and 15°C, and under two oxygenation conditions, i.e. in microaerobiosis where DOT was constantly kept below

5% saturation (DOT< 5%), and in "extreme" aerobiosis, where DOT was kept above 80% saturation (DOT> 80%).
As shown in Fig. 1 and Table 1, regardless of temperature, growth of the *Ph*TAC125-*30* mutant in extreme aerobiosis is lower than that of wild-type cells, both in terms of biomass production and specific growth rate.

	Extreme aerobiosis				**Microaerobiosis**			
	4°C		**15°C**		**4°C**		**15°C**	
	Wild type	**Mutant**	**Wild type**	**Mutant**	**Wild type**	**Mutant**	**Wild type**	**Mutant**
μmax	**0.11±0.01**	**0.07±0.01**	**0.37±0.02**	**0.29±0.01**	**-**	**-**	**-**	**-**
OD_{600} max	**4.3±0.3**	**3.5±0.2**	**7.2±0.3**	**5.4±0.3**	**1.25±0.01**	**1.01±0.01**	**0.38±0.03**	**0.6±0.03**

Table 1. Main growth parameters of *Ph*TAC125 wild-type and mutant cells calculated from the growth kinetics displayed in Fig.1 Each growth kinetic was carried out in triplicate and at least in two independent experiments

.
These results indicate that the presence of *Ph*HbO protein in wild-type cells is a clear advantage when cells are grown at high concentration of oxygen. In microaerobiosis, both strains were slowed down in their replication kinetics. At the lower temperature (4°C), wild-type cells resulted better suited to the challenging conditions, reaching higher biomass production with respect to the mutant cells. In contrast, when grown at 15°C, *Ph*TAC125-*30* mutant cells grow better, reaching higher biomass yield (Fig. 1, Table 1). To investigate on this contradictory behaviour, a transcriptional analysis of the three trHbs and flavoHb encoding genes was performed.

Transcriptional analysis of genes encoding 2-on-2 Hbs and flavoHb in *Ph*TAC125-*30* mutant and wild-type cells.
Samples were withdrawn at different times during growth of *Ph*TAC125-*30* mutant and wild type cells at 4°C and 15°C, in microaerobiosis and extreme aerobiosis. Total RNA was extracted and subjected to RT-PCR analysis. Besides the determining of PSHAa*0030* gene expression profile, transcription analysis of the PSHAa*0458* and PSHAa*2217* genes encoding the other 2-on-2 Hbs present in *Ph*TAC125 genome, and of the flavoHb encoding gene PSHAa*2880* was performed (Table 2). As expected, the PSHAa*0030* gene was not transcribed in the mutant strain confirming its knock-out. In *Ph*TAC125 wild-type cells, the PHSAa*0030* gene was always expressed. PSHAa*0458* and PSHAa*2217* encoding the other 2-on-2 Hbs were expressed in both strains under all condition, whereas transcription of the flavoHb-encoding gene was detected only in mutant cells grown at 4°C in microaerobiosis, starting from the late exponential phase (i.e. 65h of incubation, data not shown).

	Extreme aerobiosis				**Microaerobiosis**			
	4°C		**15°C**		**4°C**		**15°C**	
	Wild type	**Mutant**	**Wild type**	**Mutant**	**Wild type**	**Mutant**	**Wild type**	**Mutant**
PSHAa*0030*	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**
PSHAa*0458*	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**
PSHAa*2217*	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**
PSHAa*2880*	**-**	**-**	**-**	**-**	**-**	**+[a]**	**-**	**-**

Table 2. Transcriptional analysis of genes encoding 2-on-2 Hbs and flavoHb in *Ph*TAC125-*30* mutant and wild-type cells. +, a specific product was obtained by RT-PCR experiment; -, no specific product was obtained +[a], a specific RT-PCR product was obtained only in the samples withdrawn after 65 hrs of growth. Each experiment was carried out in triplicate

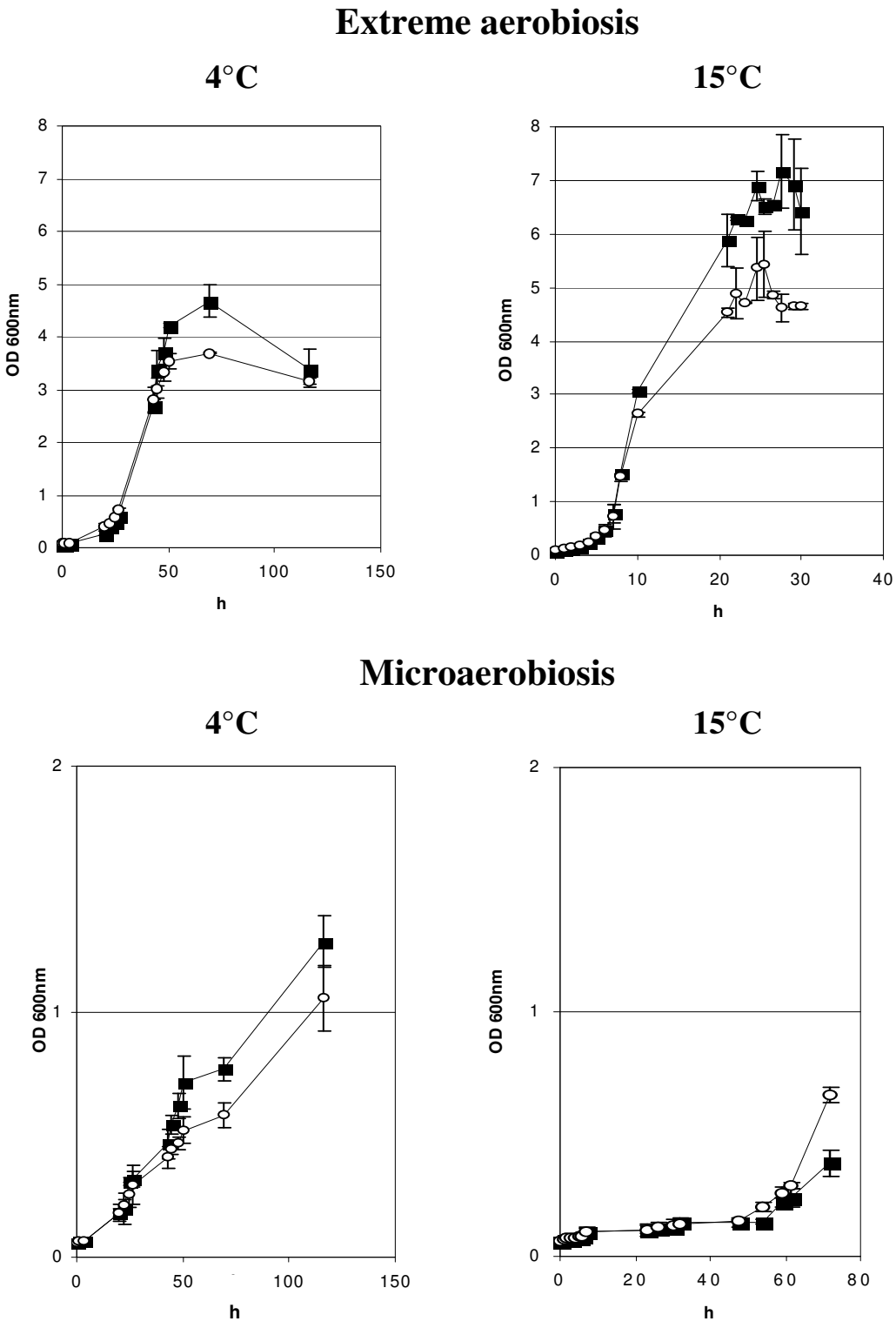

Fig. 1. Growth kinetics of *Ph*TAC125 wild type (■) and *Ph*TAC125-*30* mutant (○) at two temperatures and two oxygenation conditions, extreme aerobiosis (DOT>80%) and microaerobiosis (DOT<5%). Standard errors were calculated on kinetics carried out in triplicate and in at least two independent experiments

Resistance to hydrogen peroxide and protection from sperimidine NONOate.

To further explore the phenotypes conferred by the globin-gene mutation, *Ph* TAC125-*30* mutant and wild type cells were exposed to a number of stressing reagents and their sensitivity was measured by disk-diffusion assay. The reagents were: H_2O_2 (265 mM), spermidine NONOate (100 mM), GSNO (100 mM) and SNP (100 mM).

Upon of treatment with GSNO or SNP, no significant differences in radial cell growth (P>0.05) were recorded (data not shown). In contrast (Table 3), the mutant strain appears sensitive to H_2O_2 and spermidine NONOate, displaying a significantly greater zone of killing when compared to wild-type growth (Fig. 2); in both cases this difference was significant (P<0.02).

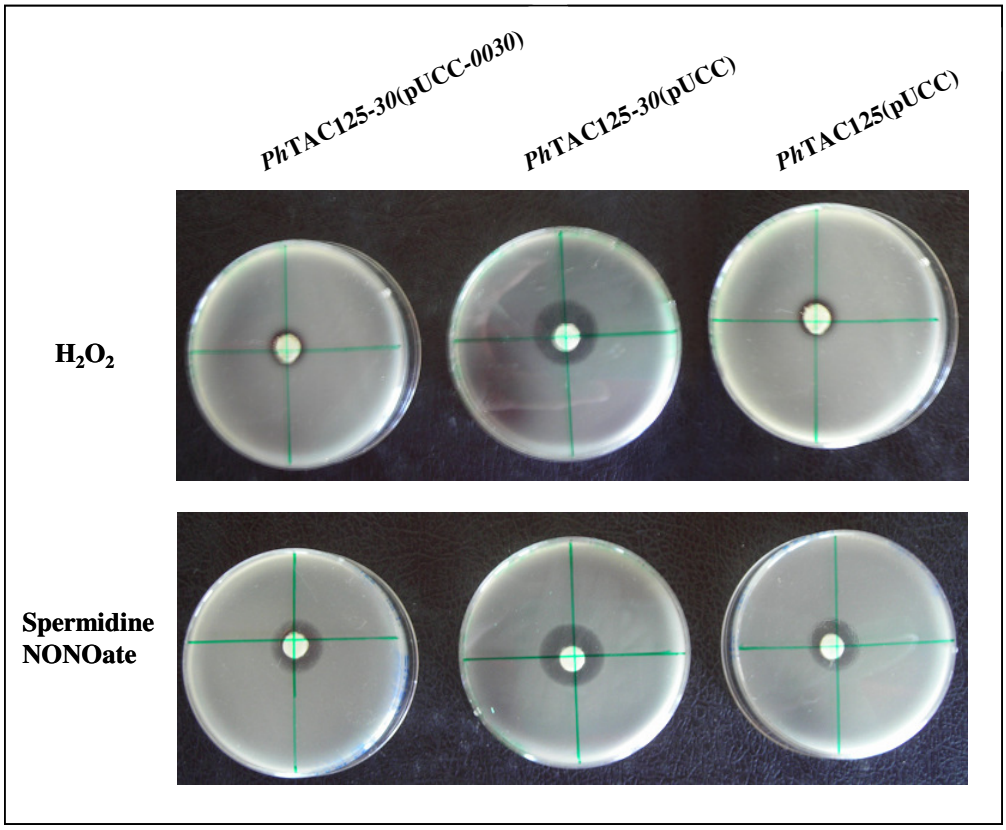

Fig. 2. Disk diffusion assay in the presence of H_2O_2 (265 mM) and spermidine NONOate (100 mM). *Ph*TAC125-*30*(pUC*0030): Ph*TAC125-*30* mutant cells transformed with pUC*0030: Ph*TAC125-*30*(pUCC), *Ph*TAC125-*30* mutant cells transformed with pUCC vector; *Ph*TAC125 wild type cells transformed with pUCC vector

To rule out the possibility that the observed sensitivity of the *Ph*TAC125-*30* mutant is due to a polar effect on the expression of PSHAa*0030* neighbouring genes, the complementing plasmid, pUC*0030*, was constructed to restore the *Ph*HbO function in *Ph* TAC125-*30* mutant cells. This plasmid contains the PSHAa*0030* gene and its upstream region (237 bp long), in which the presence of a putative promoter sequence was predicted by SoftBerry BPROM - Prediction of bacterial promoters software (http://softberry.com/berry). As shown in Table 3 and Fig. 2, the complemented strain *Ph*TAC125-*30*(pUC*0030*) was much less sensitive to H_2O_2 and spermidine NONOate, since it displayed a zone of killing comparable with that of the wild type. These results demonstrate restoring of wild-type phenotype in the complemented cells.

	Diameter of disk inhibition zone (cm)		
Stress inducer	***Ph*TAC125-**	***Ph*TAC125-**	***Ph*TAC125(pUCC)**
H_2O_2 (265 mM)	**0.82±0.13**	**0.31±0.08**	**0.26±0.05**
Spermidine NONOato	**0.84±0.15**	**0.59±0.16**	**0.56±0.11**

Table 3. Sensitivity of *Ph*TAC125 wild type and *Ph*TAC125-*30* mutant cells to H_2O_2 and spermidine NONOate. Values are expressed as mean diameter of the zone of killing ± SEM obtained from five independent experiments. The *t*-test, assuming equal variance (two tail), was used to analyse the significance of data and for all data resulted to be P<0.02.

Peroxidase activity of *Ph*HbO.

The peroxidase activity of *Ph*HbO was assessed in the purified protein *Ph*HbO and as shown in table 4 the protein clearly displayed peroxidase activity at all assayed temperatures with an optimum at 4°c and pH 7 (Table 4). Regardless the assay pH, *Ph*HbO peroxidase activity is reduced at higher temperature.

	pH 5.0	pH 7.0	pH 8.0
4°C	3,0±0,9	11,3±0,9	8,6±0,5
15°C	2,0±0,2	9,8±0,6	9,8±0,5
25°C	2,1±0,2	5,9±0,2	2,6±0,1

Table 4. Peroxidase activity of purified *Ph*HbO. Peroxidase activity values were reported as specific activities (IU/mg of purified protein). The specific activity was determined in each case as the mean value of triplicate samples.

Discussion

Amongst psychrophilic bacteria whose genome has been sequenced so far, *Ph* TAC125 is the only one having three distinct genes encoding 2-on-2 Hbs. The unusually high number of trHbs is highly suggestive that these proteins are bound to important physiological roles, which may be related to the extreme features of the Antarctic enviroment.

The physiological role fulfilled by one of them, *Ph*HbO, was investigated by a multidisciplinary approach, taking advantage of the availability of genetic tools suitably evolved for this Antarctic bacterium [25] combined with the possibility to study the purified protein [9].

*Ph*TAC125 is able to thrive in pelagic form, where cells experience high concentration of oxygen and other gases which characterizes cold waters. However, cells tend to concentrate at the air-water interface, forming a dense biofilm effective in scavenging organic particles that concentrate in the wave foam [18]. Due to the high cell density in the biofilm and the intense metabolic activity occurring within the latter, it is likely that oxygen concentration in the biofilm approaches the values of microaerobiosis experiments.

The observed capability of *Ph*TAC125 to reach very high growth yields, when provided with sufficient amounts of nutrients and aeration, indicates that respiration must be particularly efficient in this bacterium [18]. The bacterium is also able to grow in anaerobiosis, although with lower yields [18]. Therefore, it was expected to observe lower duplication speed and poor bacterial growth when *Ph*TAC125 was cultivated in microaerobiosis, especially at the higher temperature (15°C), due to the lower oxygen solubility with respect to that at 4°C. Surprisingly, the growth profiles of the *Ph*TAC125-*30* mutant and the wild type cells showed a contradictory trend. At 4°C, the wild type reached higher cell density whereas at 15°C the mutant cells showed higher growth yield. To find out the reasons of this contradictory behaviour, a transcriptional analysis of the three trHbs and flavoHb encoding genes was carried out.

The transcription of the flavoHb encoding gene (PSHAa*2880*) was observed only in *Ph*TAC125-*30* mutant when grown at 4°C in microaerobiosis. Since the transcription of flavoHb encoding genes is usually directly or indirectly induced by NO [12, 17, 31], the observed flavoHb-gene expression is suggestive of the occurrence of an NO-induced stress possibly related to the *Ph*HbO absence. As for the NO origin, non-enzymatic production should be excluded because it is reported to occur only in a nitrite-containing medium at acidic pH [7], whereas in our experimental setups the medium pH was always kept around the value of 7.0 (±0.05). Under anaerobic conditions, in *E. coli* and other bacteria, NO is endogenously produced by nitrite reductase [4, 14]. *Ph*TAC125 genome contains a gene encoding a nitrite reductase (PSHAa*1477*), and therefore endogenous production of NO is likely to occur in microaerobiosis. As for NO accumulation, it can be spontaneously oxidized if enough amount of oxygen is present in solution. In microaerobiosis, oxygen availability is further reduced when the biomass is increased, i.e. in the late exponential phase, and NO accumulation may become a real threat for cell viability. Induction of flavoHb gene may be viewed as a suitable strategy aimed at counteracting the NO-induced stress made worse by enhanced NO solubility at low temperature and in the absence of *Ph*HbO.

*Ph*HbO plays a role in the cellular protection against NO-induced stress, as shown by the higher sensitivity of the *Ph*TAC125-*30* mutant then the wild type when exposed to an NO releaser, such as spermidine NONOate.
The other nitrosating agents tested in the disk diffusion assay (SNP and GSNO) do not affect mutant cell viability. Looking at their respective mechanism of action, SNP is able to release NO only after nitrosation, while GSNO can act as a transnitrosating agent. In contrast, spermidine NONOate directly release NO with a specified half-life and is often used to study the effect of NO *per se* [28]. The observed sensitivity of *Ph* TAC125-*30* mutant cells only to sperimidine NONOate indicates that the *Ph*HbO action is related to the presence of NO *per se*.
The data do not allow to define the mechanism of action, i.e. whether the protein alleviates the NO-induced stress by simply trapping NO or whether it is able to perform enzymatic NO scavenging. In this respect, a recent paper on *Mycobacterium tuberculosis* HbN highlighted the importance of the Pre-A region in its O_2-dependent NO dioxygenase activity [16]. *Ph*HbO is characterised by the presence of an extra N-terminal sequence (20 residues long) as compared with other HbO's. Although its sequence does not display any homology to *M. tuberculosis* HbN Pre-A motif, it would be interesting to correlate the presence of this N-terminal sequence with the *Ph*HbO ability to relieve spermidine NONOate-induced toxicity in *Ph* TAC125-*30* mutant cells. Further work is required to clarify this aspect.
In extreme aerobiosis, the mutant growth profile is worse than that of the wild type, suggesting requirement of *Ph*HbO during all growth phases. This observation indicates that *Ph*HbO may be involved in protection against the stress likely induced by high oxygen concentration, and therefore its function is always necessary during growth. This hypothesis is supported by the disk diffusion assays, which correlates the sensitivity to H_2O_2 of *Ph*TAC125-*30* mutant to its specific genome mutation, since the complemented strain (*Ph*TAC125-*30*(pUC*0030*)) displays a H_2O_2 sensitivity similar to that exhibited by the wild type. The above results suggest that *Ph*HbO may be endowed of peroxidase activity. *In vitro* assays on purified *Ph*HbO demonstrated that the protein has a peroxidase activity that is higher at under likely physiological condition i.e. the lower tested temperature (4°C). Peroxidase activity has been previously reported for some tetrameric Hbs, such as human and bovine Hb [10], monomeric Hbs [15], and trHbs [1, 21]. According to replacement studies on *M. tuberculosis* HbO, the ability of 2-on-2 Hbs to act as peroxidases was related to electron-rich, potentially oxidizable, Tyr (B10), Trp (G8) and Tyr (CD1) [21] in the vicinity of the haem. These residues take a 3D conformation, which allows electron transfer from an exogenous molecule to H_2O_2. The alignment of the *Ph*HbO sequence with that of two other HbO's known to display peroxidase activity, i.e. *M. tuberculosis, Mt*HbO, and *M. leprae, Ml*HbO [1, 21], highlights the presence of these three residues TyrCD1, TyrB10 and TrpG8 involved in the peroxidase activity (Fig. 3A). Preliminary results of molecular modelling and molecular dynamic simulation on *Ph*HbO (Estrin. and Boechi, unpublished results) suggest that the spatial organization is compatible with their involvement in the observed *Ph*HbO peroxidase activity (Fig. 3B). The results herewith reported are in agreement with previous data [1, 6, 21], which suggest that the active site of group II trHbs is probably not designed to perform gaseous ligand exchange, but rather to carry out a redox process involving oxygen or perhaps H_2O_2.
The results illustrated in this paper provide the first insight into the function played by *Ph*HbO in *Ph*TAC125. The involvement of *Ph*HbO in the cellular response to NO-induced stress was demonstrated, although further biochemical and physiological studies are needed to clarify the underlying molecular mechanism, especially in relation to the presence of a canonical flavoHb in the psychrophilic cells. The observation that *Ph*HbO displays a strong peroxidase activity suggest its role in conferring remarkable resistance to H_2O_2 to *Ph* TAC125, widening our understanding of the possible strategies adopted by psychrophilic marine bacteria to cope with the challenges imposed by their environment.

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A

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                                                                  B10
Mt-HbO  .......... ......MPKS F...YDAVGG AKTFDAIVSR F[Y]AQVAEDEV
Ml-HbO  .......... ......MQQS F...YDAIGG AETFKAIVSR F[Y]AQVPEDEI
Ph-HbO  MIKRLFSKSK PATIEQTPTP EKTPYEILGG EAGALAIANR F[Y]DIMATDEY

                          CD1
Mt-HbO  LRRV[Y]PEDDL AGAEERLR.. M[F]LEQYWGGP RTYSEQRGHP RLRMRHAPFR
Ml-HbO  LREL[Y]PADDL AGAEERLR.. M[F]LEQYWGGP RTYSSQRGHP RLRMRHAPFR
Ph-HbO  AKPL[Y]DMHPL PLDRIRQVFF E[F]LSGWLGGP DLFVAKHGHP MLRKRHMPFT

                                  G8
Mt-HbO  ISLIERDA[W]L RCMHTAVASI DSETLDDEHR RELLDYLEMA AHSLVNSPF.
Ml-HbO  ITAIERDA[W]L RCMHTAVASI DSHTLDNEHR RELLDYLEMA AHSLVNSAS.
Ph-HbO  IDQDLRDQ[W]M YCMNKTL... DLEVDNPLLR EGLKQSFGQL ASHMINQH..
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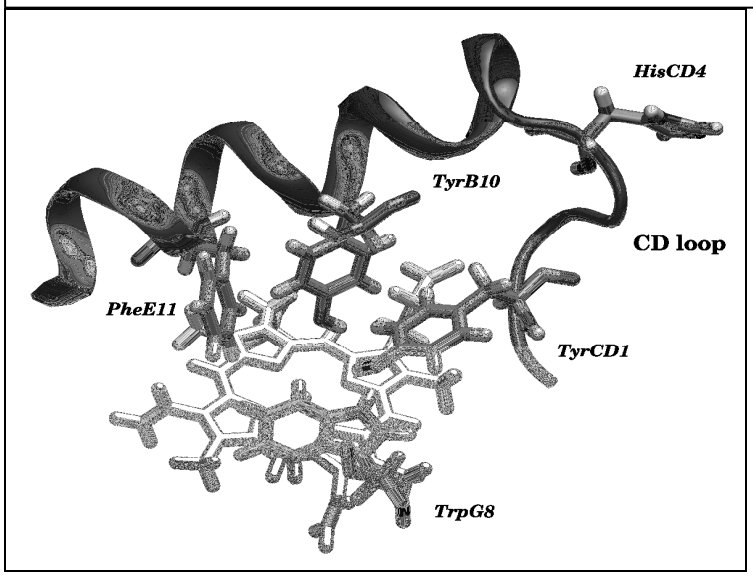

Fig. 3. Structural determinants of *PhHbO* peroxidase activity. Panel A, amino acid sequence alignments of *Mycobacterium tuberculosis* HbO (*Mt*-HbO), *Mycobacterium leprae* HbO (*Ml*-HbO), and *PhHbO*. Conserved residues in position B10, CD1, and G8 are reported in bold. Panel B, spatial organization of *PhHbO* protein residues B10, CD1, and G8 in the surrounding of heme cofactor (light grey) as derived from molecular modelling and molecular dynamic simulation (courtesy of Boechi L and Estrin D.)

References

1 Ascenzi, P., De Marinis, E., Coletta, M., Visca, P. (2008). H_2O_2 and NO scavenging by Mycobacterium leprae truncated haemoglobin O. Biochem Biophys Res Commun 373, 197-201.

2 Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., & Struhl, K. (1994). Current Protocols in Molecular Biology. New York: John Wiley & Sons.

3 Baillon, M. L., van Vliet, A. H., Ketley, J. M., Constantinidou, C. & Penn, C. W. (1999). An iron-regulated alkyl hydroperoxide reductase (AhpC) confers aerotolerance and oxidative stress resistance to the microaerophilic pathogen Campylobacter jejuni. J Bacteriol 181, 4798–4804.

4 Corker, H. & Poole, R.K. (2003) Nitric oxide formation by Escherichia coli. Dependence on nitrite reductase, the NO-sensing regulator Fnr, and flavohaemoglobin Hmp. J Biol Chem 278, 31584-31592.

5 Couture, M., Yeh, S-R., Wittenberg, B.A., Wittenberg, J.B., Ouellet, Y., Rousseau, D.L., Guertin, M. (1999). A cooperative O2-binding haemoglobin from Mycobacterium tuberculosis. Proc Natl Acad Sci U.S.A. 96, 11223-11228.

6 Feis, A., Lapini, A., Catacchio, B., Brogioni, S., Foggi, P., Chiancone, E., Boffi, A., Smulevich, G. (2008). Unusually strong H-bonding to the heme ligand and fast geminate recombination dynamics of the carbon monoxide complex of Bacillus subtilis truncated haemoglobin. Biochemistry 47, 902-910.

7 Frey, A.D., Kallio, P.T. (2005) Nitric oxide detoxification--a new era for bacterial globins in biotechnology? Trends Biotechnol 23, 69-73.

8 Giangiacomo, A., Ilari, L., Boffi, A., Morea, V., and Chiancone, E. (2005) The truncated oxygen-avid haemoglobin from Bacillus subtilis: X-ray structure and ligand binding properties, J Biol Chem 280, 9192-9202.

9 Giordano, D., Parrilli, E., Dettaï, A., Russo, R., Barbiero, G., Marino, G., Lecointre, G., di Prisco, G., Tutino, L., Verde, C. (2007). The truncated haemoglobins in the Antarctic psychrophilic bacterium Pseudoalteromonas haloplanktis TAC125. Gene 398, 69-77.

10 Giulivi, C., Davies, KJ.(1990). A novel antioxidant role for haemoglobin. The comproportionation of ferrylhaemoglobin with oxyhaemoglobin J Biol Chem. 265, 19453-19460.

11 Hanahan, D. (1983). Studies on transformation of Escherichia coli with plasmids. J Mol Biol. 166, 557-580.

12 Hausladen, A., Gow, AJ., Stamler, JS. (1998). Nitrosative stress: metabolic pathway involving the flavohaemoglobin. Proc Nal. Acad Sci, 95, 14100–14105.

13 Hughes, MN. (2008). Chemistry of nitric oxide and related species. Methods Enzymol 436, 3-19.

14 Ji, X.B. & Hollocher, T.C. (1988). Reduction of nitrite to nitric oxide by enteric bacteria. Biochem Biophys Res Commun 157, 106–108.

15 Kvist, M., Ryabova, E.S., Nordlander, E., Bülow, L. (2007). An investigation of the peroxidase activity of Vitreoscilla haemoglobin. J Biol Inorg Chem 12, 324-334

16 Lama, A., Pawaria, S., Bidon-Chanal, A., Anand, A., Gelpí, J.L., Arya, S., Martí, M., Estrin, D.A., Luque, F.J., Dikshit. K.L. (2009). Role of Pre-A motif in nitric oxide scavenging by truncated hemoglobin, HbN, of Mycobacterium tuberculosis. J Biol Chem. 284, 14457-14468.

17 Liu, L., Zeng, M., Hausladen, A., Heitman, J., Stamler, JS (2000). Protection from nitrosative stress by yeast flavohaemoglobin. Proc Natl Acad Sci. 97, 4672–4676.

18 Médigue, C., Krin, E., Pascal, G., Barbe, V., Bernsel, A., Bertin, P.N., Cheung, F., Cruveiller, S., D'Amico, S., Duilio, A., Fang, G., Feller, G., Ho, C., Mangenot, S., Marino, G., Nilsson, J., Parrilli, E., Rocha, E.P.C., Rouy, Z., Sekowska, A., Tutino, M.L., Vallenet, D., von Heijne, G., and Danchin, A. (2005). Coping with cold: the genome of the versatile marine Antarctica bacterium Pseudoalteromonas haloplanktis TAC125. Genome Research. 15, 1325-1335.

19 Methe, B.A., Nelson, K.E., Deming, J.W., Momen, B., Melamud, E., Zhang, X., Moult, J., Madupu,R., Nelson, W.C., Dodson, R.J., Brinkac, L.M., Daugherty, S.C., Durkin, A.S., DeBoy, R.T.,Kolonay, J.F., Sullivan, S.A., Zhou, L., Davidsen, T.M., Wu, M., Huston, A.L., Lewis, M.,Weaver, B., Weidman, J.F., Khouri, H., Utterback, T.R., Feldblyum, T.V., Fraser, C.M., (2005). The psychrophilic lifestyle as revealed by the genome sequence of Colwellia psychrerythraea34H through genomic and proteomic analyses. Proc NatlAcad Sci. 102, 10913-10918.

20 Milani, M., Pesce, A., Nardini, M., Ouellet, H., Ouellet, Y., Dewilde, S., Bocedi, A., Ascenzi, P.,Guertin, M., Moens, L., Friedman, J.M., Wittenberg, J.B., Bolognesi, M., (2005). Structural bases for heme binding and diatomic ligand recognition in truncated haemoglobins. J Inorg Biochem. 99, 97-109.

21 Ouellet, H., Juszczak, L., Dantsker, D., Samuni, U., Ouellet, Y.H., Savard, P.Y. Wittenberg, J.B.,Wittenberg, B.A., Friedman, J.M., Guertin, M., (2003). Reactions of Mycobacterium tubercolosis truncated haemoglobin O with ligands reveal a novel ligand-inclusive hydrogen bond network. Biochemistry 42, 5764-5774.

22 Ouellet, H., Ranguelova, K., La Barre, M., Wittenberg, J. B.,Wittenberg, B. A., Magliozzo, R. S., and Guertin, M. (2007). Reaction of Mycobacterium tuberculosis truncated haemoglobin O with hydrogen peroxide: evidence for peroxidatic activity and formation of protein-based radicals. J Biol Chem 282, 7491-7503.

23 Papa R., Rippa V., Sannia G., Marino G., Duilio A. (2007). An effective cold inducible expression system developed in Pseudoalteromonas haloplanktis TAC125 Journal of Biotechnology 127(2), 199-210.
24 Parrilli, E., De Vizio, D., Cirulli, C., Tutino, M.L. (2008a). Development of an improved Pseudoalteromonas haloplanktis TAC125 strain for recombinant protein secretion at low temperature. Microbial Cell Factories 7, 2.
25 Parrilli, E., Duilio, A., Tutino, M.L. (2008b). Heterologous protein expression in psychrophilic hosts. 365-379. In: Psychrophiles: from Biodiversity to Biotechnolgy. Edited by Margesin R, Schinner F, Marx JC, Gerday C. Springer-Verlag Berlin Heidelberg.
26 Pesce, A., Couture, M., Dewilde, S., Guertin, M., Yamauchi, K., Ascenzi, P., Moens, L., Bolognesi,M., (2000). A novel two-over-two alpha-helical sandwich fold is characteristic of the truncated haemoglobin family. EMBO J 19, 2424-2434.
27 Poole RK (2008). Microbial Haemoglobins: Proteins at the Crossroads of Oxygen and Nitric Oxide Metabolism 241-257. In Dioxygen Binding and Sensing Proteins. Edited by Bolognesi; di Prisco; Verde. Publisher Springer Milan
28 Poole, RK (2005). Nitric oxide and nitrosative stress tolerance in bacteria. Biochem Soc Trans 33, 176-80.
29 Rabus, R., Ruepp, A., Frickey, T., Rattei, T., Fartmann, B., Stark, M., Bauer, M., Zibat, A.,Lombardot, T., Becker, I., Amann, J., Gellner, K., Teeling, H., Leuschner, W.D., Glockner,F.O., Lupas, A.N., Amann, R., Klenk, H.P., (2004). The genome of Desulfotalea psychrophila, a sulfate-reducing bacterium from permanently cold Arctic sediments. Environ Microbiol 6, 887-902.
30 Sambrook, J. & Russell, D.W. (2001). Molecular Cloning inA Laboratory Manual, 3rd Ed. Edited by Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
31 Spiro, S. (2007). Regulators of bacterial responses to nitric oxide. FEMS Microbiol Rev 31,193-211
32 Tutino, M.L., Parrilli, E., Giaquinto, L., Duilio, A., Sannia, G., Feller, G. & Marino, G. (2002). Secretion of α-amylase from Pseudoalteromonas haloplanktis TAB23: two different pathways in different hosts. J Bacteriol 184, 5814-5817.
33 Vinogradov, S.N., Hoogewijs, D., Bailly, X., Arredondo-Peter, R., Gough, J., Dewilde, S., Moens, L., Vanfleteren, J.R. (2006). A phylogenomic profile of globins. BMC Evol Biol 6, 31-67.
34 Vuletich, D.A.,& Lecomte, J.T.J. (2006). A phylogenetic and structural analysis of truncated haemoglobins. J Mol Evol 62, 196-210.
35 Wittenberg, J.B., Bolognesi, M., Wittenberg, B.A., Guertin, M., (2002). Truncated haemoglobins: a new family of haemoglobins widely distributed in bacteria, unicellular eukaryotes and plants. J Biol Chem 277, 871-874.