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Engineering low-temperature expression systems for heterologous production of cold-adapted enzymes

Gro Elin Kjæreng Bjerga^{a,†}, Rahmi Lale^b, and Adele Kim Williamson^a

^aUniversity of Tromsø, Norstruct, Department of Chemistry, Faculty of Science and Technology, Tromsø, Norway; ^bNorwegian University of Science and Technology, Department of Biotechnology, Trondheim, Norway

ABSTRACT

Production of psychrophilic enzymes in the commonly used mesophilic expression systems is hampered by low intrinsic stability of the recombinant enzymes at the optimal host growth temperatures. Unless strategies for low-temperature expression are advanced, research on psychrophilic enzymes may end up being biased toward those that can be stably produced in commonly used mesophilic host systems. Two main strategies are currently being explored for the development of low-temperature expression in bacterial hosts: (i) low-temperature adaption of existing mesophilic expression systems, and (ii) development of new psychrophilic hosts. These developments include genetic engineering of the expression cassettes to optimize the promoter/operator systems that regulate heterologous expression. In this addendum we present our efforts in the development of such low-temperature expression systems, and speculate about future advancements in the field and potential applications.

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Our latest work involves the investigation of low-temperature expression of putatively cold-adapted (psychrophilic) gene candidates.¹ Psychrophilic enzymes present significant economic potential for industrial applications. For example, introduction of psychrophilic proteases, lipases, cellulases and amylases into laundry detergents can decrease the demand for both chemical additives and enzyme concentrations during production, but can also reduce the need for heating and the water consumption for end-users.^{2,3} In 2014, DuPont and P&G received the 2014 Sustainable Bio Award for their innovative protein modification producing a protease that has optimal performance at temperatures as low as 15°C. Although this was an engineered protease, basic research on psychrophilic proteases, such as subtilisins from Antarctic *Bacillus* species,^{4,5} have contributed to their economic value.

Bioprospecting, the process of exploring the biodiversity of our planet as a potential source of products,

has in recent decades turned to cold habitats such as the polar regions, glaciers and oceans to discover cold-acting enzymes that can be exploited for industrial applications. These enzymes originate from psychrophiles that have optimal growth temperatures below 15°C.⁶ The intrinsic instability of psychrophilic enzymes makes their heterologous expression in commonly used bacterial hosts such as *E. coli*, where growth limits to around 15°C, particularly challenging, and presents a significant barrier to their exploitation in biotechnology.⁷ In cases where psychrophilic enzymes must be produced as zymogens, such as proteases, activation or autoprocessing may also need to be addressed in the chosen expression systems.⁸ Unless strategies for recombinant protein expression at low temperatures are advanced, research on psychrophilic enzymes may end up being biased by production in mesophilic host systems, such as the commonly used *E. coli* (Gram-negative bacteria) and

CONTACT Gro Elin Kjæreng Bjerga  gro.bjerga@uni.no  University of Tromsø, Norstruct, Department of Chemistry, Faculty of Science and Technology, N-9037 Tromsø, Norway.

[†] Present address: Uni Research AS, Center for Applied Biotechnology, Thormøhlensgt. 55, N-5008 Bergen, Norway

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Bacillus subtilis (Gram-positive bacteria). This may limit the diversity of possible enzyme products to those that can be stably produced in these systems.

Psychrophilic enzymes are generally less stable compared to their mesophilic homologues, having high structural flexibility which enables them to function at low temperatures and imparts a decreased thermal stability.² Their active sites are even less thermostable than the whole protein structure due to high local flexibility. The properties of psychrophilic enzymes that are useful in industrial applications include their high activity at low and moderate temperatures as lower enzyme concentrations are required to achieve the same performance compared to their higher-temperature acting homologues.² Furthermore, their heat-lability can be exploited to selectively inactivate the enzyme by heat treatment.² The use of the alkaline phosphatase and nuclease from the Arctic shrimp *Pandalus borealis* in molecular biology applications^{9,10} provide 2 examples of psychrophilic enzymes that can be stably produced by recombinant technologies, both of which are commercially available.

Successful protein production systems for expression of psychrophilic enzymes will depend on being able to produce functional proteins at low enough temperatures to ensure correct folding and to maintain their structural integrity. This presents 2 possible bioengineering strategies for cell-based production: (i) low-temperature adaptations of existing mesophilic expression systems, and (ii) development of new psychrophilic hosts. The first strategy includes engineering the genetic tools in the mesophilic expression systems to improve low-temperature growth and to promote correct folding of recombinant proteins at low temperature. The best-known example of such a strategy is the co-expression of cold-adapted chaperonines to enable correct folding of recombinant proteins. Co-expression of cold-adapted homologues of the *E. coli* GroELS chaperonines, Cpn60 and Cpn10 from *Oleispira antarctica*, allows the *E. coli* host to have an operational folding system at 4–12°C.¹¹ This resulted in improvement of host growth at low temperatures and enhanced the solubility of the resulting recombinant proteins. In our case, co-expression of cold-adapted chaperonines was not sufficient for soluble expression of genes derived from Arctic-sources metagenomes in combination with the commonly used T7 expression system (T7 RNA polymerase/T7 promoter).¹ We therefore targeted optimization of low-temperature

expression at the transcription level by investigating the utility of a cold-shock inducible promoter for low-temperature expression.¹ In this *E. coli* based system, expression of recombinant proteins is regulated by a temperature-inducible promoter, the *E. coli* cold shock protein A (*cspA*) promoter, which allows induction by a downshift in growth temperature.^{12,13} Analogous systems have been investigated for *Bacillus subtilis* where the cold-inducible promoter of the desaturase encoding gene (*des*) in combination with other 5' UTR regulatory elements was shown to generate higher expression levels.^{14,15} Although the success of both expression systems had previously been demonstrated for mesophilic proteins, from bacteria to plants and humans,^{16,17} their potential for psychrophilic enzymes had not been systematically explored. By expressing the putatively psychrophilic genes in this *E. coli* system, we found that expression levels were generally high, and comparable to the commonly used T7 system which functions optimally at 37°C.¹ In contrast to the T7 system some soluble proteins were detected, however in most cases the yield was still too low for further experimentation. We therefore optimized *cspA*-driven expression by fusing the maltose-binding protein (MBP), thioredoxin (TRX), the small ubiquitin-like modifier (SUMO) and trigger factor (TF) N-terminally to these enzymes, in an attempt to improve solubility. Consistent with a recent report,¹⁷ we found that combining low-temperature induction with these solubility partners improved solubility. This has recently allowed us to perform preliminary characterization of the selected enzymes. For example, activity assays on the His-SUMO fused chitinase described in our paper¹ suggests that the enzyme is truly psychrophilic with an optimal temperature around 20°C (Fig. 1). Adding this result to our previous data¹ indicates that the *E. coli/cspA* expression system is a viable approach for production of psychrophilic chitinase, which was not feasible with the T7 system.

Although the commonly used MBP and SUMO fusion proteins were utilized successfully in the *E. coli/cspA* system, we and others found that the TRX fusion partner negatively impacted cell viability.^{1,17} In the course of developing low-temperature expression systems, attention should be given to new solubility partners that may be more compatible to psychrophilic target proteins. With this in mind, we also investigated the utility of the TF protein from the psychrophilic *Pseudoalteromonas haloplanktis* as an alternative fusion partner. The TF from *P. haloplanktis* has a

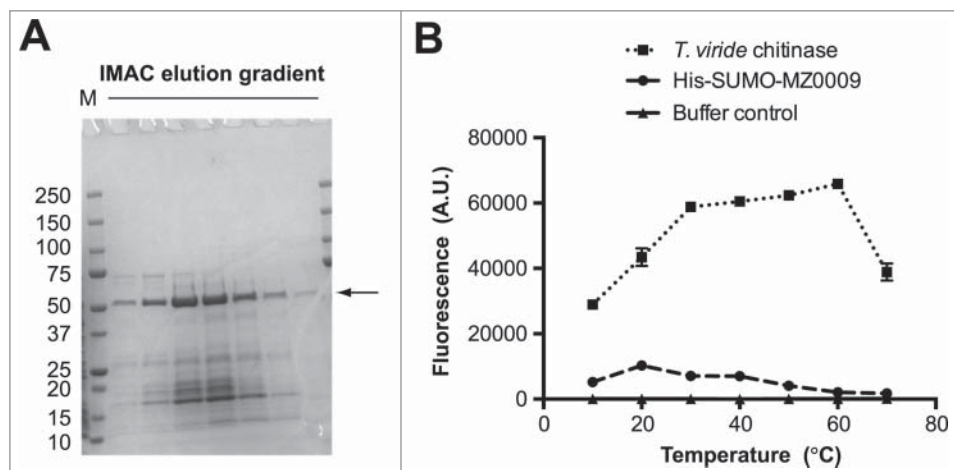


Figure 1. Temperature optimum of a recombinant chitinase (MZ0009) of Arctic origin. (A) Recombinant His-SUMO fusion of the chitinase crudely purified by immobilized affinity chromatography (IMAC). The arrow points to the recombinant protein (49kDa), which was subsequently identified by mass spectrometry. The chitinase part of the fusion protein is 36kDa; note that the His-SUMO partner is known to migrate slower (about 20kDa) than its corresponding monomeric mass of 13 kDa, thus generating a larger shift as a fusion protein. (B) The activity of the crudely purified His-SUMO-MZ0009 is expressed as the degree of fluorescence (arbitrary units) from hydrolysis of a synthetic chitin analog substrate, 4MU- β -D-N,N',N''-triacetylchitotriose, and compared to equal amounts of the commercially available *Trichoderma viride* chitinase. Error bars show the variation between 2 parallel samples in one experiment.

low-temperature activity and is strongly upregulated in the native host at 4°C in conditions where protein folding is a rate-limiting step for growth.¹⁸ We assumed that the psychrophilic TF fusion partner would ensure a faster and more efficient folding assistance of newly synthesized polypeptides during low-temperature expression in *E. coli*, however we were unable to observe any difference in the solubility of our target enzymes using the 2 different TF partners (data not shown). As we reported, removal of the *E. coli* TF fusion partner was ineffective,¹ and we again want to highlight the need for a systematic study of optimal linker length which has not, to our knowledge, been carried out for fusion proteins with the TF as a fusion partner. This is required to exploit its potential as a solubility and co-folding partner in recombinant protein expression. As an alternative to the fusion strategy, co-expression of the TF from *P. haloplanktis* in *E. coli* may be applied to improve solubility of psychrophilic enzymes, analogous to the co-expression of the chaperonins from *Oleispira antarctica*.¹¹

As mentioned, co-expression of cold-adapted chaperonins was not sufficient for production of soluble enzymes with the T7 system.¹ In line with another report,¹⁹ regulation of protein expression by the *cspA* promoter in combination with the co-expression of cold-adapted chaperonins was successful in producing soluble psychrophilic enzymes in *E. coli*.¹ Taken together, our data indicate that engineering of *E. coli*

is an attractive route for soluble low-temperature expression, but further advances in the genetic tools for protein expression will be required to improve its performance as a production host for psychrophilic enzymes.

The second strategy we propose to ensure correct folding of functional psychrophilic enzymes is based on the development of psychrophiles as production hosts. The first reported and most-studied psychrophilic host is the Gram-negative *Pseudoalteromonas haloplanktis* TAC125,^{20–22} which grows at low temperatures (4–25°C). With the availability of the complete genomic sequence²³ it is possible to inactivate or delete genes selectively, but the *Pseudoalteromonas* host systems currently lack effective protocols for gene engineering and transformation.²⁴ In the absence of better tools, conjugation is currently used to introduce foreign DNA into the host, and the most commonly used donor organism for conjugation is the mesophilic *E. coli*. During the conjugative mating a mixture of both donor and recipient must be incubated on a non-selective agar surface, which may limit the success of conjugation if the recipient (psychrophilic) organism require growth conditions (such as high salt or low growth temperature) that is not preferred by *E. coli*.²⁴ A recently developed host-independent plasmid system, pTA-Mob,²⁵ may eliminate these conjugation challenges as this plasmid carries all necessary conjugative functions on a pBBR1-based broad-host-range

replicon. Utilization of this plasmid, given that the plasmid is replicative in the chosen host, will allow any bacterial host to function as a donor in conjugative mating. By choosing a donor host with similar growth preferences to the recipient psychrophilic host, conjugation efficiency can be increased. A continued effort in genetic manipulations is however still needed to address problems with restriction of recombinant DNA, protease-degradation of the encoded proteins and multidrug resistance.²⁴ Although the *Pseudoalteromonas* system has been successful in producing recombinant proteins where *E. coli* failed, it is still limited by low cell densities.²⁶ As a recent example, the production yield of 2 recombinant proteins in *Pseudoalteromonas* sp. SM20429 was shown to be more than 5 times lower than in *E. coli*.⁸

Alongside our explorations of the *E. coli/cspA* system, we pursued engineering of new hosts for low-temperature expression. As part of this work, more than 70 isolates of psychrophilic *Pseudomonas* spp. isolates originating from the marine Arctic environment were investigated for their growth at

4–30°C; in most cases the shortest generation time was achieved at around 20°C. Two of the isolates were further explored for their potential as psychrophilic hosts in combination with both the XylS/ P_m and AraC/ P_{BAD} transcription regulator/promoter system on a broad-host-range mini-RK2 replicon (manuscript in preparation). As proof of principle, several proteins, including the above-mentioned TF protein from *P. haloplanktis* (by AraC/ P_{BAD}) and the Arctic-sourced chitinase (by XylS/ P_m), could be expressed from a mini-RK2 plasmid in *Pseudomonas* at a higher level than in the *E. coli*/T7-system (data not shown). Subsequently, we also introduced the T7 system into 2 *Pseudomonas* spp. strains by regulating the expression of a T7 bacteriophage *gene 1* (T7 RNA polymerase) from the chromosome under the control of the XylS/ P_m promoter. This system was used to express a red fluorescent reporter protein (mCherry) in the mini-RK2 plasmid under the control of T7 promoter with a *lac* operator sequence (Fig. 2A and B). As the system was found to be

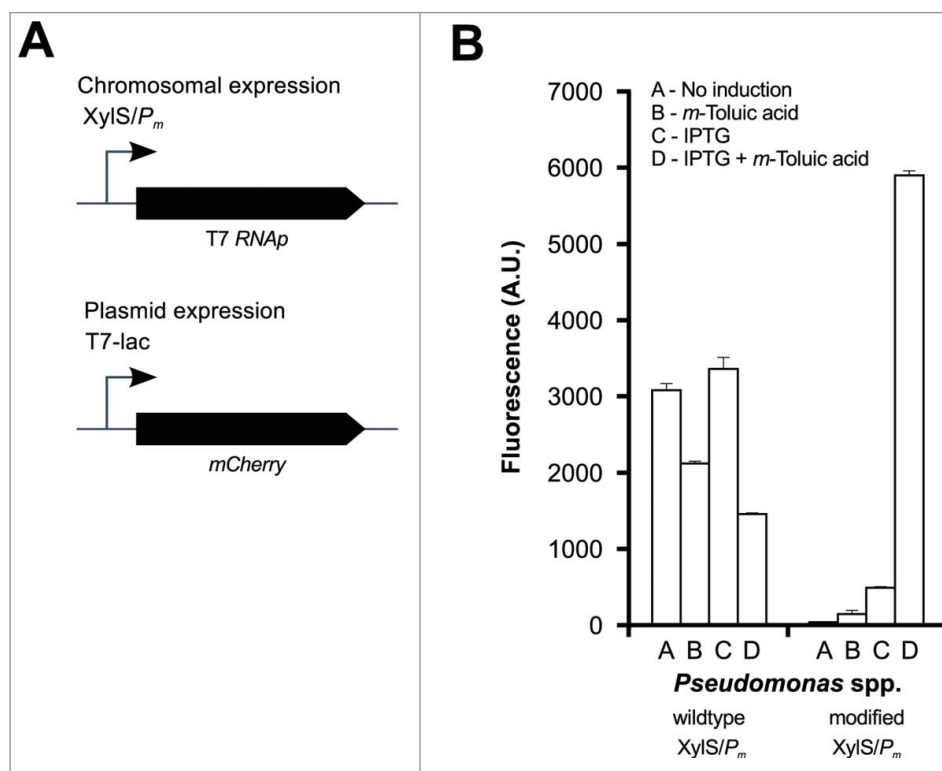


Figure 2. Expression of mCherry using the T7 system in Arctic marine *Pseudomonas*. (A) The figure depicts the organization of the relevant expression cassettes. The chromosomal expression of T7 RNA polymerase T7 RNAP is driven by the XylS/ P_m system, and the expression of the mCherry reporter protein is driven by the T7 promoter with the *lac* operator sequence (T7-lac). (B) Degree of fluorescence (arbitrary units) from mCherry expression at 20°C in the wildtype and the modified XylS/ P_m system in *Pseudomonas*. Isopropyl β -D-1-thiogalactopyranoside (IPTG) serves as the inducer of the T7 promoter, whereas *m*-Toluic acid is the inducer of the XylS/ P_m system.

leaky, the XylS/ P_m system was further modified in the 5' UTR region, based on an earlier finding,²⁷ to reduce the leakiness of the system. This modification led to a tightly regulated system in these *Pseudomonas* hosts as exemplified by the heterologous expression of mCherry (Fig. 2B). As these expression systems were carried out at the fastest growing temperature, 20°C, further studies are needed to investigate their performance at lower temperature.

To conclude, advances in generating efficient low-temperature expression systems is required for the continued effort in both basic research and industrial exploration of psychrophilic enzymes. Currently, 2 main strategies are being explored for development of such low-temperature systems in bacterial hosts: (i) low-temperature adaptations of existing mesophilic expression systems, and (ii) development of new psychrophilic hosts. Besides benefiting the exploitation of psychrophilic enzymes, a low-temperature expression system may also be advantageous for mesophilic and thermophilic proteins. In particular, expression at lower temperature may be beneficial where properties of the enzymes are deleterious to the host cell growth. In the case of potent meso- and thermophilic proteases a low-temperature expression system, where the thermal stability is retained but temperature condition is sub-optimal for the activation of the zymogens, could be advantageous to prevent short half-life due to autoproteolysis.²⁸⁻³⁰

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Authors' contributions

GEKB and RL performed the experiments for the presented data. GEKB coordinated and drafted the manuscript. All authors edited the manuscript and approved the final version.

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