SUSTAINABLE USE OF MARINE BIODIVERSITY AS SOURCE OF NOVEL ANTI-BIOFILM AGENTS IN INDUSTRIAL AND CLINICAL SETTINGS

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As the Greek philosopher Aristotle said, "Man is by nature a social animal",
and as such, it tends to associate with other individuals and form societies.
Well, microorganisms too.

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RIASSUNTO

❖ Introduzione

Durante la storia della microbiologia, i microrganismi sono stati principalmente studiati nella loro forma planctonica, in cui le singole cellule fluttuano liberamente in un mezzo liquido. Solo a partire dal XVII secolo ha iniziato a diffondersi la consapevolezza che i microrganismi sono capaci di colonizzare superfici di varia natura e formare, ad esse associati, delle vere e proprie comunità, i biofilm.
La nostra concezione di biofilm si è evoluta nel tempo; ad oggi, esso viene definito come "un aggregato di microrganismi in cui le cellule, inglobate in una matrice autoprodotta di sostanze polimeriche extracellulari (EPS), aderiscono l'una all'altra e/o ad una superficie".
La formazione di un biofilm non è la risultante di un processo casuale ma, al contrario, lo è di una sequenza ben definita di passaggi che, idealmente, possono essere descritti in tre fasi principali: una fase di adesione, regolata da interazioni elettrostatiche, interazioni idrofobiche e forze di Van der Waals, durante la quale le cellule prendono contatto con una superficie e successivamente la legano tenacemente; una fase di maturazione, in cui le cellule irreversibilmente adese alla superficie iniziano a duplicarsi, formando microcolonie, e producono EPS, permettendo così la formazione delle prime strutture tridimensionali del biofilm, ovvero le macrocolonie, che andranno poi a formare la complessa matrice del biofilm; infine, una fase di dispersione, che prevede il distacco di singole cellule o di gruppi di cellule dalla struttura del biofilm, al fine di colonizzare siti circostanti o a distanza e promuovere l'origine di un nuovo biofilm. Un giusto equilibrio tra adesione, maturazione e dispersione è importante per la produzione e il mantenimento di una comunità funzionale all'interno del biofilm.
La straordinaria capacità di diffusione di un microrganismo spiega come mai i biofilm riescono a formarsi nei luoghi più disparati. Le carene delle imbarcazioni sono spesso ricoperte di biofilm, i quali aumentano l'attrito durante la navigazione, compromettendo la velocità. Alcuni biofilm danneggiano pesantemente l'industria petrolifera facilitando la corrosione microscopica dei metalli e riducendo la durata degli oleodotti. Infine, ma non per importanza, i biofilm microbici assumono un ruolo rilevante in campo sanitario, in quanto implicati nella patogenesi di molte infezioni croniche. Si va da malattie come le prostatiti e le infezioni renali a patologie associate all'impianto di dispositivi medici come articolazioni artificiali e cateteri, fino alla carie e alle infiammazioni gengivali. In quasi tutti i casi, il biofilm ha un ruolo di primo piano nell'aiutare i microrganismi a sopravvivere o a diffondersi all'interno dell'ospite perché la matrice dei biofilm svolge un ruolo di barriera, proteggendo le cellule dalle risposte del sistema immunitario dell'ospite e dall'azione di agenti antimicrobici.
Purtroppo, ad oggi, data l'elevata complessità della struttura dei biofilm e dei loro meccanismi di difesa da insulti esterni, l'arsenale di terapie disponibili per il trattamento di queste infezioni risulta nella maggior parte dei casi inefficace.
In uno studio recente, sono stati identificati le 5 specie batteriche più frequenti tra oltre 3 milioni di isolati clinici da degenti ospedalieri, e *Staphylococcus epidermidis* è risultato una delle cause più comuni di infezioni nosocomiali in Nord America, con una frequenza del 11,5%, in America latina, con il 13,3% ed in Europa, con il 14,6%. In particolare, *S. epidermidis* è più comunemente causa di infezioni associate a dispositivi medici, come cateteri vascolari ed urinari e protesi ortopediche e cardiache. Uno dei contributi maggiori al meccanismo etio-patogenetico di queste infezioni, risiede proprio nella capacità di *S. epidermidis* di formare biofilm su superfici biotiche e abiotiche.

Data la persistenza di infezioni biofilm-associate, riluttanti ai classici trattamenti antibiotici, risulta evidente la necessità di sviluppare nuove terapie anti-infettive.
A tal proposito, nel tentativo di trovare fonti valide di agenti anti-biofilm, molti ricercatori hanno iniziato ad esplorare l'ambiente marino, in quanto rappresenta una inestimabile risorsa di biodiversità. Di circa 2 milioni di specie marine stimate, sono attualmente conosciute circa il 10%. Tra gli organismi marini, quelli che vivono in ambienti estremi, anche detti estremofili, hanno sviluppato numerosi sistemi di adattamento per sopravvivere a condizioni ambientali difficili. E date le loro differenti capacità metaboliche, sono in grado di sintetizzare numerosissimi metaboliti primari e secondari dotati di attività interessanti dal punto di vista biotecnologico e farmacologico.
In studi recenti, è stato osservato che il batterio marino antartico *Pseudoalteromonas haloplanktis* TAC125 produce e secerne molecole che inibiscono il processo di formazione del biofilm del patogeno umano *Staphylococcus epidermidis.* Inoltre, è stato dimostrato che i sovrarnatanti ottenuti da colture batteriche di diversi batteri polari appartenenti a generi *Pseudoalteromonas*, *Psychrobacter* e *Psychromonas* hanno mostrato attività anti-biofilm contro diversi ceppi patogeni. Le valutazioni preliminari sulla natura fisico-chimica delle molecole responsabili dell'attività anti-biofilm ha sottolineato la loro diversa natura. Questa capacità dei batteri marini polari di produrre diverse molecole anti-biofilm potrebbe indicare che la capacità di evitare la colonizzazione e la formazione di biofilm di altri microrganismi è un vantaggio selettivo in questi ambienti estremi, assicurando che questi batteri possano diventare una fonte interessante di nuovi composti anti-biofilm.

❖ Obiettivi

Il mio progetto di dottorato si inserisce nell'ambito della ricerca di nuove molecole ad attività anti-biofilm che agiscano contro *Staphylococcus epidermidis*. In dettaglio, mi sono occupata dello studio del batterio marino antartico *Pseudoalteromonas haloplanktis* TAC125, della sua capacità di produrre molecole anti-biofilm quando esso stesso cresce in biofilm e, più in generale, della purificazione, identificazione e caratterizzazione della molecola attiva prodotta (Capitolo I). Inoltre, dato il loro potenziale solo parzialmente esplorato, mi sono occupata anche dello studio di altri batteri polari appartenenti ai generi *Flavobacterium, Pseudoalteromonas*, *Pseudomonas*, *Psychrobacter* e *Psychromonas*, caratterizzando la loro attività anti-biofilm contro *Staphylococcus epidermidis* e mettendo a punto degli appropriati protocolli di purificazione per piccoli metaboliti e per molecole di natura proteica, al fine di identificare nuove molecole attive (Capitolo II).

❖ Risultati

Capitolo I

E' stato precedentemente dimostrato che il batterio marino antartico *Pseudoalteromonas haloplanktis* TAC125 è capace di produrre molecole anti-biofilm solo quando è coltivato in condizioni sessili, probabilmente perché le specifiche condizioni ambientali che prevalgono all'interno del biofilm inducono un profondo ricablaggio genetico e metabolico dei batteri che vivono nel biofilm, portando alla produzione di metaboliti biofilm-specifici. Una preliminare caratterizzazione fisico-chimica del sovranatante di *P. haloplanktis* TAC125 ha dimostrato che l'attività anti-biofilm è dovuta a una piccola molecola idrofobica, in grado di influenzare la formazione e la stabilità del biofilm di *S. epidermidis*. Il protocollo di purificazione inizialmente proposto ha portato all'ottenimento di una frazione arricchita della

molecola anti-biofilm, ma che era ancora una miscela di diversi composti. Pertanto, era chiaro che per la purificazione e la successiva caratterizzazione della molecola anti-biofilm era necessaria una produzione su larga scala. A tal proposito, è stata messa a punto una strategia di coltivazione di biofilm in bioreattore che ci ha permesso di ottenere una produzione su scala più ampia del sovranatante di *P. haloplanktis* TAC125, cresciuto in biofilm, indispensabile per la purificazione della molecola attiva contro il biofilm di *S. epidermidis*. L'ottimizzazione del protocollo di purificazione ha portato infine all'ottenimento di una frazione in cui erano presenti soltanto due molecole, il 2-tridecanone e il pentadecanale, ma data la presenza del chetone in una delle frazioni non attive, abbiamo concluso che l'attività fosse da attribuire al pentadecanale. L' analisi dell'attività anti-biofilm del corrispondente prodotto commerciale ha confermato la capacità inibitoria del pentadecanale sulla formazione del biofilm di *S. epidermidis*, sia in statico che in dinamico ed in maniera dose-dipendente.
Pur essendo una molecola dotata di un'interessante attività, il pentadecanale è un'aldeide e, come tale, potrebbe facilmente subire reazioni di ossidazione, polimerizzazione o idratazione, e quindi risultare chimicamente non compatibile con tutte le strategie anti-biofilm. Per cui, alcuni analoghi chimici del pentadecanale sono stati sintetizzati, purificati e saggiati per la loro capacità di inibire la formazione del biofilm di *S. epidermidis*: l'acido pentadecanoico, il pentadecanoato di metile e il 1,1dimetossipentadecano, ovvero i corrispondenti acido, estere e acetale del pentadecanale. Sebbene il meccanismo molecolare dettagliato che sottende l'attività anti-biofilm dell'aldeide e dei suoi derivati sia ben lontano dall'essere chiarito, il loro effetto sulla struttura del biofilm di *S. epidermidis* è piuttosto evidente, poiché il biofilm formato in loro presenza è fortemente ridotto e caratterizzato da una struttura apparentemente destrutturata, contenente molti canali e spazi vuoti. Questi risultati hanno suggerito di esplorare l'uso dell'aldeide e dei suoi derivati in combinazione con antibiotici per trattare le infezioni da biofilm. Analisi di microscopia confocale hanno rivelato la capacità del pentadecanale e dell'acido pentadecanoico di indebolire la struttura del biofilm di *S. epidermidis*, rendendola meno compatta ed omogenea, consentendo così la penetrazione dell'antibiotico all'interno della matrice del biofilm, rendendo anche le cellule inglobate più in profondità facili da raggiungere. Questo potrebbe essere un grande vantaggio in caso di una ipotetica strategia terapeutica che preveda una combinazione di agenti antibiotici e anti-biofilm. Infine, per esplorare le potenzialità cliniche del pentadecanale e dei suoi analoghi, la loro biocompatibilità è stata studiata su fibroblasti murini e cheratinociti umani, linee cellulari eucariotiche che rappresentano il bersaglio delle infezioni da *S. epidermidis*. I dati indicano chiaramente che le molecole risultano biocompatibili con tutte le linee cellulari analizzate.
Al fine di creare una nuova strategia anti-biofilm contro le infezioni associate a dispositivi medici causate da *S. epidermidis*, è stato sviluppato un sistema di rivestimento della superficie attraverso l'adsorbimento del pentadecanale e dei suoi analoghi su una superficie di polidimetilsilossano (PDMS), un materiale appartenente alla famiglia dei siliconi, frequentemente utilizzato per la fabbricazione di dispositivi medici come protesi fonatorie e mammarie, cateteri e valvole cardiache. In dettaglio, sono stati utilizzati il pentadecanale, l'acido pentadecanoico e l'esadecanale, al fine di capire se la lunghezza della catena alchilica e il gruppo funzionale influenzassero l'attività anti-biofilm del sistema di derivatizzazione proposto. La bagnabilità delle superfici, derivatizzate e non, è stata analizzata attraverso prove di angolo di contatto, dimostrando come l'adsorbimento delle molecole anti-biofilm ha modificato le proprietà fisicochimiche del PDMS. Inoltre, sono state effettuate analisi

topografiche delle superfici, derivatizzate e non, mediante microscopio a forza atomica, misurando a scala nanometrica la rugosità superficiale. I dati ottenuti hanno rivelato una superficie quasi perfettamente piana per il PDMS non modificato e una rugosità molto più elevata della superficie dopo l'adsorbimento delle molecole anti-biofilm. Successivamente, sono state analizzate le capacità antiadesive e anti-biofilm delle superfici derivatizzate in celle di flusso a piastre parallele, che permette di valutare in condizioni dinamiche l'adesione cellulare e il processo di formazione del biofilm su superfici di varia natura. I risultati hanno dimostrato che le superfici derivatizzate con le tre molecole anti-biofilm, nonostante le loro differenti caratteristiche, sono tutte in grado di ridurre l'adesione delle cellule di *S. epidermidis* sul PDMS e di inibire fortemente la formazione del biofilm patogeno, senza interferire con la vitalità cellulare. In conclusione, è possibile affermare che la strategia di derivatizzazione del PDMS con il pentadecanale e i suoi analoghi, attraverso adsorbimento fisico, è risultata vincente.
Un'ultima parte di questo capitolo è stata dedicata allo studio del biofilm di *P. haloplanktis* TAC125, risultato una "factory" di molecole d'interesse biotecnologico. In dettaglio, è stata analizzata l'influenza delle condizioni di crescita, quali temperatura e terreno di coltura, sulla sua capacità di formare biofilm. In dettaglio, *P. haloplanktis* TAC125 è stato cresciuto in condizioni sessili in due terreni differenti, uno ricco e uno sintetico e definito, a due temperature, 0°C e 15°C, e la sua cinetica di formazione del biofilm è stata analizzata nel tempo. Una prima valutazione quantitativa, attraverso un saggio colorimetrico, ha fatto evincere la capacità del batterio Polare di rispondere a differenti temperature e livelli di abbondanza di nutrienti, producendo biofilm in diverse quantità e forma. Infatti, in terreno sintetico il batterio ha prodotto un quantitativo di biofilm superiore rispetto a quello prodotto in terreno ricco. Questo fenomeno è probabilmente una strategia di sopravvivenza innescata dal batterio in presenza di una minore disponibilità di nutrienti, il quale risponde con una maggiore produzione di biofilm, data la capacità della matrice di catturare sostanze nutritive. Inoltre, la probabile combinazione di temperatura bassa e scarsità di nutrienti ha indotto la colonizzazione da parte del batterio dell'interfaccia solido-liquido e la formazione di un biofilm sommerso, contrariamente a quanto accade nelle altre combinazioni di terreno e temperatura, in cui troviamo un biofilm galleggiante, definito pellicola, all'interfaccia aria-liquido. Probabilmente, la ridotta motilità del batterio cresciuto in terreno sintetico a 0°C spiega la mancata formazione di pellicole all'interfaccia aria-liquido, e la sovra-espressione di pili chiarifica che la formazione di un biofilm sommerso è anch'essa frutto di un fenomeno di adattamento non casuale.
Analisi di microscopia confocale e microspettroscopia Raman hanno dimostrato che la composizione del terreno di coltura ha un ruolo chiave anche nella definizione della struttura del biofilm, risultando differenti in termini di contenuto di proteine, polisaccaridi e acidi nucleici. Lo studio del biofilm di *P. haloplanktis* TAC125, oltre a dare informazioni puramente fisiologiche in risposta a cambiamenti di condizioni ambientali, è da considerarsi come un approfondimento della conoscenza di un sistema che è in grado di produrre metaboliti bioattivi, con l'obiettivo ultimo di potenziarne l'utilizzo sostenibile come risorsa di composti d'interesse biotecnologico.

Capitolo II

Dato l'enorme potenziale dei batteri marini adattati al freddo come fonte di composti bioattivi utilizzabili in settori biotecnologici, industriali e clinici, una selezione di batteri appartenenti ai generi *Flavobacterium, Pseudoalteromonas*, *Pseudomonas*, *Psychrobacter* e *Psychromonas* sono stati analizzati per la loro capacità di produrre molecole ad attività anti-biofilm contro *S. epidermidis*. In dettaglio, sono state costruite due librerie di composti, una di piccoli metaboliti, considerando che i prodotti

naturali come i metaboliti secondari sono spesso coinvolti nella modulazione del sensing del quorum e nell'inibizione della formazione del biofilm, e una di proteine/peptidi, poiché è riportato in letteratura che ci sono molte molecole di natura proteica, come ad esempio enzimi idrolitici, capaci di degradare la matrice dei biofilm microbici.

- *Libreria di piccoli metaboliti.* Una selezione di batteri marini Polari è stata analizzata per la capacità di produrre piccoli metaboliti dotati di attività anti-biofilm contro *S. epidermidis*. Gli estratti organici ottenuti dalle crescite planctoniche in terreno sintetico e definito sono stati saggiati e preliminarmente caratterizzati, al fine di identificare la provenienza intracellulare o extracellulare del composto attivo. I due campioni più promettenti sono risultati gli estratti intracellulari ottenuti da *Pseudomonas sp*.TAA207 e *Psycrobacter* sp.TAD1, attivi entrambi con effetto dose-dipendente, ma dotati di specificità d'azione differente nei confronti dei diversi ceppi di *S. epidermidis* analizzati. I protocolli di purificazione proposti hanno portato all'identificazione di alcune frazioni attive. L'analisi attraverso spettroscopia NMR della frazione attiva identificata per *Pseudomonas sp*.TAA207, ha rivelato la presenza del pentadecanale, ovvero la molecola anti-biofilm prodotta dal batterio marino antartico *Pseudoalteromonas haloplanktis* TAC125, precedentemente identificata e caratterizzata. Purtroppo dall'analisi dell'attività anti-biofilm di *Pseudomonas sp*.TAA207 non è stata identificata nessuna nuova molecola attiva, ma è interessante notare come due batteri appartenenti a generi differenti siano in grado di produrre la stessa molecola. Al contrario, il protocollo di purificazione dell'estratto intracellulare di *Psycrobacter* sp.TAD1 ha portato all'ottenimento di due frazioni attive, contenenti molecole di natura differente, ma soprattutto, in cui non è stata identificata la presenza del pentadecanale. Studi futuri saranno dedicati all'identificazione delle molecole attive prodotte da *Psycrobacter* sp.TAD1.
- *Libreria di proteine/peptidi*. Una selezione di batteri marini polari è stata cresciuta in terreno ricco in condizioni planctoniche. I sovranatanti ottenuti dopo allontanamento delle cellule sono stati saggiati su *S. epidermidis* al fine di identificare i ceppi produttori di molecole anti-biofilm. Per discriminare se le molecole attive fossero di natura proteica, i sovranatanti sono stati sottoposti a trattamento con proteinasi K e saggiati nuovamente su *S. epidermidis*. I campioni che dopo trattamento con la proteasi hanno perso la loro attività anti-biofilm sono stati identificati come campioni proteici attivi. I due campioni più promettenti sono risultati i sovranatanti di *Pseudoalteromonas haloplanktis* TAB23, che ha mostrato la maggiore capacità inibente su *S. epidermidis*, e *Pseudoalteromonas haloplanktis* TAE80, dotato di una particolare capacità di modificare le proprietà della superficie del polistirene ed impedire in questo modo l'adesione delle cellule batteriche e la conseguente formazione del biofilm. Preliminari protocolli di purificazione attività-guidati sono stati messi a punto al fine di identificare le molecole attive. I risultati ottenuti hanno evidenziato che *P. haloplanktis* TAB23 e *P. haloplanktis* TAE80 sono in grado di produrre proteine anti-biofilm attive contro *S. epidermidis* con modalità d'azione differenti, ma tutte dotate di una potentissima attività biologica, in quanto attive a concentrazioni molto basse. Purtroppo, non sono state ancora identificate le proteine attive, in quanto i protocolli proposti hanno portato all'ottenimento di frazioni sicuramente arricchite delle proteine attive, ma che risultassero ancora delle miscele complesse di più proteine. L'analisi *in silico* del genoma di *P. haloplanktis* TAE80 ha permesso l'identificazione di alcune sequenze geniche potenzialmente responsabili dell'espressione della/e proteina/e anti-biofilm da esso prodotta/e. La generazione di mutanti di delezione permetterà di capire se quelli ipotizzati risultano effettivamente essere i responsabili della produzione delle proteine dotate di attività anti-biofilm.

SUMMARY

Amongst marine bacteria, cold-adapted microorganisms represent an untapped reservoir of biodiversity endowed with an interesting chemical repertoire able to synthesize a broad range of potentially valuable bioactive compounds, including antimicrobial activity. The rapid emergence of resistant bacteria is occurring worldwide, endangering the efficacy of antibiotics. One of the main causes of antibiotic resistance is the capability of microorganisms to associate into communities of cells called biofilms. These complex structures provide protection from potential stressors, including the lack of water, high or low pH, or the presence of substances toxic to microorganisms such as antibiotics, antimicrobials or heavy metals. Therefore, coordinated efforts to implement the arsenal of novel anti-infective treatments are greatly needed.

In this contest, my PhD project aimed to the sustainable exploitation of Polar marine biodiversity in an attempt to find viable sources of novel anti-biofilm agents, in particular acting against *Staphylococcus epidermidis*, one of the most common causes of infections associated with medical devices.

In detail, during the first part of my project, I focused on the study of the Antarctic marine bacterium *Pseudoalteromonas haloplanktis* TAC125 and of its ability to produce anti-biofilm molecules, then, on the purification, identification and characterization of the active molecule produced. By setting up of a strategy for the large scale biofilm cultivation of the Antarctic bacterium, the production yield of *P. haloplanktis* TAC125 anti-biofilm agent was improved, so as to allow the purification and the identification of the active molecule, the pentadecanal. However, as the pentadecanal is a chemically reactive agent, it could easily undergo oxidation reactions, therefore it could not be suitable for all possible anti-biofilm strategies. Therefore, some chemical analogues were synthesized and characterized for their anti-biofilm activity and their possible use in combination with antibiotics were investigated. Then, as a possible clinical application, an anti-biofilm coating system, active against *S. epidermidis*, was developed, by physical adsorption of pentadecanal and its analogues on polydimethylsiloxane (PDMS), a silicon-based material commonly used for the manufacturing of medical devices. Finally, some physiological studies were dedicated to *P. haloplanktis* TAC125 biofilm formation in relation with environmental adaptations, with the purpose to explore the potentiality of *P. haloplanktis* TAC125 in biotechnological field.

In the second part of my PhD project, given their only partially explored potential, I have also studied other Polar bacteria belonging to different genera, looking for novel anti-biofilm agents against *S. epidermidis*.

Through the screening of small metabolites and proteins/peptides libraries designed starting from planktonic cultures of Polar bacteria, some promising producer strains were identified and their anti-biofilm activities were characterized. Preliminary purification protocols were set up for each kind of molecules, according to their physico-chemical characteristics. Further studies are still ongoing to identify the structure of the active molecules.

INTRODUCTION

1. Biofilms: definition, structure and development

For most of the history of Microbiology, microorganisms have primarily been characterized as planktonic, freely suspended cells, and described on the basis of their growth characteristics in nutritionally rich culture media. Later there was the discovery of a microbiological phenomenon in which microorganisms are able to attach to and grow universally on exposed surfaces forming biofilms. The first description dates back to the 17th century, when the Dutch scientist Anthony van Leeuwenhoek analyzed the material scraped from his own teeth and, using his primitive microscope, noticed "very many small living Animals, which moved themselves very extravagantly"[1].

Our definition of biofilm has evolved over the last 40 years. The term *biofilm* was coined by Bill Costerton in 1978, who stated that "bacteria stick, tenaciously and often with exquisite specificity, to surfaces … by means of a mass of tangled fibers of polysaccharides, or branching sugar molecules, that extend from bacterial surface and form a feltlike "glycocalyx" surrounding an individual cell or a colony of cells"[2].

To date, the concept of biofilm has been widely consolidated and defined by the International Union of Pure and Applied Chemistry (IUPAC) as "an aggregate of microorganisms in which cells, that are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS), adhere to each other and/or to a surface"[3].

It is clear that microorganisms undergo profound changes during their transition from planktonic (free-swimming) organisms to cells that are part of a complex, surface-attached community. These changes are reflected in the new phenotypic characteristics developed by biofilm bacteria and occur in response to a variety of environmental signals. Recent genetic and molecular approaches used to study microbial biofilms have identified genes and regulatory circuits important for initial cell-surface interactions, biofilm maturation, and the return of biofilm microorganisms to a planktonic mode of growth. In short, the planktonic-biofilm transition is a complex and highly regulated process[4]. Basically, the biofilm development has been characterized as a well-defined succession of different stages (Fig. 1). First, an attachment phase, regulated by electrostatic interactions, hydrophobic interactions and Van der Waals forces, which involves an initial and reversible attraction of bacterial cells to the surface, and a subsequent but more tenacious and irreversible adhesion of the same. Then, a maturation phase, during which cells irreversibly attached to surfaces begin cell division forming microcolonies, and produce EPSs that allows the formation of the first three-dimensional structures of biofilm, macrocolonies, providing the matrix structure for the biofilm. Finally, a dispersal phase, which involves the detachment of single cells or groups of cells that can colonize surrounding or remote sites and promote the origin of a new biofilm[5].

The formation and maintenance of structured multicellular microbial communities crucially depend on the production and quantity of EPS. The chemical features, abundance and three-dimensional arrangement of each component of EPS determine the biofilm morphology, provide mechanical stability and form a cohesive polymer network that interconnects and transiently immobilizes biofilm cells[6].

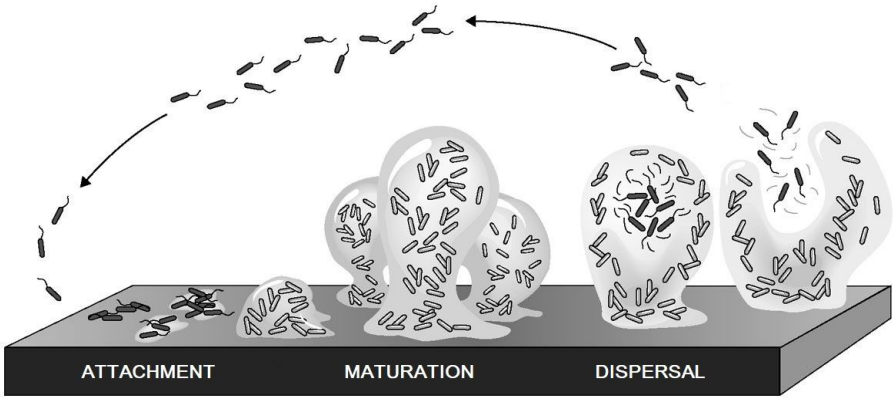

Figure 1: Biofilm development stages: adhesion of the cells to a surface (attachment), cell division and EPS production (maturation), matrix disaggregation and cell detachment (dispersal).

EPS compounds belong to such different classes of macromolecules as polysaccharides, a wide variety of proteins, glycoproteins, and glycolipids, and in some cases, surprising amounts of extracellular DNA (eDNA)[7].
The extracellular secreted proteins determine the microbial attachment process to different solid surfaces. Firstly these macromolecules are accumulated on the cell surface. After the secretion process to the external environment, the proteins can be adsorbed to contact surfaces. The formation of a protein layer on solid surface, named conditioning film, is conducive to the bacterial adhesion process. During more advanced phases of the microbial adhesion process, the *in situ* secretion of extracellular proteins leads to the intensification of the microbial attachment process by anchoring the single cells on the contact surface[8].
The polysaccharide component of the matrix can provide many diverse benefits to the cells in the biofilm, including adhesion, protection, and structure. Aggregative polysaccharides act as molecular glue, allowing the bacterial cells to adhere to each other as well as to surfaces and thus to resist physical stresses imposed by fluid movement that could separate the cells from a nutrient source[9]. Polysaccharides can also provide protection from a wide range of stresses and external assaults, such as immune responses or antibiotics[10]. Finally, polysaccharides can provide structure to biofilms, allowing stratification of the bacterial community and establishing gradients of nutrients and waste products[11]. This can be advantageous for the bacteria by establishing a heterogeneous population that is prepared to endure stresses created by the rapidly changing environments that many bacteria encounter. The diverse range of polysaccharide structures, properties, and roles highlight the importance of this matrix constituent to the successful adaptation of bacteria to nearly every niche[12].
Extracellular DNA has recently been described as one of a major structural components of the biofilm matrix[13]. However, the role of this extracellular molecules in the Gram-positive and Gram-negative biofilm development process is still unclear. To date, it is generally accepted that eDNA plays a role in cell surface attachment, cellular aggregation and even cell dispersal in the biofilm life cycle. Experiments demonstrating binding between eDNA and polysaccharides[14], proteins[15] or extracellular metabolites[16] by different mechanisms have generated an understanding of the central role of DNA as a scaffold offering structural integrity to biofilms.

Nevertheless, our understanding of biofilms is still limited. Research on microbial biofilms is proceeding on many fronts, with particular emphasis on elucidation of the genes specifically expressed by biofilm-associated organisms, in order to reach a more complete understanding of what makes the biofilm phenotype so different from the planktonic phenotype.

2. Quorum sensing, cell-cell communication in biofilms

Within the biofilm community, bacteria communicate with each other by using chemical signal molecules in response to population density in a process that is called quorum sensing (QS). The cell-cell communication via QS involves the production, release and group-wide detection of extracellular signalling molecules, which are called autoinducers (AIs). AIs accumulate in the environment as the bacterial population density increases, and bacteria monitor this information to track changes in their cell numbers and to collectively alter global patterns of gene expression[17].

Three main QS systems can be distinguished: the acyl-homoserine lactone (AHL) QS system in Gram-negative bacteria, the autoinducing peptide (AIP) QS system in Gram-positive bacteria and the autoinducer-2 (AI-2) QS system in both Gram-negative and -positive bacteria. AHL-type QS systems predominately foster intra-species cell-cell communication in Gram-negative bacteria; AHLs diffuse through the bacterial membrane and interact with specific cytoplasmic receptors, which can regulate the target genes expression acting as transcriptional activators. Because peptides are impermeable to biological membranes, AIP QS system in Gram-positive typically use secreted oligopeptides and two-component systems, which consist of membrane-bound receptors and cytoplasmic transcription factors that direct alterations in gene expression. The AI-2 QS system, shared by Gram-positive and Gram-negative bacteria, involves the production of a family of interconverting compounds synthesized by the LuxS enzyme, widely present in bacteria, leading to the suggestion that AI-2 is a universal language for interspecies communication[18].

These QS systems control genes that direct activities that are beneficial when performed by groups of bacteria acting in synchrony, including bioluminescence, sporulation, competence, antibiotic production, biofilm formation and virulence factor secretion[19]. Beyond controlling gene expression on a global scale, QS allows bacteria to communicate within and between species. In fact, high cell density and close proximity of diverse species of microorganisms, that are typical of life in natural biofilms, let the organisms get involved in complex social interactions that occur both within and between species and can be either competitive or cooperative[20]. Competition for nutrients and other growth parameters is certainly an important driving force for the development of biofilm structure. But, on the other hand, in such a complex organization, bacteria could benefit from division of labor, collective actions, and other forms of cooperative activities with their neighbors[21].

These sophisticated interactions represent good examples of microbial social activities in natural microbial biofilms. If from a certain point of view these social activities play important roles in balancing competition and coexistence of different organisms within a microbial community, maintaining biodiversity and homeostasis of microbes in the same ecosystem[22], from another perspective several problems arise from bacterial social activities, first of all the establishment of chronic biofilm-associated infections, such as medical device or implant infections, frequently

resistant to the highest deliverable levels of antibiotics[23]. Nowadays, the arsenal of therapeutics available to treat these infections are in most cases ineffective, because biofilms underlying these infections cannot be resolved with standard antibiotic treatments. Therefore, understanding bacterial social behaviours and their molecular mechanisms in the development of biofilms will greatly facilitate the development of novel strategies in the prevention and treatment of biofilm infections.

3. Biofilm and pathogenesis

Industrial pipelines, nuclear power stations, space stations, air conditioning systems, water distribution systems and one of the fastest technologically advancing settings, the hospital, are all susceptible to colonization by microorganisms growing in biofilms[24]. In clinical settings, biofilms have been implicated in a variety of human infections, such as endocarditis, osteomyelitis, chronic otitis media, gastrointestinal ulcers, urinary tract infections, chronic lung infections in cystic fibrosis patients, caries, and periodontitis[25]. Among all, the most common clinical infections associated with biofilm formation are medical device-related infections. Pacemakers, electrical dialysers, joint prosthetics, intravenous catheters, urinary catheter present high risk of biofilm-associated infection.

The causative agents of biofilm-associated infections are different Gram-positive species of *Staphylococcus, Streptococcus*, and *Enterococcus* as well as Gram-negative bacteria, such as *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumoniae.* Infections caused by biofilm-forming bacteria are often difficult to treat. Biofilm formation almost always leads to a large increase in resistance to antimicrobial agents (up to 1000-fold decrease in susceptibility) in comparison with planktonic cultures grown in conventional liquid media[26] and to the triggering of evasion mechanisms of the bacteria from immune system responses of the host[27].

Three mechanisms have been proposed to explain the general resistance of biofilms to biocidal agents. The first is the barrier properties of the slime matrix[28]. This mechanism might be more relevant for reactive (bleach or superoxides), charged (metals) or large (immunoglobulin) antimicrobial agents that are neutralized or bound by the EPS and are effectively reduced to sub-lethal concentrations before they can reach all of the individual bacterial cells within the biofilm. The barrier properties of the EPS hydrogel might also protect against UV light and dehydration, and might localize enzymatic activity. The second protective mechanism could involve the physiological state of biofilm organisms. Although many antibiotics can freely penetrate the EPS, cells within the biofilm are often still protected. The creation of stationary phase dormant zones in biofilms seems to be a significant factor in the resistance of biofilm populations to antimicrobials[29-30], particularly against antibiotics, such as β−lactams, which are effective against rapidly dividing Gram-positive bacteria by interruption of cell-wall synthesis. However, obviously all antibiotics require at least some degree of cellular activity to be effective, because the mechanism of action of most antibiotics involves disruption of a microbial process. Therefore, the creation of zones of cells in a biofilm in stationary phase quiescence might represent a general mechanism of antibiotic resistance. A third mechanism of protection is due to the existence of subpopulations of resistant phenotypes in the biofilm, which have been referred to as “persisters”[31]. Persister cells are highly multidrug-tolerant cells that constitute a small fraction of the population. They are

transiently refractory to killing, without having acquired resistance through genetic modification[32]. Consequently, when the antibiotic pressure drops, the cells will give rise to a population that is as susceptible as the original one, and that again possesses a similarly small proportion of persister cells. This discriminates persister cells from resistant mutants, which exhibit stable, inheritable drug insensitivity. Persisters have a significantly reduced growth rate[33]. Their indifference to the presence of antibiotics can therefore be explained by a global shutdown of processes essential for active growth, as the very processes that are targeted by antibiotics are no longer operational and hence not subject to inhibition any more. This also corroborates the non-specific nature of persister cell drug tolerance. So, based both on these findings and on modelling studies[34], it was suggested that persisters may well represent the long-looked-for explanation for biofilm tolerance to antibiotics.

4. *Staphylococcus epidermidis*, the opportunistic pathogen

Coagulase-negative staphylococcal species are the predominant bacterial species that colonize normal human skin. In particular, *Staphylococcus epidermidis* is the most frequently isolated species from human epithelia and, as part of the human epithelial microflora, it usually has a benign relationship with its host[35].

Although the specific mechanisms through which skin surface microbes influence host function are incompletely understood, specific strains of coagulase-negative staphylococcal species have been shown to produce proteins that work together with endogenous host antimicrobial peptides to provide direct protection against infectious pathogens. For example, it was demonstrated that *S. epidermidis* produces toxins that can selectively kill bacterial pathogens such as *S. aureus*[36]. Further evidence that commensal *Staphylococcus* species provide host defense has come from observations that nasal colonization with a specific strain of *S. epidermidis* that produces a particular serine protease can inhibit nasal colonization by *S. aureus*[37].

Despite the evidence that the skin microbiome has an important role in promoting host defense, the research and the information on the non-infectious lifestyle of *S. epidermidis* are still relatively scarce, whereas, on the other hand, the interest in *S. epidermidis* infections and mechanisms by which it promotes diseases is continuously increasing.

The main defined virulence factor associated with *S. epidermidis* is its ability to form biofilm and colonize biotic and abiotic surfaces. Particularly, *S. epidermidis* represents the most frequent causative agent involved with infections of any type of indwelling medical devices[38]. The onset of these infections usually start with the introduction of bacteria from the skin of the patient or that of health care personnel during device insertion and have increased in number most likely owing to the increased use of such devices.

The American “Centers for Disease Control and Prevention” estimates that 2 million patients suffer from hospital-acquired infections every year. About 65% of these nosocomial infections are biofilm related and the treatment of these biofilm-associated infections costs greater than $1 billion annually only in the United States[39]. In a recent study, the 5 most frequently occurring bacteria have been identified among more than 3 million clinical isolates from hospital inpatients, and *S. epidermidis* was one of the most common cause of nosocomial bacteremia in North America (prevalence, 11.5%), Latin America (prevalence, 13.3%) and Europe (prevalence, 14.6%)[40].

S. epidermidis infections are recalcitrant to the deleterious action of antibiotics and impedes the host immune response, therefore, the treatment of patients with *S. epidermidis* biofilm-related infections typically involves removal of the offending device and subsequent replacement, causing an increase in morbidity and cost.
Fortunately, the utilization of newer technologies enables investigators to probe mechanisms of virulence within *S. epidermidis*, particularly those factors that mediate biofilm formation, so as to identify new strategies to inhibit *S. epidermidis* colonization and infections.

5. Polar marine bacteria as source of new anti-biofilm molecules

Biodiversity in the seas is only partly explored, although marine organisms are excellent sources for many industrial products. Of the 2 million marine species on Earth, the total number of currently known marine species is only 230,000[41-42]. Through close cooperation between industrial and academic partners, it is possible to successfully collect, isolate and classify marine organisms, such as bacteria, fungi, micro- and macroalgae, cyanobacteria, and marine invertebrates from the oceans and seas globally.
Marine organisms possess a vast diversity of metabolic capabilities, because of their varied adaptations to a diverse range of physical and chemical conditions of marine ecosystems[43]. In fact, the specific habitat where organism is growing has influence on the chemical nature of the marine primary and secondary metabolites.
Not by chance, microorganisms that inhabit extreme environments, referred to as extremophiles, have an enormous potential as sources of bioactive compounds with applications in biotechnology and pharmaceutical industry[44]. In particular, marine microorganisms living in Polar regions, to survive under the constant influence of low temperatures, strong winds, low nutrient and high UV radiation or combinations of these factors, require a diverse array of biochemical and physiological adaptations that are essential for survival. These adaptations are often accompanied by modifications to both gene regulation and metabolic pathways, increasing the possibility of finding unique functional metabolites of pharmaceutical importance[45].
One of the developed survival strategies may be represented by the production of molecules with anti-biofilm activity, which might be exploited to fight the biological competition of other bacteria.
Several natural products were isolated from polar species, endowed with disparate biological activity, varying from cytotoxic, enzyme inhibitory, antioxidant, antiparasitic, antiviral to antibacterial[46]. Recently, research is focusing on the discovery of novel anti-biofilm agents, like quorum sensing inhibitors[47] or biosurfactants[48].
The Antarctic marine bacterium *Pseudoalteromonas haloplanktis* TAC125 is considered as a model for the study of adaptation to cold marine conditions. The study of its exceptional genomic and metabolic features revealed the ability of the bacterium to grow, to a very high cell density under laboratory settings, in a wide range of temperatures (+20°C/-2.5°C)[49], its capability to colonize biotic and abiotic surfaces, with a propensity to make a water/air biofilm[50], and its validity either as a non-conventional system for production of recombinant protein[49] and as a rich source of bioactive compounds[51-52].
Furthermore, it was observed that *P. haloplanktis* TAC125 is able to produce and secrete molecule/s inhibiting the biofilm of the human pathogen *Staphylococcus epidermidis* biofilm formation[53-54]. In parallel, it was demonstrated that supernatants

obtained from bacterial cultures of several Polar bacteria belonging to *Pseudoalteromonas*, *Psychrobacter* and *Psychromonas* genera showed anti-biofilm activity against different pathogenic strains[55] and preliminary evaluations on the physicochemical nature of the molecules responsible for anti-biofilm activity emphasized their different nature.
This ability of Polar marine bacteria to produce several anti-biofilm molecules could suggest that the capacity to avoid the colonization and biofilm formation of competitors is a selective advantage in this extreme environment, ensuring that these bacteria may become an interesting source of novel anti-biofilm compounds.

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CHAPTER I

1. *Pseudoalteromonas haloplanktis* TAC125 biofilm formation: physiological studies and anti-biofilm agents discovery

Pseudoalteromonas haloplanktis TAC125 anti-biofilm activity has been discovered and characterized during the last years[1-2]. The Antarctic bacterium is able to synthesize and secrete in the environment in which it lives a small anti-biofilm molecule, active against *Staphylococcus epidermidis*. In detail, it was demonstrated that the cell-free supernatant of *P. haloplanktis* TAC125 inhibits the biofilm formation of different *S. epidermidis* strains, even on the mature forms. On the basis of these results, in this part of my PhD project I focused my work on the identification of *P. haloplanktis* TAC125 anti-biofilm agent and its possible clinical applications.

This chapter is organized in three paragraphs:

- the first paragraph describes the set-up of a biofilm cultivation of *P. haloplanktis* TAC125 in automatic bioreactor, for a large scale production of the anti-biofilm molecule, and the subsequent characterization and identification of the active compound;
- the second paragraph describes the design of new anti-biofilm compounds, based on the chemical structure of the identified *P. haloplanktis* TAC125 anti-biofilm molecule, but endowed with different physicochemical properties, and their possible clinical application as anti-biofilm coating for medical devices;
- the third paragraph describes some physiological studies on *P. haloplanktis* TAC125 biofilm formation in relation with environmental adaptations, with the purpose to explore the potentiality of *P. haloplanktis* TAC125 in biotechnological field.

1.1. Characterization and identification of *Pseudoalteromonas haloplanktis* TAC125 anti-biofilm agent

From a previous study[1], *P. haloplanktis* TAC125 has been shown to produce the anti-biofilm molecule only when it is grown in sessile condition, probably because the specific environmental conditions prevailing within the biofilm may induce profound genetic and metabolic rewiring of the biofilm-dwelling bacteria[3], and this could led to the production of biofilm-specific metabolites. A preliminary physicochemical characterization of the supernatant of *P. haloplanktis* TAC125 demonstrated that the anti-biofilm activity is due to a small hydrophobic molecule, able to affect *S. epidermidis* biofilm formation and stability. The purification protocol initially proposed led to the obtainment of a fraction enriched in the anti-biofilm molecule, but which was still a mixture of several compounds[2]. Therefore, it was clear that for the purification and the subsequent characterization of the anti-biofilm molecule a large-scale production was necessary.

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METHOD PAPER

Large-scale biofilm cultivation of Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 for physiologic studies and drug discovery

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Abstract Microbial biofilms are mainly studied due to detrimental effects on human health but they are also well established in industrial biotechnology for the production of chemicals. Moreover, biofilm can be considered as a source of novel drugs since the conditions prevailing within biofilm can allow the production of specific metabolites. Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 when grown in biofilm condition produces an anti-biofilm molecule able to inhibit the biofilm of the opportunistic pathogen *Staphylococcus epidermidis*. In this paper we set up a *P. haloplanktis* TAC125 biofilm cultivation methodology in automatic bioreactor. The biofilm cultivation was designated to obtain two goals: (1) the scale up of cell-free supernatant production in an amount necessary for the anti-biofilm molecule/s purification; (2) the recovery of *P. haloplanktis* TAC125 cells grown in biofilm for physiological studies. We set up a fluidized-bed reactor fermentation in which floating polystyrene supports were homogeneously mixed, exposing an optimal air–liquid interface to let bacterium biofilm formation. The proposed methodology allowed a large-scale production of anti-biofilm molecule and paved the way to study differences between *P. haloplanktis* TAC125 cells grown in biofilm and in planktonic conditions. In particular, the modifications occurring in the lipopolysaccharide of cells grown in biofilm were investigated.

Keywords Biofilm cultivation · *Pseudoalteromonas haloplanktis* TAC125 · Anti-biofilm · Bacterial biofilm phenotype

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Introduction

Biofilm is a structured aggregation of microorganisms associated with a surface and it is the predominant mode of growth for bacteria in most environments. The transition from the planktonic state to biofilm growth occurs as a consequence of environmental changes that trigger the activation of multiple regulatory networks (Hall-Stoodley et al. 2004; de la Fuente-Núñez et al. 2013). Thus, upon sensing a proper signal, free-living (planktonic) cells will initiate attachment to a surface, which will lead to the formation of a biofilm that has a greater ability to withstand environmental challenges. Bacteria possess a biofilm genetic program that could be triggered by stressful conditions, aiming at the adaptation to transiently hostile environments (de la Fuente-Núñez et al. 2013). This program involves the switch on of regulatory circuits that cause transient genetic alterations rather than permanent. Proteomic and transcriptomic studies have shown a global shift in metabolism when growth switches from planktonic to biofilm (de la Fuente-Núñez et al. 2013).

Although bacterial biofilm has drawn increasing attention due to many detrimental effects on human health (Percival et al. 2015), it can be used in many biotechnological

applications (Rosche et al. 2009). Indeed, the specific environmental conditions prevailing within biofilms induce profound genetic and metabolic rewiring of the biofilm-dwelling bacteria that can allow the production of metabolites different from those obtained in planktonic condition. From this point of view, the biofilm could be considered as a source of novel drugs. Furthermore, many bacterial biofilms secrete molecules such as quorum sensing signals, surfactants, enzymes, and polysaccharides that act by regulating biofilm architecture or mediating the release of cells from biofilms during the dispersal stage of the biofilm life cycle (Valle et al. 2006; Qin et al. 2009; Ni et al. 2009; Kiran et al. 2010; Papa et al. 2015). While understanding of these aspects of biofilms has increased, further work is needed, especially in the field of in vitro systems development for growing and studying microbial biofilms.

Two major biofilm models are studied in the laboratory, biofilms grown without a continuous flow of fresh medium and biofilms grown with a continuous flow of fresh medium. These systems generally provide a surface that can be removed and examined once it is colonized to assess biofilm formation. Along with the development of these systems for biofilm physiology studies at small scale, the biotechnological applications of microbial biofilm, such as water purification and wastewater treatment and enhanced production of added-value fermentation products (Pongtharangku and Demirci 2007, Cheng et al. 2010) fostered the set-up of biofilm reactors for large-scale industrial production. Biofilm reactors have been proven quite effective in enhancing productions of added-value products, such as bioethanol, organic acids, enzymes, antibiotics, and polysaccharides as they can generate increased volumetric productivity rates by maintaining high biomass concentration in the bioreactors (Cheng et al. 2010).

In general, biofilm reactors can be categorized into two groups: fixed-bed and expanded-bed reactors. Fixed-bed reactors include all processes in which the biofilm develops on static media (Cheng et al. 2010; Szilágyi et al. 2013). Expanded-bed reactors include biofilm with continuously moving media driven by high air or liquid velocity, or by mechanical stirring (Cheng et al. 2010). A great variety of solid supports have been developed and designed to increase the specific surface area per volume of reactor to obtain higher efficiency and compactness (Cheng et al. 2010).

We previously (Papa et al. 2013b; Parrilli et al. 2015) demonstrated that the cell-free supernatant of Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 (*P. haloplanktis* TAC125) inhibits *Staphylococcus epidermidis* (*S. epidermidis*) biofilm formation. Interestingly *P. haloplanktis* TAC125 shows this activity only when it is grown in sessile condition (Papa et al. 2013b). Recent investigation (Parrilli et al. 2015) on chemical nature of *P. haloplanktis* TAC125 anti-biofilm molecule demonstrated that the anti-biofilm activity is due to small hydrophobic molecule that likely works as signal. These results are highly suggestive of actual differences in *P. haloplanktis* TAC125 cells physiology when sessile rather than planktonic lifestyle is adopted, and pave the way to several open questions about the biofilm-specific pathway involved in the anti-biofilm molecule synthesis. To answer these questions it is necessary to have a methodology to investigate the characteristics of *P. haloplanktis* TAC125 cell grown in biofilm. Moreover, the previously proposed purification protocol (Parrilli et al. 2015), even if it allowed to obtain an active fraction enriched in anti-biofilm compound, is characterized by a poor purification yield. Therefore, the purification and characterization of the anti-biofilm molecule requires a larger scale production.

In this paper, we set up a biofilm cultivation methodology of *P. haloplanktis* TAC125 in automatic bioreactor. The Gram-negative psychrotolerant marine bacterium *P. haloplanktis* TAC125 is one of the best-studied cultivable representatives of the marine bacterioplankton and it is considered a model organism of bacterial cold-adaptation (Medigue et al. 2005). Indeed, in the last few years the increasing interest in *P. haloplanktis* TAC125 has led to the accumulation of different data types, including its complete genome sequence (Medigue et al. 2005), its intracellular and extracellular proteome (Piette et al. 2010, 2011; Papa et al. 2006), detailed growth phenotypes (Wilmes et al. 2010; Giuliani et al. 2011), and a genome-scale metabolic model (Fondi et al. 2015). A fermentation scheme to upscale *P. haloplanktis* TAC125 growth in automatic bioreactors, at a laboratory scale, was developed and used for batch (Giuliani et al. 2011), chemostat cultivation (Giuliani et al. 2011) and fed-batch fermentation (Wilmes et al. 2010). However, no strategy was established to obtain a biofilm cultivation of *P. haloplanktis* TAC125 in bioreactor.

In this paper, we proposed a biofilm cultivation of *P. haloplanktis* TAC125 in automatic bioreactor aimed at a larger scale production of *P. haloplanktis* TAC125 supernatant grown in biofilm condition, indispensable for the purification of the molecule active against *S. epidermidis* biofilm. The set up process also allowed a consistent enhancement of the recovery yield of *P. haloplanktis* TAC125 biomass, making now possible to carry out comparative physiologic studies of Antarctic bacterial cells grown in biofilm and in planktonic conditions.

Materials and methods

Bacterial strains and culture conditions

Bacterial strains used in this work were: *S. epidermidis* O-47 isolated from clinical septic arthritis and kindly

provided by Prof. Gotz (Heilmann et al. 1996); *P. haloplanktis* TAC125 (Medigue et al. 2005) collected in 1992 from seawater near French Antarctic Station Dumont d'Urville. Bacteria were grown in Brain Heart Infusion broth (BHI, Oxoid, UK). Biofilm formation was assessed in static condition while planktonic cultures were performed under vigorous agitation (180 rpm). All strains were maintained at −80 °C in cryovials with 15 % of glycerol.

Biofilm formation of *P. haloplanktis* TAC125 on supports in flask

Polystyrene supports were hand made using pieces of expanded polystyrene with two different shapes. The mean length of the outer side is 2 cm, the mean thickness is 1 cm and the mean surface exposed area is 12 cm^2 in case of support A. Type B are characterized by a mean thickness of 1 cm, a mean diameter of 0.5 cm and a mean surface exposed area of 2.1 cm^2.

The supports were placed in water and sterilized in autoclave at 121 °C for 20 min. After sterilization, the polystyrene supports were added to cell cultures. In detail, 250 ml glass wide-neck flasks were filled with 25 ml of BHI medium and an appropriate dilution of Antarctic bacterial culture in exponential growth phase (about 0.1 OD 600 nm) was added into each flask. Several replicates of autoclaved polystyrene supports were separately placed onto the surface of the broth with a sterile forceps to allow a homogeneous soaking of the surfaces and to enable biofilm formation at air–liquid interface. In detail, four replicates of support A (Fig. 1) and 24 replicates of support B (Fig. 1) were added in each flask, respectively. *P. haloplanktis* TAC125 cultures were incubated at 15 and at 4 °C for 96 h in static condition. After incubation, supernatants were separated from polystyrene supports and sterilized by filtration through membranes with a pore diameter of 0.22 µm, and stored at 4 °C until use. Supports were recovered and rinsed twice with 40 ml of PBS. Then adhered cells on supports were stained with 0.1 % crystal violet, rinsed twice with double-distilled water, and thoroughly dried. The dye bound to adherent cells on supports was solubilized with 20 ml of 20 % (v/v) glacial acetic acid and 80 % (v/v) ethanol and the absorbance at 590 nm was measured.

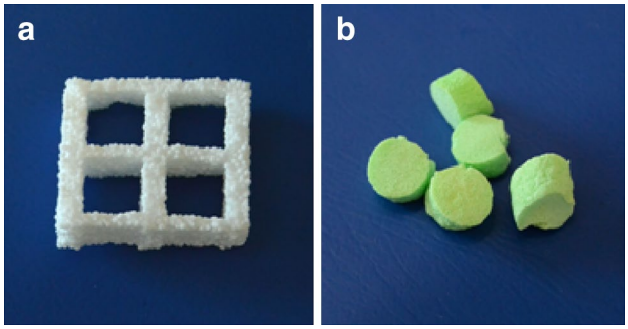

Fig. 1 Autoclaved polystyrene supports. **a** Support A: the mean length of the outer side is 2 cm, the mean thickness is 1 cm and the mean surface exposed area is 12 cm^2. **b** Support B: the mean thickness is 1 cm, the mean diameter is 0.5 cm and the mean surface exposed area is 2.1 cm^2

Recovery of attached cells by sonication

250 ml glass wide-neck flasks were filled with 25 ml of BHI medium and an appropriate dilution of Antarctic bacterial culture in exponential growth phase (about 0.1 OD 600 nm) was added into each flask. Then four replicates of support A and 24 replicates of support B were added in each flask, respectively. *P. haloplanktis* TAC125 cultures were incubated at 4 °C for 96 h in static condition, after incubation supports were transferred with a sterile forceps to a 50 ml Falcon tube filled with 10 ml of BHI broth, with the supports completely immersed into the broth. In detail, one single support A and six supports B in each Falcon tube were transferred, respectively. Samples were sonicated at three different exposure times (5, 10 and 15 min). Sonication was performed by using an ultrasound bath (Elmasonic S 30/H) at a constant ultrasound frequency of 37 kHz. The supports floated to the top of the liquid level while the detached biomass settled. Sonication fluid obtained after three different exposure times was transferred to 10 ml Falcon tubes, respectively. Samples were centrifuged at 13,000 rpm, supernatant was discarded and cell pellets were resuspended in a small volume of fresh broth. Cell suspensions were transferred in 2 ml microcentrifuge tubes and centrifuged at 13,000 rpm. Then supernatant was discarded and cell pellets were dried in an oven at 55 °C for 24 h. Dry weight of recovered cell pellets was measured by using an analytical balance.

Anti-biofilm compound/s production in automatic bioreactor

P. haloplanktis TAC125 bacterial culture was grown in BHI medium in a Stirred Tank Reactor 3 L fermenter (Applikon) connected to an ADI-1030 Bio Controller (Applikon) with a working volume of 1 L. The bioreactor was equipped with the standard pH-, pO_2-, level- and temperature sensors for the bioprocess monitoring. To allow the biofilm formation, autoclaved solid polystyrene supports A were added into the bioreactor (33 supports in 1 L). The culture was carried out at 15 °C for 48 h, or at 4 °C for 96 h, in aerobic conditions using an airflow of 6 L h^{-1}, without stirring. Supernatant was recovered and separated from supports and cells by a centrifugation at 13,000 rpm. Then, it was sterilized by filtration through membranes with a pore

diameter of 0.22 µm, and stored at 4 °C until use. Supports were recovered and subjected to biofilm detachment procedure previously described.

Biofilm formation of staphylococci

Quantification of in vitro biofilm production was based on the method described by Christensen with slight modifications (Papa et al. 2013a; Artini et al. 2013). Briefly, the wells of a sterile 48-well flat-bottomed polystyrene plate were filled with 400 µl of BHI medium. 1/100 Dilution of overnight bacterial cultures was added into each well (about 5.0 OD 600 nm). The first row contained the untreated bacteria, while each of the remaining rows contained serial dilutions of supernatant (SN) starting from 1:2. The plates were incubated aerobically for 24 h at 37 °C.

Biofilm formation was measured using crystal violet staining. After treatment, planktonic cells were gently removed; each well was washed three times with PBS and patted dry with a piece of paper towel in an inverted position. To quantify biofilm formation, each well was stained with 0.1 % crystal violet and incubated for 15 min at room temperature, rinsed twice with double-distilled water, and thoroughly dried. The dye bound to adherent cells was solubilized with 20 % (v/v) glacial acetic acid and 80 % (v/v) ethanol. After 30 min of incubation at room temperature, OD 590 nm was measured to quantify the total biomass of biofilm formed in each well. Each data point is composed of three independent experiments each performed at least in 3-replicates.

LPS extraction and characterization

P. haloplanktis TAC125 cells were grown in planktonic and in biofilm conditions at 15 and 4 °C, respectively. Then the cells were extracted by phenol/chloroform/light petroleum ether (PCP) method to isolate the lipopolysaccharide (LPS) fractions (Galanos et al. 1969) and visualized by electrophoresis. For the extraction of LPS from *P. haloplanktis* TAC125 grown in planktonic conditions, bacterial cells were treated as already reported (Corsaro et al. 2001). PAGE was performed using the system of Laemmli et al. (1970) with sodium deoxycholate (DOC) as detergent. The separating gel contained final concentrations of 16 % acrylamide, 0.1 % DOC, and 375 mM Tris/HCl pH 8.8; the stacking gel contained 4 % acrylamide, 0.1 % DOC, and 125 mM Tris/HCl pH 6.8. LOS samples were prepared at a concentration of 0.05 % in the sample buffer (2 % DOC and 60 mM Tris/HCl pH 6.8 25 % glycerol, 14.4 mM 2-mercaptoethanol, and 0.1 % bromophenol blue). All concentrations are expressed as mass/volume percentage. The electrode buffer was composed of SDS (1 g L^{-1}), glycine (14.4 g L^{-1}), and Tris (3.0 g L^{-1}). Electrophoresis was performed at constant amperage of 30 mA. Gels were fixed in an aqueous solution of 40 % ethanol and 5 % acetic acid. LOS bands were visualized by silver staining (Tsai and Frasch 1982).

Monosaccharides were analyzed as acetylated methyl glycosides. The LPS samples (1 mg) were treated with HCl/CH_3OH (1.25 M, 1 mL) and the methanolysis was performed as reported (Carillo et al. 2015). After acetylation the samples were analyzed on an Agilent Technologies gas chromatograph 6850A equipped with a mass selective detector 5973 N and a Zebron ZB-5 capillary column (Phenomenex, 30 m × 0.25 mm i.d., flow rate 1 ml min^{-1}, He as carrier gas), accordingly with the following temperature program: 140 °C for 3 min, 140 °C → 240 °C at 3 °C min^{-1}.

Results and discussion

Polystyrene supports selection

As previously reported (Papa et al. 2013b; Parrilli et al. 2015), *P. haloplanktis* TAC125 can be efficiently grown in biofilm condition using sterile 24-well flat-bottomed polystyrene plates. Therefore, the first step to assess a proper strategy to scale up the biofilm growth condition was the identification of suitable polystyrene supports for biofilm formation. *P. haloplanktis* TAC125 biofilm formation mostly occurs at air–liquid interface (Medigue et al. 2005), therefore we selected supports able to float and characterized by a large surface area per volume unit to promote microorganism adhesion. Two types of polystyrene supports were tested (Fig. 1).

To assess the ability of *P. haloplanktis* TAC125 to form biofilm on selected polystyrene supports, Antarctic bacterium was grown in BHI medium using glass wide-neck flasks in the presence of the supports A or B, at 15 and 4 °C in static condition for 96 h. *P. haloplanktis* TAC125 resulted to be able to form biofilm on both types of polystyrene supports and a qualitative measurement of formed biofilm was performed as described in "Materials and methods". The amount of biofilm formed on 5 type A supports or on 30 type B supports resulted to be almost the same (data not shown). This result is not surprising as type A supports have a mean exposed surface area of 12 cm^2, about six times higher than that of type B supports (mean exposed surface area 2.1 cm^2).

Moreover, supernatants of *P. haloplanktis* TAC125 grown in the presence of tested supports were found to be able to inhibit *S. epidermidis* O-47 biofilm formation (data not shown).

In view of a possible characterization of cells in biofilm, supports were treated with ultrasounds for three different exposure times (5, 10 and 15 min) to allow biofilm

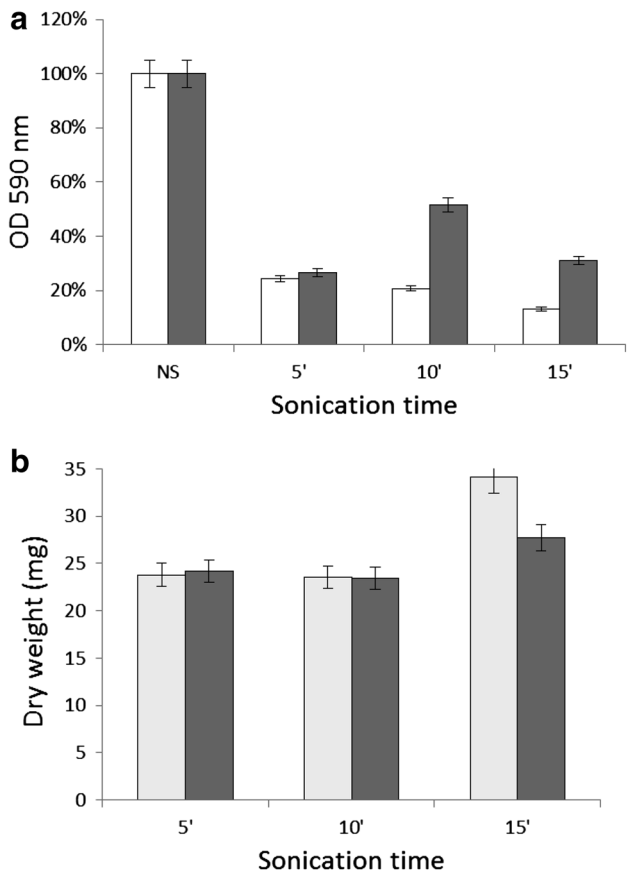

Fig. 2 Evaluation of *P. haloplanktis* TAC125 biofilm removal from polystyrene supports by sonication. Data reported are referred to treatment of five supports A (*white bar*) and 30 supports B (*gray bar*). **a** Crystal violet staining: supports A and B stained before (NS) and after sonication for biofilm mass evaluation. **b** Dry weight of cell pellets recovered from supports A and B after sonication

detaching and biomass recovery. Sonication was performed using an ultrasound bath at a constant ultrasound frequency of 37 kHz. After treatments, a qualitative analysis of residual biofilm biomass was determined using crystal violet staining (Fig. 2a).

For a quantitative evaluation, the dry weight of cell pellets recovered after sonication of the supports was determined (Fig. 2b). As shown in Fig. 2, both supports were suitable for biofilm formation, but support A allowed a better biofilm detachment and cell recovery after sonication. Therefore, we selected the support A for the set-up of biofilm cultivation of Antarctic bacterium in automatic bioreactor.

Set up of *P. haloplanktis* TAC125 biofilm cultivation in automatic bioreactor

To develop a scalable process for *P. haloplanktis* TAC125 growth in biofilm, Antarctic bacterium was grown in bioreactor in the presence of supports A, without stirring and keeping a low air inflow (see "Materials and methods"). The experimental conditions were chosen to obtain a sort of fluidized-bed reactor, in which the supports were homogeneously mixed and properly exposed to air–liquid interface, where *P. haloplanktis* TAC125 biofilm formation mostly occurs. Moreover, all parameter were selected to avoid the shearing detachment of biofilm. To attain these aims, several airflow conditions and ratios between medium volume and number of added supports were tested (data not shown). The best results were obtained using 33 supports A in 1 L of BHI medium using an airflow of 6 L h^{-1} without stirring.

To evaluate the molecule/s anti-biofilm production in the selected conditions, Antarctic bacterium was grown in BHI in a 3L-stirred tank reactor (Applikon ADI 1030) at two different temperatures (4 and 15 °C) in the presence of supports A. Corresponding supernatants were recovered for each condition after 24, 48, 72 and 96 h at 4 °C and after 24, 48 and 72 h at 15 °C, respectively, and their effect on *S. epidermidis* strain O-47 biofilm was evaluated (Fig. 3). Anti-biofilm effect is reported as percentage of residual biofilm after treatment in comparison with biofilm formation by untreated bacteria. As shown in Fig. 3, the anti-biofilm compound was produced at both tested temperatures and in all tested conditions. In particular, at 15 °C the greatest production occurs at 72 h of cultivation, while at lower temperature (4 °C) 96 h of incubation are needed to achieve the best anti-biofilm compound production (Fig. 3). This result is in perfect agreement with previously reported production conditions (Parrilli et al. 2015) where sterile 24-well flat-bottomed polystyrene devices were used. Therefore the proposed *P. haloplanktis* TAC125 biofilm cultivation is suitable for a larger scale production of anti-biofilm molecule and it allows to obtain an amount of cell-free supernatant sufficient for the future purification.

Comparison between *P. haloplanktis* TAC125 lipopolysaccharide extracted from planktonic and biofilm associated cells

It is widely accepted that the choice of biofilm lifestyle imposes profound physiological, metabolic, and morphological changes to planktonic growing bacteria. It is very likely that also Antarctic marine bacteria do not escape this general rule. However, only very limited information, essentially coming from the study of *P. haloplanktis* TAC125, are available on cold-adapted bacteria grown in sessile conditions. Therefore, the second—but not less important—aim of this work was to demonstrate that the optimized fermentation process allowed the easy recovery of *P. haloplanktis* TAC125 biomass grown in biofilm, ready to be used in comparative structural analyses with respect to

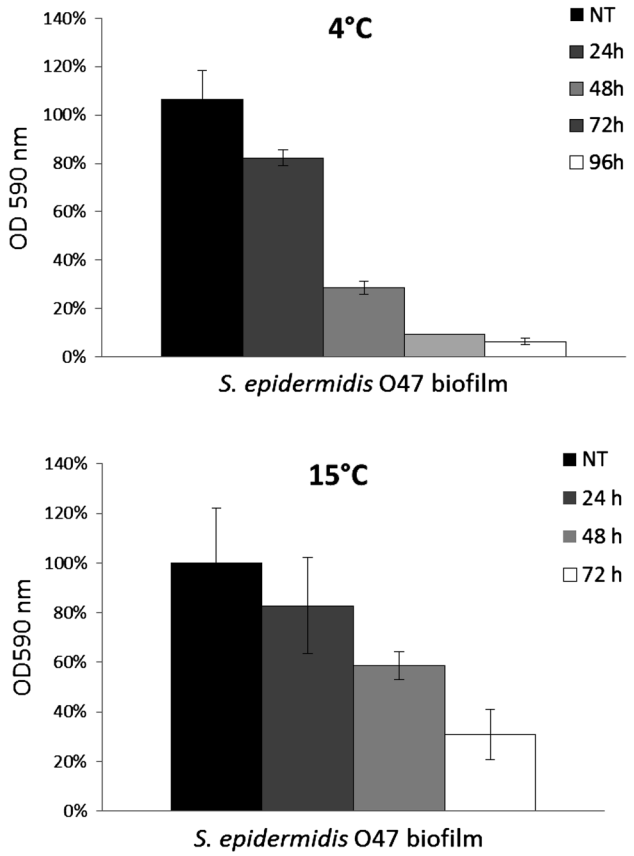

Fig. 3 *S. epidermidis* biofilm formation in the presence of *P. haloplanktis* TAC125 cell-free supernatants. *P. haloplanktis* TAC125 was grown in an automatic bioreactors in the presence of supports A at 4 °C and at 15 °C. Corresponding supernatants recovered at different times were tested. Data are reported as percentage of residual biofilm after the treatment. Each data point represents the mean ± SD of three independent experiments each performed at least in 3-replicates of three independent samples

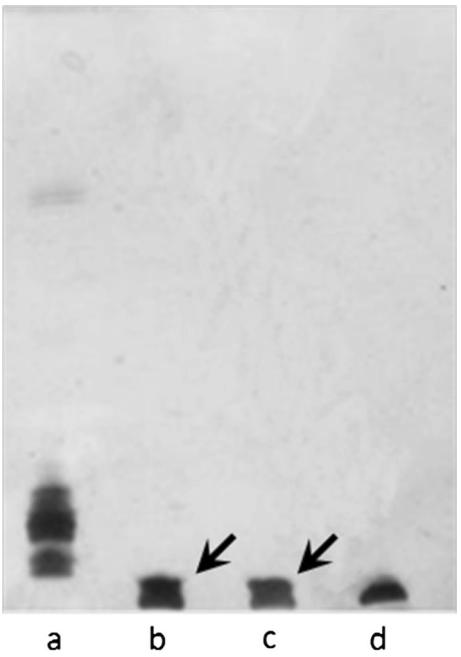

Fig. 4 16 % DOC-PAGE analysis of *Escherichia coli* O55:B5 LPS used as standard (**a**), *P. haloplanktis* TAC125, LPSb4 (**b**), LPSb15 (**c**), and *P. haloplanktis* TAC125 LPSp15 (**d**). The *arrows* indicate the LPS bands extracted from cells grown in biofilm

cells grown in planktonic conditions. Amongst the cellular components that were structurally characterized in planktonic *P. haloplanktis* TAC125 cells, LPS was the object of the comparative analysis. LPS is the major constituent of all Gram-negative outer membranes, and although their structure varies in response to certain environmental stimuli (Raetz and Whitfield 2002), few studies have investigated changes in structure of LPSs extracted from cells grown in biofilms (Hansen et al. 2007; Ciornei et al. 2010; Chalabaev et al. 2014). In particular, reversible loss of lipopolysaccharide O-antigen and alteration of lipid A have been observed in some *Pseudomonas aeruginosa* strains when grown in biofilm (Ciornei et al. 2010). Recently, Chalabaev and coworkers demonstrated that *Escherichia coli* LPS displays some modifications, in particular occurring in the lipid A moiety, when the molecules extracted from the two growth conditions were compared (Chalabaev et al. 2014). Here, we compared *P. haloplanktis* TAC125 LPS obtained during biofilm growth at 15 °C (LPSb15) and 4 °C (LPSb4) with previously characterized LPS extracted by planktonic bacteria grown at 15 °C (LPSp15) (Corsaro et al. 2001). Dried cells embedded in the biofilm were extracted by PCP procedure to isolate the crude LPS. The purified samples were analyzed by DOC-PAGE electrophoresis and visualized with silver nitrate staining (Fig. 4).

Electrophoresis analysis revealed that all the samples contain bands at low molecular masses indicating the rough nature of LPS. This latter revealed that *P. haloplanktis* TAC125, grown in static condition, produced a lipopolysaccharide with the same nature of the LPS previously described for planktonic condition (Corsaro et al. 2001). Nevertheless, a difference was observed between the samples grown in sessile (Fig. 4, lanes b and c) and planktonic conditions (Fig. 4, lane d). Indeed, while the planktonic LPS showed only one band, two bands were clearly detectable for LPS molecules extracted from cells grown in biofilm. A glycosyl analysis was then performed on the three different LPS samples (LPSb15, LPSb4 and LPSp15) to assess if this difference could be due to a modification in the sugar composition.

The analysis was carried out by GC–MS of the acetylated methyl glycosides and revealed for all the samples the presence of galactose (Gal), 2-amino-2-deoxymannose (ManN), 2-amino-2-deoxyglucose (GlcN) and heptose (Fig. S1-S3). Since all these monosaccharides were already

described to be the components of *P. haloplanktis* TAC125 LPS (Corsaro et al. 2001), it can be deduced that the glycosyl composition of LPSb15 and LPSb4 is the same of LPSp15 (Fig. S1–S3). Then, the detection of an additional band in LPS molecules extracted from cells grown in biofilm conditions suggests that the modification could be present in the lipid A moiety (Ciornei et al. 2010; Chalabaev et al. 2014). Further characterization of LPS from *P. haloplanktis* TAC125 biofilm cells by NMR and mass spectrometry will help in the identification of the punctual differences with respect to planktonic bacteria.

Conclusions

Cold-adapted marine bacteria represent an untapped reservoir of biodiversity endowed with an interesting chemical repertoire. A preliminary characterization of molecules isolated from cold-adapted bacteria revealed that these compounds display antimicrobial, anti-fouling and various pharmaceutically relevant activities (Bowman et al. 2005). *P. haloplanktis* TAC125 is considered to be one of the model organisms of cold-adapted bacteria and it resulted to be a source of bioactive metabolites of biotechnological relevance, such as anti-biofilm molecules (Papa et al. 2013b, Parrilli et al. 2015). In particular *P. haloplanktis* TAC125 produces this activity only when it is grown in sessile condition (Papa et al. 2013b), confirming that the study of biofilm lifestyle allows to analyze a different associated chemical diversity. The proposed strategy for a biofilm cultivation of the Antarctic bacterium in automatic bioreactor makes also possible the isolation of bioactive compounds produced at very low amount. Beside this "drug discovery" aspect, this paper reports for the first time the possibility to recover Antarctic bacteria biomass grown in biofilm, paving the way to study the features of a cold-adapted biofilm.

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Anti-Biofilm Activity of a Long-Chain Fatty Aldehyde from Antarctic *Pseudoalteromonas haloplanktis* TAC125 against *Staphylococcus epidermidis* Biofilm

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Staphylococcus epidermidis is a harmless human skin colonizer responsible for ∼20% of orthopedic device-related infections due to its capability to form biofilm. Nowadays there is an interest in the development of anti-biofilm molecules. Marine bacteria represent a still underexploited source of biodiversity able to synthesize a broad range of bioactive compounds, including anti-biofilm molecules. Previous results have demonstrated that the culture supernatant of Antarctic marine bacterium *Pseudoalteromonas haloplanktis* TAC125 impairs the formation of *S. epidermidis* biofilm. Further, evidence supports the hydrophobic nature of the active molecule, which has been suggested to act as a signal molecule. In this paper we describe an efficient activity-guided purification protocol which allowed us to purify this anti-biofilm molecule and structurally characterize it by NMR and mass spectrometry analyses. Our results demonstrate that the anti-biofilm molecule is pentadecanal, a long-chain fatty aldehyde, whose anti-*S. epidermidis* biofilm activity has been assessed using both static and dynamic biofilm assays. The specificity of its action on *S. epidermidis* biofilm has been demonstrated by testing chemical analogs of pentadecanal differing either in the length of the aliphatic chain or in their functional group properties. Further, indications of the mode of action of pentadecanal have been collected by studying the bioluminescence of a *Vibrio harveyi* reporter strain for the detection of autoinducer AI-2 like activities. The data collected suggest that pentadecanal acts as an AI-2 signal. Moreover, the aldehyde metabolic role and synthesis in the Antarctic source strain has been investigated. To the best of our knowledge, this is the first report on the identification of an anti-biofilm molecule form from cold-adapted bacteria and on the action of a long-chain fatty aldehyde acting as an anti-biofilm molecule against *S. epidermidis*.

Keywords: anti-biofilm, *Staphylococcus epidermidis*, long fatty acid aldehyde, quorum sensing, *Pseudoalteromonas haloplanktis* TAC125

INTRODUCTION

Staphylococcus epidermidis (*S. epidermidis*) is now being recognized as an important opportunistic pathogen that can cause significant problems when breaching the epithelial barrier, especially during the biofilm-associated infection of indwelling medical devices (Otto, 2008; Arciola et al., 2012). Most diseases caused by *S. epidermidis* are of a chronic character and occur as device-related infections (such as intravascular catheter or prosthetic joint infections) and/or their complications (Dohar et al., 2009; Artini et al., 2013). Implantations of medical devices are steadily increasing and thus heightening the relevance of *S. epidermidis* as a human pathogen. The ability of *S. epidermidis* to adhere on both eukaryotic cells and abiotic surfaces and to form biofilm is an essential virulence factor that contributes to the chronicization of infections particularly difficult to eradicate. Biofilms are sticky, surface-attached agglomerations of bacteria that are embedded in an extracellular matrix and provide protection for single cells from antibiotics and mechanisms of host defense (Epstein et al., 2012).

S. epidermidis infections are estimated to reach 250,000 cases per year in the USA with a mortality rate of up to 25%. The interest in the development of innovative approaches for the prevention and treatment of staphylococcal adhesion and biofilm formation capabilities has therefore increased. A viable approach should target the staphylococcal adhesive properties without affecting the bacterial viability in order to avoid the rapid appearance of escape mutants (Papa et al., 2015).

From another point of view, biofilm could be considered as a source of novel drugs. Indeed, the specific environmental conditions prevailing within biofilms may induce a profound genetic and metabolic rewiring of biofilm-dwelling bacteria and therefore may allow the production of metabolites different from those obtained in the planktonic condition. Indeed, many bacterial biofilms secrete molecules such as quorum sensing signals (Ni et al., 2009), surfactants (Kiran et al., 2010), enzymes (Kaplan, 2010), and polysaccharides (Valle et al., 2006; Qin et al., 2009) that act by regulating the biofilm architecture or mediating the release of cells from biofilms during the dispersal stage of the biofilm life cycle (Kaplan, 2010). Furthermore, the production of extracellular molecules that degrade adhesive components in the biofilm matrix is a basic mechanism used in the biological competition between phylogenetically different bacteria (Kaplan, 2010).

Starting from the idea that the production of an anti-biofilm compound might result from the selective pressure exerted on bacteria living in oligotrophic and extreme environments like Antarctica, cold-adapted marine bacteria have been investigated as a possible source of anti-biofilm molecules. Previous papers (Klein et al., 2011; Papa et al., 2013b, 2015; Parrilli et al., 2015, 2016; Sun et al., 2015) have confirmed that cold adapted bacteria represent an untapped reservoir of biodiversity able to synthesize a broad range of potentially valuable bioactive compounds (Klein et al., 2011), including anti-biofilm molecules (Papa et al., 2013b; Parrilli et al., 2015, 2016).

In particular, the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 (*P. haloplanktis* TAC125), when grown with a sessile life-style, proved to be able to secrete an anti-biofilm molecule capable of inhibiting *S. epidermidis* biofilm formation (Papa et al., 2013b; Parrilli et al., 2015, 2016). This anti-biofilm compound impairs biofilm development and disaggregates the mature biofilm of *S. epidermidis*, even in dynamic conditions, without affecting the bacterial viability, showing that its action is specifically directed against biofilm (Papa et al., 2013b; Parrilli et al., 2015, 2016).

In this research study the anti-biofilm molecule produced by *P. haloplanktis* TAC125 has been purified and characterized. In detail, a *P. haloplanktis* TAC125 biofilm cultivation in automatic bioreactor (Parrilli et al., 2016) has been used to obtain a cell-free supernatant in a sufficient amount to purify and characterize the anti-biofilm molecule. A suitable purification protocol was developed using an activity-guided fractionation strategy. The structure of the purified molecule, obtained by NMR and mass spectrometry, corresponded to pentadecanal, a long chain fatty aldehyde. Several experiments were performed to assess the chemical features responsible for its activity, by testing chemical analogs differing in the length of the aliphatic chain and in their functional group properties. Therefore, the anti-biofilm activity of different long chain alcohols and aldehydes on *S. epidermidis* biofilm was evaluated. As for the anti-biofilm mode of action, the results reported demonstrated that the long-chain fatty aldehyde works as an AI-2 signal, suggesting that it may interfere with the *S. epidermidis* quorum sensing system. Moreover, in this paper we have investigated the role of pentadecanal in the metabolism of the source strain, in order to clarify if the molecule regulates biofilm development also in the Antarctic bacterium.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Bacterial strains used in this work were: *S. epidermidis* O-47 isolated from clinical septic arthritis and kindly provided by Prof. Gotz (Heilmann et al., 1996); *S. epidermidis* RP62A reference strain isolated from infected catheter (ATCC collection no. 35984); *P. haloplanktis* TAC125 (Médigue et al., 2005) collected in 1992 from seawater near French Antarctic Station Dumont d'Urville. Bacteria were grown in Brain Heart Infusion broth (BHI, Oxoid, UK) and in Tryptic Soy Broth (TSB, Oxoid, UK). Biofilm formation was assessed in static condition while planktonic cultures were performed under vigorous agitation (180 rpm).

Vibrio harveyi BB170 (luxN::Tn5kan) (ATCC®BAA-1117™) was grown as suggested by suppliers, cultivated in the Autoinducer Bioassay (AB) Medium (Taga, 2005) and incubated aerobically on a rotary shaker at 30°C under vigorous agitation (180 rpm).

All strains were maintained at −80°C in cryovials with 15% of glycerol.

Bioluminescence Assay

To screen the ability of the compounds to interfere with QS, the test products were serially diluted in the AB medium using sterile, black, clear-bottom 96-well microtiter plates (Greiner Bio-one

#655090) as assay platform. An overnight culture of *V. harveyi* BB170 was diluted 1:100 in fresh AB medium and incubated at 30°C up to a visible increase in basal bioluminescence. 100 μl of the diluted culture were dispensed inside each well in the microtiter plate starting from a cellular density of about 0.05 $(OD)_{600nm}$; 100 μl of opportune dilutions of pentadecanal in AB medium were added into each well. Luminescence and $(OD)_{600nm}$ were monitored every 15 min over 18 h with a high-performance multimode plate reader (GloMax® Discover System, Promega) in order to correlate the effects of the products on both the growth and bioluminescence kinetics. Data are reported as light units (LU).

Large Scale Biofilm Cultivation of *P. haloplanktis* TAC125 in Automatic Bioreactor for the Anti-Biofilm Compound/s Production

P. haloplanktis TAC125 bacterial culture was grown in BHI medium in a Stirred Tank Reactor 3 L fermenter (Applikon) connected to an ADI-1030 Bio Controller (Applikon) with a working volume of 1 L. The bioreactor was equipped with the standard pH-, pO_2-, level-, and temperature sensors for the bioprocess monitoring. To allow the biofilm formation, autoclaved solid polystyrene supports were added into the bioreactor (33 supports in 1 L). The culture was carried out at 15°C for 48 h, or at 4°C for 96 h, in aerobic conditions using airflow of 6 L/h, without stirring. After incubation, supports were removed from the supernatant and the adherent cells were recovered by sonication as previously described (Parrilli et al., 2016). In parallel the supernatant was recovered and further separated from cells by centrifugation at 13,000 rpm, sterilized by filtration through membranes with a pore diameter of 0.22 μm and stored at 4°C until use.

P. haloplanktis TAC125 Growth in Aerobiosis and Microaerobiosis

Batch cultivations were performed in a computer-controlled bioreactor (Sixfors System, Infors) equipped with control units for pH and temperature, an oxygen sensing electrode measuring the solution oxygen pressure, and mechanical stirring (200 rpm) at 4°C. Each fermentation unit, filled with medium and sterilized by autoclaving, was equilibrated at process temperature. In order to allow oxygenation of the medium, the stirring speed was set up at the maximum value to be used during the fermentation, and then sterile air supply was switched on. The system was left to stabilize for at least 30 min to guarantee the saturation of the medium with air. In these conditions 100% of measured oxygen pressure was assigned. The zero-point set calibration was performed by saturating the medium with sterile nitrogen gas. Under aerobiosis conditions, measured oxygen pressure was maintained always above 20% by modifying stirring speed and aeration rate. In microaerobiosis (measured oxygen pressure 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.

Molecular Methods and Reagents Suppliers

Standard methods were employed for DNA manipulation and isolation, amplification by PCR, and DNA sequencing. 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.

Vector pVSb0219 and *P. haloplanktis* TAC125-b0219 Mutant Construction

PCR was employed to amplify a DNA fragment of PSHAb0219 gene. *P. haloplanktis* TAC125 genomic DNA was used as PCR template and two primers were designed to amplify a 275 bp-long region of the PHSAb0219 gene and to introduce an *Eco*RI and a *Sph*I site (Oligo b0219*Eco*RIfw 5′-CTATGAATTCAAGAAGATATTTACGAGC-3′ and Oligo b0219*Sph*Irv 5′-AATACCCGCATGCCGTTGGTGCC-3′). The amplified DNA fragment was digested by *Eco*RI and *Sph*I, and inserted into the pVS plasmid (Giuliani et al., 2012) corresponding site, thus obtaining the pVSb0219 vector. The vector was mobilized by intergeneric conjugation into *P. haloplanktis* TAC125 cells and insertion mutants were screened on plates at 4°C containing carbenicillin (30 μg/ml) as selection agent.

RNA Preparation and RT-PCR

Total RNA was isolated from 500 μl aliquots of *P. haloplanktis* TAC125 cells recovered from both planktonic growth and, after sonication of supports, biofilm growth as reported in literature (Rippa et al., 2012). RNA was reverse transcribed using SuperScript II RNase H- reverse transcriptase (Invitrogen) according to the manufacturer's instructions. PHSAb0219 was reverse transcribed starting from total RNA (∼5 μg) using as primer-specific oligonucleotide designed on the 3′ region of the gene (RT-PCR-Rev 5′-AATACCCGCATGCCGTTGGTGCC-3″). The reaction mix was denatured at 65°C for 5 min and reverse transcribed at 45°C for 50 min. cDNA (275 bp), the cDNA was amplified using primers Oligo b0219 *EcoRI*fw 5′-CTATGAATTCAAGAAGATATTTACGAGC-3′ and Oligo b0219 *Sph*Irv 5′-AATACCCGCATGCCGTTGGTGCC-3′, and Taq polymerase (Promega, Madison, WI, USA) according to the manufacturer's instructions. The reaction mix 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). For each reverse transcriptase amplification, an additional PCR reaction on DNA-free total RNA was performed as control, in order to exclude false positive signals (Figure S3).

Determination of Minimal Inhibitory Concentration (MIC)

MIC was performed according to the guidelines of Clinical Laboratory Standards Institute. Pentadecanal was added directly from mother stock and solutions were prepared by two-fold

serial dilutions. A total of 5 concentrations were used within the 1.6–0.1 μg/ml range. Experiments were performed in quadruplicate. The MIC was determined as the lowest concentration at which the observable bacterial growth was inhibited. No inhibition of the bacterial growth was evidenced at testedconcentrations.

BIOFILM FORMATION OF STAPHYLOCOCCI

Static Biofilm Assay

The quantification of *in vitro* biofilm production was based on the method described by Christensen with slight modifications (Artini et al., 2013; Papa et al., 2013a). Briefly, the wells of a sterile 48-well flat-bottomed polystyrene plate were filled with 400 μl of BHI or TSB medium. 1/100 Dilution of overnight bacterial cultures was added into each well. The first row contained the untreated bacteria, while each of the remaining rows contained serial dilutions of the supernatant (SN) starting from 1:2. The plates were incubated aerobically for 24 h at 37°C.

The biofilm formation was measured using crystal violet staining. After treatment, planktonic cells were gently removed; each well was washed three times with PBS and patted dry with a piece of paper towel in an inverted position. To quantify the biofilm formation, each well was stained with 0.1% crystal violet and incubated for 15 min at room temperature, rinsed twice with double-distilled water, and thoroughly dried. The dye bound to adherent cells was solubilized with 20% (v/v) glacial acetic acid and 80% (v/v) ethanol. After 30 min of incubation at room temperature, (OD) was measured at 590 nm to quantify the total biomass of biofilm formed in each well. Each data point is composed of three independent experiments, each performed at least in 3-replicates.

Dynamic Biofilm Assay

To continuously monitor the biofilm development in dynamic condition, we utilized a BioFlux 2,000 microfluidic system (Fluxion Biosciences Inc., San Francisco, CA), which allows the acquisition of microscopic images over time using the experimental protocol previously set up (Iebba et al., 2014). Each flow channel connects to an input well (inlet) and an output well (outlet) on the plate. To grow the biofilm in the BioFlux system, the channels were first primed. We have filled the outlet with 100 μl of sterile distilled water and set the flow at a share setting of 1 dyne/cm^2 for 2 min. Coating with 100 μl of 10 μg/ml fibronectin was made for 2 min at 1 dyne/cm^2. The fibronectin binding was performed for 30 min without flow. After priming, fibronectin was aspirated from the output wells and replaced with 100 μl of fresh overnight cultures diluted to an $(OD)_{600}$ of 0.8. The channels were seeded by pumping from the output wells to the input wells at 2.0 dyne/cm^2 for 4 sec. Bacterial adhesion was performed for 30 min at 37°C without flow. 2.0 mL of BHI was added to the input well and pumped at 1 dyne/cm^2 for 12 h. We used two inlet wells; in the first well we added only BHI. In the second well we added pentadecanal at a concentration of 120 μg/ml. Bright-field images were taken at 40X magnification at 1-min intervals for a total of 720 time points.

Anti-Biofilm Molecule Purification and Identification

As reported in the previous paper (Parrilli et al., 2016), *P. haloplanktis* TAC125 supernatant deriving from sessile growth was dialyzed, and the permeated water (2 g) was then purified on a gel-filtration column (Biorad, Biogel P-2) eluted with MilliQ water. The active fractions were collected and fractionated on a C_{18} reverse phase column (Sigma, 30 mL, 40 × 0.5 cm, fraction volume 4 mL), eluted with CH_3CN:H_2O ranging from 10 to 95% of CH_3CN. The fraction resulted to be active (**S**) was analyzed on a Agilent Technologies gas chromatograph 6850A equipped with a mass selective detector 5973N and a Zebron ZB-5 capillary column (Phenomenex, 30 m × 0.25 mmi.d., flow rate 1 cm^3/min, He as carrier gas), by using the following temperature program: 150°C for 3 min, from 150 to 300°C at 15°C/min, at 300°C for 5 min.

NMR Spectroscopy

NMR spectra were performed by using a Bruker Avance-DRX 600 MHz spectrometer equipped with a cryoprobe. ^{1}H-^{1}H DQF-COSY, ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C DEPT-HSQC, and ^{1}H-^{13}C HMBC experiments were recorded at 298 K in $CDCl_3$. The mixing time for TOCSY experiment was 100 ms.

Fatty Aldehydes Chemical Synthesis

C14-C16 aldheydes were purchased from TCI. All the other tested compounds were synthesized starting from the corresponding alcohols (Sigma). 1- heptadecanol (30 mg, 0.12 mmol) was charged into a 10 mL round-bottom flask equipped with a magnetic stir bar. The solid was then dissolved in toluene (2 mL) at 20°C, and an aqueous solution of sodium bicarbonate (0.2 g, 2.38 mmol in 2 mL of deionized water) was prepared and charged into the toluene slurry. Solid iodine (2.0 eq., 60 mg, 0.24 mmol) was then charged to the alcohol followed by solid TEMPO (0.1 eq., 1.87 mg, 12.0 μmol; Miller and Hoerrner, 2003). The reaction mixture was then left under stirring for 16 h at 20°C. The batch was cooled to 5°C, diluted with ethyl acetate (2 mL), and quenched at 5°C by adding an aqueous solution of sodium sulfite 10% (312 mg sodium sulfite in 2 mL of deionized water). The quenched reaction mixture was transferred into a separatory funnel, rinsed with additional ethyl acetate (10 mL) and deionized water (10 mL), and the aqueous layer was cut away. The organic layer was then washed with 10 mL of saturated aqueous sodium bicarbonate, followed by 10 mL of brine. The organic layer was then dried over sodium sulfate, filtrated, and concentrated in vacuum to a volume of 4 mL. Then, the solution was completely dried under a stream of argon, to give the aldehyde with a 95% yield.

The same procedure was then utilized for the alcohols 1-octadecanol, 1-nonadecanol, and 1-eicosanol for the obtainment of the corresponding aldehydes.

Statistics and Reproducibility of Results

The data reported were statistically validated using the Student's *t*-test comparing the mean absorbance of treated and untreated samples. The significance of differences between the mean

absorbance values was calculated using a two-tailed Student's *t*-test. A $p < 0.05$ was considered significant.

Bacterial Viability and Biofilm Thickness Determined by Confocal Laser Scanning Microscopy

For confocal microscopy biofilms were formed on Nunc™ Lab-Tek® 8-well Chamber Slides (n° 177445; Thermo Scientific, Ottawa, ON, Canada). Briefly, overnight cultures of *S. epidermidis* O-47 and RP62A grown in BHI were diluted and inoculated into each well of the chamber slide to a cell concentration of about 0.001 $(OD)_{600nm}$. The bacterial cultures were incubated at 37°C for 20 h in presence of pentadecanal (100 μg/ml) in order to assess its anti-biofilm activity and its influence on cell viability.

The biofilm cell viability was determined with the FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (Molecular Probes, Invitrogen, Carlsbad, California) following the manufacturer's instructions. After rinsing with filter-sterilized PBS, each well of the chamber slide were filled with 300 μl of working solution of fluorescent stains, containing the SYTO® 9 green fluorescent nucleic acid stain (10 μM) and Propidium iodide, the red-fluorescent nucleic acid stain (60 μM), and incubated for 20–30 min at room temperature, protected from light. All excess stain was removed by rinsing gently with filter-sterilized PBS.

All microscopic observations and image acquisitions were performed with a confocal laser scanning microscope (CLSM; LSM700-Zeiss, Germany) equipped with an Ar laser (488 nm), and a He-Ne laser (555 nm). Images were obtained using a 20X/0.8 objective. The excitation/emission maxima for these dyes are ∼480/500 nm for SYTO® 9 stain and 490/635 nm for propidium iodide. Z-stacks were obtained by driving the microscope to a point just out of focus on both the top and bottom of the biofilms. Images were recorded as a series of.tif files with a file-depth of 16 bits. For each condition, two independent biofilm samples were used.

RESULTS

Anti-Biofilm Production and Purification

We have previously (Papa et al., 2013b; Parrilli et al., 2015) demonstrated that the cell-free supernatant of the Antarctic bacterium *P. haloplanktis* TAC125 inhibits *S. epidermidis* biofilm formation and that the bacterium produces the active molecule only when it is grown in a sessile condition (Papa et al., 2013b). To obtain a sufficient amount of cell-free supernatant, we adopted a recently described (Parrilli et al., 2016) biofilm cultivation strategy for *P. haloplanktis* TAC125. This consists in a fluidized-bed reactor fermentation in the presence of floating polystyrene supports used to increase the biofilm formation (Parrilli et al., 2016). In this work the Antarctic bacterium has been grown in the presence of floating polystyrene supports in BHI in a 3 L tank reactor at 4°C without stirring, using an airflow of 6 L h^{-1} (see materials and Methods Section). The obtained cell-free supernatant was recovered after 96 h and separated from the floating polystyrene supports and the cells by a centrifugation at 13,000 rpm. Subsequently the supernatant was sterilized by filtration through membranes with a pore diameter of 0.22 μm, and then underwent dialysis treatment using a semipermeable membrane with a cut-off of 3.5 KDa. The permeate product after lyophilization was fractionated on a Biogel P-2 column and the anti-biofilm activity of each fraction was evaluated (data not shown). The fraction endowed with anti-biofilm activity on *S. epidermidis* O-47 was further purified on a C_{18} reverse phase column eluted with acetonitrile/water. Several fractions (A–S) were collected, and the anti-biofilm activity of each fraction was tested as reported in **Figure 1A**. Fraction S, eluted with 95% acetonitrile, showed the highest inhibitory activity on *S. epidermidis* biofilm. The analysis of the ^{1}H NMR spectrum of this fraction revealed a signal at δ 9.77 ppm, which immediately suggested the presence of an aldehyde (MOLBASE.COM CAS. No. 2765-11-9). This was believed to be aliphatic, due to the presence of intense signals in the range between δ 0.1–2.5 ppm (data not shown). To confirm this hypothesis, and to check the purity of the sample, a GC-MS analysis was performed (**Figure 1B**). The chromatogram clearly indicated two different compounds, named **A** and **B**, the EI mass spectra of which are reported (**Figures 1C,D**). Both spectra showed signals at *m/z* 43, 57, 71, and 85, that are typical of a straight chain hydrocarbon backbone, thus confirming the aliphatic character of the two molecules. As this type of fragmentation can be attributed to a broad range of compounds, a research in the NIST library was required. The results indicated with a high score a 2-tridecanone and a pentadecanal for **A** and **B**, respectively. As the inactive fraction Q mainly contains the ketone whereas fraction S shows a higher content of the pentadecanal, we concluded that the activity should be assigned to the aldehyde. To exclude the possibility that the mass spectrum of **B** could belong to an aldehyde with a short difference in the carbon chain length, we injected into the gas chromatograph commercial C14-C16 aldehydes. The retention time of compound **B** proved to correspond to that of pentadecanal.

To confirm the data obtained from GC-MS, the **S** fraction was analyzed by 2D-NMR spectroscopy. In particular, two-dimensional ^{1}H-^{1}H DQF-COSY (double quantum-filtered correlation spectroscopy), ^{1}H-^{1}H TOCSY (total correlation spectroscopy), ^{1}H-^{13}C DEPT-HSQC (distortionless enhancement by polarization transfer-heteronuclear single quantum coherence), and ^{1}H-^{13}C HMBC (heteronuclear multiple bond correlation) experiments were performed. The proton at δ 9.79 ppm, that clearly indicated an aldehyde functional group on the molecule, displayed a correlation with a carbon at δ 203.1 ppm (**Figure 2A**; data not shown). In turn, this carbon showed a long range scalar connectivity with protons at δ 2.43 ppm (CH_2,C2; **Figure 2B**). Starting from this proton signal, the correlations revealed in the COSY and TOCSY spectra allowed us to attribute all the CH_2(C3-C14) that constitute the aliphatic chain. The last signal, attributable to the CH_2C14 (δH/C 1.36/22.9), showed a long-range cross-peak, with the methyl group at δH/C 0.9/13.6 ppm (**Figure 2C**).

A confirmation of the correct assignment of the pentadecanal structure was obtained by testing the commercial product on

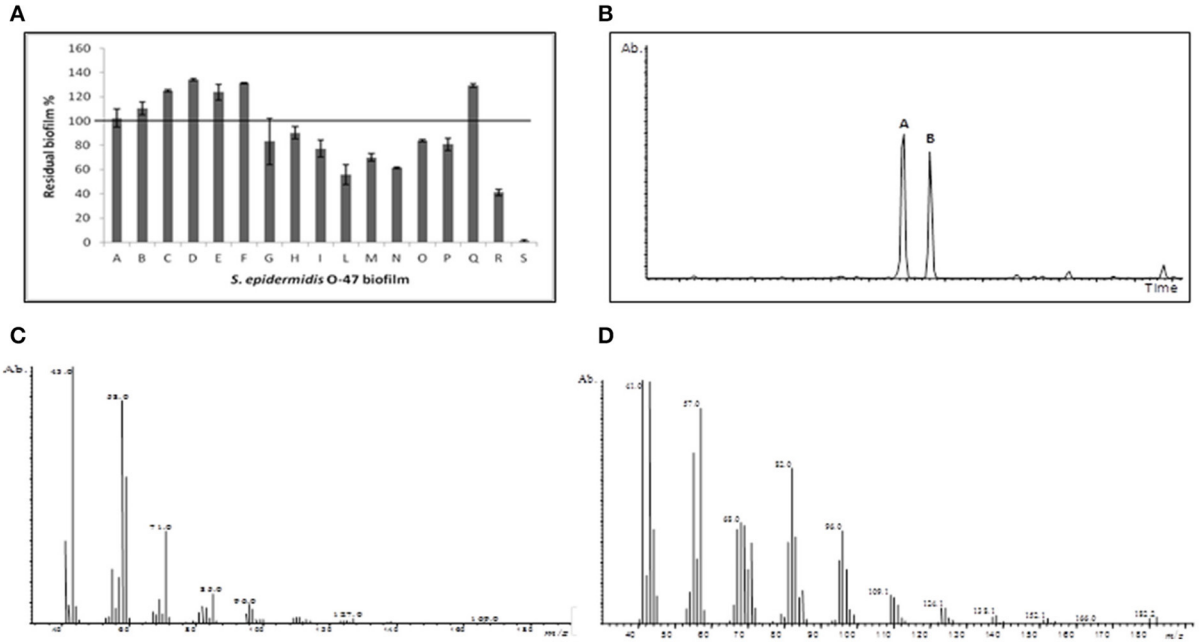

FIGURE 1 | Anti-biofilm assay and identification by GC-MS analysis. (A) The anti-biofilm activity of different fractions obtained from reverse phase C_{18} column. The fraction S eluted with 95% acetonitrile showed the highest inhibition activity. **(B)** GC-MS chromatogram of the fraction S. **(C)** Mass spectrum of compound A, and **(D)** compound B.

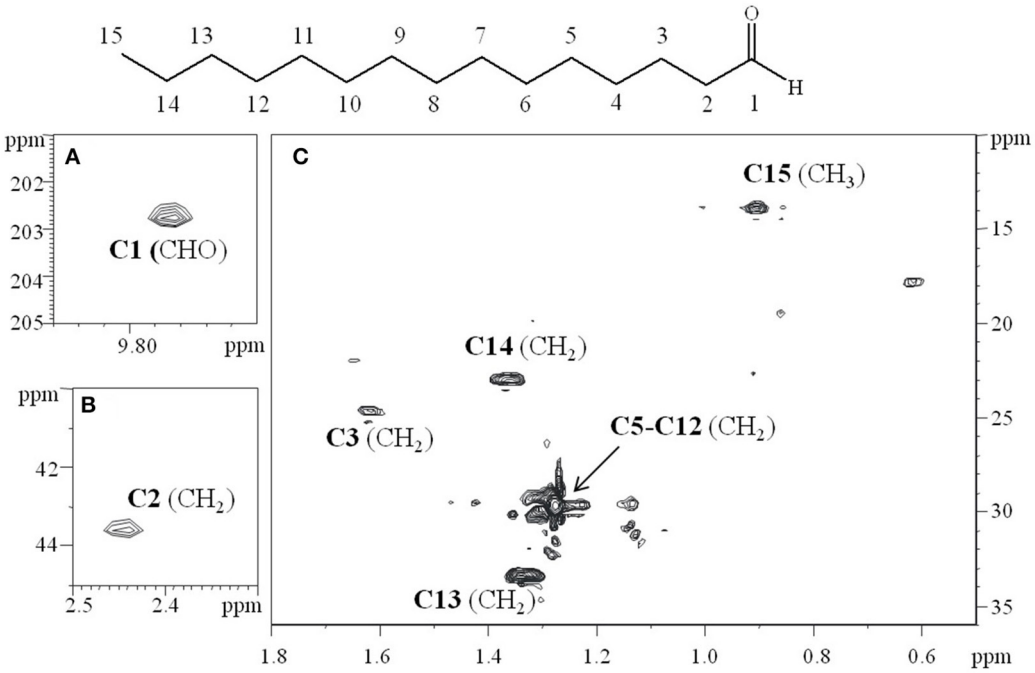

FIGURE 2 | Relevant sections of ^{1}H-^{13}C HSQC spectrum, recorded in $CDCl_3$ at 298K at 600 MHz. (A) The correlation at δ 9.79/203.1 ppm, clearly indicated the aldehydic functional group of the molecule. **(B)** The carbon signal at δ 203.1 ppm is in turn correlated in the HMBC experiment (data not shown), with the protons at δ 2.43 ppm (CH_2 C2). **(C)** The attribution of the aliphatic chain (C3–C15).

S. epidermidis biofilm, that was found to be active (**Figure 3A**). Consequently, commercial pentadecanal was used for all further experiments.

Anti-Biofilm Activity and Specificity of Pentadecanal on *S. epidermidis* Biofilm

Once the structure of the anti-biofilm molecule had been identified, the first step was to confirm the absence of any antimicrobial activity of pentadecanal on the staphylococcal reference strains by determining the minimum inhibitory concentration. The data obtained indicated no bacteriostatic and no bactericidal activity within the range of concentrations explored (the highest concentration explored was 1,6 mg/ml). Preliminary experiments were also carried out to assess the effects of the molecule on the planktonic growth rate of *S. epidermidis*. The results obtained showed that pentadecanal did not affect the staphylococcal duplication rate. Bacterial growth curves were superimposable both in the presence and absence of pentadecanal (data not shown).

The pentadecanal activity on biofilm formation was assessed on two different *S. epidermidis* strains (**Figure 3A**), according to previously reported data that demonstrated the cell-free

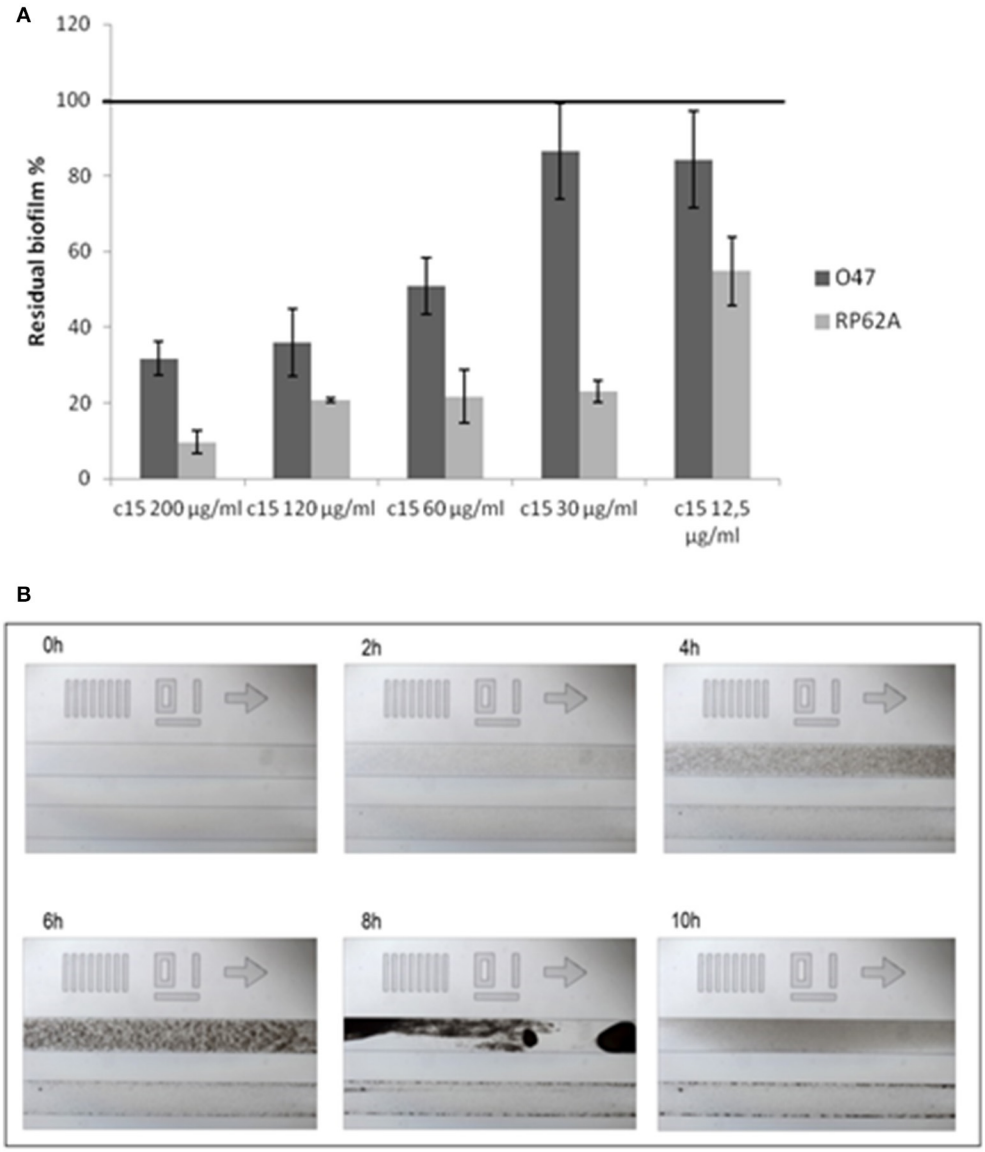

FIGURE 3 | Pentadecanal anti-biofilm activity on *S. epidermidis* biofilm formation. (A) The effect of pentadecanal at different concentrations on biofilm formation of *S. epidermidis* O-47 and *S. epidermidis* RP62A. The data are reported as percentages of residual biofilm. Each data point is composed of three independent samples. **(B)** Biofilm formation of *S. epidermidis* O-47 in a BioFlux system in the presence of pentadecanal. Each image contains two channels: the bottom channel is the pentadecanal-treated sample and the top channel is the control. Bright-field microscopic images were collected at 1-min intervals. The images presented were taken from the complete set of 720 images taken at 40x magnification.

supernatant activity of *P. haloplanktis*TAC125 against the *S. epidermidis* O-47 and *S. epidermidis* RP62A biofilms (Papa et al., 2013b). As expected, the pentadecanal inhibitory effect was clearly dose-dependent with an efficacy higher than 50% already at 60 μg/ml (50.9% residual biofilm for *S. epidermidis* O-47 and 21.8% for *S. epidermidis* RP62A, respectively). All previously described experiments were performed in BHI medium. The pentadecanal anti-biofilm activity was also tested in TSB medium to verify its effectiveness in function of growth medium composition. The results demonstrated that this latter did not influence the anti-biofilm ability of pentadecanal (untreated bacteria 0.761 ± 0.137; treated bacteria 0.152 ± 0.020).

The pentadecanal anti-biofilm efficacy was also tested in dynamic conditions with the BioFlux system, a microfluidic device that precisely controls the flow of growth medium between two interconnected wells of a microtiter plate. By positioning the channel connecting the two wells over a window accessible for viewing by microscopy, biofilm growth can be monitored in a time-course assay in which images are collected at 1-min intervals. In **Figure 3B** selected images of the BioFlux analysis are reported, showing the biofilm development of *S. epidermidis* O-47 at different times, in the absence or in the presence of pentadecanal (top and bottom lanes of each panel, respectively). The bacteria were seeded in both channels visible in each frame and after 30 min the flow was applied. The images collected showed an initial rapid growth of the bacteria, resulting in a confluent "lawn" of cells that was followed by a period of detachment. Pentadecanal clearly impaired the biofilm formation confirming the results obtained in the static system.

The pentadecanal effect on *S. epidermidis* O-47 biofilm was further investigated by confocal laser scanning microscopy (**Figures 4A–D**). CLSM was used to analyse the biofilm structure and viability, as shown in **Figures 4B,D**. In the presence of pentadecanal, as expected, the biofilm thickness decreased significantly with a notable alteration in the architecture. This effect was evident both on *S. epidermidis* O-47 biofilm and on *S. epidermidis* RP62A biofilm. Moreover, the viability of cells encapsulated in the biofilm in the presence and in the absence of pentadecanal was evaluated by live/dead staining. As shown in **Figure 4C**, cells exposed to pentadecanal were alive (green indicates viable cells while red indicates dead cells) confirming that the long-chain fatty aldehyde had no bactericidal activity on the *S. epidermidis* cells living in the biofilm.

In order to investigate the chemical features (the length of the chain and the functional group nature) that are essential for the anti-biofilm activity, the effect of similar aldehydes and corresponding alcohols, characterized by different lengths of the aliphatic chain in the range from C-14 to C-20, were analyzed and the results are summarized in **Figure 5**. The data are reported as percentages of residual biofilm after the treatment in comparison

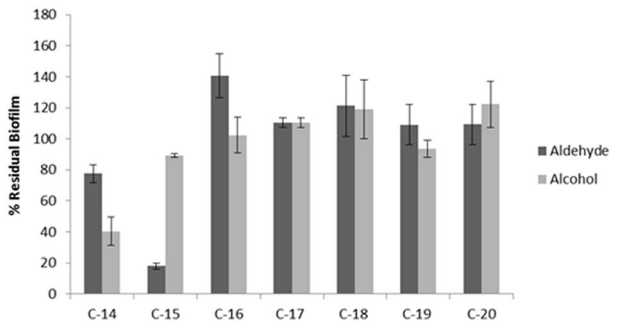

FIGURE 5 | Anti-biofilm activity of different aldehydes and alcohols on *S. epidermidis* O-47. The anti-biofilm activity of different long-chain aldehydes and alcohols on *S. epidermidis* O-47. The data are reported as percentages of residual biofilm. Each data point is composed of four independent samples.

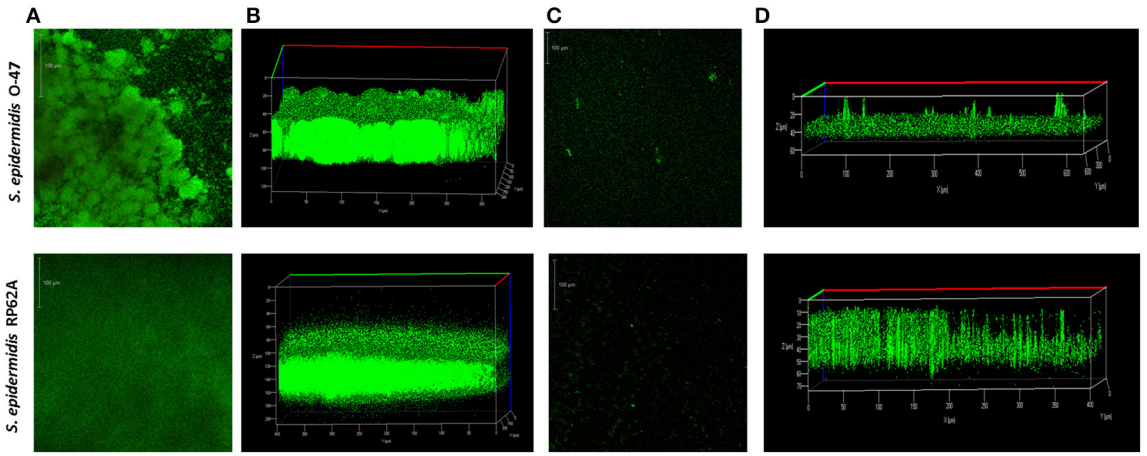

FIGURE 4 | CLSM of *S. epidermidis* O-47 and *S. epidermidis* RP62A biofilms in the presence and absence of pentadecanal. (A) CLSM of *S. epidermidis* O-47 and *S. epidermidis* RP62A biofilms in BHI medium without pentadecanal and **(C)** with pentadecanal 100 μg/ml. The bacteria were grown in chamber slides for 20 h and then stained with LIVE/DEAD reagents. The green fluorescence (SYTO9) indicates viable cells PI and the red fluorescence (PI) indicates dead cells. **(B)** Z-stack analysis of *S. epidermidis* O-47 and RP62A biofilms without pentadecanal. **(D)** Z-stack analysis of *S. epidermidis* O-47 and RP62A biofilms treated with 100 μg/ml pentadecanal.

with the untreated biofilm of *S. epidermidis* O-47. Our results clearly showed that, except for a partial reduction observed after treatment with the alcohol at the C-14 chain, the anti-biofilm activity was limited to pentadecanal.

Pentadecanal As an AI-2 Signaling Molecule in the LuxS/AI-2 QS System

To test whether the anti-biofilm activity displayed by pentadecanal is correlated with a modulation/activation of the AI-2 quorum sensing system, the effect of pentadecanal was investigated on a *V. harveyi* BB170 strain (luxN::tn5Kan), a mutant sensor strain that responds only to AI-2 autoinducers. The *V. harveyi* BB170 bioluminescence was monitored (**Figure 6**) after adding two different concentrations of pentadecanal. At a low concentration (12.5 μg/ml) the pentadecanal was able to increase the *V. harveyi* bioluminescence (**Figure 6**), indicating that the *V. harveyi* LuxS/AI-2 QS system responds to pentadecanal. At a higher concentration (200 μg/ml) pentadecanal reduced the bioluminescence (**Figure 6**). In this condition, pentadecanal probably inhibits the *V. harveyi* luciferase. Since the *V. harveyi* luciferase physiological substrate is tetradecanal (Ulitzur and Hastings, 1979), the bioluminescence in *V. harveyi* BB170 was also monitored after adding two different concentrations of tetradecanal (Figure S1). As expected, a higher concentration (200 μg/ml) of tetradecanal reduced the *V. harveyi* bioluminescence due to the luciferase substrate inhibition, at low concentrations tetradecanal had no effect on the *V. harveyi* bioluminescence (Figure S1).

The Role of Pentadecanal in the *P. haloplanktis*TAC125 Physiology

To assess the role of pentadecanal in the *P. haloplanktis*TAC125 physiology, we investigated the conditions necessary for pentadecanal production and the possible synthetic pathway involved.

The *in silico* analysis of the *P. haloplanktis*TAC125 genome (EMBL under accession nos. CR954246 and CR954247). did not allow us to identify the genes coding for enzymes known to be able to synthesize fatty aldehydes, like fatty acid reductase (EC 1.2.1.50 (Gahan, 2012) or fatty acid peroxidase (EC1.11.1.3, Martin and Stumpf, 1959). However, the analysis allowed the identification of a gene (PSHAb0219) coding for an aldehyde dehydrogenase B (EC1.2.1.3), an enzyme annotated as a reversible aldehyde oxidoreductase $NAD(P)^+$-dependent with a wide specificity (http://www.genoscope.cns.fr/agc/microscope/mage/viewer.php). The aldehyde oxidoreductase, in specific conditions, could be able to produce pentadecanal by reducing the corresponding fatty acid. To assess if this was a correct hypothesis, we first investigated the presence of the pentadecanoic acid in *P. haloplanktis* TAC125 cells. A GC-MS analysis of fatty acid methyl esters from *P. haloplanktis* TAC 125 cells, grown either in sessile or in planktonic conditions, revealed the presence of pentadecanoic acid (Figure S2).

Once the presence of the possible substrate had been verified, both in sessile and in planktonic conditions, a RT PCR analysis of the PSHAb0219 gene was performed to assess if the gene transcription is growth condition-dependent. *P. haloplanktis* TAC125 cells were cultured in planktonic or in sessile conditions and total RNA was extracted from the two different samples. The PSHAb0219 gene amplification was obtained with specific oligonucleotide pairs (see Material and Methods Section). The results of the RT-PCR experiments (Figure S3) showed that a PSHAb0219 gene transcription was clearly detected in the RNA samples extracted from *P. haloplanktis* TAC125 cells grown in both conditions. Several experiments were then attempted to delete the PSHAb0219 gene by using genetic tools for the creation of insertion/deletion

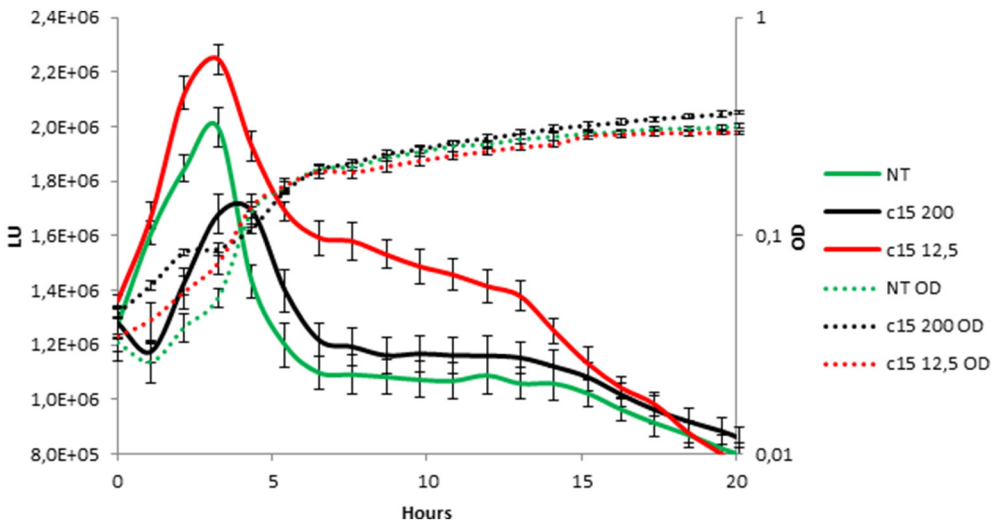

FIGURE 6 | *V. harveyi* LuxS/AI-2 QS system responds to the presence of pentadecanal. Bioluminescence (solid lines) and growth curves (dotted lines) of the *V. harveyi* BB170 strain incubated for 20 h in the presence of only the AB medium (green lines), 12.5 μg/mL pentadecanal (black lines) and 200 μg/mL pentadecanal concentrations (red lines).

mutants already available (Parrilli et al., 2010). However, these experiments were not successful probably due to the fact that PSHAb0219, in tested conditions, is likely to be an essential gene.

Since the pentadecanoic acid was produced in both sessile and planktonic conditions and the PSHAb0219 gene was transcribed in both settings, a possible explanation of the presence of pentadecanal only in the supernatant of the sessile form could be that the aldehyde oxidoreductase catalyses the reduction of the acid to aldehyde only in the biofilm condition. In particular, in oxygen limitation, a condition occurring in biofilm (Wessel et al., 2014), bacterial cells could reduce the fatty acids in order to obtain the oxidized cofactor ($NAD(P)^+$) necessary for cell metabolism. To verify this hypothesis *P. haloplanktis* TAC125 cells were grown, in an automatic bioreactor, at 4°C in microaerobiosis under agitation, a condition where the measured oxygen pressure is kept constantly below 5% saturation (measured oxygen pressure <5%)(see Materials and Methods Section). The anti-biofilm activity of the cell-free supernatant of *P. haloplanktis* TAC125 grown in microaerobiosis (SN-M) was tested on *S. epidermidis* biofilm. The SN-M proved to be able to inhibit the *S. epidermidis* O-47 biofilm formation (**Figure 7**), thus indicating that the pentadecanal production occurs also in microaerobiosis. This experiment demonstrates that oxygen availability is a crucial parameter for pentadecanal synthesis and proves to be the main difference between sessile and planktonic conditions, so determining the anti-biofilm production.

Although pentadecanal could be not defined as a strictly biofilm-specific metabolite, its effect on *P. haloplanktis* TAC125 biofilm formation and development was tested. The aldehyde presence proved to have no effect (Figure S4) on the Antarctic bacterium biofilm development. Moreover, pentadecanal proved to be inactive against the mature biofilm of *P. haloplanktis* TAC125 (data not shown).

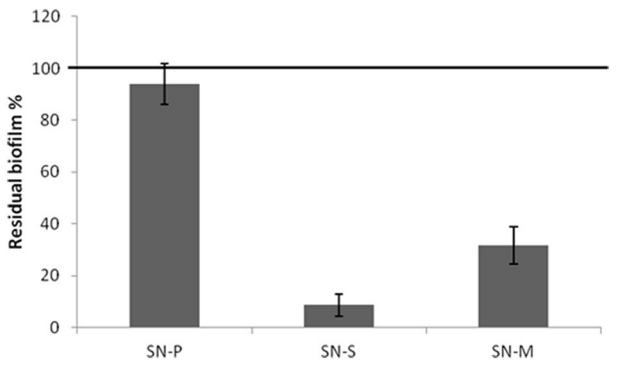

FIGURE 7 | The cell-free supernatant of *P. haloplanktis* TAC125 grown in microaerobiosis has an anti-biofilm activity *S. epidermidis* O-47 biofilm formation after incubation with *P. haloplanktis* TAC125 cell-free supernatants obtained from sessile (SN-S) and planktonic growths (SN-P) and from microaerobiosis growth (SN-M). The data are reported as percentages of residual biofilm. Each data point represents the mean ± SD of at least three independent samples.

DISCUSSION

The emergence of *Staphylococcus epidermidis* as an opportunistic pathogen is closely related to the biofilm forming capability of this bacterial species, especially during the biofilm-associated infection of indwelling medical devices. The increasing use of implanted medical devices heightens the importance of *S. epidermidis* as a human pathogen. Our research has been aimed at discovering anti-biofilm molecules from natural sources, such as marine microbiota, since we are convinced that this approach will lead to the discovery of novel and unforeseen compounds. Cold-adapted marine bacteria represent a still underexploited source of biodiversity able to synthesize a broad range of bioactive compounds, including anti-biofilm molecules. Previous results (Papa et al., 2013b; Parrilli et al., 2015) have demonstrated that the culture supernatant of Antarctic marine bacterium *P. haloplanktis* TAC125 impairs the formation of *S. epidermidis* biofilm.

In this paper we applied a *P. haloplanktis* TAC125 biofilm cultivation strategy in automatic bioreactor (Parrilli et al., 2016) and an efficient activity-guided purification protocol to produce and purify the anti-biofilm molecule. The structure of the anti-biofilm molecule, obtained by NMR and mass spectrometry, corresponds to pentadecanal, a long-chain fatty aldehyde.

The pentadecanal activity on biofilm formation was confirmed on two different *S. epidermidis* strains in static condition and, by the BioFlux system, also in dynamic conditions. BioFlux can reproduce environmental or physiological conditions by precisely controlling shear flow, bridging the gap between *in vitro* and *in vivo* assays. In Bioflux conditions bacteria progress through a series of developmental steps, ultimately forming a multicellular structure containing differentiated cell populations. The observation of the growing biofilm at various time-points throughout this process provides a glimpse of the temporal changes that occur. Flow biofilm is more closely related to natural biofilms and can differ from static biofilms in terms of the hydrodynamic influences on cell signaling. The finding that pentadecanal is active also in dynamic conditions demonstrates that this molecule shows great promise in terms of its use *in vivo* systems.

It is interesting to note that most of the known anti-biofilm molecules also display an antibacterial activity (bactericidal or bacteriostatic). In contrast, pentadecanal lacks any antibacterial activity against free-living bacteria and against bacteria entrapped in biofilm matrix. Indeed as demonstrated by CLSM analysis the presence of pentadecanal reduces the biofilm thickness without affecting the viability of *S. epidermidis* cells living in the biofilm. Therefore, pentadecanal targets the adhesive properties without affecting the bacterial viability, a type of behavior which should prevent the development of escape mutants. This property makes pentadecanal particularly interesting and suggests its possible use in combination with conventional antibiotic therapy.

The specificity of pentadecanal action on *S. epidermidis* biofilm has been demonstrated by testing chemical analogs differing either in the length of the aliphatic chain or in their functional group properties. The results reported demonstrate that both the length of the aliphatic chain and the functional

group properties are essential for the pentadecanal activity against *S. epidermidis* biofilm.

With regard to the anti-biofilm mode of action, several observations prompted us to explore the idea that the action of pentadecanal on *S. epidermidis* biofilm could be related to its possible interference in the quorum sensing system. The most important and best-characterized quorum-sensing system in staphylococci is the accessory gene regulator (*agr*) system. Remarkably, the *P. haloplanktis* TAC125 anti-biofilm molecule is active on the clinical isolate O-47, which is a naturally occurring *agr* mutant (Vuong et al., 2003); therefore a possible effect mediated by quorum sensing should be directed at the LuxS/AI-2 quorum sensing system (Xu et al., 2006; Li et al., 2008), the second quorum sensing system present in *S. epidermidis*. The role of the LuxS/AI-2 QS system in *S. epidermidis* biofilm regulation is under debate. Xu and co-authors have suggested that the autoinducer 2 (AI-2) negatively regulates the expression of the *ica* gene [*ica* operon encodes enzymes responsible for the production of polysaccharide intercellular adhesion PIA (Otto, 2008)] at the transcriptional level, reducing the PIA synthesis and biofilm formation (Xu et al., 2006). In contrast, a recent paper of Xue et al. (2015) has reported that AI-2 increased biofilm formation in *S. epidermidis* RP62A by enhancing the transcription of the *ica* operon. Moreover, in a paper (Li et al., 2008) concerning AI-2 dependent gene regulation in *S. epidermidis,* it has been demonstrated that AI-2 controls also the levels of phenol-soluble modulins (PSMs), key molecules able to lead to biofilm detachment of *S. epidermidis* biofilm *in vivo* e *in vitro* (Otto, 2013). In any case, the crucial role of the LuxS/AI-2 QS system in *S. epidermidis* biofilm formation is evident, while its role in *Staphylococcus aureus* biofilm formation is less clear; indeed, several studies suggest that the LuxS/AI-2 quorum sensing system has no role in *S. aureus* biofilm formation (Doherty et al., 2006; Cluzel et al., 2010). The differences in the involvement of AI-2 molecules in biofilm formation between *S. aureus* and *S. epidermidis* could explain why the *P. haloplanktis* TAC125 anti-biofilm compound is effective against the *S. epidermidis* biofilm but is not able to inhibit the *S. aureus* biofilm (Papa et al., 2013b). To test whether the anti-biofilm activity displayed by pentadecanal is correlated with a modulation/activation of the AI-2 quorum sensing system, the effect of pentadecanal was investigated on a *V. harveyi* mutant sensor strain that responds only to the AI-2 autoinducers.

The results reported demonstrate that at low concentration the pentadecanal is able to increase the *V. harveyi* bioluminescence, indicating that the *V. harveyi* LuxS/AI-2 QS system responds to pentadecanal, at a higher concentration it reduces the bioluminescence. In this condition, pentadecanal probably inhibits the *V. harveyi* luciferase. The key reaction in bacterial bioluminescence is the oxidation of the luciferase-catalyzed $FMNH_2$ and the long chain aliphatic aldehyde (Byers et al., 1988), and, consequently, the bacterial luciferase, that can utilize fatty aldehydes of varying chain lengths, is inhibited by the high levels of aldehydes (Francisco et al., 1993). Therefore, it is reasonable to assume that also pentadecanal could be an inhibitor of the *V. harveyi* luciferase. Since the *V. harveyi* luciferase physiological substrate is tetradecanal (Ulitzur and Hastings, 1979), the bioluminescence in *V. harveyi* mutant was also monitored in presence of tetradecanal, and, as expected, a higher concentration reduced the *V. harveyi* bioluminescence due to the luciferase substrate inhibition. It is interesting to note that at low concentrations tetradecanal had no effect on the *V. harveyi* bioluminescence.

The results reported demonstrate that the *V. harveyi* LuxS/AI-2 QS system responds to pentadecanal. These data suggest that the pentadecanal anti-biofilm activity in *S. epidermidis* could be due to an interference in the AI-2 quorum sensing system. Although several studies will be necessary to clarify the molecular details of the pentadecanal action on *S. epidermidis* biofilm formation, this is the first report on the action of a long-chain fatty aldehyde as an anti-biofilm molecule that works as a signaling molecule in an AI-2 QS system. Further investigations will be necessary to clarify if this modulation occurs also in the *S. epidermidis* AI-2 quorum sensing system.

Besides the characterization of pentadecanal anti-biofilm activity on *S. epidermidis* biofilm, we have investigated the metabolic role and synthesis of this long fatty compound in the Antarctic source strain. In a previous work we examined the anti-biofilm activity of cell-free supernatants obtained from the *P. haloplanktis* TAC125 grown in sessile or in planktonic conditions on different staphylococci (Papa et al., 2013b). Our results demonstrated that only when *P. haloplanktis*TAC125 is grown in sessile condition the cell-free supernatant inhibit the biofilm formation of *S. epidermidis.* This latter result was explained by considering that biofilm specific environmental conditions may induce a profound genetic and metabolic rewiring allowing the production of biofilm-specific metabolites (Beloin and Ghigo, 2005). To evaluate if pentadecanal can be considered a biofilm-specific metabolite, we investigated the conditions necessary for pentadecanal production and the possible synthetic pathway involved. The results reported strongly suggest that a *P. haloplanktis*TAC125 reversible aldehyde oxidoreductase NAD(P)+-dependent catalyses, in oxygen limitation, the reduction of the pentadecaonic acid to aldehyde to obtain the oxidized cofactor (NAD(P)+) necessary for cell metabolism. Therefore, the pentadecanal production occurs in the biofilm condition and in microaerobiosis due to the reduced oxygen availability that characterizes these two growth conditions. The oxygen availability results a crucial parameter for pentadecanal synthesis and proves to be the main difference between sessile and planktonic conditions, so determining the anti-biofilm production. Moreover, the results described in this paper indicate that the long-chain fatty aldehyde is not involved in the control/modulation of the Antarctic bacterium biofilm development.

In conclusion, this is the first report both on the action of a long chain fatty aldehyde as an anti-biofilm molecule and on an aldehyde working as a signaling molecule in the AI-2 QS system. We believe that this paper endorses the potential of cold-adapted marine bacteria as a source of bioactive compounds of interest, and contributes to the development of innovative approaches for the prevention and treatment of *S. epidermidis* biofilm-associated infections.

AUTHOR CONTRIBUTIONS

AC: Performed the experiments, suggested critical parameters in design of experiments and co-wrote the paper. RP: Performed the experiments, suggested critical parameters in design of experiments and co-wrote the paper. AR: Performed the experiments and co-wrote the paper. FS, MZ and MT: Performed the experiments. LS: Provided advice in performance of experiments and edited paper. GM: Provided advice in performance of experiments and co-wrote paper. MC: Suggested critical parameters in design of experiments and co-wrote paper. MLT: Suggested critical parameters in design of experiments and co-wrote paper. MA: Provided advice in performance of experiments and edited paper. EP: Designed the experiments, provided advice in performance of experiments and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2017.00046/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

1.2. Pentadecanal inspired anti-biofilm molecules: from the synthesis towards the clinical applications

A large-scale production and an optimization of the purification protocol have been decisive for the identification of the *P. haloplanktis* TAC125 anti-biofilm molecule, the pentadecanal. It was demonstrated that pentadecanal interferes with the *S. epidermidis* quorum sensing system and reduces the biofilm formation without affecting bacterial viability[4]. This capability of preventing the biofilm formation could be exploited for the development of new materials, or surface coatings, which avoid the adhesion of viable bacteria. However, as the pentadecanal is an aldehyde, and thus a chemically reactive agent, it could easily undergo oxidation reactions, therefore it could be not suitable for all the possible anti-biofilm strategies. One of the aims of this work was the design of some pentadecanal derivatives, in order to enrich the arsenal of weapons to fight the biofilms, and, with these molecules, the development of a valid anti-biofilm strategy.

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Pentadecanal inspired molecules as new anti-biofilm agents against *Staphylococcus epidermidis*

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ABSTRACT

Staphylococcus epidermidis, a harmless human skin colonizer, is a significant nosocomial pathogen in predisposed hosts because of its capability to form a biofilm on indwelling medical devices. In a recent paper, the purification and identification of the pentadecanal produced by the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125, able to impair *S. epidermidis* biofilm formation, were reported. Here the authors report on the chemical synthesis of pentadecanal derivatives, their anti-biofilm activity on *S. epidermidis*, and their action in combination with antibiotics. The results clearly indicate that the pentadecanal derivatives were able to prevent, to a different extent, biofilm formation and that pentadecanoic acid positively modulated the antimicrobial activity of the vancomycin. The cytotoxicity of these new anti-biofilm molecules was tested on two different immortalized eukaryotic cell lines in view of their potential applications.

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Introduction

Although *Staphylococcus epidermidis* is a harmless human skin colonizer, it has emerged as an important opportunistic pathogen in infections associated with medical devices (such as urinary and intravascular catheters, orthopaedic implants), causing approximately 30–43% of joint prosthesis infections (Valour et al. 2013) and fracture fixation infections (Morgenstern et al. 2016). The capability of *S. epidermidis* to adhere on both eukaryotic cells and abiotic surfaces and to form biofilm is an essential virulence factor (Otto 2012) that contributes to the chronicization of infections caused by this bacterium as particularly difficult to eradicate. *S. epidermidis* can migrate from the skin along the surface of the device into the body, forming a highly organized bacterial community known as biofilm. A biofilm is a microbially derived sessile community, characterized by cells attached either to a substratum, or to an interface, or to each other, embedded in a self-produced matrix of extracellular polymeric substance, which exhibit an altered phenotype with regard to growth, gene expression and protein production (Donlan and Costerton 2002).

In a biofilm, cells are embedded and protected from external assaults and in this condition bacteria have been found to be more resistant to conventional antibiotic treatments, thus resulting in recalcitrant biofilm-associated infections (Koo et al. 2017). Biofilm resistance to antimicrobials is a complex phenomenon, not only correlated merely with the genetic resistance that arises from mutations, although the increased microbial cell density may help the transfer of resistance genes. Indeed, other mechanisms are involved, such as: (1) the low penetration of the antimicrobial agent due to the barrier function performed by the biofilm matrix; (2) the presence of persister cells exhibiting a high multidrug tolerance; and (3) a reduced susceptibility to antibiotics as a consequence of stress adaptive responses or changes in the chemical biofilm microenvironment (Satpathy et al. 2016). Accordingly, the strategies adopted to treat these challenging infections are rapidly changing, according to the increasing understanding of the structure and functions of the biofilm. Nonetheless, the prevention of biofilm formation and the treatment of existing biofilms is currently a difficult challenge

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and therefore the discovery of new multi-targeted or combinatorial therapies is increasingly urgent (Koo et al. 2017).

In recent years, several authors working in this field have focused their studies on the search for anti-biofilm molecules in extreme environments, such as Antarctica, since it has been reported that cold-adapted bacteria represent an unexploited source of chemical biodiversity, able to produce a wide range of high added-value compounds (Papaleo et al. 2013; Sun et al. 2015; De Santi et al. 2016, Sannino et al. 2017; Sannino et al. 2018), including anti-biofilm molecules (Papa et al. 2015).

In a recent paper, it was demonstrated that Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 produces a long-chain fatty aldehyde, the pentadecanal (Casillo et al. 2017), endowed with a strong anti-biofilm activity against *S. epidermidis* (Papa et al. 2013, Parrilli et al. 2015). The pentadecanal interferes with the quorum sensing system of *S. epidermidis* and reduces biofilm formation without affecting bacterial viability (Casillo et al. 2017). It is interesting to note that the pentadecanal is also found in the essential oils of different plant species (Gao et al. 2017). This mechanism of action should prevent the development of escape mutants, making the pentadecanal a good candidate for combined therapies with conventional antibiotics. Moreover, the pentadecanal's capability of preventing biofilm formation could be exploited for the development of new materials, or surface coatings, which avoid the adhesion of viable bacteria. In the literature, the incorporation of an antibiotic or biocide within a 'reservoir' coating is being studied as a possible approach to inhibit bacterial adhesion and biofilm formation (Busscher et al. 2012). An ideal anti-biofilm molecule must be endowed with several characteristics described by Batoni et al. (2016); amongst the other things it should interfere with the bacterial cell communication machinery, penetrate the extracellular matrix and/or interfere with its production, and synergize with other conventional and unconventional antimicrobial compounds. Moreover, an anti-biofilm molecule, to have a concrete therapeutic application, should be active following its incorporation within a 'reservoir' coating and, in any case, the coating should not alter the biocompatibility of devices or delivery systems. However, as the pentadecanal is an aldehyde (a chemically reactive agent), it could easily undergo oxidation reactions, therefore it may not be suitable for all possible anti-biofilm strategies. One of the aims of this work was the design of some pentadecanal derivatives to enrich the arsenal of weapons to fight biofilm development. With the purpose of obtaining new anti-biofilm molecules endowed with different physico-chemical properties, the dimethyl acetal of the pentadecanal, the pentadecanoic acid, and its methyl ester, were synthesized.

The capability of the pentadecanal derivatives to prevent biofilm formation by *S. epidermidis* and their possible use in combination with antibiotics was investigated. Finally, to explore the possible clinical applications, their biocompatibility on eukaryotic cells was analysed on two different immortalized cell lines.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains used in this work were: *S. epidermidis* RP62A, a reference strain isolated from an infected catheter (ATCC collection no. 35984); and *S. epidermidis* O-47, a strong biofilm producer (Figure S1 in Supplemental material) *agr*-mutant, isolated from clinical septic arthritis and kindly provided by Prof. Gotz (Heilmann et al. 1996). Bacteria were grown in brain heart infusion broth (BHI, Oxoid, UK). Biofilm formation was assessed in static conditions at 37 °C, whereas planktonic cultures were performed under vigorous agitation (180 rpm) at 37 °C. All strains were maintained at −80 °C in cryovials with 15% glycerol. Q1

Derivatives design and synthesis and purification

To generate new anti-biofilm molecules endowed with physico-chemical properties different from those of pentadecanal, pentadecanoic acid (carboxylic acid), pentadecanoic acid methyl ester (ester), and 1,1-dimethoxypentadecane (acetal) were synthesized. The choice of these derivatives was mainly suggested by the well-known reactivity of an aldehyde, which is able to be oxidized by oxygen in the air, even in the presence of a catalytic amount of Fe^{3+} (Carey and Sundberg 2000; Farquhar 1961; Jiang et al. 2018).

Pentadecanoic acid was purchased from Sigma (Sigma-Aldrich, Inc., © 2018 Merck KGaA, Darmstadt, Germany). Q4

The acetal was obtained by treating the pentadecanal (50 mg) by reaction with 6 ml of 1.25 M HCl/CH_3OH at 80 °C for 16 h. After overnight reaction, the reaction was quenched with anionic exchange resin (HCO_3^- form), and the product recovered with a yield of 98%.

To obtain the methyl ester, 50 mg of pentadecanoic acid were treated with 5 ml of 1.25 M HCl/CH_3OH at 80 °C for 16 h. The methanol layer was extracted three times with hexane to recover the methyl ester with a yield of 99%. The mixture was then treated with Dowex 1X8 resin (Sigma, HCO_3^- form) and dried under argon flow. As for the methyl ester, the recovery of the acetal was 98%.

All the synthesized compounds were characterized by ^{1}H-NMR spectroscopy and gas chromatography-mass spectrometry (GC-MS). Furthermore, the purity of the synthesized derivatives was confirmed by GC-MS analysis (Figure S2). All the compounds were analysed on an Agilent 7820 A GC System-5977B MSD spectrometer equipped with the automatic injector 7693Aand a Zebron ZB-5 capillary column (Phenomenex, Toornace, CA, USA; flow rate 1 ml min^{-1}; He as carrier gas), using the following temperature program: 150 °C for 3 min, 150 °C → 300 °C at 15 °C/min, 300 °C for 5 min. As for the ^{1}H NMR experiments, each sample was dissolved in 0.55 ml of dimethyl sulfoxide-d6 (DMSO-d6), and the spectra were recorded at 298 K using a Bruker Avance 600 MHz spectrometer equipped with a cryoprobe (Bruker Italia, Milano, Italy).

Biofilm formation by staphylococci

Static biofilm assay

The quantification of *in vitro* biofilm production was based on the method described by Christensen with slight modifications (Papa et al. 2013).

Briefly, the wells of a sterile 96-well flat-bottomed polystyrene plate (tissue culture treated by vacuum gas plasma, Falcon) were filled with *S. epidermidis* RP62A or *S. epidermidis* O-47 cultures in exponential growth phase diluted in BHI with a final concentration of about 0.1 and 0.001 OD_{600nm} respectively. The derivatives were added at different concentrations and the final volume was 200 µl. Q3

The first row contained the untreated bacterial cells, while each of the remaining rows contained different concentrations (from 12.5 µg ml^{-1} to 100 µg ml^{-1}) of the derivatives samples. Dried derivatives were first re-solubilized in DMSO and a working solution of each derivative to be tested was diluted in BHI (0.8% DMSO final concentration at the maximal concentration used). From this stock solution, serial two-fold dilutions in BHI broth were carried out in 96-well plates over the concentration range 100–12.5 µg ml^{-1}. A control using BHI broth with 0.8% DMSO was also included in each experiment. The plates were incubated aerobically for 24 h at 37 °C. After the incubation, planktonic cells were gently removed; each well was washed three times with double-distilled water and patted dry with a piece of paper towel in an inverted position. To quantify the biofilm formation, each well was stained with 0.1% (w v^{-1}) crystal violet and incubated for 15 min at room temperature, rinsed twice with double-distilled water, and thoroughly dried. The dye bound to adherent cells was solubilized with 20% (v v^{-1}) glacial acetic acid and 80% (v v^{-1}) ethanol. After incubation at room temperature for 30 min, the OD was measured at 590 nm to quantify the total biomass of biofilm formed in each well. Each data point is composed of three independent experiments, each performed in six replicates. The data reported were statistically validated using the Student's *t*-test comparing the mean absorbance of treated and untreated samples. The significance of differences between the mean absorbance values was calculated using a two-tailed Student's *t*-test. A *p*-value of <0.05 was considered significant.

CLSM analysis and COMSTAT

For confocal microscope analysis, biofilms were formed on Nunc™ Lab-Tek® 8-well Chamber Slides (n° 177445; Thermo Scientific, Ottawa, Canada). Briefly, the wells of the chamber slide were filled with *S. epidermidis* RP62A or *S. epidermidis* O-47 cultures in exponential growth phase diluted in BHI at a final concentration of ~0.1 and 0.001 OD_{600nm} respectively and incubated in the presence of derivatives (100 µg ml^{-1} – 0.8% DMSO). The final volume was 300 µl. The bacterial cultures were incubated at 37 °C for 20 h in the presence and absence of derivatives in order to assess their anti-biofilm activity and their influence on cell viability. The biofilm cell viability was determined by the FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (Molecular Probes, Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. After rinsing with filter-sterilized PBS, each well of the chamber slide was filled with 300 µl of a working solution of fluorescent stains, containing the SYTO® 9 green fluorescent nucleic acid stain (10 µM) and propidium iodide, and the red-fluorescent nucleic acid stain (60 µM), and incubated for 20–30 min at room temperature, protected from light. Excess stain was removed by gently rinsing with filter-sterilized PBS. All microscopic observations and image acquisitions were performed with a confocal laser scanning microscope (CLSM; LSM700-Zeiss, Q2

Germany) equipped with an Ar laser (488 nm), and a He-Ne laser (555 nm). Images were obtained using a 20×/0.8 objective. The excitation/emission maxima for these dyes are ~480/500 nm for SYTO® 9 stain and 490/635 nm for propidium iodide. Z-stacks were obtained by driving the microscope to a point just out of focus on both the top and bottom of the biofilms. Images were recorded as a series of .tif files with a file-depth of 16 bits. A COMSTAT software package was used to determine biomasses (μm^3 μm^{-2}), average thicknesses (μm) and roughness coefficient (Ra*). For each condition, two independent biofilm samples were used.

MIC and MBC of vancomycin determination

The conventional MIC of vancomycin was determined for *S. epidermidis* RP62A in duplicate, by twofold serial dilution (CLSI 2017). Briefly, an overnight culture was diluted at 0.5 McFarland and 90 μl of this dilution were added to each well of a 96-well microtitre dish. Ten μl vancomycin previously diluted to the desired final concentration were added to the wells. Ten μl water were added for the control lane. The microtitre plate was incubated at 37 °C for 24 h. After the incubation the MIC was determined. Furthermore, the minimal bactericidal concentration (MBC) of vancomycin was determined as reported. Using the replica plater for 96-well plate (Sigma Aldrich) ~3 μl of planktonic culture from each well of the microtitre plate were transferred to the surface of a BHI agar plate. Plates were incubated for 16 h at 37 °C. The MBC was determined by identifying the cut off for bacterial growth.

MBIC and MBEC of vancomycin determinations

The minimum biofilm inhibitory concentration (MBIC) and the minimum biofilm eradication concentration (MBEC) of vancomycin for *S. epidermidis* RP62A were determined. Briefly, the wells of a sterile 96-well flat-bottomed polystyrene plate (Falcon) were filled with 100 μl of BHI with an appropriate dilution of overnight cultures of *S. epidermidis* RP62A (0.1 OD_{600nm}) and incubated overnight at 37 °C without shaking, to allow bacterial attachment. Non-adherent cells were removed by gentle washing three times with sterile PBS. The plates were left to air dry for 15 min. Serial twofold dilutions of vancomycin (initial concentration 240 μg ml^{-1}) in cation-adjusted Mueller–Hinton broth (CAMHB) were added to the microplates followed by incubation at 37 °C for 24 h. The MBIC was defined as the minimal antimicrobial concentration at which there was no observable bacterial growth in wells containing adherent microcolonies, ie the minimal concentration that inhibited the release of planktonic bacteria from the biofilm (Reiter et al. 2013).

After MBIC measurement, the content of each well was spotted on an agar plate using a replica plater for 96-well plate (Sigma-Aldrich). The agar plate was incubated for 24 h at 37 °C. The MBEC was defined as the minimal antimicrobial concentration at which bacteria fail to regrow after antimicrobial exposure, ie the minimal concentration required for eradicating the biofilm (Reiter et al. 2013). All the determinations were performed in duplicate.

Synergy testing by checkerboard assay

Synergism between the pentadecanoic acid or the pentadecanal and vancomycin was assessed by the so-called 'checkerboard' assay against *S. epidermidis* RP62A. Twofold serial dilutions of pentadecanoic acid or the pentadecanal and vancomycin were tested in combination. To do this, the pentadecanoic acid and the pentadecanal were tested at concentrations ranging from 250 to 31.25 μg ml^{-1} and vancomycin at concentrations ranging from 240 to 7.5 μg ml^{-1}, respectively. All the determinations were performed in duplicate.

MBIC and MBEC experiments were performed as previously described.

Synergy testing by CLSM analysis

For synergy evaluation between the anti-biofilm molecules and vancomycin by confocal microscopy, biofilm formation was assessed as previously described. Briefly, an overnight culture of *S. epidermidis* RP62A grown in BHI was diluted and inoculated into each well of an eight-well chamber slide at an appropriate cell concentration, ~0.1 OD_{600nm}. The bacterial cultures were incubated at 37 °C for 2 h in presence of 120 μg ml^{-1} of vancomycin, with or without the anti-biofilm molecules, pentadecanal and pentadecanoic acid (250 μg ml^{-1}), in order to assess whether the anti-biofilm molecules were able to improve the antimicrobial activity of vancomycin. The biofilm cell viability was determined by the FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (Molecular Probes, Invitrogen) as previously described (Casillo et al. 2017).

Cytotoxicity assay

Immortalized human keratinocyte cells (HaCaT), and mouse fibroblasts (BALB/c3T3) were from ATCC. These cell lines are commonly used for biocompatibility and cytotoxicity assay, as reported by Wiegand and Hipler (2009), and are particularly appropriate cell lines for use in cytotoxicity and tolerance tests concerning the skin because they represent dermal fibroblasts and epidermal keratinocytes, both targets of *S. epidermidis*. Cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich), supplemented with 10% foetal bovine serum (HyClone, Logan, UT, USA), 2 mM L-glutamine and antibiotics. Cells were grown in a 5% CO_2 humidified atmosphere at 37 °C. To test the biocompatibility of the molecules, cells were seeded in 96-well plates at a density of 2.5×10^3 well^{-1}. Twenty-four hours after seeding, cells were incubated with increasing amounts of the molecule under test (from 10 to 100 µg ml^{-1}) for 24, 48 and 72 h. At the end of incubation, cell viability was assessed by the MTT assay as previously described (Arciello et al. 2011). Cell survival was expressed as a percentage of viable cells in the presence of the analysed molecule, with respect to the control cells. Control cells were represented by cells grown in the absence of the molecule and by cells supplemented with identical volumes of DMSO. Two-way ANOVA was performed as a statistical analysis.

Results

Anti-biofilm activity of derivatives

To evaluate the anti-biofilm activity of pentadecanal derivatives, molecules were tested at different concentrations against the reference strain *S. epidermidis* RP62A and against *S. epidermidis* O-47, a strong biofilm producer *agr*-mutant (Heilmann et al. 1996). As shown in Figure 1, all the molecules reduced biofilm formation by the reference strain *S. epidermidis* RP62A and, surprisingly, the carboxylic acid, at low concentrations (25 µg ml^{-1} and 12.5 µg ml^{-1}), was more active than the pentadecanal. Besides the pentadecanal, the most effective derivatives on *S. epidermidis* O-47 biofilm was the ester and then the acetal. It is interesting to note that the acid was not able to induce a significant reduction in the *S. epidermidis* O-47 biofilm, contrary to what found on *S. epidermidis* RP62A. The anti-biofilm activity of pentadecanal derivatives was further confirmed by CLSM. CLSM analysis of *S. epidermidis* O-47 and RP62A biofilms were performed after 24 h incubation in the presence

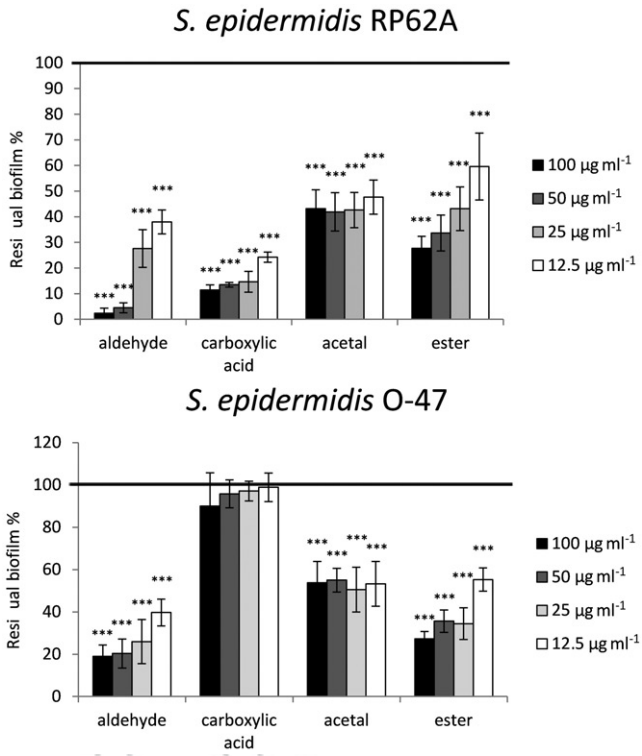

Figure 1. The anti-biofilm activity of pentadecanal and its derivatives on *S. epidermidis* biofilm formation. The anti-biofilm effect was evaluated on biofilm formation of *S. epidermidis* O-47 and *S. epidermidis* RP62A using different concentrations of the tested molecules. The data are reported as percentages of residual biofilm. Each data point represents the mean ± the SD of three independent samples; the mean values were compared to the untreated control and considered significant when $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) according to the Student *t*-test. Change. Q6

and absence of the derivatives, respectively. The LIVE/DEAD staining was used to analyse the biofilm structure and cell viability. As shown in Figure 2A, cells exposed to derivatives were alive (green indicates viable cells and red indicates dead cells), confirming that the new molecules, exactly like the pentadecanal (Casillo et al. 2017), had no bactericidal activity on either of the *S. epidermidis* bacterial strains living in the biofilm. To obtain more detailed information on the biofilm structure, the collected three-dimensional files were analysed using the COMSTAT image analysis software package (Heydorn et al. 2000). The results demonstrated that biofilms formed in the presence of the derivatives were characterized by a lower biomass (Figure 2B) and this effect was clearly evident in both the *S. epidermidis* strains using all the derivatives. It is interesting to note that the carboxylic acid by CLSM and COMSTAT analysis was active, contrary to what was demonstrated when its anti-biofilm activity was evaluated by crystal violet assay (Figure 1). As shown in Figure 2C and D, the

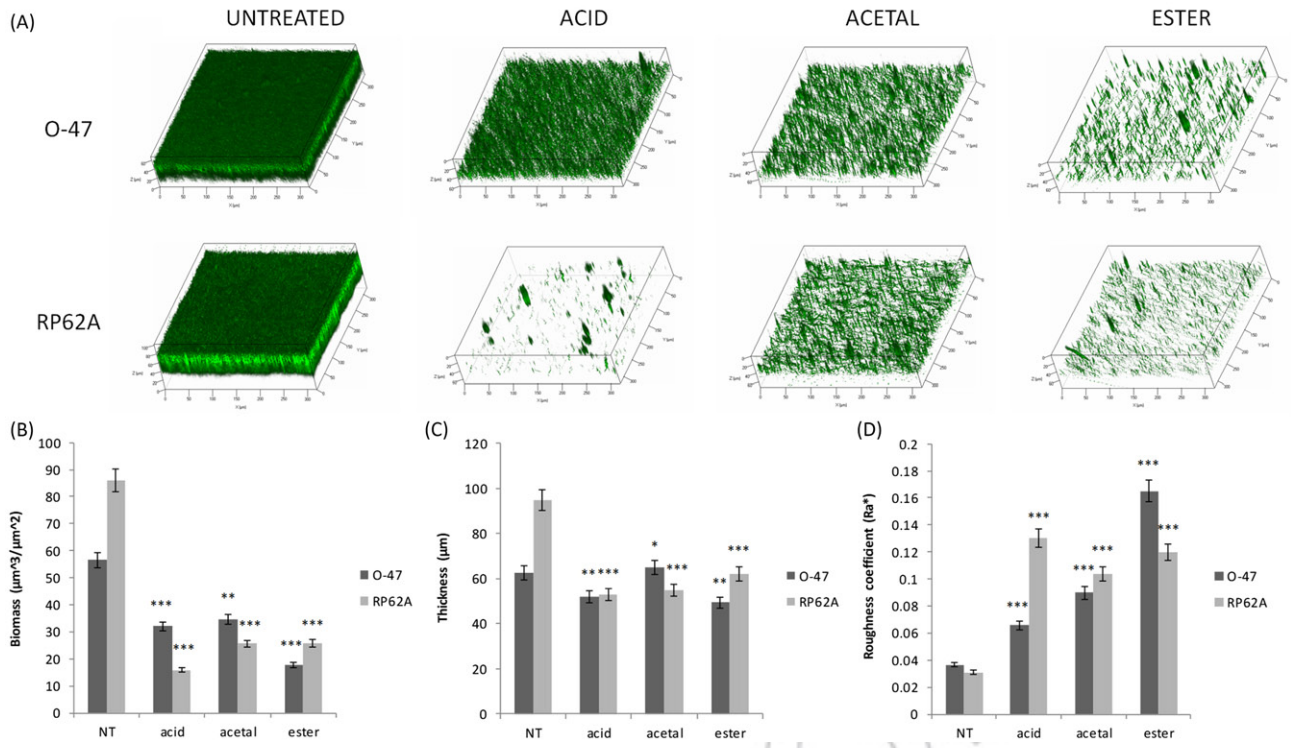

Figure 2. Analysis of the effect of pentadecanal derivatives on *S. epidermidis* O-47 and *S. epidermidis* RP62A biofilm structure. (A) CLSM analysis of *S. epidermidis* O-47 and *S. epidermidis* RP62A biofilms were performed after 24 h incubation at 37 °C in the presence and in absence of the derivatives. Three-dimensional biofilm structures were obtained using the LIVE/DEAD® Biofilm Viability Kit. COMSTAT quantitative analysis of (B) biomass, (C) average thickness and (D) roughness coefficient of *S. epidermidis* O-47 and *S. epidermidis* RP62A biofilms were performed for all the tested conditions.

biofilms obtained in the presence of the derivatives were quite similar in terms of thickness, but the roughness coefficient indicated significant differences. In particular, an increase in the roughness coefficient was observed. This dimensionless factor provides a measure of how much the thickness of a biofilm varies, and it is thus used as a direct indicator of biofilm heterogeneity. The same reported trend was observed also for biofilm obtained in presence of the pentadecanal (Figure S3).

The synergy of derivatives with vancomycin by checkerboard assay

The pentadecanal or its synthetic derivatives were tested in combination with an antibiotic on mature biofilm in order to assess whether the anti-biofilm activity could modulate the antibiotic activity by weakening the *S. epidermidis* biofilm structure, making it less compact and rougher, and thus allowing the penetration of the antimicrobial agent. These experiments were performed using vancomycin as antibiotic and *S. epidermidis* RP62A as the target strain.

To exclude any interference between the anti-biofilm molecules and the vancomycin, the MBC of this antibiotic on *S. epidermidis* RP62A was calculated in the presence and in the absence of anti-biofilm compounds (up to 250 µg ml^{-1}). The MBC value was evaluated for the vancomycin on *S. epidermidis* RP62A planktonic cells according to CLSI (2017) (Table 1) and, in all the tested conditions, the vancomycin efficacy was not affected by the presence of the anti-biofilm molecules (data not shown). Measurement of the MBIC and of the MBEC was used to evaluate the vancomycin antimicrobial activity against the mature biofilms (Reiter et al. 2013). The *S. epidermidis* RP62A 24 h mature biofilm was subjected to antimicrobial exposure and, as expected, the MBEC and MBIC values were extremely high when compared to the MBC value. The MBIC and MBEC were equivalent and significantly higher than the value obtained for the MBC on planktonic growth (Table 1). The possible synergistic effect of anti-biofilm molecules and vancomycin was evaluated on 24 h mature biofilm using the pentadecanoic acid or the pentadecanal as only these two molecules showed a slight, but significant effect on mature *S. epidermidis* RP62A biofilm (data not shown). The synergy was evaluated by using the checkboard method, as described in the Materials and methods section. The results are reported in Table 1. The addition of pentadecanal did not prompt a reduction

Table 1. Antimicrobial activities of vancomycin and vancomycin in the presence of pentadecanoic acid and pentadecanal against *S. epidermidis* RP62A in planktonic and biofilm growth.

	Vancomycin	Vancomycin + pentadecanoic acid 250 μg ml^{-1}	Vancomycin + pentadecanoic acid 125 μg ml^{-1}	Vancomycin + pentadecanal 250 μg ml^{-1}	Vancomycin + pentadecanal 125 μg ml^{-1}
MBC (μg ml^{-1})	7.5	7.5	7.5	7.5	7.5
MBIC (μg ml^{-1})	60	15	15	60	60
MBEC (μg ml^{-1})	60	15	15	60	60

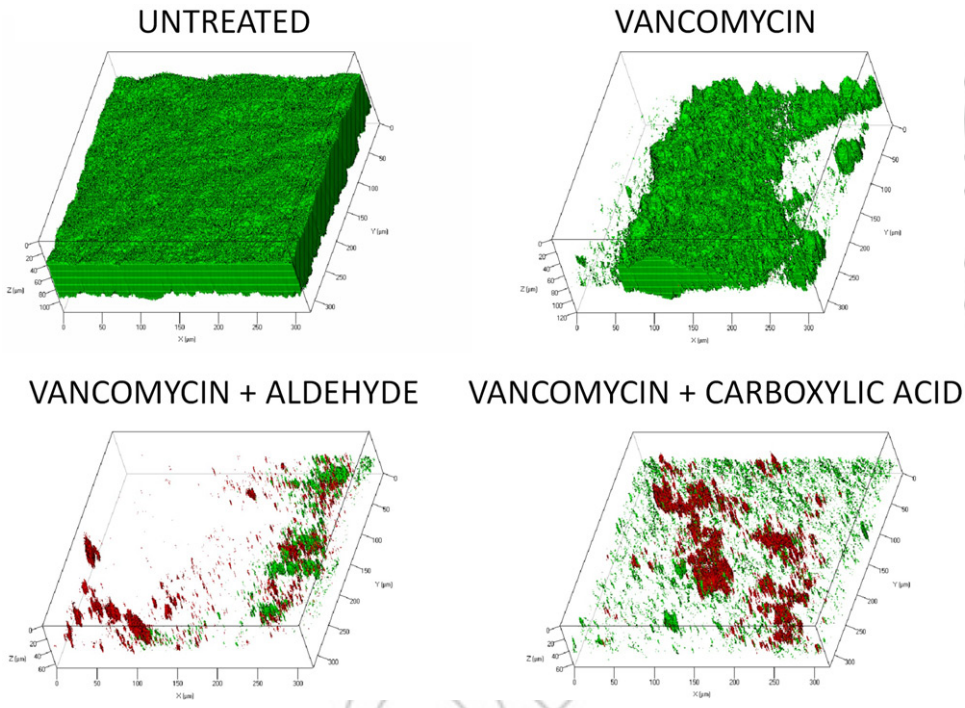

Figure 3. Analysis of the synergistic effect of derivatives with vancomycin on *S. epidermidis* RP62A biofilm structure. CLSM analysis of *S. epidermidis* RP62A biofilms was performed on mature biofilms after 2h incubation at 37 °C in the presence and absence of derivatives and vancomycin. Three-dimensional biofilm structures were obtained using the LIVE/DEAD® Biofilm Viability Kit.

in the MBIC or MBEC values of vancomycin on *S. epidermidis* RP62A. On the contrary, the pentadecanoic acid modulated the antimicrobial activity of the vancomycin. In particular, the MBIC and MBEC values, obtained after 24 h incubation, were reduced twofold in combination with 125 μg ml^{-1} and 250 μg ml^{-1} of pentadecanoic acid, respectively.

In order to collect information on the mechanism of action of the pentadecanoic acid in combination with the vancomycin and to understand the reasons for the inefficiency of pentadecanal, a CLSM analysis of *S. epidermidis* RP62A mature biofilm treated with vancomycin in the absence or in the presence of either pentadecanal or pentadecanoic acid was performed.

In detail, 24 h mature biofilm of *S. epidermidis* RP62A was incubated for 2 h in the presence of 120 μg ml^{-1} of vancomycin and in the presence or absence of 250 μg ml^{-1} of the pentadecanal or the pentadecanoic acid.

As shown in Figure 3, the vancomycin led to a slight reduction in the biofilm biomass, but it was not able to kill the cells embedded in the biofilm matrix. Whereas, when the vancomycin was combined with either the pentadecanal or the carboxylic acid, it was able to penetrate the biofilm structure, showing a substantial bactericidal activity even against the deepest cells in the biofilm structure (Figure 3, red signal).

Effect of pentadecanal derivatives on eukaryotic cell viability

The biocompatibility of the pentadecanal and its derivatives was investigated on keratinocytes and fibroblasts. These two cell lines were chosen as they represent the skin, in which keratinocytes are the outer part of the skin and cover the fibroblasts layer and are a target for *S. epidermidis* infections. As shown in Figure 4, after 24 h incubation, in the case of keratinocytes, all the analysed molecules were fully biocompatible, as no toxicity was

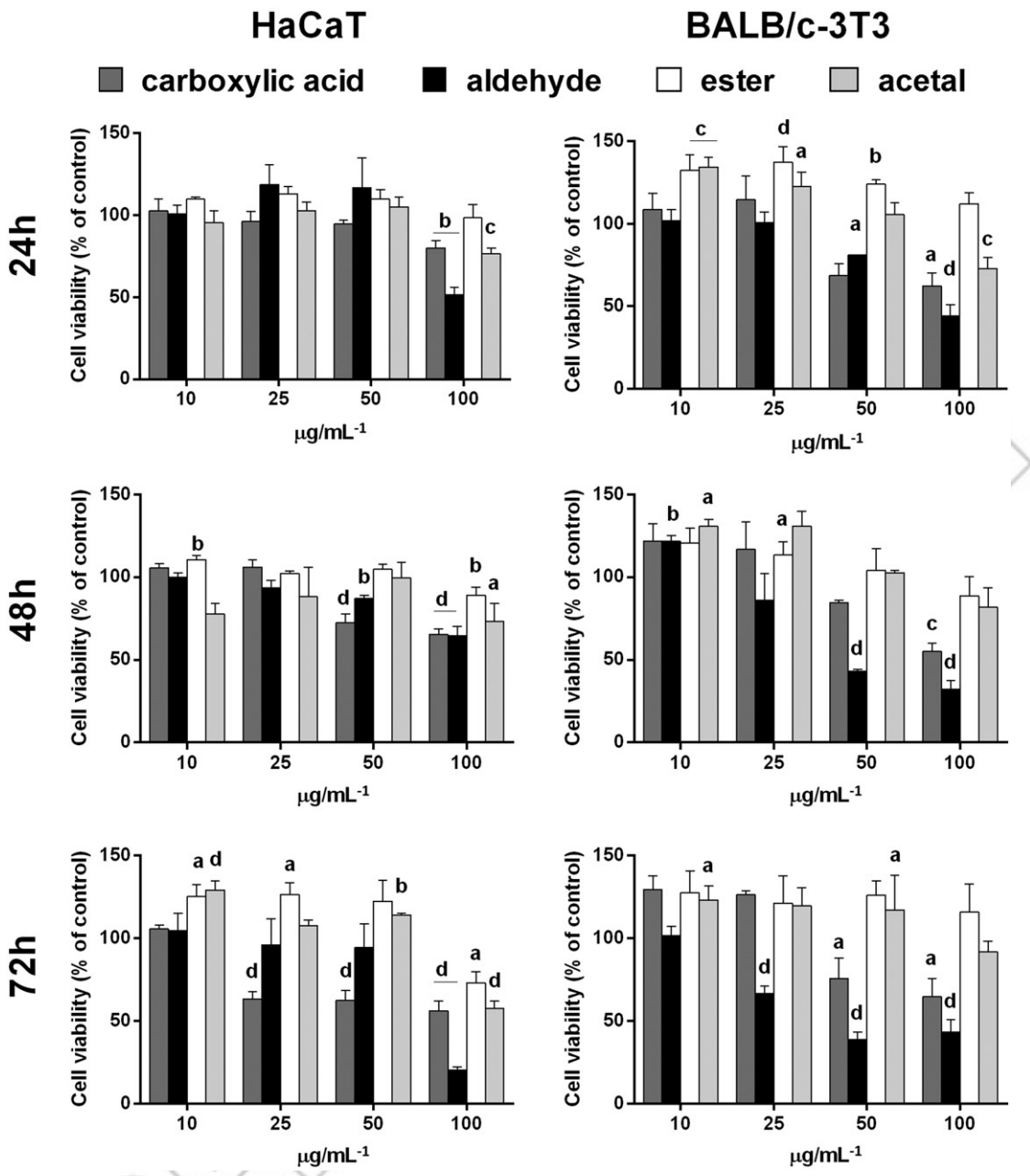

Figure 4. Effect of pentadecanal and its derivatives on immortalized cell lines. Dose-response plot of cells after 24, 48 and 72 h incubation with increasing concentrations (10–100 μg ml^{-1}) of molecules. Cell viability was assessed by the MTT assay and expressed as described in the Materials and methods section. Values are given as means ± SD ($n \geq 3$); a indicates $p < 0.05$, b indicates $p < 0.01$, c indicates $p < 0.005$, d indicates $p < 0.001$, with respect to control cells.

observed, except for the pentadecanal, which was biocompatible up to 50 μg ml^{-1}. After 48–72 h the ester and acetal were safe for HaCaT cells up to 50 μg ml^{-1}, whereas a toxicity was observed for the acid (after 72 h incubation). The pentadecanal was fully biocompatible when used at concentrations up to 50 μg ml^{-1}. These results were similar also in the case of BALB/c-3T3 cells.

Altogether, these data clearly indicate that the ester derivative is fully biocompatible, followed by acetal.

Discussion

Recently, the identification of the pentadecanal as a new anti-biofilm molecule against *S. epidermidis* was reported (Casillo et al. 2017). This molecule, produced by the Antarctic bacterium *P. haloplanktis* TAC125, is a very promising anti-biofilm molecule. Nonetheless, the pentadecanal is a chemically reactive agent which could easily undergo reactions of oxidation, polymerization, and/or hydration, and therefore it could be not chemically compatible with

all anti-biofilm strategies. Therefore, the aim of this work was to produce and characterize novel pentadecanal derivatives to be used in anti-biofilm applications.

In a previous paper, the specificity of the pentadecanal action on *S. epidermidis* biofilm was explored by testing chemical analogues differing either in the length of the aliphatic chain or in their functional group properties (Casillo et al. 2017). In particular, similar aldehydes and corresponding alcohols, characterized by different lengths of the aliphatic chain (C-14/C-20), were analysed, and the results demonstrated that both the length of the aliphatic chain and the functional group properties were important for the pentadecanal activity against *S. epidermidis* biofilm (Casillo et al. 2017). Since the hexadecanal and the tetradecanal did not show anti-biofilm activity (Casillo et al. 2017), whereas the pentadecanol showed a slight but significant activity, the new anti-biofilm molecules were designed preserving the length of the aliphatic chain and modifying the functional group.

The pentadecanal derivatives synthesized were: pentadecanoic acid, the pentadecanoic acid methyl ester and 1,1-dimethoxypentadecane. In particular, the acetal could offer the opportunity to generate the aldehyde when treated with an electrophilic agent. Therefore, the acetal could be a good choice in treating *in vivo* infections, especially those close to the implant surface, where the bacterial metabolic activity generates a local acidic pH (4–5) (Vroom et al. 1999; Wang et al. 2014). The ester and the carboxylic acid were selected since both are less reactive than aldehyde in oxidation and polymerization reactions.

All the synthesized molecules were tested for their anti-biofilm activity and, unexpectedly, all of them showed that they were able to prevent, to a different extent, biofilm formation by *S. epidermidis* RP62A. This result clearly demonstrates that the functional group has a key role in the anti-biofilm activity, even if they are not strictly related. Casillo et al. (2017) demonstrated that the pentadecanal interfered in the AI-2 quorum sensing system of *S. epidermidis*. It is interesting to note that the AI-2 quorum sensing molecular pathway in Gram-positive bacteria is not completely clarified, and a putative receptor for AI-2 molecules has not yet been identified (Ma et al. 2017). Thus, it is not clear how AI-2 signals are detected, or which functions are regulated by them in many luxS-containing bacteria. Moreover, in *S. epidermidis*, no potential AI-2 receptors have been found. Therefore, it is not possible to exclude the possibility that the pentadecanal and its derivatives cross the bacterial cell wall by diffusion since their physico-chemical properties are compatible with this mechanism.

Moreover, the AI-2 mechanism of action in *S. epidermidis* has not yet been thoroughly characterized, and probably, this unexpected result will be clarified when all the details of the AI-2 quorum sensing molecular mechanism in *S. epidermidis* have been explained.

The efficacy of the pentadecanoic acid addition on *S. epidermidis* RP62A and *S. epidermidis* O-47 biofilm formation was different. Indeed, *S. epidermidis* O-47 biofilm formation was poorly affected by the carboxylic acid addition, compared to the strong effect shown on the reference strain.

S. epidermidis O-47 is a naturally occurring *agr* mutant (Vuong et al. 2003). As previously reported (Vuong et al. 2003; Kohler et al. 2014), the *agr*-negative genotype enhanced biofilm formation on polymer surfaces by increasing expression of the surface protein AtlE, a bifunctional adhesin/autolysin abundant in the cell wall of *S. epidermidis*. The amount of AtlE present in the cell envelope is one of the reported differences between the *S. epidermidis* RP62A and *S. epidermidis* O-47 (Vuong et al. 2003). It is possible to speculate that the overexpression of AtlE renders the *S. epidermidis* O-47 cell envelope less permeable, compared to RP62A, to a hypothetical diffusion of charged molecules, such as the pentadecanoic acid, the only charged molecule tested here. Moreover, AtlE induces significant changes in the hydrophobicity of the bacterial surface (Büttner et al. 2015); this effect could explain the inefficacy of the pentadecanoic acid, that is less hydrophobic compared to the pentadecanal. Nevertheless, the *S. epidermidis* O-47 genome sequence is not available, and this information will be instrumental in understanding the observed difference.

Although the detailed molecular mechanism that underpins the anti-biofilm activity of aldehyde derivatives is far from being elucidated, their effect on the *S. epidermidis* biofilm structure is quite evident, as the biofilm formed in their presence is strongly reduced and characterized by a porous structure containing many channels and voids. These results suggested exploring the use of the aldehyde and its derivatives in combination with antibiotics to treat biofilm infections. *S. epidermidis* RP62A is a strongly adherent, slime-producing pathogenic strain isolated from a patient with intravascular catheter-associated sepsis and, for this reason, it represents the ideal candidate for these experiments since it is a reference biofilm positive/*ica*-positive strain and methicillin-resistant (Qin et al. 2007). As for the antibiotic, the

glycopeptide vancomycin was chosen since it is considered one of the most reliable therapeutic agents against infections caused by multidrug-resistant staphylococci (Qin et al. 2007). Moreover, it is the primary antibiotic used to cure infections caused by coagulase-negative staphylococci and is used to treat prosthetic valve endocarditis caused by *S. epidermidis* (Nailor and Sobel 2009). On the other hand, vancomycin displays a reduced penetration into the biofilm matrix and a low microbicidal effect on *S. epidermidis* within the biofilm (Zhang et al. 2015).

To investigate a possible synergy between the pentadecanal or the pentadecanoic acid and vancomycin, the MBEC and the MBIC in the presence of the anti-biofilm molecules were evaluated, since these methodologies have been suggested as a laboratory assay to evaluate antimicrobial activity against mature biofilm (Reiter et al. 2013). The results demonstrated that the pentadecanoic acid modulated the antimicrobial activity of vancomycin, with a twofold reduction in MBIC and MBEC values at a concentration of anti-biofilm molecules of 125 μg ml^{-1}, whereas no effect was detected using the aldehyde. As the MBIC and MBEC assays were usually performed on mature biofilm treated with the anti-biofilm and antibiotic for 24 h, this condition could not be optimal for a chemically reactive agent such as the pentadecanal. Therefore, CLSM analyses of mature biofilm of *S. epidermidis* RP62A treated with vancomycin and pentadecanal were performed, selecting experimental conditions which allowed the detection of a possible effect in a short incubation time. The results obtained revealed the capacity of the pentadecanal and the carboxylic acid to weaken the *S. epidermidis* biofilm structure, making it less compact and homogeneous, and thus allowing the penetration of the vancomycin into the structure of the biofilm, making cells deeply embedded into the matrix easier to reach. This could be a great advantage in the case of a possible therapeutic strategy providing an antibiotic/anti-biofilm combination.

To explore the clinical potential of pentadecanal and its derivatives, their biocompatibility was investigated on fibroblasts and keratinocytes, eukaryotic cell lines which represent the target of *S. epidermidis* infections. The data clearly indicate that the pentadecanal derivatives are biocompatible with all the cell lines analysed at all the concentrations tested, while the aldehyde is fully biocompatible when used at concentrations lower than 50 μg ml^{-1}.

In conclusion, this work endorses pentadecanal and its derivatives as useful molecules for the development of innovative approaches mainly aimed at the prevention of biofilm formation. Nevertheless, these molecules could also be useful to fight established infections in combination with an opportune antibiotic therapy, thus suggesting that the use of pentadecanoic acid in combination with vancomycin could improve the efficacy of therapy for the treatment of *S. epidermidis* biofilm-associated infections.

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1.3. Physiological studies of *Pseudoalteromonas haloplanktis* TAC125 biofilm for biotechnological applications

The biofilm mode of growth of *Pseudoalteromonas haloplanktis* TAC125 resulted to be the crucial variable for the production of the pentadecanal, underlying the hypothesis that the specific environmental conditions prevailing within biofilms could induce profound genetic and metabolic rewiring of the biofilm-associated bacteria, which might allow the production of metabolites different from those obtained in planktonic condition.
From this point of view, microbial biofilms could be considered as cell factories. In fact, many biofilm-secreted molecules, such as surfactants[5], enzymes[6], polysaccharides[7], may be of biotechnological utility for, among others, the cosmetics, food, and pharmaceutical industries.
These considerations strongly suggest the existence of differences in *P. haloplanktis* TAC125 cells physiology between the sessile and the planktonic lifestyle and pave the way to several open questions about the biofilm-specific pathway involved in the pentadecanal synthesis. Therefore, to better understand the mechanisms undergoing the anti-biofilm production, and, especially, to explore the potentiality of *P. haloplanktis* TAC125 in other biotechnological fields, the structural characterization of the biofilm of the Antarctic bacterium, produced in response to different nutrient abundance and temperatures, was investigated.

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Environmental conditions shape the biofilm of the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125

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ABSTRACT

Biofilms are the most widely distributed and successful microbial modes of life. The capacity of bacteria to colonize surfaces provides stability in the growth environment, allows the capturing of nutrients and affords protection from a range of environmental challenges and stress. Bacteria living in cold environments, like Antarctica, can be found as biofilms, even though the mechanisms of how this lifestyle is related to their environmental adaptation have been poorly investigated. In this paper, the biofilm of *Pseudoalteromonas haloplanktis* TAC125, one of the model organisms of cold-adapted bacteria, has been characterized in terms of biofilm typology and matrix composition. The characterization was performed on biofilms produced by the bacterium in response to different nutrient abundance and temperatures; in particular, this is the first report describing the structure of a biofilm formed at 0 °C. The results reported demonstrate that *Ph*TAC125 produces biofilms in different amount and endowed with different physico-chemical properties, like hydrophobicity and roughness, by modulating the relative amount of the different macromolecules present in the biofilm matrix. The capability of *Ph*TAC125 to adopt different biofilm structures in response to environment changes appears to be an interesting adaptation strategy and gives the first hints about the biofilm formation in cold environments.

1. Introduction

Bacteria in nature are most often found associated with surfaces in communities known as biofilms (Hall-Stoodley et al., 2004). Biofilm has been defined as an aggregate of microorganisms in which the cells are surrounded by a self-produced matrix constituted of extracellular polymeric substances (EPS) and adhere to each other and/or to a surface. The intermolecular interactions between the EPS molecules determine the mechanical properties of the matrix, and the physiological activity of the organisms in the biofilm (Flemming and Wingender, 2010). Although the precise chemical and physical composition of the EPS varies according to the species and the growth conditions, the main biofilm matrix building blocks are bacterial proteins, extracellular DNA (eDNA) (Whitchurch et al., 2002), lipids and polysaccharides (Flemming and Wingender, 2010). The eDNA is a critical component of the biofilm matrix of several bacteria (Whitchurch et al., 2002; Seper et al., 2011), and its amount in the biofilms varies greatly from strain to strain. It has multiple functions in biofilm formation (Okshevsky et al., 2015) and influences the three-dimensional biofilm architecture and stability by acting as a cell-cell interaction polymer (Allesen-Holm et al., 2006).

The importance of proteins in the biofilm structure and function is increasingly being recognized. Matrix proteins include not only the outer membrane proteins and the secreted proteins, but also particular classes of proteins, such as the adhesins, or motility organelles, such as curli or type IV pili proteins (Flemming and Wingender, 2010; Johnson et al., 2014).

Extracellular polysaccharides are important structural components of the biofilm matrix. Most of the exopolysaccharides of the matrix are very long, with a molecular weight range of 500–2000 kDa; they can be homopolymers, such as cellulose, curdlan, and dextran, or heteropolymers, such as alginate, emulsan, gellan, and xanthan (Yildiz et al., 2014).

Biofilm architectures are highly variable, ranging from open structures, containing channels and columns of bacteria, to structures with no obvious pores and densely packed regions of cells (Wimpenny et al., 2000; Doghri et al., 2015). To date, most attention has been focused on biofilms arising from the colonization of solid-liquid (S–L) interfaces

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(i.e. submerged biofilms), but several other kinds of interface, as the air-liquid (A–L), also provide ecological opportunities for bacterial colonization. The biofilms formed on the surface of static liquids are usually referred to as pellicles or floating biofilms (Wimpenny et al., 2000).

Irrespective of the interface where the bacteria aggregate, the biofilm formation consists of several stages and involves numerous conserved and/or species-specific factors (Hall-Stoodley et al., 2004). In any case, the biofilm formation can be described as a developmental process with distinct stages: an 'initial adhesion', in which the microorganisms adhere to biotic or abiotic surfaces; an 'early biofilm formation', during which the microorganisms begin to produce extracellular polymeric substances (EPS); a 'biofilm maturation', involving the development of three-dimensional structures where the EPS component provides a multifunctional and protective scaffold; and finally a 'dispersal', whereby the cells leave the biofilm to reenter the planktonic phase (Sauer et al., 2002). This is a dynamic and complex process and requires a considerable energetic cost (Saville et al., 2011); however, this cost may be evolutionarily acceptable due to the structural and physico-chemical advantages deriving from the biofilm formation. First, the capacity to colonize a surface provides a high level of stability in the growth environment. Secondly, the matrix enables the biofilm to capture resources such as the nutrients that are present in the environment or that are associated with the substratum on which the biofilm is growing (Flemming and Wingender, 2010). Thirdly, the biofilm formation affords protection from a wide range of environmental challenges (Flemming et al., 2016). Moreover, the spatial organization of the cells in biofilms allows a high degree of biodiversity and complex, dynamic and synergistic interactions, including cell-to-cell communication and enhanced horizontal gene transfer (Flemming et al., 2016). Therefore, the ability to form biofilm is a selective advantage for bacteria.

Correspondingly, bacteria living in extreme environments, like Antarctica, can be found as biofilms and this ability is believed to aid their adaptation and survival in the environment (Liao et al., 2016). The capability of cold-adapted bacteria to live and proliferate at low temperatures is the result of a wide range of adaptive features (Margesin and Feller, 2010; Carillo et al., 2015; Casillo et al., 2017) but how and if the capability to form biofilm can be included into these features is poorly investigated (Smith et al., 2016; Liao et al., 2016).

In this paper, our attention has been focused on the biofilm structure of *Pseudoalteromonas haloplanktis* TAC125 (*Ph*TAC125) (Médigue et al., 2005). *Pseudoalteromonas* strains played a significant role in marine ecosystems and they have been frequently isolated from natural biofilms or surfaces of eukaryote (Holmström and Kjelleberg, 1999). They are also known to produce a wide array of compounds with pharmaceutical and antifouling potential (Isnansetyo and Kamei, 2003; Klein et al., 2011). Moreover, *Ph*TAC125 is one of the model organisms of cold-adaptation and is one of the most intensively investigated psychrophilic bacteria. The increasing interest in *Ph*TAC125 has led to the accumulation of different data types for this bacterium in the last few years, including its complete genome sequence (Médigue et al., 2005), its proteome (Piette et al., 2011), its growth phenotypes description in different conditions (Giuliani et al., 2011; Sannino et al., 2017; Wilmes et al., 2010) and the construction of a genome-scale metabolic model (Fondi et al., 2015).

The main aim of this paper was the characterization of *Ph*TAC125 biofilm in different environmental conditions to asses if and how the *Ph*TAC125 biofilm structure is shaped by the environment. In detail, the response to different temperatures (15 °C vs 0 °C) and nutrient abundance (rich medium vs synthetic medium) has been analyzed. The *Ph*TAC125 biofilm has been characterized in terms of biofilm typology and matrix composition by means of several classic experimental approaches, such as confocal laser scanning microscopy, and Raman Microspectroscopy a technique recently used to provide molecular details of the chemical composition of bacterial biofilms (Carey et al., 2017; Henry et al., 2017; Takahashi et al., 2017).

2. Materials and methods

2.1. Bacterial strains and culture conditions

Pseudoalteromonas haloplanktis TAC125 was isolated in 1992 from an Antarctic coastal seawater sample collected near the French Antarctic station Dumont d'Urville, Terre Adélie (66°40′ S; 140° 01′ E). It was grown in Brain Heart Infusion broth (BHI, Oxoid, UK) and the synthetic medium GG (10 g/L D-Gluconic acid sodium, 10 g/L glutamic acid, SCHATZ salt mixture) (Sannino et al., 2017). The biofilm formation was assessed in the static condition in the BHI medium and GG medium at 15 °C and 0 °C at different times (24 h, 48 h, 72 h, and 96 h).

2.2. Biofilm formation and assays

The quantification of the *in vitro* biofilm production was based on the method described by Christensen with slight modifications (Christensen et al., 1985). Briefly, the wells of a sterile 24-well flat-bottomed polystyrene plate were filled with 1 mL of a medium with a suitable dilution of the Antarctic bacterial culture in the exponential growth phase (about 0.1 OD 600 nm). The plates were incubated at 15 °C and 0 °C for different times (24 h, 48 h, 72 h, and 96 h). After rinsing with PBS, the adherent cells were stained with 0.1% (w/v) crystal violet, rinsed twice with double-distilled water, and thoroughly dried. To analyze the typology of the biofilm formed at the different tested conditions, the stained biofilms were photographed by a camera from the top (the submerged biofilms) and from the front (the pellicles) of the plates. Subsequently, the dye bound to the adherent cells was solubilized with 20% (v/v) acetone and 80% (v/v) ethanol. After 10 min of incubation at room temperature, the OD 590 nm was measured to quantify the total biomass of biofilm formed in each well; the OD590 values reported was obtained by subtracting the OD590 value of the control obtained in absence of bacteria. Each data point was composed of six independent samples.

2.3. Biofilm recovery

Pellicles. The biofilm formation assay was performed in BHI medium and GG medium at 15 °C, since in these conditions the biofilm formation occurred at the air-liquid interface. Briefly, the wells of a sterile 24-well flat-bottomed polystyrene plate were filled with 1 mL of a medium with a suitable dilution of a *Ph*TAC125 culture in the exponential growth phase (about 0.1 OD600 nm). Plates were incubated at 15 °C for 96 h to allow the formation of compact and resistant pellicles at the air-liquid interface. After incubation, the pellicles were recovered using a pipette and stored at −20 °C. The samples were freeze-dried for further analysis.

Submerged biofilm. The biofilm formation assay was performed in the GG medium at 0 °C, since in this condition the biofilm formation occurred at the solid-liquid interface. Briefly, sterile plastic Petri dishes (90 × 15 mm) were filled with 25 mL of the GG medium with a suitable dilution of a *Ph*TAC125 culture in the exponential growth phase (about 0.1 OD 600 nm). The plates were incubated at 0 °C for 48 h to allow a strong biofilm formation at the solid-liquid interface on the bottom of the plates. After incubation, the supernatant and the cells were discarded, and the plates were rinsed twice with filter-sterilized PBS. The submerged biofilm was recovered by scraping with a sterile cell scraper, centrifuged to remove the supernatant and stored at −20 °C. The samples were freeze-dried for further analysis.

2.4. Microbial adhesion to hydrocarbons (MATH) assay

Solution chemistry and cleaning procedure. Analytical reagent grade chemicals were used throughout and Milli-Q deionized (DI) water was used to prepare all the solutions. The electrolyte solutions were prepared with 200 mM NaCl. All the experiments were performed at

room temperature (20 ± 1 °C). The cleaning procedure for the glassware was rinsed with acetone, washing with DI water, acid-washing with 12 M HCl and finally rinsing repeatedly with DI water.

Preparation of bacteria. The *Ph*TAC125 biofilm formation was performed in the BHI medium and GG medium at 15 °C. After 96 h incubation, the biofilms (pellicles) were recovered, separated from the supernatant by centrifugation and then resuspended in a 0.2 M NaCl electrolyte solution. To remove traces of the growth medium, the biofilms were centrifuged and resuspended in a fresh electrolyte solution three times to obtain a final bacterial suspension suitable for use.

Hydrocarbons. Three different hydrocarbons were chosen: *n*-hexadecane (97–99%, Sigma), *n*-dodecane (≥99%, Sigma), and toluene (99.8%, Sigma). The partition coefficients were: *n*-hexadecane, log Kow = 8.20; *n*-dodecane, log Kow = 6.10; and toluene, log Kow = 2.73.

MATH assay. The cell surface hydrophobicity (CSH) of the biofilms (pellicles), recovered in the BHI medium and GG medium as previously described, was determined through the Microbial Adhesion to Hydrocarbons (MATH) assay, as a measure of their hydrophobic adherence, by following the method of (Rosenberg et al., 1980) with slight modifications. The bacterial suspension was adjusted to an absorbance (A0) of 0.6 at 600 nm. In a clean borosilicate round-bottom glass tube (16 × 150 mm), 1 mL of the test hydrocarbon was added to 4 mL of the bacterial suspension. The tube was vortexed for 2 min and set aside to rest for 30 min to allow for the phase separation. Next, a sample of the bacterial suspension was retrieved with a clean Pasteur pipette, with great care taken to avoid allowing the hydrocarbon layer to enter the pipette. The sample was then transferred to a cuvette for the final absorbance measurement (Af) at 600 nm. The adhesion of the bacteria to the hydrocarbons was evaluated as the fraction partitioned in the hydrocarbon phase, FPC. This was calculated as (FPC = 1 − Af /A0).

2.5. Motility assay

For the swarming motility assay, BHI and GG soft agar plates (0.3% agar) were used. An appropriate volume of a saturated cell culture (about 50 µL) of *Ph*TAC125, grown in the BHI medium and GG medium, was spotted on autoclaved circular pieces of Whatman Filter Paper with an average diameter of approx. 1.5 cm. Once the cells had been absorbed, the filters were placed in the center of the BHI and GG soft-agar plates and then the plates were incubated at 15 °C and at 0 °C, in parallel. To evaluate the ability of *Ph*TAC125 to swarm into a semi-solid medium, the cell spots were measured every 24 h in terms of distance from the filter. In this work, the motility is expressed as the length of the path per unit of time (mm/h).

2.6. Mannose effect on the biofilm formation

To test whether the presence of mannose affects the ability of *Ph*TAC125 to form a biofilm, a static biofilm formation assay was performed in the GG medium for 24 h at 0 °C in the presence of mannose. In detail, 200 µL of the medium with a suitable dilution of a *Ph*TAC125 culture in the exponential growth phase (about 0.1 OD 600 nm) were added into each well of a sterile 96-well flat-bottomed polystyrene plate in the absence and presence of 200 mM D-mannose (Merck KGaA) and 200 mM D-galactose (VWR International) as a control. After the incubation, the biofilm quantification was performed by means of the crystal violet method, as previously described.

2.7. Confocal laser scanning microscopy

For the confocal microscopy analysis, the biofilm formation was performed on Nunc™ Lab-Tek® 8-well Chamber Slides (n° 177445; Thermo Scientific, Ottawa, ON, Canada) in the BHI medium and GG medium at 15 °C and 0 °C for 24 h. All the microscopic observations and image acquisitions were performed with a confocal laser scanning microscope (CLSM) (LSM700-Zeiss, Germany) equipped with an Ar laser (488 nm), and a He-Ne laser (555 nm).

Bacterial Viability and Biofilm Thickness Determination. The biofilm cell viability was determined by the FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (Molecular Probes, Invitrogen) following the manufacturer's instructions. Briefly, 300 µL of the medium with a suitable dilution of a *Ph*TAC125 culture in the exponential growth phase (about 0.1 OD 600 nm) were added to each well of a sterile Chamber Slide. After 24 h incubation, the plates were rinsed with filter-sterilized PBS. Then each well of the chamber slide was filled with 300 µL of a working solution of fluorescent stains, containing the SYTO® 9 green fluorescent nucleic acid stain (10 µM) and Propidium iodide, the red-fluorescent nucleic acid stain (60 µM), and incubated for 20–30 min. at room temperature, protected from light. All the excess staining was removed by rinsing gently with filter-sterilized PBS. The images were obtained using a 20X/0.8 objective. The excitation/emission maxima for these dyes are approximately 480/500 nm for the SYTO® 9 stain and 490/635 nm for propidium iodide. Z-stacks were obtained by driving the microscope to a point just out of focus on both the top and bottom of the biofilms. The images were recorded as a series of. tif files with a file-depth of 16 bits. The COMSTAT software package(Heydorn et al., 2000) was used to determine the biovolume ($\mu m^3/\mu m^2$), mean thickness (µm) and roughness coefficient (Ra*). Biovolume was defined as the number of biomass pixels in all images of a stack, multiplied by the voxel size and divided by the substratum area of the image stack. Biovolume represents the overall volume of the biofilm, and also provides an estimate of the biomass in the biofilm. Average thickness of biofilm provided a measure of the space size of the biofilm and is the most common variable in biofilm literature. Roughness coefficient is calculated from the thickness distribution of the biofilm and provides a measure of how much the thickness of the biofilm varies and is an indicator of biofilm heterogeneity. For each condition, two independent biofilm samples were used and at least two Z-stacks were performed.

2.8. Raman microspectroscopy

Raman is a technique appropriate for the investigation of biopolymer compositions in media containing high concentrations, such as in biopolymer crystals (Vergara et al., 2013, 2005) or cells. Indeed, biofilm media and substrates have been investigated by Raman microspectroscopy in order to describe the chemical composition of bacterial biofilms (Henry et al., 2017; Carey et al., 2017). A confocal Raman microscope (Jasco, NRS-3100) was used to obtain the Raman spectra. The 514 nm line of an air-cooled Ar^+ laser (Melles Griot, 35 LAP431 220) was injected into an integrated Olympus microscope and focused to a spot diameter of approximately 1 µm by a 100x objective with a final 6 mW power at the sample. A holographic notch filter was used to reject the excitation laser line. The Raman backscattering was collected using a diffraction lattice of 1200 grooves/mm and 0.1 mm slits. Typically, it took 60 s to collect a complete data set by means of a Peltier-cooled 1024 × 128 pixel CCD photon detector (Andor DU401BVI). The Raman measurements were at least triplicated for reasons of reproducibility for each spot sampled. The wavelength calibration was performed by using cyclohexane as standard.

2.9. DOC-PAGE

PAGE was performed using the system of Laemmli (Laemmli, 1970) with sodium deoxycholate (DOC) as the detergent. The separating gel contained final concentrations of 14% acrylamide, 0.1% DOC, and 375 mM Tris/HCl (pH 8.8); the stacking gel contained 4% acrylamide, 0.1% DOC, and 125 mM Tris/HCl (pH 6.8). The biofilm samples were prepared at a concentration of 0.05% in the sample buffer (2% DOC and 60 mM Tris/HCl [pH 6.8], 25% glycerol, 14.4 mM 2-mercaptoethanol, and 0.1% bromophenol blue). All the concentrations are expressed as the mass/vol percentage. The electrode buffer was composed of SDS

(1 g/L), glycine (14.4 g/L), and Tris (3.0 g/L). The electrophoresis was performed at a constant amperage of 30 mA. The gels were fixed in an aqueous solution of 40% ethanol and 5% acetic acid. The biofilm sample bands were visualized by silver staining as previously described (Tsai and Frasch, 1982).

2.10. Sugar analysis

The glycosyl analysis was performed as already reported (Carillo et al., 2014). Briefly, biofilm samples (0.5 mg) were mixed with 1 mL of HCl/CH_3OH, subjected to methanolysis for 16 h at 80 °C and then acetylated. Fatty acids were extracted twice with hexane and the methanol layer was dried and acetylated. Both the acetylated methyl glycosides and methyl esters of the fatty acids were injected into the GC–MS. All the sample derivatives were analyzed on an Agilent Technologies gas chromatograph 6850 A equipped with a mass selective detector 5973 N and a Zebron ZB-5 capillary column (Phenomenex, 30 m × 0.25 mm i.d., flow rate 1 mL/min, He as carrier gas). The acetylated methyl glycosides were analyzed using the following temperature program: 140 °C for 3 min, and 140 °C → 240 °C at 3 °C/min. The acetylated alditols were analyzed using the following temperature program: 150 °C for 3 min, and 150 °C → 330 °C at 3 °C/min.

2.11. Calcofluor binding assay

The cellulose production was detected by growing bacteria on GG or BHI agar supplemented with 200 μg/ml Calcofluor (Sigma, Milan). The plates were incubated at 0 °C or 15 C for 96 h. The colonies were visualized under a 366 nm light source.

2.12. Cellulase assay

20 mg of each sample was incubated with 0.4 mL of acetic acid 0.05 M, 0.1 mL of water, and 0.1 mL of cellulase from *Trichoderma reesei* ATCC 26921, at 37 °C for 2 h. The reaction was quenched by transferring the tube into an ice bath and centrifuged at 4 °C, 3000 rpm, 10 min. The supernatant was freeze-dried and analyzed as acetylated alditols, as reported (Pieretti et al., 2010).

2.13. DNase I and proteinase K treatment on biofilm formation

To understand if DNase I and proteinase K are able to affect the *Ph*TAC125 biofilm formation process, a static biofilm assay was performed in the presence of DNase I or proteinase K. The biofilm formation was assessed in the BHI medium and GG medium at 15 °C and 0 °C for 24 h. In detail, 200 μL of the medium with a suitable dilution of a *Ph*TAC125 culture in the exponential growth phase (about 0.1 OD 600 nm) were added into each well of a sterile 96-well flat-bottomed polystyrene plate in the absence and presence of DNase I or proteinase K at 100 μL/mL. After incubation, biofilm quantification was performed by means of the crystal violet method, as previously described (Christensen et al., 1985).

3. Results

3.1. Characterization of PhTAC125 biofilm obtained in different culture conditions

To investigate the capability of *Ph*TAC125 to form biofilm at different growth temperatures, the bacterium was grown in BHI, a rich culture medium, or in GG, a synthetic minimum medium, at 15 °C and 0 °C in static conditions, and the biofilm was evaluated at different incubation times. As shown in Fig. 1, the Antarctic bacterium was able to form biofilm in all the tested conditions and the amount of the biofilm formed in the diverse conditions was different (statistically significative shown Table S1); in the synthetic medium GG, the amount of biofilm produced by the bacterium was higher than that produced in the rich medium BHI both at 15 °C and at 0 °C (Fig. 1). Moreover, the kinetics of the biofilm formation proved to be slightly different in the analyzed conditions, considering that, at 15 °C in GG the amount of biofilm increased over time while at 0 °C decreased (Fig. 1), suggesting that the temperature strongly influences the kinetics of the biofilm formation.

Furthermore, as shown in Fig. 1 the *Ph*TAC125 biofilm accumulated mainly at the air-liquid interface forming pellicles at 15 °C in both BHI and GG media and at 0 °C only in BHI, while, interestingly, it formed biofilm mainly at the solid-liquid interface at 0 °C in the GG medium.

3.2. MATH assay on pellicles obtained in different culture conditions

The macroscopic inspection of the pellicles deriving from A–L biofilms obtained when *Ph*TAC125 was grown in BHI or GG medium was suggestive of changes in their respective structural features. In fact, pellicles from the bacterial growth in BHI medium at 15 °C after 96 h incubation proved to be stickier and slimier than those obtained in the GG in the same conditions, which, in contrast, presented more compact. Therefore, the microbial adhesion to hydrocarbons (MATH) assay was performed to assess the hydrophobicity of the *Ph*TAC125 pellicles by examining their solubility in three different solvents: toluene, *n*-dodecane, and *n*-hexadecane. As shown in Fig. 2, the *Ph*TAC125 pellicles obtained in GG medium exhibited a significant degree of solubility in all the hydrocarbon solvents examined. In contrast, the *Ph*TAC125 pellicles formed in BHI medium demonstrated negative partitioning fractions in the tested solvents, indicating that the *Ph*TAC125 GG-grown pellicles exhibited a higher hydrophobicity with respect to the *Ph*TAC125 BHI-grown ones.

3.3. Motility assay in different culture conditions

The different motility of the *Ph*TAC125 cells in the four studied conditions was evaluated (Table 1) by means of an LB soft agar motility assay (Liu et al., 2010) and was expressed as the length of the path travelled on the BHI and GG soft-agar plates per unit of time. The reduction of the flagellar motility at low temperatures has previously been reported (De Maayer et al., 2014), and indeed, as expected, at 0 °C the bacterium displayed a lower ability to move in a semi-solid medium than at 15 °C.

3.4. CLSM analysis of biofilm obtained in different culture conditions

The biofilms produced by *Ph*TAC125 in the diverse conditions were further investigated by confocal laser scanning microscopy (CLSM) to analyze the biofilm structure and the biomass distribution by means of three-dimensional biofilm images. Live/dead staining was used to visualize the *Ph*TAC125 cells encapsulated in the biofilm matrix (Figs. 3a and S1) (green indicates viable cells and red indicates damaged cells). Although the four images were clearly different, it is difficult to obtain detailed and accurate descriptions of the differences among the four biofilms, based on visual inspection only. Therefore, all the CLSM image stack data were further analyzed using the COMSTAT image analysis software package (Heydorn et al., 2000) to evaluate the different variables describing the biofilm structure. As expected, the values of the biomass and the average thickness of the biofilm obtained in the rich medium BHI were significantly lower if compared to those of the biofilm produced by *Ph*TAC125 in the GG medium at the same temperature (Fig. 3b). The analysis revealed that in the GG medium the biofilm proved to be more compact, both at 15 °C and at 0 °C, as indicated by a lower roughness coefficient (Fig. 3b). This dimensionless factor provides a measure of how much the thickness of a biofilm varies, and it is thus used as a direct indicator of biofilm heterogeneity, suggesting that the medium composition has a key role in the definition of this characteristic of the biofilm structure (Toyofuku et al., 2016; Bester et al.,

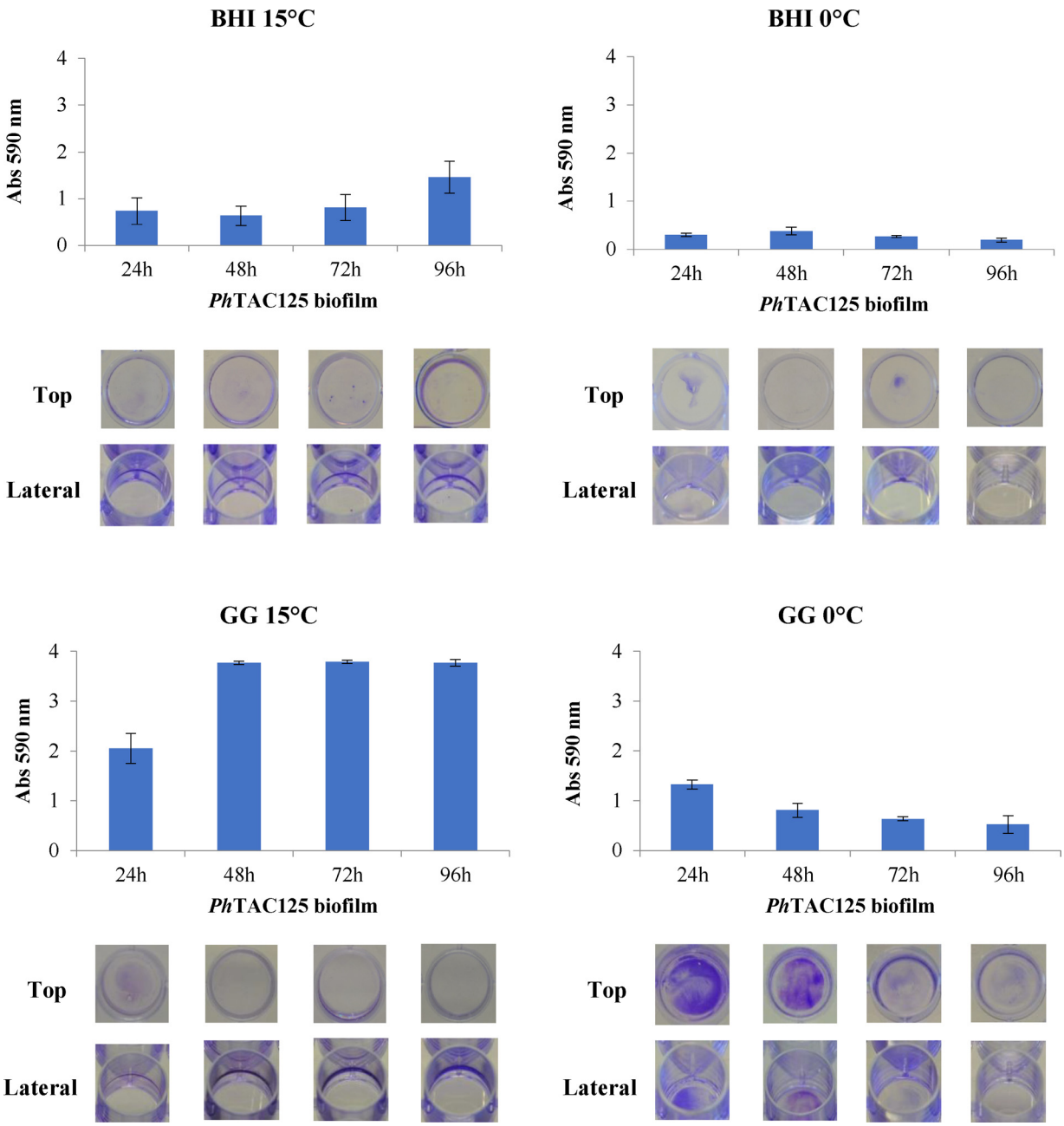

Fig. 1. Analysis of the effect of temperature and growth medium on the *Ph*TAC125 biofilm formation at different times. *Ph*TAC125 biofilm obtained at 15 °C or 0 °C in the BHI medium or in the GG medium. The biofilms were analyzed at 24 h, 48 h, 72 h, and 96 h with the crystal violet assay. Each data point was composed of six independent samples. (Top) Stained biofilms: the top view of wells allows a better vision of the submerged biofilms at the solid/liquid interface, (Lateral) whereas the lateral view is more useful to see the biofilms at the air/liquid interface.

2011; Shrout et al., 2006).

3.5. Raman microspectroscopy of PhTAC125 biofilms

High-quality Raman spectra were collected for biofilms grown at 0 °C in the GG and BHI media (spectra a and b in Fig. 4). Eventual signals coming from growth medium and/or the polystyrene supports were explored by recording the Raman spectra of the uninoculated media and of polystyrene, from which the GG-grown biofilm at 0 °C was scraped. As shown in Fig. 4, the GG medium presented several Raman bands in the 800-1500 cm^{-1} region (spectrum c in Fig. 4) that can be mainly assigned to carbohydrate-related vibrations. BHI medium was instead not active at Raman analysis (spectrum d in Fig. 4). The observed Raman bands recorded in the reference experiments were taken into consideration and not mentioned during the following analysis of microbial biofilms.

Both biofilms grown at 0 °C in the GG and BHI media showed Raman bands assignable to proteins, nucleic acids, and carbohydrates (Henry et al., 2017; Carey et al., 2017). The visual inspection of the obtained Raman spectra indicated that they were significantly different. Therefore, these spectral changes do indeed reflect the different chemical composition of the *Ph*TAC125 biofilms. For the purpose of a quantitative comparison, the spectra of the biofilms in Fig. 4 were normalized with respect to the amide I band (~ 1665 cm^{-1}). Therefore, the following comparison between the biofilms refers to the relative content of nucleic acids or carbohydrates with respect to the protein content (Henry et al., 2017).

The strongest evidence was the presence of Raman bands at

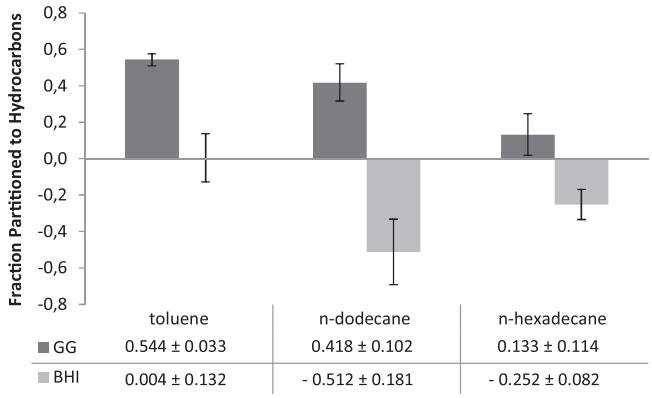

Fig. 2. MATH assay of *Ph*TAC125 A–L biofilms. The fraction of the biofilms partitioned in the hydrocarbon phase measured by means of MATH assay. The bacterial suspensions are contacted with toluene, n-dodecane, and n-hexadecane. The cells were suspended in 200 mM NaCl for all the measurements. Each data point represents the mean ± SD of four independent samples.

Table 1
Motility of *Ph*TAC125 in different conditions. Analysis of the ability of *Ph*TAC125 to swarm into a semi-solid medium. The motility was expressed as the length of the path travelled on the BHI and GG soft-agar plates per unit of time (mm/h). The data are reported as the mean ± SD of three independent experiments.

	MOTILITY (mm/h)	
	0 °C	15 °C
BHI	0.092 ± 0.008	0.375 ± 0.02
GG	0.016 ± 0.002	0.104 ± 0.003

674 cm^{-1} (the guanine–ring mode), 786 cm^{-1} (mostly the DNA phosphodiester backbone mode), and 815 cm^{-1} (mostly the RNA phosphodiester backbone mode) only in the sample grown in the GG medium. These bands, which represent important nucleic acid fingerprints (Carey et al., 2017), were, on the contrary, not detectable in the biofilm obtained in the BHI. Other nucleic acid-related bands can be compared between the two samples, consistently indicating a much higher nucleic acid relative content in the GG-grown biofilms. Indeed, the Raman bands at 1588 cm^{-1} (the adenine/guanine ring) and at 1178 cm^{-1} (the guanine-ring) presented a higher intensity in the latter sample. Moreover, the band at 1488 cm^{-1} (the adenine/guanine ring), after normalization to amide I (1665 cm^{-1}), was ~5 times higher in the GG- than in the BHI- grown biofilms.

The relative content of carbohydrates can be estimated by the ratio I1130/I1007, related to the carbohydrate and Phe-ring bands, respectively (Carey et al., 2017; Henry et al., 2017). Other carbohydrate-related Raman bands were located at 1366 and 1400 cm^{-1}. All these markers consistently indicated a slightly higher carbohydrate/protein content in the biofilms grown in the GG medium compared to those obtained in the BHI medium.

Concerning the analysis of biofilms grown at 15 °C, as a high fluorescence was observed for biofilms grown in both media, a further washing step was carried out to get rid of this interference. Unfortunately, a Raman spectrum of modest quality was collected only for the sample grown in the GG medium (data not shown). Despite its modest quality, the Raman spectrum recorded allowed us to see at least detectable nucleic acid-related Raman bands at 674 cm^{-1}, 786 cm^{-1}, and 815 cm^{-1} with a relative nucleic acid/protein Raman intensity similar to that observed for the biofilms grown at 0 °C, indicating that the specific growth temperature did not influence the relative nucleic/protein ratio.

3.6. Sugar analysis of PhTAC125 biofilms

In order to obtain information on the polysaccharides present in the different *Ph*TAC125 biofilm, samples were analyzed by 14% DOC-PAGE and visualized by using the silver nitrate method (Tsai and Frasch, 1982) (Fig. S2). The silver nitrate showed the presence in all the samples of one band at low molecular masses, corresponding to LPS, the

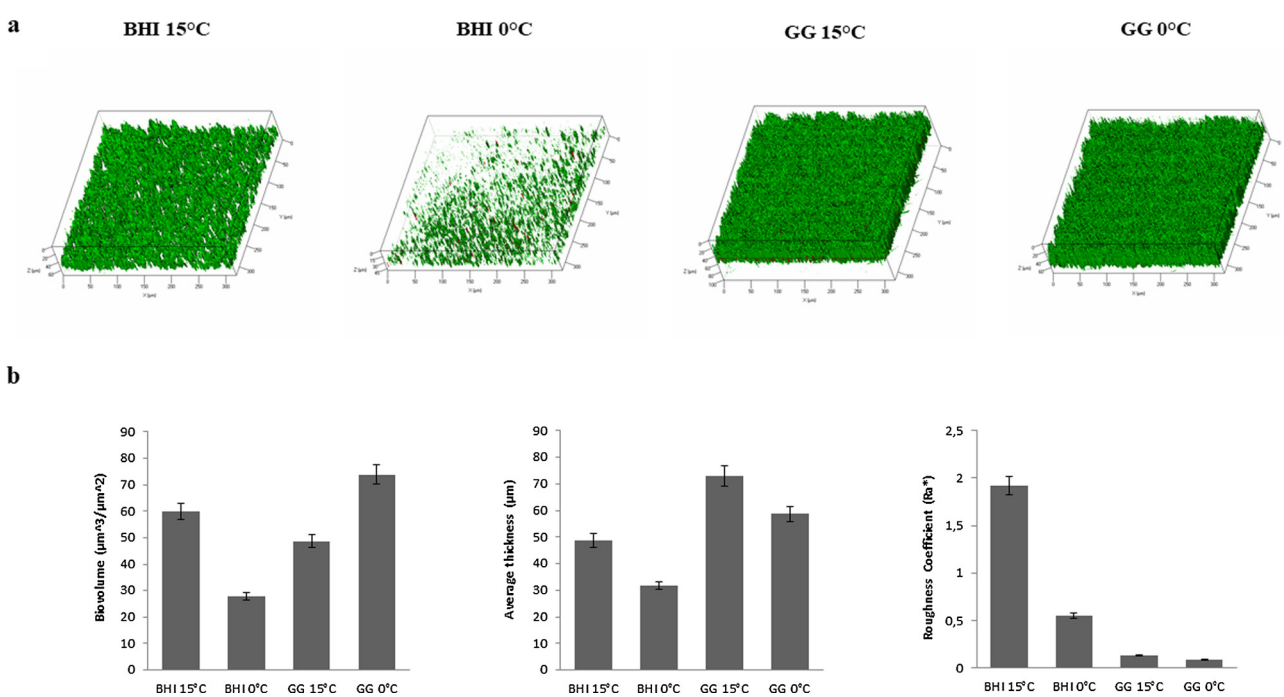

Fig. 3. CLSM analysis of *Ph*TAC125 biofilms. Analysis of the effect of temperature and growth medium on the *Ph*TAC125 biofilm structure. (a) CLSM analysis of *Ph*TAC125 biofilm grown in chamber slides at 15 °C and 0 °C in the BHI medium and GG medium for 24 h. The three-dimensional biofilm structures were obtained using the LIVE/DEAD® Biofilm Viability Kit. (b) COMSTAT quantitative analysis of the biomass, average thickness and roughness coefficient of the *Ph*TAC125 biofilms at all the tested conditions. (For interpretation of the references to colour in the text, the reader is referred to the web version of this article).

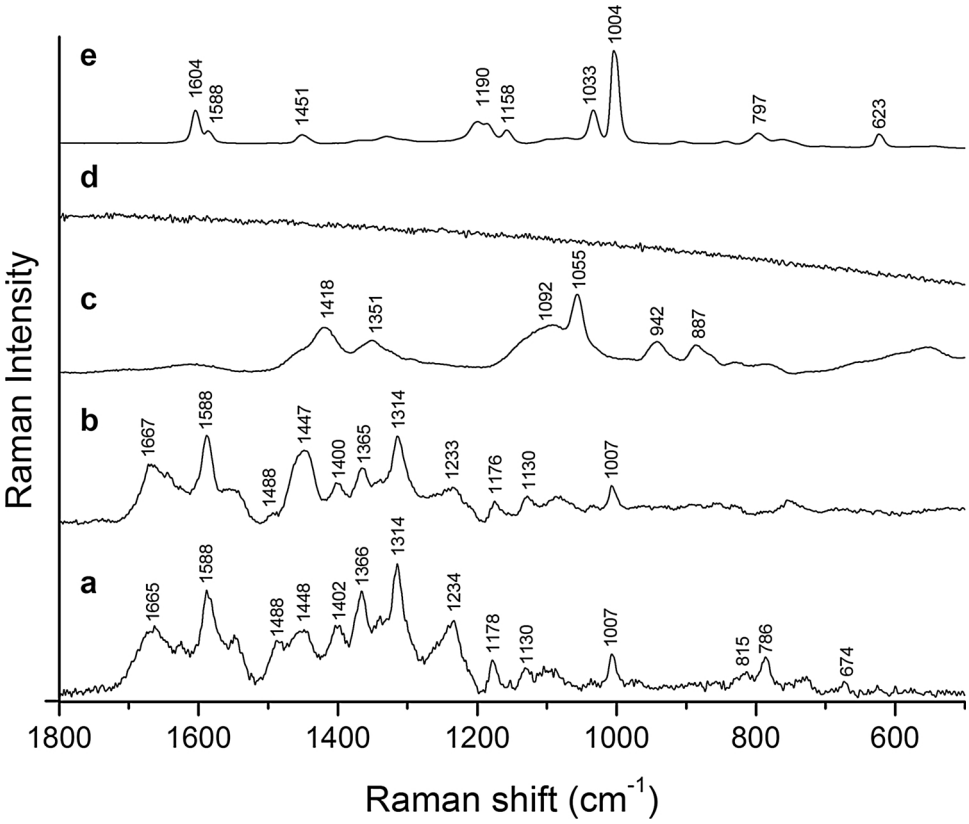

Fig. 4. Raman analysis of *Ph*TAC125 biofilms. Raman spectra (500–1800 cm^{-1}) collected with 514 nm laser, 60 s exposure, and 1 µm spot size: (a) GG-grown biofilm at 0 °C, (b) BHI-grown biofilm at 0 °C, (c) GG medium, (d) BHI medium, and (e) polystyrene substrate. The spectra a and b are normalized with respect to the amide I band (1665 cm^{-1}).

Table 2
Biofilm sugar analysis profile. Monosaccharides percentage calculated for each biofilm growth condition referred to the relative abundance of each component compared to the total sugars.

Carbohydrate	*Ph*TAC125 S-L Biofilm 0 °C GG	*Ph*TAC125 A-L Biofilm 0 °C BHI	*Ph*TAC125 A-L Biofilm 15 °C BHI	*Ph*TAC125 A-L Biofilm 15 °C GG
Ribose	37.6%	3.4%	–	22.5%
Mannose	–	1.1%	1.8%	1.2%
Galactose	13.1%	36.1%	36.2%	15.7%
Glucose	4.9%	5.3%	4.5%	7.4%
GlcN	18.1%	18.2%	19.3%	24.6%
Heptose	22.8%	35%	37.5%	22.7%
NAM	3.5%	0.9%	0.7%	5.9%

structure of which had already been characterized (Michela Corsaro et al., 2001). In addition, samples corresponding to GG-grown biofilm and the BHI-grown biofilm, both developed at 0 °C, showed several bands attributable to nucleic acids (Fig. S2 lanes a and b). Glycosyl analysis performed on the *Ph*TAC125 biofilms confirmed the presence of sugars attributable to the lipopolysaccharide (Michela Corsaro et al., 2001) like galactose, glucosamine, heptose, and traces of mannosamine; indeed, in the biofilm a lot of biomass is constituted by cells. Moreover, N-Acetyl-muramic acid (MurNAc, NAM), ribose and glucose were found (Table 2). The presence of ribose confirmed the occurrence of nucleic acids in the samples.

The presence of cellulose in *Ph*TAC125 biofilm was preliminarily investigated by staining cells, grown on GG or BHI agar plates, with the Calcofluor (Fig. S3). Although it is routinely used for this purpose (Zogaj et al., 2001), Calcofluor is not absolutely specific for cellulose (it is known to bind also β[1–4] and β[1–3]-linked glucosyl polymers). Therefore, to confirm the presence of cellulose, the samples of the *Ph*TAC125 biofilms were treated with a cellulase, an enzyme which has the capability to specifically hydrolyze cellulose, leading to free glucose formation. The released glucose was revealed as acetylated alditol, demonstrating the presence of cellulose in all the tested samples (Fig. S4).

3.7. DNase I and proteinase K effect on biofilm formation in different environmental conditions

The role of proteins and eDNA in the 'initial adhesion' and 'early biofilm formation' steps was studied to collect more information on molecular mechanisms involved in *Ph*TAC125 biofilm formation in the four explored conditions. *Ph*TAC125 was grown in the BHI and GG media at 15 °C and 0 °C in static conditions, in the presence of either DNase I or proteinase K, and a quantification of the formed biofilm was performed by means of crystal violet staining (Fig. 5). The results obtained revealed that the treatment with DNase I did not lead to any reduction in the biofilm development but, interestingly, in the GG at 0 °C the nuclease addition led to an increase in the biofilm biomass. The proteinase K treatment, on the other hand, strongly affected the Antarctic bacterium biofilm formation in the BHI medium, whereas the protease addition did not have a great impact on the biofilm formation in the GG medium (Fig. 5).

4. Discussion

The ability of bacteria to form biofilms in many environments is undoubtedly related to the selective advantage that the surface association offers. As previously reported the Antarctic bacterium *Ph*TAC125 is able to form biofilm at 4 °C in BHI and in GG broths and in these conditions, the biofilm formation generally occurs at the air-liquid interface (Papa et al., 2013; Parrilli et al., 2015).

In this paper, the structural characterization of the biofilm of the Antarctic bacterium *Ph*TAC125, produced in response to different nutrient abundance and temperatures, was investigated. In particular, the capability of *Ph*TAC125 to form biofilm was investigated at 15 °C and 0 °C in BHI and GG media.

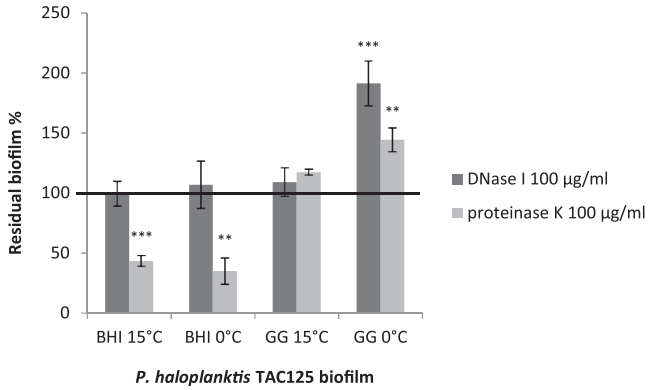

Fig. 5. Analysis of the effect of DNase I and proteinase K on *Ph*TAC125 biofilm formation. The *Ph*TAC125 biofilm formation obtained in the BHI medium or GG medium at 15 °C or 0 °C for 24 h in the absence and presence of DNase I or proteinase K at a concentration of 100 µg/ml. The data are reported as a percentage of the residual biofilm after the treatment. Each data point represents the mean ± the SD of six independent samples; the mean values were compared to the untreated control and considered significant when $p < 0.05$(* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) according to the Student t-test.

The Antarctic bacterium resulted to be able to form biofilm and the majority of cells unbudded in the matrix resulted vital (Fig. S1) in all the tested conditions. This result is not surprising since its genomic and metabolic features indicate that this bacterium is adapted to periodic changes in temperature and nutrient availability (Médigue et al., 2005). Data described demonstrating that the cold-adapted bacterium responds to different temperatures (15 °C vs 0 °C) and levels of nutrients abundance (rich medium vs synthetic medium) producing different amount of biofilm, indeed in the synthetic medium, the biofilm quantity was higher than that produced in the rich medium. It's important to underline that in planktonic growth condition the biomass reached in BHI is always higher than that obtained in GG medium (data not shown). Likely, the presence of a lower availability of nutrients could induce a greater production of biofilm since the biofilm matrix can improve the capture of nutrients (Flemming et al., 2016; Shrout et al., 2012; Tolker-Nielsen and Tolker-Nielsen, 2015), and therefore the higher biofilm production could be a strategy to survive in poor nutrient conditions. It's interesting to note that at 0 °C in the GG medium, the bacterium produced biofilm to the same extent as in BHI at 15 °C, indicating that also at low temperatures the energetic cost related to the biofilm production is adequate to the advantages deriving from the biofilm formation. The CLSM analysis on the biofilms produced by *Ph*TAC125 in GG and BHI revealed that the medium composition has a key role also in the definition of the biofilm structure. Actually, the investigation revealed that the biofilm obtained in the GG medium was more compact than the biofilm developed in the rich medium.

It is useful to remark that the temperature and the media composition not only affect the amount and the compactness, but also the typology, of the biofilm. Indeed, the *Ph*TAC125 biofilm accumulates mainly at the air-liquid interface at 15 °C in both BHI and GG media and at 0 °C only in BHI, while, interestingly, it forms biofilm mainly at the solid-liquid interface at 0 °C in the GG medium. Although a correlation between the typology of a biofilm and the growth medium has recently been published (Paytubi et al., 2017), to the best of our knowledge, this is the first report of the effect of the temperature on the typology of biofilm (i.e. in the spatial localization of biofilm). Indeed, the bacterium in GG medium produced a liquid-air biofilm if grown al 15 °C, while in the same medium it produced a biofilm on solid-liquid interface when grown at 0 °C. Anyway, it is remarkable to note that *Ph*TAC125 is able to efficiently produce both submerged biofilms and pellicles and that the *Ph*TAC125 pellicles obtained in the two media resulted to be different. The pellicles obtained in the BHI was slimier and stickier than those obtained in the GG which, in contrast, seemed to be more compact and better structured. Moreover, the *Ph*TAC125 GG pellicles displayed a higher hydrophobicity compared to the *Ph*TAC125 BHI pellicles.

The A–L biofilm formation has been described for various bacteria and proceeds through several stages (Armitano et al., 2013). In the early stages, a thin layer of cells appears at the air-liquid interface (Armitano et al., 2013) and, subsequently, the three-dimensional structures develop as the pellicle grows and thickens (O'Toole et al., 2000). The exact mechanisms underpinning the initial stages of the pellicle formation (i.e. how the cells reach the interface) have not been well-characterized. However, the cell motility seems to strongly influence the A–L biofilm formation, which requires upward swimming capabilities (Kobayashi, 2007). In fact, it has been reported that many bacteria show defects in pellicle formation when the genes involved in the flagellum synthesis are mutated, and, in some species, the ability to form a pellicle is completely abolished (Lemon et al., 2007; Serra et al., 2013). Since several authors have reported that motility might facilitate the bacteria localization near the A–L interface and thereby contribute to the A–L biofilm formation, the different motility of the *Ph*TAC125 cells in the four studied conditions was explored and the bacterium resulted to be slower in the GG than in the BHI medium. This result could explain why the bacterium was unable to form pellicles at 0 °C in the GG medium, but it does not completely clarify why in this condition *Ph*TAC125 was able to form an S-L biofilm. Unpublished proteomic data from our laboratory revealed that the major pilin MshA of type IVa mannose-sensitive hemagglutinin pili is overexpressed when *Ph*TAC125 is grown at 0 °C in a GG medium. Interestingly, type IVa mannose-sensitive hemagglutinin pili are critical for the initial attachment to a surface and for the S-L biofilm formation in several bacteria, such as *Vibrio cholerae* (Watnick et al., 1999) and *Pseudoalteromonas tunicata* (Dalisay et al., 2006). In particular, MshA pili are crucial for the "orbiting" of near-surface motility trajectories (Utada et al., 2014) and it has been demonstrated that when the mannose is added to the medium, in order to saturate the MshA pili binding, the orbiting motility of *V. cholerae* is ablated and the initial surface attachment is impaired (Utada et al., 2014). To assess if the capability of *Ph*TAC125 to form an S-L biofilm in the GG medium at 0 °C was related to the type IV mannose-sensitive hemagglutinin pili, the bacterium was grown in the GG medium at 0 °C in the presence and absence of mannose and the biofilm formation was evaluated (Fig. S5). The presence of mannose reduced the formed biofilm suggesting that, as in *V. cholera*, also in *Ph*TAC125 at 0 °C in the GG medium the surface attachment was mediated by type IV pili and it occurred mainly in these conditions due to the type IV pili overexpression and the reduced motility.

All the data obtained on the biofilm characterization have demonstrated that the specific features like the typology, hydrophobicity, biomass, thickness, and compactness, of *Ph*TAC125 biofilms obtained in different conditions, were deeply different. To assess if these differences might be related to a possible different chemical composition of the biofilm matrix components, the biofilm structures were further investigated by Raman microspectroscopy. This technique has been recently proposed and applied to the chemical characterization of similar biofilms (Carey et al., 2017; Henry et al., 2017; Takahashi et al., 2017). Overall, the Raman investigation of the *Ph*TAC125 biofilms indicated a much higher nucleic acid/protein ratio and an only slightly higher carbohydrate/protein ratio in the biofilms obtained in the GG medium compared to those grown in the BHI medium. The high nucleic acid/protein ratio, reported for the biofilms grown in the GG, could be in accordance with the observation that the *Ph*TAC125 GG pellicles displayed a higher hydrophobicity than the *Ph*TAC125 BHI pellicles. Indeed, several studies have suggested that eDNA increases the hydrophobicity of bacterial cells (Okshevsky and Meyer, 2015). Therefore, it's possible to correlate the hydrophobicity of GG pellicles with a higher content of eDNA, or better with a higher nucleic acid/protein ratio in the biofilm obtained in GG. Moreover, the different nucleic acid/protein relative ratio could also account for the reported

differences in the *Ph*TAC125 biofilm structure (Fig. 3b, roughness coefficient) due to the known influence of eDNA on biofilm three-dimensional architectures (Okshevsky and Meyer, 2015).

Polysaccharides are considered as a significant structural component of the biofilm matrix. As predictable, sugar analysis done on the *Ph*TAC125 biofilms demonstrated the presence of sugars characteristic of the *Ph*TAC125 lipopolysaccharide (Michela Corsaro et al., 2001) like galactose, glucosamine, heptose, and mannosamine. Moreover, N-Acetyl-muramic acid (MurNAc, NAM), ribose and glucose were found. Muramic acid is one of the components of the peptidoglycan structure but also found in LPS structures (Zych et al., 1998; Casillo et al., 2015). As far as we know, it has never been reported as a biofilm component. Instead, ribose has already been found in biofilms (Hung et al., 2005). The presence of glucose in all the samples and the occurrence of a cellulose synthase gene cluster in the *Ph*TAC125 genome (Römling and Galperin, 2015) prompted us to investigate the presence of cellulose in the *Ph*TAC125 biofilm matrix. Cellulose is a significant extracellular matrix component of the biofilms of several ecologically diverse bacteria, and it mediates cell-cell interactions, cell adherence and biofilm formation on biotic and abiotic surfaces (Römling and Galperin, 2015). Four principal types of cellulose synthase operon have been found in various bacterial genomes, in the *Ph*TAC125 genome was found (Römling and Galperin, 2015) an *E. coli*-like type of *bcs* operon which is widespread among the members of beta and gamma subdivisions of proteobacteria, the expression of the cellulose synthase gene cluster is generally stimulated during biofilm formation (Zogaj et al., 2001; Prigent-Combaret et al., 2012). The data reported demonstrated that the cellulose is a constituent of *Ph*TAC125 biofilm matrix in all the tested conditions.

Although the Antarctic bacterium synthesizes cellulose as polysaccharidic component present in the matrix in all the tested conditions, when the different relative content of eDNA and proteins is modulated, it produces biofilm matrices with different characteristics in terms of hydrophobicity, porosity, and roughness.

Last part of the research work was dedicated to assessing if the environmental conditions also influence the *Ph*TAC125 biofilm development process. In particular, the role of proteins and eDNA in the 'initial adhesion' and 'early biofilm formation' steps were studied when the bacterium was grown in the GG or BHI media. The results obtained revealed that the treatment with DNase I did not lead to any reduction in the biofilm development but, interestingly, in the GG at 0 °C the nuclease addition led to an increase in the biofilm biomass. For some bacteria, eDNA, besides its structural role, is required for the initial attachment to surfaces, whereas in other bacteria it plays a role during the transition from the attachment phase to the biofilm maturation phase (Okshevsky et al., 2015). Data reported suggested that the eDNA has a marginal role in the *Ph*TAC125 early biofilm formation both in the GG or BHI. On the other hand, proteins have a key role in Antarctic bacterium biofilm formation in the BHI medium, whereas they did not have a great impact on the biofilm formation in the GG medium. The reported differences in the role of proteins and of eDNA in early biofilm formation steps suggest that the environmental conditions also influence the molecular mechanisms responsible for biofilm establishment and formation.

In conclusion, the capability of *Ph*TAC125 to adopt different biofilm structures in response to changes in its environment appears to be an interesting adaptation strategy and the results herein described give the first hints in biofilm formation in cold environments.

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Declaration of interest

None.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.micres.2018.09.010.

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CHAPTER II

2. Polar marine bacteria as source of novel anti-biofilm molecules

Marine microorganisms from Antarctica can synthesize a broad range of potentially valuable bioactive compounds. Previous studies revealed that supernatants obtained from bacterial cultures of cold-adapted bacteria belonging to *Flavobacterium, Pseudoalteromonas*, *Pseudomonas*, *Psychrobacter* and *Psychromonas* genera showed anti-biofilm activity against different pathogenic strains[1].
With the aim of identifying new anti-biofilm molecules, two different libraries were constructed, starting from a selection of Polar marine bacteria (Tab. 1), and screened against *Staphylococcus epidermidis*: a "small metabolite" library, considering that natural products like secondary metabolites are often involved in quorum sensing modulation and biofilm inhibition[2], and a "protein/peptide" library, since it is reported that there are many molecules of proteinaceous nature with anti-biofilm activity[3-5].

Table 1: Polar marine bacteria used in this study

Strain	Origin	Reference and/or source
Flavobacterium sp. TAB 87	Antarctic sea water	Liège collection
Pseudoalteromonas haloplanktis TAB23	Antarctic sea water	[6]Feller G. et al., 1992
Pseudoalteromonas haloplanktis TAE56	Antarctic sea water (algae necrosed suspended in sea water)	Liège collection
Pseudoalteromonas haloplanktis TAE57	Antarctic sea water (algae necrosed suspended in sea water)	Liège collection
Pseudoalteromonas haloplanktis TAE79	Antarctic sea water (algae necrosed suspended in sea water)	Liège collection
Pseudoalteromonas haloplanktis TAE80	Antarctic sea water (algae necrosed suspended in sea water)	Liège collection
Pseudomonas sp.TAA207	Antarctic sea water (marine sediment)	Liège collection
Pseudomonas sp.TAD18	Antarctic sea water	Liège collection
Psychrobacter sp.TAD1	Antarctic sea water	Liège collection
Psychrobacter arcticus 273-4	Siberian permafrost sediment cores	[7]Bakermans et al., 2006
Psychromonas arctica	Arctic sea water (Svalbard islands, Arctic)	[8]Groudieva et al., 2003

This chapter is organized in two paragraphs:

- the first paragraph describes the construction of the "small metabolite" library and its screening against *S. epidermidis*;
- the second paragraph describes the construction of the "protein/peptide" library and its screening against *S. epidermidis*.

Polar bacterial extracts inhibiting *Staphylococcus epidermidis* biofilm formation: looking for novel anti-biofilm agents

Abstract

With the aim to identify new anti-biofilm molecules, a “small metabolite” library was constructed starting from Polar marine bacteria. In this study, total organic extracts were obtained from the planktonic cultures of several Polar strains and their anti-biofilm activity was analysed on *Staphylococcus epidermidis*. A preliminary analysis revealed the great potential of the extracts obtained from *Pseudomonas sp.* TAA207 and *Psycrobacter sp.* TAD1, able to inhibit *S. epidermidis* biofilm formation with different specificity of action. A fractionation of the total organic extracts in intracellular and extracellular samples demonstrated, for both the analyzed bacteria, the localization of the active molecule/s in the intracellular compartment. Suitable purification strategies were set up for the selected samples. Obtained results revealed that the active molecule produced by *Pseudomonas sp.* TAA207 was the pentadecanal, the same molecule identified to be responsible for the anti-biofilm activity of *Pseudoalteromonas haloplanktis* TAC125. The purification protocol proposed for *Psycrobacter sp.* TAD1 led to the obtainment of two different active fractions, likely containing molecules of different nature. Further analysis are still ongoing aiming to the identification of the active molecules.

Introduction

In nature, bacteria predominantly exist as communities of sessile cells that develop as biofilms[1-2]. By generating a matrix, bacteria in biofilms create a physically distinct habitat that provides shelter, promotes the accumulation of nutrients and fundamentally alters both the physicochemical environment of the biofilm and interactions among the organisms therein[3]. In the biofilm state, bacteria are significantly more resistant to external assault, including attack by antibiotics, biocides and disinfectants. In industrial fields, biofilms are responsible for biofouling and contamination of process water[4], deterioration of the hygienic quality of drinking water[5] and microbially influenced corrosion[6]. Within a host environment, they are insensitive to antiseptics and basic host immune responses. This enables bacteria within biofilms to be the cause, in clinical settings, of many persistent infections of live tissue and contaminations of medical device surfaces. For all these reasons, microbial biofilms have great negative impacts on the world’s economy and pose serious problems to industry, public health and medicine.

Staphylococci are the most important etiological agents of biofilm associated-infections on indwelling medical devices. The capacity of biofilm production is dependent on the pathogenic potential of staphylococcal strains as well as the host’s resistance to infections. The colonized devices become the focus of infection and can cause localized and generalized infections[7].

Within the past two decades, the dramatically rapid and continuous emergence of antibiotic resistance has directed the interest of research towards the discovery of novel anti-biofilm compounds[8].

In an effort to find viable sources of anti-biofilm agents, many researchers have started to extract and analyze natural products from a myriad of plants and marine organisms[9-11]. Only recently scientists have begun to understand the extreme

diversity present in marine microorganisms such as viruses, bacteria, fungi and phytoplankton. Whole genome functional annotation of marine bacteria has highlighted that 20-33% of the genome encoding capacity encodes for unknown or orphan proteins. Moreover, marine organisms possess a vast diversity of metabolic capabilities, because of their varied adaptations to a diverse range of physical and chemical conditions of marine ecosystems, and thus may represent a unique source of biomolecules that holds great potential[12].
Interestingly, recent studies have shown that there is a remarkably high hit rate of marine-based compounds with anti-biofilm activity[13-14]. Within the marine environment, cold-adapted marine bacteria are able to produce compounds endowed with antimicrobial, anti-fouling and various pharmaceutically relevant activities[15].
In this work, Polar marine bacteria belonging to *Flavobacterium, Pseudoalteromonas*, *Pseudomonas*, *Psychrobacter* and *Psychromonas* genera were analyzed for their ability to produce anti-biofilm molecules active against *Staphylococcus epidermidis.* In particular, total organic extracts obtained from the planktonic cultures of Polar marine bacteria were tested on *Staphylococcus epidermidis* biofilm. For the most promising samples suitable purification strategies were set up in order to identify the molecules responsible for the anti-biofilm activity.

Materials and Methods

Bacterial strains and culture conditions
Bacterial strains used in this work are listed in Table 1. Polar bacteria were grown in flasks in synthetic medium GG[16] (D-Gluconic acid, sodium salt 10 g/L, L-Glutamic acid, sodium salt 10 g/L, NaCl 10 g/L; NH_4NO_3 1 g/L; KH_2PO_4•$7H_2O$ 1 g/L; $MgSO_4$•$7H_2O$ 200 mg/L; $FeSO_4$•$7H_2O$ 5 mg/L; $CaCl_2$•$2H_2O$ 5 mg/L) at 15°C under vigorous agitation (180 rpm) until the stationary phase of growth (72-90 h). *S. epidermidis* O-47 was grown in Brain Heart Infusion broth (BHI, Oxoid, UK) at 37°C. Planktonic cultures were performed under vigorous agitation (180 rpm) while biofilm formation was assessed in static condition. All strains were maintained at -80°C in cryovials with 15% of glycerol.

Organic extracts preparation
Polar bacterial cultures were collected and subjected to a liquid-liquid extraction as initial separation technique to process the whole cultures, to obtain “total organic extracts”. Subsequently, for the most promising samples, cell-free supernatants and cell pellets were processed separately, to obtain “extracellular extracts” and “intracellular extracts”.
In detail, to prepare total organic extracts, the whole Polar bacterial cultures, obtained as previously described, were recovered and frozen at -80°C until use. Liquid-liquid extraction was performed without adding cryoprotectants. Polar bacterial cultures were thawed and then stirred with an equal volume of ethyl acetate (Assay Percent Range ≥ 99.5%) (Sigma-Aldrich) and mixed at 1% with acid formic (Assay Percent Range = 90%) (JT Baker). Each solution was stirred at least for 30 min and then it was centrifuged at 3000 rpm for 30 min. The resulting two phases were separated and the organic phase was recovered and dried using a rotary evaporator Rotavapor (Buchi R-210) at a temperature lower than 40°C. The resulting total organic extracts, named TE, were aliquoted in 4 mg dry weight samples and stored at -20°C.

To prepare extracellular and intracellular extracts, supernatants and cell pellets deriving from the Polar bacterial cultures previously described were recovered by centrifugation at 6,000 rpm at 4°C for 50 min. After centrifugation, the supernatants were separated from the cells and were sterilized by filtration through membranes with a pore diameter of 0.22 µm. Cell pellets were resuspended in an opportune volume of sterile distilled water. Resuspended cell pellets and cell-free supernatants were frozen at -80°C until use. Liquid-liquid extraction was performed on cell pellets and supernatants of cold-adapted bacteria separately, without adding cryoprotectants, as previously described. The resulting intracellular and extracellular extracts, named IE and EE respectively, were aliquoted in 4 mg dry weight samples and stored at -20°C.

Biofilm formation of Staphylococcus epidermidis

Organic extracts were screened for their anti-biofilm activity against *S. epidermidis* O-47. Quantification of *in vitro* biofilm production was based on the method described by Christensen with slight modifications[17]. The organic extracts were first solubilized in dimethylsulfoxide (DMSO), subsequently a working solution of each extract to be tested was diluted in BHI. Briefly, the wells of a sterile 96-well flat-bottomed polystyrene plate were filled with 100 µl of an opportune dilution of an overnight culture of *S. epidermidis* O-47. 100 µl of working solution of organic extracts were added into each well with a final concentration of 4 mg ml^{-1} and a DMSO concentration of 4% v/v, since this DMSO concentration does not interfere with cell growth and bacteria viability[18]. The plates were incubated aerobically for 20 h at 37°C in static condition. Biofilm formation was measured using crystal violet staining. After incubation, planktonic cells were gently removed; each well was washed three times with double-distilled water and patted dry with a piece of paper towel in an inverted position. To quantify biofilm formation, each well was stained with 0.1% crystal violet and incubated for 15 min at room temperature, rinsed twice with double-distilled water, and thoroughly dried. The dye bound to adherent cells was solubilized with 20% (v/v) glacial acetic acid and 80% (v/v) ethanol. After 30 min of incubation at room temperature, OD_{590nm} was measured to quantify the total biomass of biofilm formed in each well. Each data point is composed of three independent experiments each performed at least in 6-replicates.

Silica gel chromatography

The intracellular extracts from *Pseudomonas sp.* TAA207 (TAA207-IE, 100 mg) and *Psycrobacter sp.* TAD1 (TAD1-IE, 113 mg) were both purified on a silica gel column. The columns (30 mL, 50 x 0.7 cm for TAA207-IE and 49 mL, 32 x 0.7 cm for TAD1-IE, respectively) were initially eluted with $CHCl_3$:CH_3OH ranging from 100 to 70 % of $CHCl_3$, and then with $CHCl_3$:CH_3OH:H_2O 9/3/0.5 v/v/v. Only for TAD1-IE, a further elution with $CHCl_3$:CH_3OH:H_2O 6/4/0.5 v/v/v was performed.

Active fractions were analyzed on a Agilent Technologies gas chromatograph 6850A equipped with a mass selective detector 5973N and a Zebron ZB-5 capillary column (Phenomenex, 30m x 0.25 mmi.d., flow rate 1 cm^3/min, He as carrier gas), by using the following temperature program: 150°C for 3 min, from 150 to 300°C at 15°C/min, at 300°C for 5 min.

Statistics and reproducibility of results

Data reported were statistically validated using Student t-test comparing mean absorbance of treated and untreated samples The significance of differences

between mean absorbance values was calculated using a two-tailed Student's t-test. A p value <0.05 (* p <0.05, ** p <0.01, *** p <0.001) was considered significant.

Results

Total organic extracts from Polar marine bacteria

A selection of nine Polar marine bacteria belonging to *Pseudoalteromonas*, *Pseudomonas*, *Psycrobacter* and *Psychromonas* genera (Tab. 1) were analyzed for their capability to produce anti-biofilm molecules active against *S. epidermidis* O-47, a strong biofilm producer *agr*-mutant, isolated from clinical septic arthritis and kindly provided by Prof. Gotz[17]. Polar marine bacteria were grown in planktonic conditions, under vigorous shaking, until the stationary phase of growth at 15°C. A synthetic medium based on gluconate and glutammate (GG)[16] was chosen as culture medium in order to minimize the potential interference of culture medium on the organic extraction step. The bacterial growths were monitored over a period of 72 h; at the achievement of stationary phase, the whole bacterial cultures were recovered and frozen at -80°C, without adding cryoprotectants, in an attempt to induce cell lysis by cold shock, and thus recover intra- and extracellular components. Polar bacterial cultures were then subjected, as reported in materials and methods section, to a liquid-liquid extraction, using ethyl acetate as extraction solvent, since it is a very useful tool for bioseparations, frequently used in the downstream recovery of fermentation products, such as antibiotics or secondary metabolites.

Analysis of the anti-biofilm activity of total organic extracts on S. epidermidis O-47

Total organic extracts, obtained as previously described, were screened for their anti-biofilm activity against *S. epidermidis* O-47. The quantification of biofilm production was based on a microtiter plate assay (MTP): an opportune dilution of bacterial culture in exponential growth phase was added into the wells of a sterile 96-well flat-bottomed polystyrene plate in absence and in presence of total organic extracts (TE). A concentration of 4 mg ml^{-1} was tested for each sample; after 20 h incubation, biofilm formation was evaluated. Figure 1 shows the percentage reduction of *S. epidermidis* O-47 biofilm production after treatment with total organic extracts.

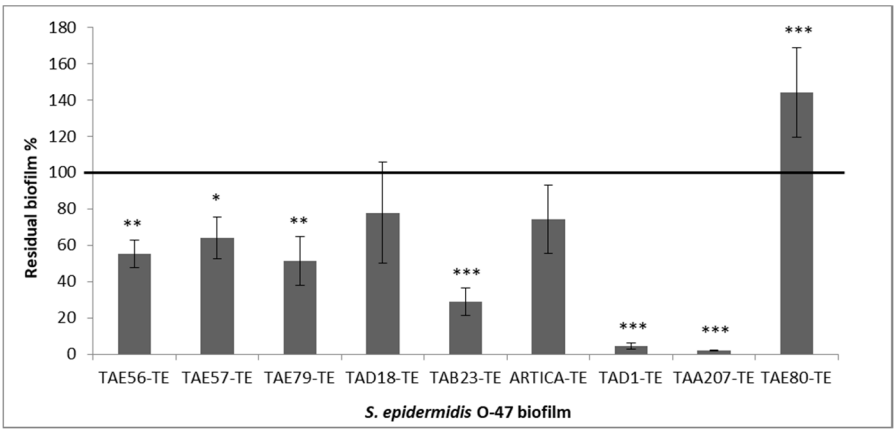

Fig 1: Effect of total organic extracts from cold-adapted bacteria on *S. epidermidis* O-47 biofilm formation. Data are reported as percentage of residual biofilm after the treatment. Each data point represents the mean ± SD of five independent samples. Biofilm formation was considered unaffected in the range 90-100%. Differences in mean absorbance were compared to the untreated control and considered significant when p< 0.05 (* p< 0.05, ** p < 0.01, *** p < 0.001) according to t-Student test.

Reported data demonstrated that most of the tested total organic extracts from Polar marine bacteria induced a reduction in biofilm formation. Among all, some samples showed a mild anti-biofilm effect, such as *P. haloplanktis* TAB23, *P. haloplanktis* TAE79 and *P. haloplanktis* TAE56 total organic extracts (TAB23-TE, TAE79-TE, TAE56-TE), while total organic extracts from *Pseudomonas sp.* TAA207 and *Psycrobacter sp.* TAD1 (TAA207-TE, TAD1-TE) resulted strongly active against *S. epidermidis* O-47 and were thus chosen for further investigations.

Analysis of dose-dependence and species specificity of total organic extracts anti-biofilm activity

Total organic extracts from *Pseudomonas sp.* TAA207 and *Psycrobacter sp.* TAD1 showed an interesting anti-biofilm activity on *S. epidermidis* O-47. Therefore, in order to investigate if TAA207-TE and TAD1-TE were able to inhibit the biofilm formation of different *S. epidermidis* strains, their anti-biofilm activity was evaluated against: *S. epidermidis* RP62A, *S. epidermidis* XX-17 and *S. epidermidis* O-47. *S. epidermidis* RP62A is a reference strain isolated from an infected catheter. The clinical isolate O-47 is a naturally occurring *agr* mutant[19] while the *S. epidermidis* XX-17 is an *ica* defective mutant[20]. These genetic differences have an impact on the ability to form biofilm of the different strains.

The analysis of the anti-biofilm activity of the selected total organic extracts (Fig. 2) revealed that TAA207-TE reduced the biofilm production of two of the tested *S. epidermidis* strains, resulting able to interfere with the biofilm formation process of *S. epidermidis* O-47 and RP62A strains, but the same sample did not display anti-biofilm effect against *S. epidermidis* XX17, suggesting the *ica* genes as possible target of this anti-biofilm compound. Whereas, TAD1-TE reduced the biofilm formation of both the three *S. epidermidis* strains, showing a non-species-specific activity, and thus indicating that the anti-biofilm compound/s produced by *Psycrobacter sp.*TAD1 is different from that produced by *Pseudomonas sp.*TAA207.

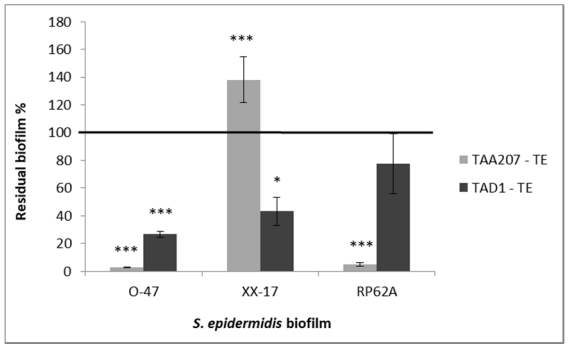

Figure 2: Effect of total organic extracts from *Pseudomonas sp.*TAA207 and *Psycrobacter sp.*TAD1 on biofilm formation of three *S. epidermidis* strains. Data are reported as percentage of residual biofilm after the treatment. Each data point represents the mean ± SD of five independent samples. Biofilm formation was considered unaffected in the range 90-100%. Differences in mean absorbance were compared to the untreated control and considered significant when $p< 0.05$ (* $p< 0.05$, ** $p < 0.01$, *** $p < 0.001$) according to t-Student test.

Because of its ability to form a strong biofilm, *S. epidermidis* O-47 strain has been used to evaluate the dose-responsive effect of total organic extracts. In particular, *S. epidermidis* O-47 was incubated in presence of serial dilutions of TAA207-TE and TAD1-TE, starting from a concentration of 4 mg ml^{-1}. Figure 3 shows a clear dose-dependence of the anti-biofilm activity of TAA207-TE, whereas TAD1-TE anti-biofilm activity was evident only at sample concentrations of 4 mg ml^{-1} and 2 mg ml^{-1}.

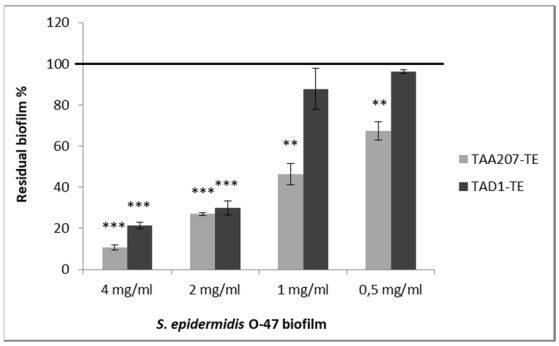

Figure 3: Dose-dependent effect of total organic extracts from *Pseudomonas sp.*TAA207 and *Psycrobacter sp.*TAD1 on *S. epidermidis* O-47 biofilm formation. Data are reported as percentage of residual biofilm after the treatment. Each data point represents the mean ± SD of four independent samples. Biofilm formation was considered unaffected in the range 90-100%. Differences in mean absorbance were compared to the untreated control and considered significant when p< 0.05 (* p< 0.05, ** p < 0.01, *** p < 0.001) according to t-Student test.

Evaluation of the anti-biofilm activity of intracellular and extracellular organic extracts

In order to reduce the composition complexity of the analyzed samples, liquid-liquid extraction was performed on cell pellets and cell-free supernatants of *Pseudomonas sp.* TAA207 and *Psycrobacter sp.* TAD1 cultures separately and then the anti-biofilm activity of the obtained extracts was evaluated. In detail, the selected Polar marine bacteria were grown in planktonic conditions at 15°C in GG medium until the stationary phase of growth. Cell pellets and supernatants were separated by centrifugation, then the liquid-liquid extraction was carried out, for each bacterial strain, on both samples independently. For sake of simplicity, the organic extracts deriving from cell pellets of bacterial cultures were defined as IE (Intracellular extract), while the extracts from cell-free supernatants were defined as EE (Extracellular extract), respectively.

Serial dilutions of *Pseudomonas sp.* TAA207 and *Psycrobacter sp.* TAD1 intracellular and extracellular extracts were tested on *S. epidermidis* O-47 starting from a concentration of 1 mg ml^{-1}, since, on the basis of the dose-dependence study, this concentration was sufficient to detect a clear anti-biofilm effect.

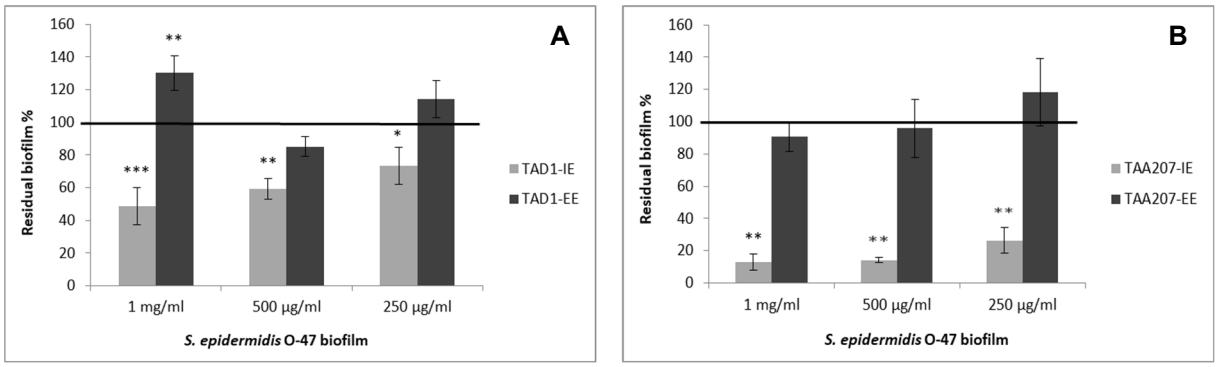

Figure 4: Effect of Intracellular and Extracellular extracts from *Pseudomonas sp.*TAA207 (A) and *Psycrobacter sp.*TAD1 (B) on *S. epidermidis* O-47 biofilm formation. Data are reported as percentage of residual biofilm after the treatment. Each data point represents the mean ± SD of five independent samples. Biofilm formation was considered unaffected in the range 90-100%. Differences in mean absorbance were compared to the untreated control and considered significant when p< 0.05 (* p< 0.05, ** p < 0.01, *** p < 0.001) according to t-Student test.

As reported in Figure 4, TAA207-IE and TAD1-IE had a strong anti-biofilm activity, inducing an evident reduction of *S. epidermidis* O-47 biofilm formation, whereas TAA207-EE and TAD1-EE did not show any inhibitory effect. Therefore, *Pseudomonas sp.*TAA207 and *Psycrobacter sp.*TAD1 intracellular extracts were subjected to further purification steps.

Fractionation of Pseudomonas sp.TAA207 and Psycrobacter sp.TAD1 intracellular extract by Silica Gel Chromatography

In order to develop a preliminary purification protocol, TAA207-IE and TAD1-IE were subjected to different elution conditions on Thin Layer Chromatography (TLC) in order to identify the condition for a preparative scale purification step.

Silica gel column chromatography was used to partition of TAA207-IE and TAD1-IE. A suspension of first eluent (100% $CHCl_3$) with the stationary phase powder of silica gel was prepared and then carefully poured into the column. Then TAA207-IE and TAD1-IE were dissolved in chloroform and loaded into the column separately. After loading phase, previously chosen solvents were slowly eluted through the column in order of their increasing polarity. A final washing elution step with $CHCl_3$:CH_3OH:H_2O 6:4:0.5 v/v was added for TAD1-IE fractionation. Collected fractions, of 4 ml each, were dried and then suspended in dimethylsulfoxide (DMSO). In the anti-biofilm assay, one chromatography fraction every three was tested against *S. epidermidis* O-47, thus simplifying the explorative analysis, at a concentration of 200 µg ml^{-1}.

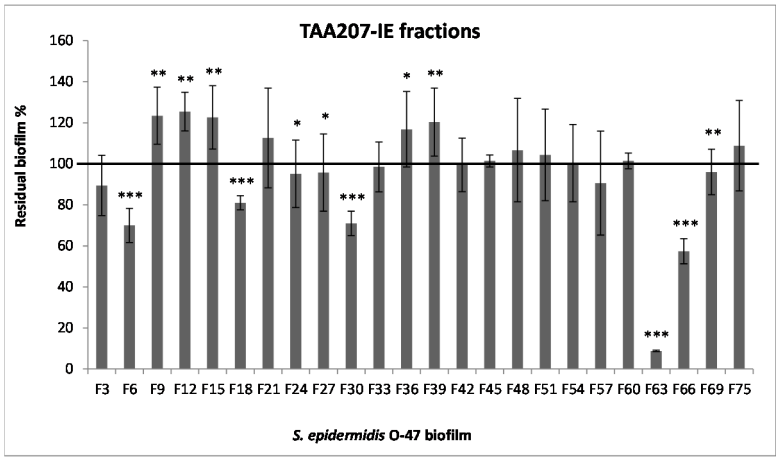

Figure 5: Effect of *Pseudomonas* sp.TAA207 intracellular extract chromatographic fractions on *S. epidermidis* O-47. Biofilm formation was evaluated in presence of 200 µg ml^{-1} of each fraction. Data are reported as percentage of residual biofilm. Biofilm formation was considered unaffected in the range 90-100%. Differences in mean absorbance were compared to the untreated control and considered significant when $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) according to t-Student test.

As shown in Figure 5, TAA207-IE chromatographic fraction F63 displayed a strong biofilm reduction, suggesting the presence of the active agent. To obtain information about the composition complexity, the fraction F63 was acetylated and analysed by GC-MS. The analysis revealed the presence of the pentadecanal, the anti-biofilm molecule produced by another Polar bacterium *Pseudoalteromonas haloplanktis* TAC125[19], thus suggesting that the anti-biofilm activity shown by this fraction is attributable to the pentadecanal. No further analysis were necessary, since the anti-biofilm activity of the pentadecanal was deeply analysed[19].

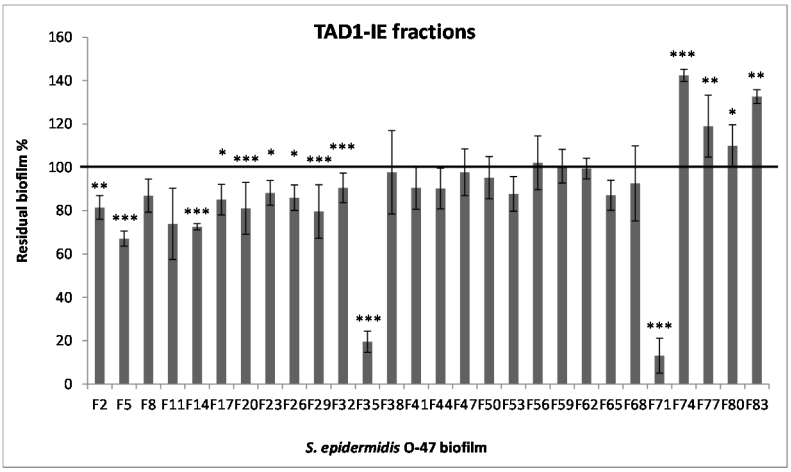

Figure 6: Effect of *Psicrobacter* sp.TAD1 intracellular extract chromatographic fractions on *S. epidermidis* O-47. Biofilm formation was evaluated in presence of 200 µg ml^{-1} (2% DMSO) of each fraction. Data are reported as percentage of residual biofilm. Biofilm formation was considered unaffected in the range 90-100%. Differences in mean absorbance were compared to the untreated control and considered significant when $p< 0.05$ (* $p< 0.05$, ** $p < 0.01$, *** $p < 0.001$) according to t-Student test.

Whereas, as shown in Fig 6, two strongly active chromatographic fractions were identified, F35 and F71. In this case, the GC-MS analysis of these fractions didn't show the presence of the pentadecanal. This result leaves hope for a forthcoming identification of new anti-biofilm molecules.

Discussion

From a previous study, it had been proved that Polar marine bacteria were able to produce molecules endowed with anti-biofilm activity against different *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* strains[20]. A physicochemical characterization of the active samples revealed that the molecules responsible for the activities were of a different nature, creating expectations of a possible identification of different anti-biofilm agents. In this work, a selection of total organic extract obtained from Polar marina bacteria were screened against *S. epidermidis* looking for small metabolites with anti-biofilm properties. Several extract showed a mild inhibiting effect, but two of them resulted extremely active, i.e. the total organic extracts obtained from *Pseudomonas sp.* TAA207 and *Psycrobacter sp.* TAD1 (TAA207-TE and TAD1-TE), which were selected for further analysis. First, intracellular and extracellular extracts from both the selected bacteria were separately obtained and tested in order to identify the origin of the active compounds. In both cases, the intracellular extracts (TAA207-IE and TAD1-IE) turned out to be the active samples. The purification protocol proposed for TAA207-IE led to the obtainment of only one active fraction, whose analysis by GC-MS revealed the presence of the pentadecanal. Pentadecanal was discovered to be a small anti-biofilm molecule, active against *S. epidermidis*, produced by the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125[19], which were able to synthesize this molecule only when grown in sessile condition in a rich culture medium[17]. Surprisingly, these culture conditions completely differ from those of *Pseudomonas sp.* TAA207, which produced the pentadecanal in planktonic conditions in a synthetic medium. Furthermore, *P. haloplanktis* TAC125 secreted the pentadecanal in the

extracellular environment, whereas, in this case, it is located in the intracellular compartment of *Pseudomonas sp.* TAA207. Finally, for *P. haloplanktis* TAC125 a putative gene sequence responsible for the pentadecanal synthesis was proposed, but an *in silico* analysis of *Pseudomonas sp.* TAA207 genome did not reveal the presence of this gene (data not shown). Taken together, these results indicated that *Pseudomonas sp.* TAA207 and *P. haloplanktis* TAC125 were able to produce the same molecule, endowed with the same anti-biofilm activity against *S. epidermidis*, but, interestingly, no connection seemed to exist between them. Further studies will be carried out on this fascinating aspect, in order to clarify the role of the pentadecanal in *Pseudomonas sp.* TAA207 and *P. haloplanktis* TAC125 physiology.
On the other hand, the purification protocol proposed for TAD1-IE led to the obtainment of two different active fractions, likely containing molecules of different nature. In this case, the GC-MS analysis didn't show the presence of the pentadecanal in these fractions. Given the capability of TAD1-TE to indiscriminately act on both the three tested *S. epidermidis* strains, species-specificity analysis of the obtained fractions will likely lead to the identification of at least one molecule with a specific anti-biofilm effect on *S. epidermidis* XX-17, or, even better, an anti-biofilm agent endowed with a broad spectrum of action. Further analysis are still ongoing trying to identify the structures of the molecules responsible for *Psycrobacter sp.* TAD1 anti-biofilm activity against *S. epidermidis*. It is essential to underline that *Psycrobacter sp.* TAD1 turned out to be one of the most promising anti-biofilm agents producer[20], showing impressive capabilities of biofilm inhibition also on *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains. Therefore, in this contest, future studies will aim at a deeper analysis of *Psycrobacter sp.* TAD1, its genomic and metabolic features, and their influence on its anti-biofilm activity.

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Anti-biofilm proteins from Polar marine bacteria to control *Staphylococcus epidermidis* biofilm formation

Abstract

The present study aims to the discovery of novel anti-infective approaches for the prevention of the biofilm formation process or the treatment of the mature biofilms. It was demonstrated that Polar marine bacteria belonging to *Pseudoalteromonas*, *Psychrobacter*, and *Psychromonas* species were able to destabilize the biofilm matrix of different *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* strains without killing cells. In this work, a protein/peptide library was constructed starting from cell-free supernatants of Polar marine bacteria, in which the anti-biofilm activity was due to molecules of proteinaceous nature. Preliminary activity-guided purification protocols were set up for the two most promising strains, *Pseudoalteromonas haloplanktis* TAB23 and *Pseudoalteromonas haloplanktis* TAE80. Obtained results demonstrated that both the Polar bacteria produce anti-biofilm proteins endowed with different and strong biological activities, but the obtained active fractions were still mixtures of several proteins. An optimization of the purification protocols is needed in order to purify and identify the anti-biofilm proteins produced.

Introduction

Microbial biofilms have great negative impacts on the world's economy and pose serious problems to industry, public health and medicine. Biofilms are communities of microorganisms that can attach to both abiotic and biotic surfaces and thus being implicated in the development of Healthcare-associated infections (HAIs)[1]. One of the main causes is recognized to be the colonization and the biofilm formation of staphylococcal species. In particular, *Staphylococcus epidermidis* represents the most common source of infections on indwelling medical devices[2]. This likely stems from the fact that *S. epidermidis* is a permanent and ubiquitous colonizer of human skin, and consequently, the device contamination during insertion is highly probable. Its ability to colonize a surface and form a biofilm is an important virulence factor[3]. The complex and resistant structure of the biofilms protect bacteria from the most common therapeutic treatment, therefore the necessity of novel anti-infective strategies is becoming increasingly urgent. The discovery of antifouling or antimicrobial surfaces can be a possible approach to prevent the bacterial adhesion, and therefore inhibiting the initial steps of the biofilm formation process[4]. Another uprising strategy is the use of effective anti-biofilm molecules, such as quorum sensing modulators[5], which interfere with bacterial signaling pathways inducing the detachment phase, or biofilm-dispersing enzymes, which degrade matrix components destabilizing the biofilm structure[6].
Extracellular matrix of bacterial biofilms is a highly hydrated set of biopolymers such as polysaccharides, lipids, proteins and nucleic acids produced by the bacteria themselves which form an extracellular polymeric substance (EPS)[7]. Therefore, enzymes like glycosidases as well as proteases or deoxyribonuclease can degrade bacterial matrix and facilitate the antimicrobial agent penetration enhancing the treatment efficacy[8-9].

Several biofilm-dispersing enzymes are implicated in active biofilm dispersal. One well-studied biofilm-dispersing enzyme is dispersin B, a glycoside hydrolase produced by *A. actinomycetemcomitans*[10], which degrades poly-N-acetylglucosamine (PNAG), a biofilm matrix polysaccharide that mediates the attachment of the cells to abiotic surfaces and facilitates the aggregation of bacteria. Another example is Deoxyribonuclease I (DNase I), which is capable of digesting the extracellular DNA (eDNA) present within the biofilm structures[11].
Reported data from our group demonstrated the capacity of Polar marine bacteria to produce and secrete anti-biofilm molecules of proteinaceous nature active against *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* strains[12]. Based on these results, in this work a "protein/peptide" library was constructed, starting from cell-free supernatants of Polar marine bacteria, and screened for anti-biofilm activity against *S. epidermidis* O-47. Preliminary activity-guided purification protocols were set up.

Materials and Methods

Bacterial strains and culture conditions
Bacterial strains used in this work are listed in Table 1. Bacteria were grown in Brain Heart Infusion broth (BHI, Oxoid, UK). Biofilm formation was assessed in static condition and planktonic cultures were performed under vigorous agitation (180 rpm). Polar marine bacterial strains were grown at 15°C and 4 °C while staphylococci were grown at 37°C. All strains were maintained at -80°C in cryovials with 15% of glycerol.

Preparation of cell-free supernatants from Polar marine bacteria
Polar marine bacteria were grown in planktonic conditions at 15°C under vigorous agitation (180 rpm) until the stationary phase of growth (30-48 h). Supernatants were recovered by centrifugation at 8,000 rpm at 4°C for 30 min, sterilized by filtration through membranes with a pore diameter of 0.22 µm and stored at 4°C until use.

Design of a "protein/peptide" library from Polar marine bacteria
Cell-free supernatants from Polar marine bacteria, obtained as previously described, were screened for their anti-biofilm activity against *S. epidermidis* O-47. The active samples were selected for the construction of the library. In order to identify the positive hits, all the active supernatants were subjected to a proteinase K treatment. In detail, cell-free supernatants were incubated in presence and in absence of proteinase K (Sigma Aldrich, St Louis, MO) at a final concentration of 1 mg ml^{-1} for 1 h at 37°C. As control, only BHI was incubated for 1 h at 37°C with the proteinase K. After the incubation, all the samples were tested against *S. epidermidis* O-47; supernatants which have partially or almost totally lost the anti-biofilm activity after the treatment were identified as the hits of the library.

Biofilm formation of staphylococci
Static biofilm assay. Biofilm formation of staphylococci was evaluated in the presence and in the absence of supernatants, as previously described[9]. In detail, the wells of a sterile 24-well flat-bottomed polystyrene plate were filled with 500 µl of BHI. An overnight cultures of *S. epidermidis* was diluted and added into each well at a cell concentration of about 0.001 OD_{600nm}. Each well was filled with 500 µl of BHI and 500 µl of each supernatant, respectively. In this way each supernatant was used diluted

1:2 with a final concentration of 50%. As control, the first row contained bacteria grown only in BHI (untreated bacteria). The plates were incubated aerobically for 24 h at 37°C. Biofilm formation was measured using crystal violet staining. After incubation, planktonic cells were gently removed; each well was washed three times with PBS and thoroughly dried. Each well was then stained with 0.1% crystal violet and incubated for 15 min at room temperature, rinsed twice with double-distilled water, and thoroughly dried. The dye bound to adherent cells was solubilized with 20% (v/v) glacial acetic acid and 80% (v/v) ethanol. After 30 min of incubation at room temperature, the OD_{590nm} was measured to quantify the total biomass of biofilm formed in each well. Each data point was composed of six independent samples.
Surface coating assay. A volume of 10 µl of cell-free supernatant, or 10 µl of BHI/Tris-HCl as control, was deposited at the center of a well of a 24-well tissue-culture-treated polystyrene microtiter plate in three-replicate. The plate was left overnight under laminar hood to allow complete evaporation of the liquid. The wells were then filled with 1 ml of BHI broth containing an opportune dilution of *S. epidermidis* O-47 (about 0.001 OD_{600nm}) and incubated at 37°C. After 18 h, wells were rinsed with water and stained with 1 ml of 0.1% crystal violet. Stained biofilms were rinsed with water and dried, and the wells were photographed by a camera.

Ammonium sulphate precipitation
Ammonium sulphate at 50% and 100% saturation (314 g l^{-1} and 761 g l^{-1}) was added slowly to the culture filtrate with stirring at 4°C respectively. After the $(NH_4)_2SO_4$ had completely dissolved it was stored overnight in the cold, to allow the protein to precipitate. The solution was then centrifuged at 13,000 rpm at 4°C for 30 min. The precipitates were resuspended in a buffer 50 mM Tris/HCl pH 8, 200 mM NaCl and then dialyzed against the same buffer to remove salts, using a semipermeable membrane with a cutoff of 3 kDa.

Analytical techniques
Centricon tubes (Millipore, Billerica, MA, USA) with 10 kDa MWCO filters were used to approximately determine the molecular weight of the anti-biofilm molecules. Freeze drying or lyophilization has been used to concentrate protein samples after dialysis, removing the water from frozen samples by sublimation and desorption.
TCA precipitation has been used to concentrate protein samples for SDS-PAGE analysis. Briefly, 10% (v/v) of TCA 100% was added into each sample and incubated over night at 4°C followed by centrifugation at 13,000 rpm for 30 minutes. Protein pellets were washed with cold acetone 90% and centrifuged at 13,000 rpm for 30 minutes. Samples were dried in a stove to eliminate any acetone residue. For SDS-PAGE, 4x Laemmli sample buffer was added, samples were boiled at 95°C for 5 min and loaded on to polyacrylamide gel. Colloidal Coomassie was used for the visualization of proteins separated by SDS-PAGE.

Chromatographic Techniques
SEC: Size-Exclusion Chromatography was performed using a HiLoad™ 16/600 Superdex™ 75 column (GE Healtcare) by using an ÄKTA Explorer FPLC (GE, Amersham). The column was washed and equilibrated with 2,5 column volumes of degassed elution buffer (50 mM Tris/HCl, 200 mM NaCl, pH 8). Sample was loaded with a flow-rate of 0,1 ml min^{-1} and eluted for 1,5 column volumes with a flow-rate of 0,7 ml min^{-1}, following the conjugated rings of tryptophan and tyrosine absorbance at 280 nm and protein absorbance at 220 nm.

HIC: Hydrophobic Interaction Chromatography was performed using a HiPrep™ Phenyl HP 16/10 column (GE Healtcare) by using an ÄKTA Explorer FPLC (GE, Amersham). The column was washed and equilibrated with 2,5 column volumes of degassed binding buffer (50 mM Tris-HCl, 1 M $(NH_4)_2SO_4$, pH 7). Sample was loaded with a flow-rate of 1 ml min^{-1} and eluted with 5 column volumes of degassed elution buffer (50 mM Tris-HCl) creating a discontinuous gradient of $(NH_4)_2SO_4$, from 100% to 0%. At last the column was washed with 3 column volumes of bi-distilled water in both the flow directions with a flow-rate of 3 ml min^{-1}.
AEC: Anion Exchange Chromatography was performed using a HiPrep™ Q HP column (GE Healtcare) by using an ÄKTA Explorer FPLC (GE, Amersham). The column was washed and equilibrated with 2,5 column volumes of degassed binding buffer (Tris-HCl 20 mM). Sample was loaded with a flow-rate of 1 ml min^{-1} and eluted with 5 column volumes of degassed elution buffer (50 mM Tris-HCl, 1 M NaCl) creating a discontinuous gradient of NaCl, from 0% to 100%.

Statistics and reproducibility of results
Reported data were statistically validated using Student t-test comparing mean absorbance of treated and untreated samples The significance of differences between mean absorbance values was calculated using a two-tailed Student's t-test.

Results

Design and screening of a "protein/peptide" library from Polar marine bacteria
Previously reported data demonstrated the capability of Polar marine bacteria to interfere with the biofilm formation process of some pathogenic strains[9]. With the purpose to identify novel anti-biofilm molecules of proteinaceous nature, active against *S. epidermidis*, the construction of a "protein/peptide" library was set up starting from our collection of Polar marine bacteria. In detail, Polar marine bacteria (Tab. 1) were grown in planktonic condition in BHI medium at 15°C until the stationary phase of growth. The anti-biofilm activity of the collected cell-free supernatants was evaluated against *S. epidermidis* O-47. Supernatants that induced a biofilm reduction were selected as library samples. To identify the samples in which the activity was due to a protein or a peptide, all the samples were subjected to a proteinase K treatment and then tested again on *S. epidermidis* O-47. Supernatants which have partially or almost totally lost the anti-biofilm activity after the treatment with proteinase K were qualified as positive hits (Tab. 2), given their likely content of an anti-biofilm molecule of proteinaceous nature.

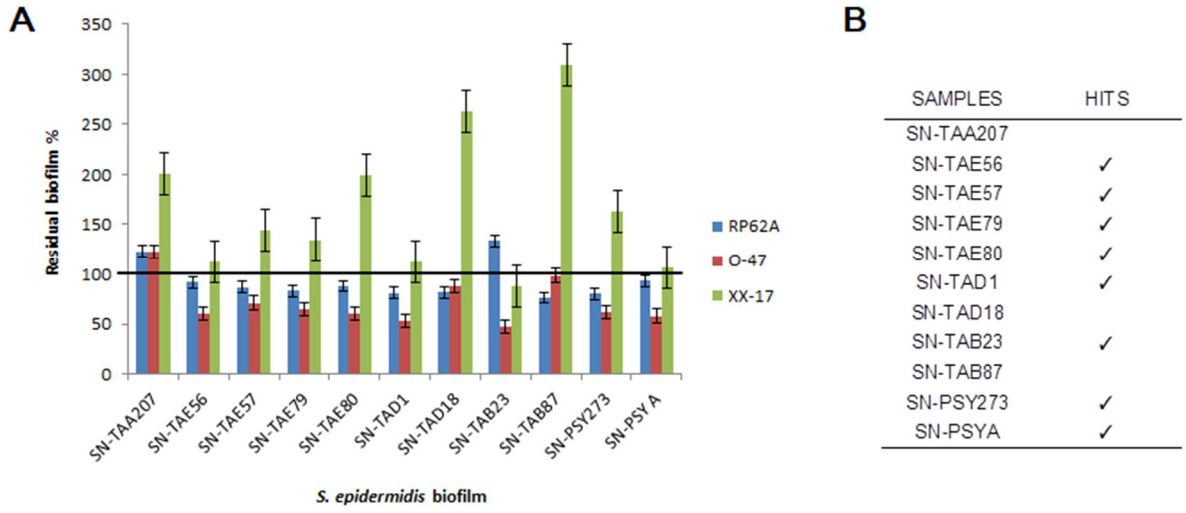

Figure 1: A) Anti-biofilm activity of cell-free supernatants obtained from Polar Marina bacteria against different *S. epidermidis* strains. Each data point represents the mean ± SD of three different samples. B) Protein/peptide library samples and hits. Hits were selected on the basis of the anti-biofilm activity lost after proteinase K treatment of the samples. Data sets resulted statistically significant according to t-Student test.

Among the identified hits, two were selected for further analysis:
- *P. haloplanktis* TAB23 supernatant (SN-TAB23), because it showed the strongest inhibitory effect on *S. epidermidis* O-47 biofilm formation;
- *P. haloplanktis* TAE80 supernatant (SN-TAE80), given its interestingly ability to prevent biofilm formation of *S. epidermidis* O-47 by modifying the polystyrene surface properties[12].

Preliminary molecular weight analysis
In order to approximately determinate the anti-biofilm compound molecular weight, SN-TAB23 and SN-TAE80 were filtered using a Centricon centrifugal tube with 10 kDa MWCO filter. Then, the retained and the rejected fractions were tested against *S. epidermidis* O-47; the analysis of the biofilm formation revealed that only the retained fractions of SN-TAB23 and SN-TAE80 showed an anti-biofilm effect (data not shown), indicating that the activity can probably be due to a protein, rather than to a small peptide.

Set up of preliminary purification strategies
To separate the proteins from the molecule mixture in the supernatants, SN-TAB23 and SN-TAE80 were subjected to an ammonium sulphate precipitation at different percentages of salt saturation. Precipitates obtained at 50% and 100% saturation of ammonium sulphate were collected by centrifugation and then suspended and dialyzed against an appropriate buffer to remove salts and then subjected to freeze-drying to obtain more concentrated samples. Lyophilized powders were finally suspended in a small volume of buffer and tested to verify the anti-biofilm activity on *S. epidermidis* O-47 biofilm. Samples so obtained were indicated as SN-TAB23 50, SN-TAB23 100, SN-TAE80 50 and SN-TAE80 100.

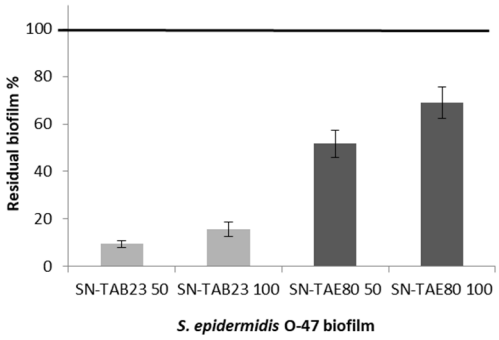

Figure 2: Effect of *P. haloplanktis* TAB23 and *P. haloplanktis* TAE80 supernatants on *S. epidermidis* O-47 biofilm after ammonium sulphate precipitation at 50% and 100% salt saturation. Data are reported as percentage of residual biofilm. Each data point represents the mean ± SD of three different samples. Data sets resulted statistically significant according to t-Student test.

Obtained data (Fig. 2) showed that the supernatants of *P. haloplanktis* TAB23 and *P. haloplanktis* TAE80 retained in both cases the anti-biofilm activity. This result suggested that ammonium sulphate precipitation was an effective step to reduce the complexity of the supernatants and thus facilitate the purification of the anti-biofilm compound produced by the selected Polar bacteria. Moreover, data indicated that 50% saturation of ammonium sulphate was enough to achieve a high recovery of the anti-biofilm compound and a good removal of interfering proteins that precipitate at 100% saturation of ammonium sulphate, therefore SN-TAB23 50 and SN-TAE80 50 were selected for the further purification steps.

***Pseudoalteromonas haloplanktis* TAB23**

P. haloplanktis TAB23 supernatant was precipitated with ammonium sulphate at 50% saturation, suspended in a small volume of buffer and subjected to a Hydrophobic Interaction Chromatography (HIC). The analysis of the anti-biofilm activity of the collected fractions (data not shown) didn't reveal the presence of a single active fraction, but, on the contrary, all fractions possessed a low, and not significative, inhibiting effect on *S. epidermidis*. Therefore, a Size-Exclusion Chromatography (SEC) was selected as first chromatographic step. Fractions F1 to F7 (Fig. 3A) were collected, normalized to protein content (10 µg ml^{-1}) and then analyzed for their anti-biofilm activity on *S. epidermidis* O-47, using an appropriate control. As shown in Fig. 3B, the fraction F1, corresponding to the first peak in chromatogram, displayed the greatest anti-biofilm activity. Interestingly, also the latest fraction F7 showed a slight inhibiting effect.

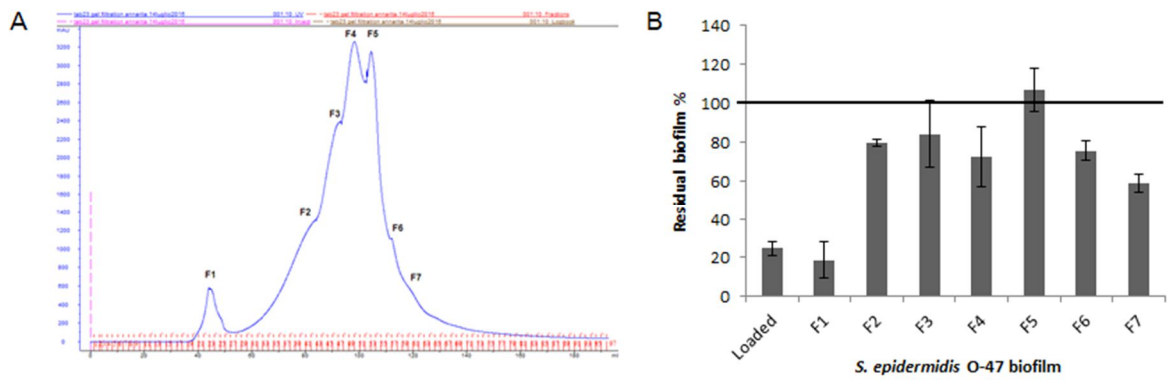

Fig 3: A) SEC chromatogram. Red line: absorbance (220 nm); blue line: absorbance (280 nm). B) Evaluation of the anti-biofilm activity of the SEC collected fractions on *S. epidermidis* O-47. Data are reported as percentage of residual biofilm. Each data point represents the mean ± SD of three different samples. Data sets resulted statistically significant according to t-Student test.

A SDS-PAGE was performed to analyze the protein separation and the profile complexity of the chromatographic fractions (data not shown). A partial separation of SN-TAB23 50 total content of proteins was obtained, but the sample corresponding to both fraction F1 and F7 were complex mixtures of different proteins. Since F1 fraction showed the greatest anti-biofilm activity, it was selected for a further purification step. F1 fraction was subjected to an Anion exchange chromatography (AEC). Proteins were eluted with an increasing percentage (0% to 100%) of NaCl. As shown in the chromatogram (Fig. 4A) four main peaks were collected, dialyzed to remove salts and freeze-dried to obtain more concentrated samples. Lyophilized powders were finally suspended in a small volume of an appropriate buffer, normalized to protein content (10 µg ml^{-1}) and tested for their anti-biofilm activity on *S. epidermidis* O-47. Collected data (Fig. 4B) showed that only the unbound sample were able to impair the pathogen biofilm formation.

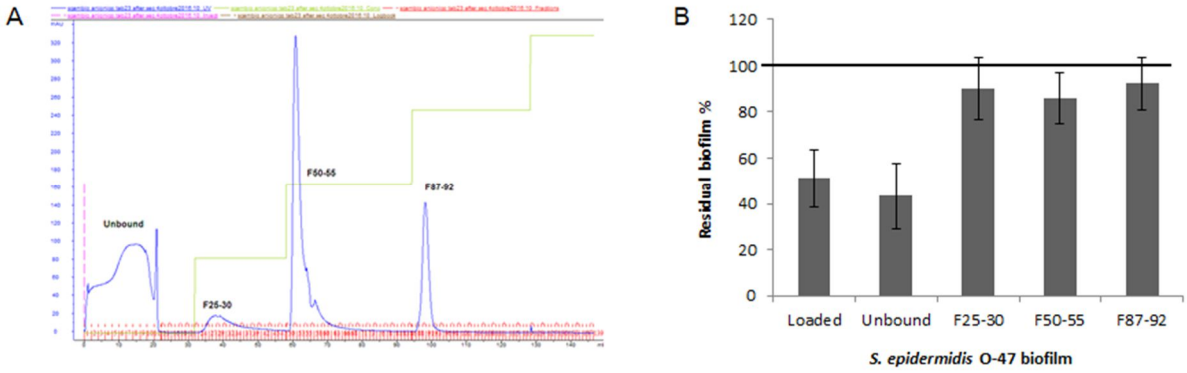

Fig 4: A) AEC chromatogram. Red line: absorbance (220 nm); blue line: absorbance (280 nm). B) Evaluation of the anti-biofilm activity of the SEC collected fractions on *S. epidermidis* O-47. Data are reported as percentage of residual biofilm. Each data point represents the mean ± SD of three different samples. Data sets resulted statistically significant according to t-Student test.

A SDS-PAGE was carried out to analyze the protein profile complexity of the chromatographic fractions, but, unfortunately, the unbound fraction, which exhibited the anti-biofilm activity, was still a mixture of several proteins.

Pseudoalteromonas haloplanktis TAE80

P. haloplanktis TAB23 supernatant was precipitated with ammonium sulphate at 50% saturation (SN-TAE80 50), suspended in an appropriate buffer, and subjected to a Hydrophobic Interaction Chromatography (HIC). As shown in chromatogram (Fig. 5A), several peaks were collected corresponding to unbound proteins and protein fractions eluted with a decreasing and discontinuous gradient of ammonium sulphate. Each collected fraction was dialyzed to remove salts and subsequently freeze-dried to obtain more concentrated samples. Lyophilized powders were finally suspended in a small volume of an appropriate buffer, normalized to protein content (10 µg ml^{-1}) and tested for their anti-biofilm activity on *S. epidermidis* O-47.

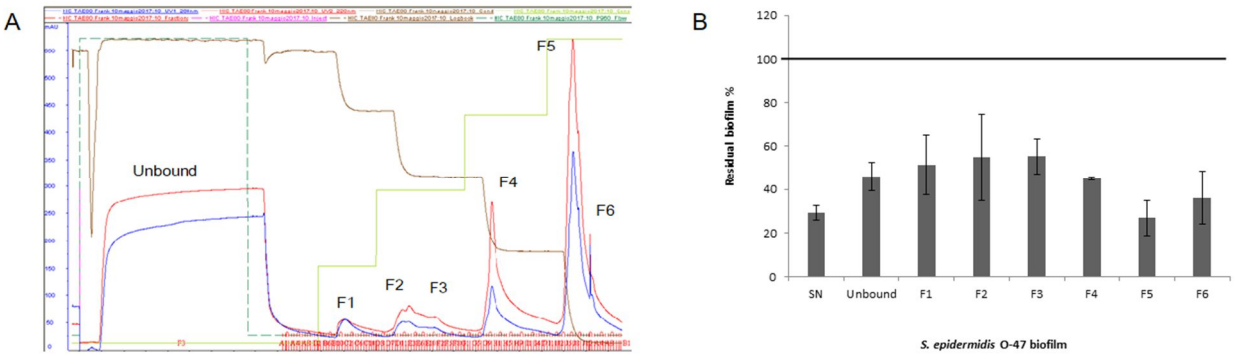

Figure 5: A) HIC chromatogram. Red line: absorbance (220 nm); blue line: absorbance (280 nm); B) Evaluation of the anti-biofilm activity of the HIC collected fractions on *S. epidermidis* O-47. Data are reported as percentage of residual biofilm. Each data point represents the mean ± SD of three different samples. Data sets resulted statistically significant according to t-Student test.

As shown in Fig. 5B, the biofilm formation assay revealed a dispersion of the anti-biofilm activity, probably due to the presence of different active proteins. Therefore, with the purpose of purify and identify the protein/s endowed with the anti-adhesive properties, each collected fraction was also evaluated by the surface coating assay. To do this, a drop of each fraction was deposited onto the surface of a well of a 24-well flat-bottomed polystyrene plate and after its evaporation, the biofilm formation capability of *S. epidermis* O-47 was evaluated.

Figure 6: Surface coating assay of SN TAE80 50 and its chromatographic fractions after HIC. NT: *S. epidermidis* O-47 untreated biofilm.

Surprisingly, the surface coating assay (Fig. 6) revealed that fraction F5, which displayed the greatest anti-biofilm activity, resulted the only fraction exhibiting anti-adhesive properties. These data suggest that the previous chromatographic step allowed a good separation of the proteins in the sample, so probably the anti-biofilm effect of all the chromatographic fractions is due to the presence of more than one active protein. Therefore, in order to purify the anti-biofilm molecule endowed also with the anti-adhesive properties, fraction F5, eluted in absence of ammonium

sulphate and thus containing high hydrophobic proteins, was selected for a further purification step. Fraction F5 was subjected to an Anion Exchange Chromatography (AEC) using a HiPrep™ Q HP column (GE Healtcare). As shown in chromatogram (Fig. 7A), several peaks were collected corresponding to unbound proteins and to protein fractions eluted with an increasing percentage (0% to 100%) of sodium chloride. Each collected fraction was dialyzed to remove salts and subsequently freeze-dried to obtain more concentrated samples. Lyophilized powders were finally suspended in a small volume of an appropriate buffer, normalized to protein content (10 µg ml^{-1}) and tested for their anti-biofilm activity on *S. epidermidis* O-47 (Fig. 7B).

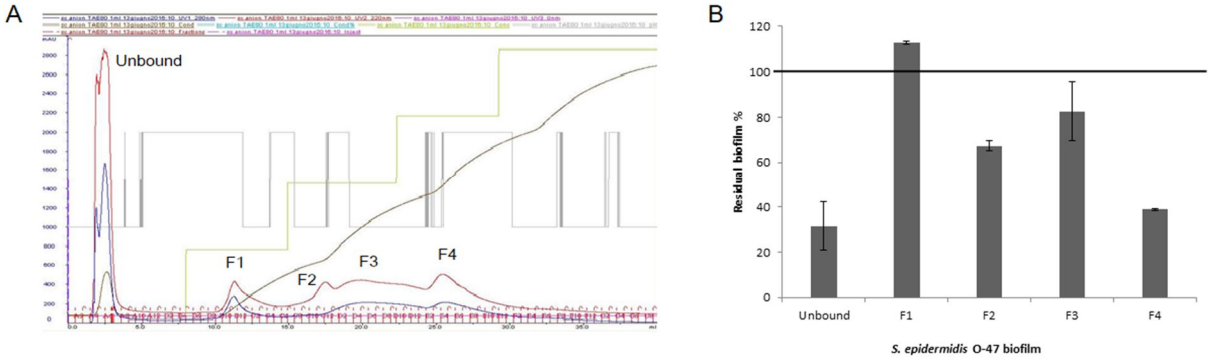

Figure 7: A) AEC chromatogram. Red line: absorbance (220 nm); blue line: absorbance (280 nm). B) Evaluation of the anti-biofilm activity of the HIC collected fractions on *S. epidermidis* O-47. Data are reported as percentage of residual biofilm. Each data point represents the mean ± SD of three different samples. Data sets resulted statistically significant according to t-Student test.

Each chromatographic fraction was also tested using the surface coating assay. As shown in Fig. 8, fraction F4 which, also in this case, displayed the greatest anti-biofilm activity, exhibited also anti-adhesive properties.

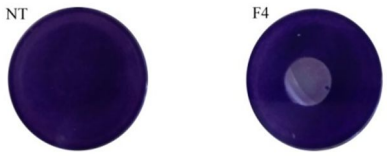

Figure 8: Surface coating assay with anionic exchange fraction F4. NT: *S. epidermidis* O47 biofilm untreated

A SDS-PAGE was carried out to analyze the protein profile complexity of the chromatographic fraction which exhibited anti-biofilm activity and anti-adhesive properties. As shown in Fig. 9, F4 fraction eluted in absence of sodium chloride and thus containing proteins with high isoelectric point, was, unfortunately, still a mixture of several proteins, therefore an optimization of the purification protocol resulted fundamental.

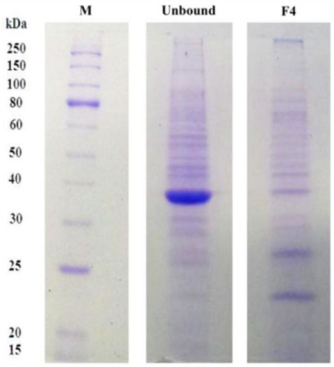

Figure 9: SDS-PAGE stained with Coomassie brilliant blue. Protein profile of chromatographic fractions after AEC. M: molecular weight marker.

Given the complexity of the samples obtained after the selected purification steps, an alternative approach has been tried to identify the anti-biofilm protein/s produced by *P. haloplanktis* TAE80. An *in silico* comparative analysis of *P. haloplanktis* TAE80 and *P. haloplanktis* TAE79 genomes was performed. *P. haloplanktis* TAE79 and *P. haloplanktis* TAE80 are philogenetically very close[13], but, as previously described[9], *P. haloplanktis* TAE79 was not able to produce any protein endowed with anti-adhesive properties. Therefore, a comparison between all putative proteins encoded by the two strains was performed looking for the sequences present only in *P. haloplanktis* TAE80. All the highlighted interesting sequences were then analyzed looking for the presence of a signal peptide, since the anti-biofilm protein/s produced *P. haloplanktis* TAE80 was found in the culture supernatant, and therefore secreted in the extracellular environment. Only two sequence were obtained from the analysis and their main features were listed below (Fig. 10).

>01153
MSAATQAMAFLLCTPAIRSVISTTSPDVVGYWNSAPKISWFTASVALPTITSKPNCSARVLTTSIVCVCTSS
AIKNALAFLAFDTRFARPIASAAAVDSSSSDALAKSRPVKSRVIVWKFNNDSKRPCAISG

Properties	Value	Unit
Average mass	13.886,10	Dalton
Monoisotopic mass	13.877,13	Dalton
Molar absorbance	1,34	cm^-1
Molar ext. coeff. (280nm)	18.590,00	cm^-1 M^-1
Isoelectric point	10,50	
Hydrophobicity index	0,34	

>02098
MAAGAKLFCVALFTLLFTAANAFVLVALLVTVAALFCVALVALSIAVAALFCVALVALSIAVAALFCVALTALL
VTALLFTALFCSLGNALFINAWLRACACKFNGAVLSVGTAGKRWLVNLNASFAASRSVIAFGCAE

Properties	Value	Unit
Average mass	14.146,24	Dalton
Monoisotopic mass	14.136,67	Dalton
Molar absorbance	0,84	cm^-1
Molar ext. coeff. (280nm)	11.860,00	cm^-1 M^-1
Isoelectric point	8,95	
Hydrophobicity index	1,76	

Figure 10: Putative proteins sequences of *P. haloplanktis* TAE80 and predicted properties.

It is interesting to note that the identified putative proteins, hypothetically encoding for the anti-biofilm protein/s of *P. haloplanktis* TAE80, resulted both characterized by similar molecular weight (~ 14 kDa) and high hydrophobicity and isoelectric point values.

Discussion

In a previous study, it was demonstrated that Polar marine bacteria belonging to *Flavobacterium, Pseudoalteromonas*, *Pseudomonas*, *Psychrobacter*, and *Psychromonas* species displayed an anti-biofilm activity against different *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* strains. Cell-free supernatants deriving from sessile and planktonic Polar bacterial

cultures showed this activity with several differences in terms of specificity and efficiency.
In this work, with the aim to identify novel anti-biofilm molecules of proteinaceous nature, a “protein/peptide” library was designed. Polar marine bacteria were grown in planktonic condition until the stationary phase of growth in a rich medium at 15°C. Cell-free supernatants were treated with proteinase K and tested against *S. epidermidis* O-47, a strong biofilm producer strain, in order to identify the samples whose anti-biofilm activity was due to a protein or a peptide. Among all, two supernatants were shown to have an interesting anti-biofilm effect: the supernatant obtained from *Pseudoalteromonas haloplanktis* TAB23, endowed with the strongest biofilm-inhibiting activity, and the supernatant obtained from *Pseudoalteromonas haloplanktis* TAE80, which was not only able to act reducing the biofilm biomass, but was also able to impair the adhesion capability of the staphylococcal cells.
A preliminary physicochemical characterization of the samples revealed that the active molecules of both *P. haloplanktis* TAB23 and *P. haloplanktis* TAE80 are of a molecular weight higher than 10 kDa, indicating that the activity could probably be due to a protein, rather than to a small peptide. Preliminary activity-guided purification protocols were set up, in order to identify the active proteins.
As regard *P. haloplanktis* TAB23, the hydrophobic interaction chromatography caused a loss of the anti-biofilm activity, which resulted scattered in all the collected fractions. This result indicated that HIC was not useful for *P. haloplanktis* TAB23 proteins separation, therefore a size-exclusion chromatography was performed as first chromatographic step. The anti-biofilm activity analysis of the collected SEC fractions denoted the presence of one strongly active fraction (F1) eluted as first peak, suggesting the large dimensions of the proteins contained therein. Moreover, the mild anti-biofilm effect showed by the last collected fraction (F7) seemed to demonstrate the ability of *P. haloplanktis* TAB23 to produce more than one active protein. Nonetheless, a ion exchange chromatography was performed on fraction F1, but from the anti-biofilm assay performed on the AEC fractions it was possible to note a reduction of the biofilm inhibiting effect of the loaded sample, compared with the stronger inhibiting effect of the F1 fraction. Apparently, the manipulation steps preceding the AEC step have partially affected the activity of the anti-biofilm agent/s present in F1 fraction, including dialysis and lyophilization and buffer exchange (different salt concentrations). These results suggested the possibility that *P. haloplanktis* TAB23 anti-biofilm activity was attributable to the action of a multimeric protein, in which the single monomeric components are probably also able to show a slight effect, thus justifying the strong activity of the F1 SEC fraction, containing proteins with high molecular weight, and the mild activity showed by the F7 SEC fraction, containing proteins with lower molecular weight. And again, this hypothesis could also explain the loss of the activity of the sample over time, during which the multimeric protein could be subject to dissociation reactions.
As regard *P. haloplanktis* TAE80, the proposed purification protocol highlighted the presence of more than one protein endowed with anti-biofilm activity, one interfering with the biofilm maturation process, and the other inhibiting the adhesion of bacteria to a surface. Therefore, in order to the identify the protein/s responsible for the anti-adhesive effect, F5 HIC fraction was subjected to a further purification step. In this case, the ion exchange chromatography allowed the obtainment of only one fraction with anti-adhesive properties, but the protein profile analysed by SDS-PAGE revealed a still high complexity of the sample.

Uunfortunately, it was difficult to purify the active protein/s produced by *P. haloplanktis* TAB23 and *P. haloplanktis* TAE80, because, in first instance, these bacteria resulted able to produce more than one active molecule, therefore the use of an activity-guided purification protocol is not a useful tool for the purification of native proteins, which, by more, are endowed with strong biological activities.
This is the reason why an alternative approach was used to identify the anti-adhesive protein/s produced by *P. haloplanktis* TAE80. Exploiting their phylogenetic proximity, a comparative analysis of *P. haloplanktis* TAE80 and *P. haloplanktis* TAE79 genomes was performed, looking for putative proteins present in one but not the other, since *P. haloplanktis* TAE79 was proved not to show anti-adhesive properties. Then, considering that *P. haloplanktis* TAE80 secreted the active protein/s in the extracellular environment, only two putative proteins, whose sequences included peptide signal for secretion, were selected. Interestingly, the predicted physicochemical properties of these putative proteins seemed to correspond to those of the anti-adhesive protein/s present in the active fractions. The creation of *P. haloplanktis* TAE80 deletion mutants for these protein sequences could allow to a lack in the anti-adhesive activity and thus to the identification of, at least, one of the active proteins produced by *P. haloplanktis* TAE80.

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Research activity in foreign laboratories

From January 10th to July 1th, in 2018, my research activities were carried out at laboratories of Prof. Henny C. van der Mei, at the Department of Biomedical Engineering of University of Groningen, in The Netherlands. My work aimed at the development of an anti-biofilm coating strategy using the identified Polar marine anti-biofilm molecules.

APPENDIX

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Hydrophobin coating prevents *Staphylococcus epidermidis* biofilm formation on different surfaces

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ABSTRACT

Staphylococcus epidermidis is a significant nosocomial pathogen in predisposed hosts because of its capability of forming a biofilm on indwelling medical devices. The initial stage of biofilm formation has a key role in *S. epidermidis* abiotic surface colonization. Recently, many strategies have been developed to create new anti-biofilm surfaces able to control bacterial adhesion mechanisms. In this work, the self-assembled amphiphilic layers formed by two fungal hydrophobins (Vmh2 and Pac3) have proven to be able to reduce the biofilm formed by different strains of *S. epidermidis* on polystyrene surfaces. The reduction in the biofilm thickness on the coated surfaces and the preservation of cell vitality have been demonstrated through confocal laser scanning microscope analysis. Moreover, the anti-biofilm efficiency of the self-assembled layers on different medically relevant materials has also been demonstrated using a CDC biofilm reactor.

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Introduction

Biofilms are structured multicellular communities of bacteria that colonize biotic and abiotic surfaces. The bacteria living in biofilms are a continuous source of infection, especially in a hospital setting, because of the cells detaching from the biofilms which cover indwelling medical devices. An encouraging approach to attract, retard or repel biofilm formation is modification of the surface properties of a material, namely its mechanical and chemical properties, structure and polarity (Rosenhahn et al. 2008). The physico-chemical properties of abiotic surfaces play a key role in cell adhesion and biofilm development. Electrostatic forces, surface wettability, roughness and Lifshitz–Van der Waals forces positively or negatively influence microbial adhesion to a surface (Palmer et al. 2007). The anti-biofilm support surfaces, functionalized by surface chemistry, can affect the biocompatibility of the materials (Wang et al. 2007; Zhang et al. 2011). A possible approach to change the surface features consists of the use of self-assembling proteins able to stably coat the surfaces (Gu et al. 2017).

Fungal hydrophobins are small amphiphilic proteins self-assembling at hydrophobic/hydrophilic interfaces and thus changing the wettability of different supports. They are involved in various phases of fungal growth, eg helping the submerged hyphae to breach the air–water interface, and facilitating the air dispersion of spores (Bayry et al. 2012). Despite the fact that they share a motif of eight conserved cysteines, involved in four-disulfide bridges and a large percentage of hydrophobic amino acids, their sequence conservation is very low. Conventionally, hydrophobins are split into two classes based on the stability of the layers that they form. In particular, Class I hydrophobins form amyloid like fibrils which can be dissolved only in the presence of strong acids (trifluoroacetic or formic acid), being stable to solvent or detergent washing (Wösten and Scholtmeijer 2015). Due to their peculiar characteristics, several potential applications of this protein family have been proposed, including the stabilization of emulsions, drug delivery and surface modification (Khalesi et al. 2012). The latter offers a non-covalent alternative to the conventional methods used to create chemical (nano)patterns on surfaces (De Stefano et al. 2007), to adsorb proteins without losing activity (Qin, Hou, et al. 2007; Wang et al. 2010; Longobardi et al. 2015) or to reduce the friction of the surfaces in order to avoid

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tissue injury in relation to biomedical applications (Misra et al. 2006). Therefore, the use of hydrophobin coatings to obtain anti-biofilm surfaces could be a good method for the prevention of biofilm formation. However, Rieder et al. (2011) showed that the coating of the Class I hydrophobins H*Protein A and H*Protein B has no impact on *Escherichia coli* biofilm or on the mixed waste water community. Nevertheless, it is worth continuing with research in this area because different hydrophobins can form layers with different superficial properties, such as hydrophobicity or charge. Furthermore, different bacterial species use different mechanisms for surface adhesion and biofilm formation. Therefore, the negative results described for *E. coli* do not exclude a possible anti-biofilm action of other hydrophobins on other bacterial species. Accordingly, in this work, two Class I hydrophobins, the well-characterized Vmh2 from *Pleurotus ostreatus* (Longobardi et al. 2012; Gravagnuolo et al. 2016) and the recently isolated hydrophobin from *Acremonium sclerotigenum* (Cicatiello et al. 2016), here named Pac3, were studied in relation to their capability of preventing *Staphylococcus epidermidis* biofilm formation on different surfaces.

S. epidermidis is a human commensal that normally colonizes the human skin and mucous membranes, representing a major component of the saprophytic bacterial flora of these habitats (Kloos 1980). In predisposed hosts, *S. epidermidis* has become a significant nosocomial pathogen. Indeed, it represents the most frequent etiologic agent involved in infections of indwelling medical devices because it is a ubiquitous saprophytic skin colonizer. It can cause indwelling medical device contamination by virtue of its capability of adhering and forming biofilms on biotic and abiotic surfaces (Otto 2008). Biofilm formation is a complex process and extensive research has led to the characterization of the biofilm lifecycle, which encompasses three main stages: initial attachment, microcolony and macrocolony formation, and detachment or disassembly. The initial attachment of *S. epidermidis* to foreign materials is a pivotal step toward the establishment of a device-associated infection. Many factors specifically involved in mediating bacterial–surface interactions have been identified and characterized. Some genetic evidence suggests that bacterial binding to unmodified polystyrene is fostered by the *S. epidermidis* autolysin AtlE (Heilmann et al. 1996), a bacterial peptidoglycan (PGN)-hydrolase. AtlE plays a key role in the degradation of the bacterial cell wall, in primary adhesion and in stable bacterial surface binding, even though the molecular mechanisms have not yet been well established (Biswas et al. 2006; Otto 2014). Furthermore, extensive research has focused on the role of extracellular DNA (eDNA) in the primary attachment of *S. epidermidis,* independently of the *S. epidermidis* genetic background (Qin, Ou, et al. 2007). Indeed, eDNA is released by means of increased cell lysis (Christner et al. 2012) that in *S. epidermidis* is determined to a large extent by the activity of the autolysin AtlE (Biswas et al. 2006).

In this paper, a new strategy for the reduction of *S. epidermidis* adhesion to abiotic surfaces has been explored by coating selected materials with hydrophobins. The study has been performed using three *S. epidermidis* strains, differing in biofilm structure and composition, and coating different surfaces of medical interest with the two hydrophobins Pac3 and Vmh2.

Materials and methods

Microbial strains, media and growth conditions

The bacterial strains used in this work were: *S. epidermidis* O-47 isolated from clinical septic arthritis, a naturally occurring non-functional agr mutant and a strong biofilm producer (Vuong et al. 2003), kindly provided by Prof.. Gotz (Heilmann et al. 1996); *S. epidermidis* RP62A, a reference strain isolated from an infected catheter (ATCC collection no. 35984) and *S. epidermidis* XX17, a clinical isolate from an infected catheter (Artini et al. 2013). The bacteria were grown in brain heart infusion broth (BHI, Oxoid, Basingstoke, Hampshire, UK). Biofilm formation was assessed in static conditions.

Pleurotus ostreatus (Jacq.: Fr.) Kummer (type: Florida; ATCC No. MYA-2306) was maintained at 4°C through a periodic transfer on potato dextrose agar (BD Difco, Franklin Lakes, NJ, USA) plates in the presence of 0.5% yeast extract. The mycelia were inoculated in 1 l flasks containing 500 ml of potato dextrose broth (24 g l^{-1}) supplemented with 0.5% yeast extract, grown for 10 days at 28°C with shaking (150 rpm). *Acremonium sclerotigenum* MUT 4872 from the Mycotheca Universitatis Taurinensis was grown for 10 days at 20°C in a 1 l flask containing 500 ml of XNST30 broth (malt extract 3 g l^{-1}, yeast extract 3 g l^{-1}, sodium chloride 30 g l^{-1}, glucose 10 g l^{-1}, peptone 5 g l^{-1}).

Hydrophobin purification

Vmh2 purification

The protein was extracted from *P. ostreatus* mycelium. The mycelium was separated from the medium by filtration, using Whatman filter paper, and abundantly washed with 60% ethanol, 2% hot sodium dodecyl sulfate (SDS) and water. Next, the mycelium was lyophilized and treated with 100% trifluoroacetic acid (TFA) in a water bath sonicator for 30 min and centrifuged for 10 min at 3,200 × *g*. The supernatant was recovered and dried under nitrogen flux, dissolved in 60% ethanol and centrifuged for 20 min at 3,200 × *g*. The ethanol was removed from the solution under vacuum at 40°C using a rotavapor, the pellet was freeze-dried, and then the lipids were extracted

in a mixture of water–methanol–chloroform 2:2:1 vv^{-1}. The protein pellet was recovered by removing the liquid phase and treated with TFA for 30 min in a bath sonicator, redried, dissolved in 60% ethanol, and centrifuged (90 min at 12,000 × *g*).

Pac3 purification

The protein was extracted from the culture broth of *A. sclerotigenum*. The culture broth was separated from the mycelia by filtration using Whatman filter paper, and agitated in a Waring blender to produce foam. Next, the recovered foam was treated with 20% trichloroacetic acid, incubated over night at 4°C in a static condition and centrifuged for 1 h at 3,300 × *g*. Afterwards, the precipitate was collected, freeze-dried, treated with 100% TFA, sonicated in a water bath sonicator for 20 min, dried in a stream of nitrogen and dissolved in 60% ethanol. Moreover, the raw extract in 60% ethanol solution was dried and the lipids were extracted in a mixture of water–methanol–chloroform 2:2:1 vv^{-1}. After centrifugation, the protein was recovered by removal of the liquid phases and treated with TFA for 20 min in a bath sonicator, dried again, dissolved in 60% ethanol, and centrifuged (20 min at 12,000 × *g*).

Atomic force microscopy (AFM)

An XE-100 AFM (Park Systems, Suwon, Korea) was used for the imaging of the biomolecules. Surface imaging was obtained in non-contact mode using silicon/aluminum coated cantilevers (PPP-NCHR 10 M; Park Systems; tip radius less than 10 nm) 125 μm long with a resonance frequency of 200 to 400 kHz and a nominal force constant of 42 N m^{-1}. The scan frequency was typically 0.5 Hz per line. When necessary, the AFM images were processed by flattening, in order to remove the background slope, and the contrast and brightness were adjusted. For sample preparation, muscovite mica with a surface area of ~1 cm^2 was used as the substratum. The mica was freshly cleaved using adhesive tape prior to each deposition in order to ensure its cleanliness. Two-μl aliquots of the samples at a protein concentration of 100 μg ml^{-1} were directly deposited by casting onto the freshly cleaved substratum, dried by evaporation at room temperature under a ventilated fume hood and washed with 60% ethanol.

Hydrophobin coating of polystyrene multiplate wells and disks of different materials

Into each well of a 96-well flat-bottomed polystyrene plate were placed 200 μl of 60% ethanol solutions of the hydrophobins at different concentrations, and the plates were kept under a laminar flow hood overnight to functionalize the bottom and the walls of the wells. Afterwards, all the solutions were evaporated and each well was washed with 60% ethanol to remove the excess of protein.

Glass, steel, titanium and Teflon (PTFE) disks (diameter 1.3 cm) were firstly cleaned with 10% trichloroacetic acid and ethanol, then functionalized with 3 ml of 60% ethanol solution of 50 μg ml^{-1} of Pac3 and 100 μg ml^{-1} of Vmh2 in a Petri dish. The support surfaces were incubated for 3 min for each side, dried and washed with 60% ethanol solution to remove the excess protein. Contact angle measurements were performed on a KSV Instruments Ltd (Helsinki, Finland) CAM 200 Optical Contact Angle Meter coupled with drop shape analysis software. Each contact angle was calculated as the average of four drops of 5 μl of water, spotted on different points of the different surfaces.

Biofilm assays

The quantification of *in vitro* biofilm production was based on the method described by Christensen et al. (1985) with slight modifications (Artini et al. 2013). In brief, an overnight culture of each of the three *S. epidermidis* strains (O-47, RP62A, XX17) grown in BHI was diluted to a cell concentration of ~ 0.001 (OD) 600 nm. A volume of 200 μl of the culture was added to each well of a 96-well flat-bottomed polystyrene plate, previously functionalized. As a negative control, the same culture was added to wells treated with only 60% ethanol solution. The plates were incubated aerobically for 24 h at 37°C. Planktonic cells were gently removed after the incubation; each well was washed three times with PBS and dried by gently tapping in an inverted position on a piece of paper towel. The biofilm formation was measured using crystal violet staining. In detail, each well was stained with 0.1% crystal violet and incubated for 15 min at room temperature, rinsed twice with double-distilled water, and thoroughly dried. The dye bound to the adherent cells was solubilized with 20% (vv^{-1}) glacial acetic acid and 80% (vv^{-1}) ethanol. After incubation for 30 min at room temperature, the optical density was measured at 590 nm to quantify the total biomass of the biofilm formed in each well. Each data point was obtained from three independent experiments, each performed at least in triplicate.

The surface coating assay was performed as follows: a volume of 10 μl of hydrophobin solution, or 60% ethanol solution as a negative control, was transferred to the center of a well of a six-well tissue-culture-treated polystyrene microtiter plate. The plate was incubated at room temperature to allow complete evaporation of the liquid in sterile conditions. The wells were then filled with 4 ml of an overnight culture of *S. epidermidis* O-47 grown in BHI diluted to a cell concentration of about 0.001 (OD) 600 nm and incubated at 37°C in a static condition. After 24 h, the

wells were rinsed with water and stained with 4 ml of 0.1% crystal violet. The stained biofilms were rinsed with water and dried, and the wells were photographed.

The biofilm initial attachment assay was carried out as previously reported (Nithya and Pandian 2010) with slight modifications. In brief, an overnight culture of *S. epidermidis* O-47 grown in BHI was diluted to a cell concentration of about 0.01 (OD) 600 nm. A volume of 200 µl of the culture was added to each well of a 96-well flat-bottomed polystyrene plate, previously functionalized with hydrophobins. As a negative control, the same culture was added to wells treated with only 60% ethanol solution. The plates were incubated aerobically for 1 h at 37°C to allow the adherence of bacterial cells to the surface. After incubation, the plates were processed as previously described. Each data point was obtained from three independent experiments, each performed at least in triplicate.

Bacterial viability and biofilm thickness determined by confocal laser scanning microscopy (CLSM)

For confocal microscopy biofilms were formed on polystyrene in Nunc™ Lab-Tek® 8-well chamber slides (no 1,77,445; Thermo Scientific, Ottawa, ON, Canada). In brief, overnight cultures of *S. epidermidis* O-47 grown in BHI were diluted to a cell concentration of about 0.001 (OD) 600 nm and inoculated into each well of a chamber slide, previously functionalized with hydrophobins. As a negative control, the same culture was added to wells treated only with 60% ethanol solution. The bacterial culture was incubated at 37°C for 20 h in the presence of hydrophobins in order to assess its anti-biofilm activity and its influence on cell viability. Biofilm cell viability was determined with the FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (Molecular Probes, Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. After rinsing with filter-sterilized PBS, each well of the chamber slide was filled with 300 µl of a working solution of fluorescent stains, containing the SYTO® 9 green fluorescent nucleic acid stain (10 µM) and propidium iodide, and the red-fluorescent nucleic acid stain (60 µM), and incubated for 20–30 min at room temperature, protected from light. All the excess stain was removed by rinsing gently with filter-sterilized PBS. All microscopic observations and image acquisitions were performed with a confocal laser scanning microscope (CLSM) (LSM700-Zeiss, Oberkochen, Germany) equipped with an Ar laser (488 nm), and a He–Ne laser (555 nm). Images were obtained using a 20×/0.8 objective. The excitation/emission maxima for these dyes were ~480/500 nm for the SYTO® 9 stain and 490/635 nm for propidium iodide. Z-stacks were obtained by driving the microscope to a point just out of focus on both the top and bottom of the biofilms. Images were recorded as a series of .tif files with a file-depth of 16 bits. For each condition two independent biofilm samples were used.

CDC biofilm reactor assay

A CDC biofilm reactor (Biosurface Technologies, Bozeman, MT, USA) was used for the biofilm growth. Small disks used for this experiment were made with the following materials: PTFE, glass, stainless steel and titanium. Two disks for each material were used as controls. They were mounted on the disk holders, and inserted into the CDC biofilm reactor; 400 ml of BHI were added and autoclaved before use. After the sterilization, the disks were functionalized with the hydrophobins (as described above) and mounted in sterile conditions.

Overnight cultures of *S. epidermidis* (grown under shaking conditions at 37°C) were diluted at a ratio of 1:100 and inoculated into the glass vessel of the CDC reactor aseptically to obtain a final concentration ~10^6 CFU ml^{-1}. Growth was maintained at 37°C under mild agitation (60 rpm) with a magnetic stirrer. After 18 h, the disks were aseptically removed and the biofilm was dissolved. Biofilm formation was measured using crystal violet staining. In detail, each disk was first washed with distilled water, stained with 0.1% crystal violet and incubated for 15 min at room temperature. Next, all the disks were rinsed twice with double-distilled water, and thoroughly dried. The dye bound to the adherent cells on each disk was solubilized with 20% (vv^{-1}) glacial acetic acid and 80% (vv^{-1}) ethanol. After incubation for 30 min at room temperature, the disks were removed and the optical density of the resulting solution was measured at 590 nm to quantify the total biomass of biofilm formed on each support surface. Three duplicate CDC biofilm chamber experiments were performed.

Statistics

The data reported were statistically validated using the Student's *t*-test comparing the mean absorbance of the treated and untreated samples. The significance of the differences between the mean absorbance values was calculated using a two-tailed Student's *t*-test. A p-value of < 0.05 was considered significant.

Results

Characterization of the hydrophobin layers

The purified Vmh2 and Pac3 hydrophobins were deposited and dried on the hydrophilic mica surface to analyze the morphology of their layers by AFM analysis. The acquired images (Figure 1) clearly show a difference in the self-assembling structures of the two proteins. On

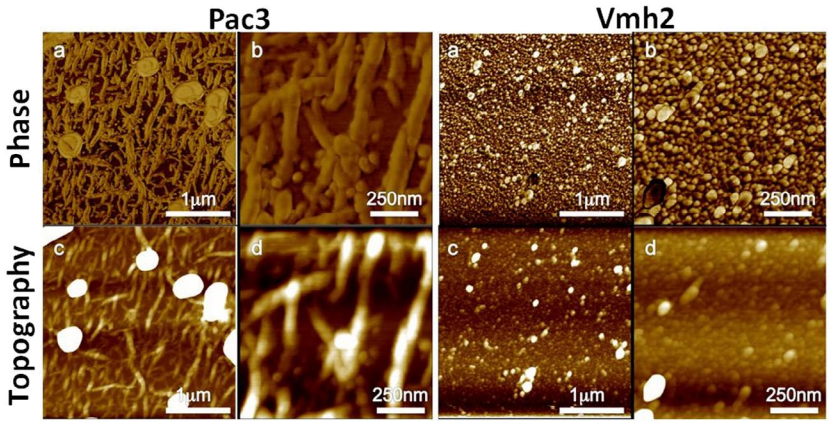

Figure 1. AFM images of Pac3 and Vmh2 at 100 µg ml^{-1} deposited on a mica surface and washed with 60% ethanol. The scale bar is 1 µm in (a) and (c) and 250 nm in images (b) and (d). For each sample the non-contact phase and morphology are reported.

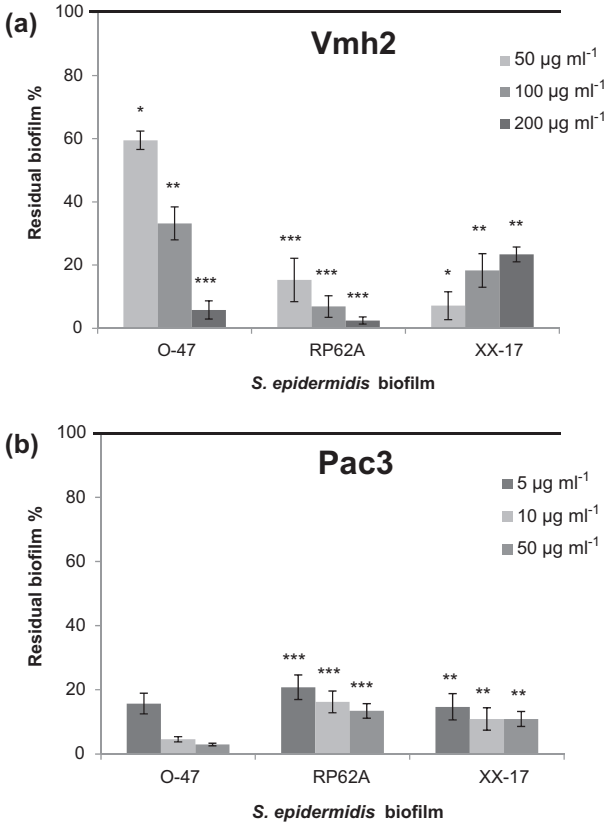

Figure 2. The effect of a Vmh2 and a Pac3 coating on the biofilm formation by different *S. epidermidis* strains. Biofilm formation was evaluated after 24 h incubation on polystyrene plates previously coated with the hydrophobin solutions. The data are reported as the percentage of residual biofilm for each tested *S. epidermidis* strain. The black line represents the biofilm obtained on uncoated polystyrene plates. Each data point represents the mean ± the SD of four independent samples; the mean values were compared to the untreated control and considered significant when $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) according to the Student *t*-test.

Pac3 amyloid-like-structures were formed, typical of Class I hydrophobins, while no fibrils were observed for Vmh2 in this condition, but only sponge-like structures. Because of the different morphology of the two protein layers, both hydrophobins were tested for *S. epidermidis* biofilm inhibition.

S. epidermidis biofilm on hydrophobin coated polystyrene surfaces

The binding of bacteria to abiotic materials is a pivotal step toward the establishment of a device-associated infection. The influence of the deposition of Vmh2 and Pac3 on polystyrene surfaces in *S. epidermidis* biofilm formation was evaluated using three *S. epidermidis* strains. The functionalization of polystyrene multi-wells was performed using Vmh2 and Pac3 solutions at concentrations from 50 to 200 µg ml^{-1}. The hydrophobin coating effect on biofilm formation was assessed on the three *S. epidermidis* strains (Figure 2). The data are reported as the percentage of residual biofilm obtained by growing *S. epidermidis* on hydrophobin coated wells with respect to that obtained on uncoated ones. Biofilm formation on Vmh2 coated surfaces was strongly inhibited for all tested *S. epidermidis* strains (Figure 2a). The inhibition was higher than 50% at 100 µg ml^{-1} (33.2% residual biofilm for *S. epidermidis* O-47, 6.9% for *S. epidermidis* RP62A, and 18.3% for XX17, respectively). In the case of Pac3, a substantial inhibition of biofilm formed was observed even at the lowest concentration used. For this reason Pac3 solutions at 5 and 10 µg ml^{-1} were tested (Figure 2b). Distinct anti-biofilm activity was also obtained using 5 µg ml^{-1} (15.7% residual biofilm for *S. epidermidis* O-47, 20.8% for *S. epidermidis* RP62A, and 14.7% for XX17, respectively). Biofilm inhibition on the Pac3 and Vmh2 coated surfaces was dose-dependent in the case of the *S. epidermidis* RP62A and O-47 but not in the case of the *S. epidermidis* XX17 biofilm.

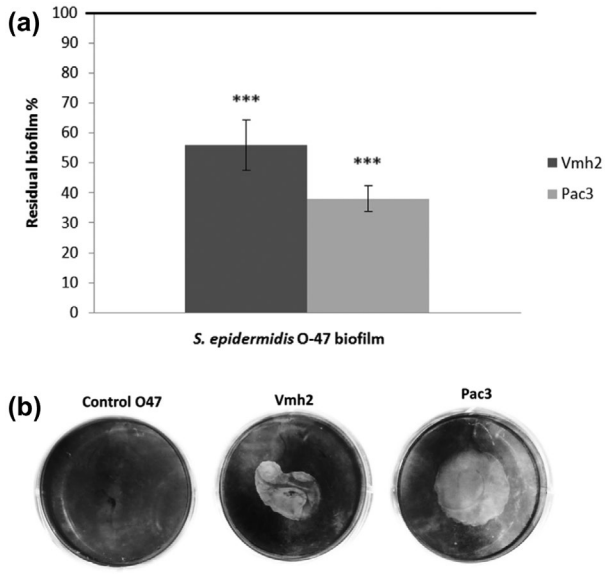

Figure 3. (a) The effect of Vmh2 and Pac3 functionalization on the initial attachment of *S. epidermidis* O-47. Biofilm formation was evaluated after incubation for 1h on polystyrene plates previously functionalized with hydrophobin solutions. The data are reported as the percentage of residual biofilm. The black line represents the biofilm obtained in uncoated polystyrene plates. Each data point represents the mean ± SD of four independent samples; the mean values were compared to the untreated control and considered significant when $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) according to the Student *t*-test. (b) Analysis of the surfactant capability of hydrophobin solutions on *S. epidermidis* O-47. The center of each well of a six-well tissue-culture-treated polystyrene microtiter plate was coated with 10 µl of each solution. Biofilm formation was evaluated after 24 h incubation using crystal violet staining and then photographed. The areas where biofilm was not present appear unstained.

Further analyses were performed to understand the hydrophobin mode of action and to determine which step biofilm formation was affected by the hydrophobin coatings. In particular, *S. epidermidis* initial attachment was explored. As reported in Figure 3a, both Vmh2 and Pac3 inhibited *S. epidermidis* biofilm formation after 1 h of bacterial growth (the biofilm was reduced by ~44% by Vmh2 and by ~62% by Pac3, respectively).

To confirm that the anti-biofilm activity of the protein layers was related to their capability to modify surface properties, a surface coating assay was performed. In this case, only the central area of the bottom of a well was coated by the deposition of a single drop of the protein solutions and the ability of the coated surfaces to repel biofilm formation by *S. epidermidis* O-47 was tested. This strain was selected because it is the strongest biofilm producer among those used in this work. As shown in Figure 3b, only the hydrophobin coated regions were able to repel biofilm formation. All these results demonstrate that the prevention of *S. epidermidis* biofilm formation by hydrophobins occurred by means of inhibition of the initial attachment of bacterial cells to the surface.

The prevention of *S. epidermidis* O-47 biofilm formation was further investigated by CLSM. CLSM was used to analyze the structure of the biofilm and to assess cell viability. As shown in Figure 4, *S. epidermidis* biofilm thickness was significantly reduced on polystyrene surfaces coated with Vmh2 at 100 µg ml^{-1}, in comparison with the uncoated surfaces. The biofilm thickness reduction was evident on Pac3 coated surfaces with a notable alteration in the *S. epidermidis* O-47 biofilm architecture at both the tested concentrations. Moreover, the viability of the cells embedded in the biofilm on the hydrophobin coated polystyrene surfaces was evaluated by live/dead staining. As shown in Figure 4, the cells exposed to the hydrophobin coated polystyrene surfaces were alive. The green stain indicates viable cells while red indicates dead cells, therefore the lack of red staining demonstrated that the hydrophobins had no bactericidal activity on the *S. epidermidis* cells. It is worth noting that most known anti-biofilm molecules display antibacterial activity (bactericidal or bacteriostatic). Therefore, the hydrophobins affected the adhesive properties of *S. epidermidis*, and did not impair cell viability, a behavior that should prevent the development of escape mutants.

Analysis of S. epidermidis biofilm formation on different hydrophobin coated surfaces

The capability of the two hydrophobins to coat four different biomedically relevant materials (PTFE, glass, stainless steel and titanium) was evaluated by WCA analysis. Indeed, a specific feature of the hydrophobin coatings is the modification of the wettability of the surfaces, due to the peculiar amphiphilic nature of these proteins. In Figure 5 measurements of the WCAs of the coated and uncoated surfaces are shown. In all cases, the change in surface wettability demonstrated the presence of the hydrophobin layers, stable also after ethanol washing.

Biofilm formation by *S. epidermidis* was first evaluated on the bare materials in a CDC biofilm reactor. This apparatus permits the growth of biofilm on the two faces of disks made of specific materials. These disks are exposed to shear forces by a swirling paddle in the base of the reactor (Williams and Bloebaum 2010). *S. epidermidis* RP62A was able to produce a variable quantity of biofilm on all the tested materials and the greater biofilm production was achieved on the hydrophilic substratum glass (Figure 6a). Next, the hydrophobin coated materials were used in the CDC biofilm reactor to test the capability of *S. epidermidis* RP62A to form a biofilm on these supporting materials (Figure 6b). The results are reported as percentages of biofilm formation on pre-coated disks

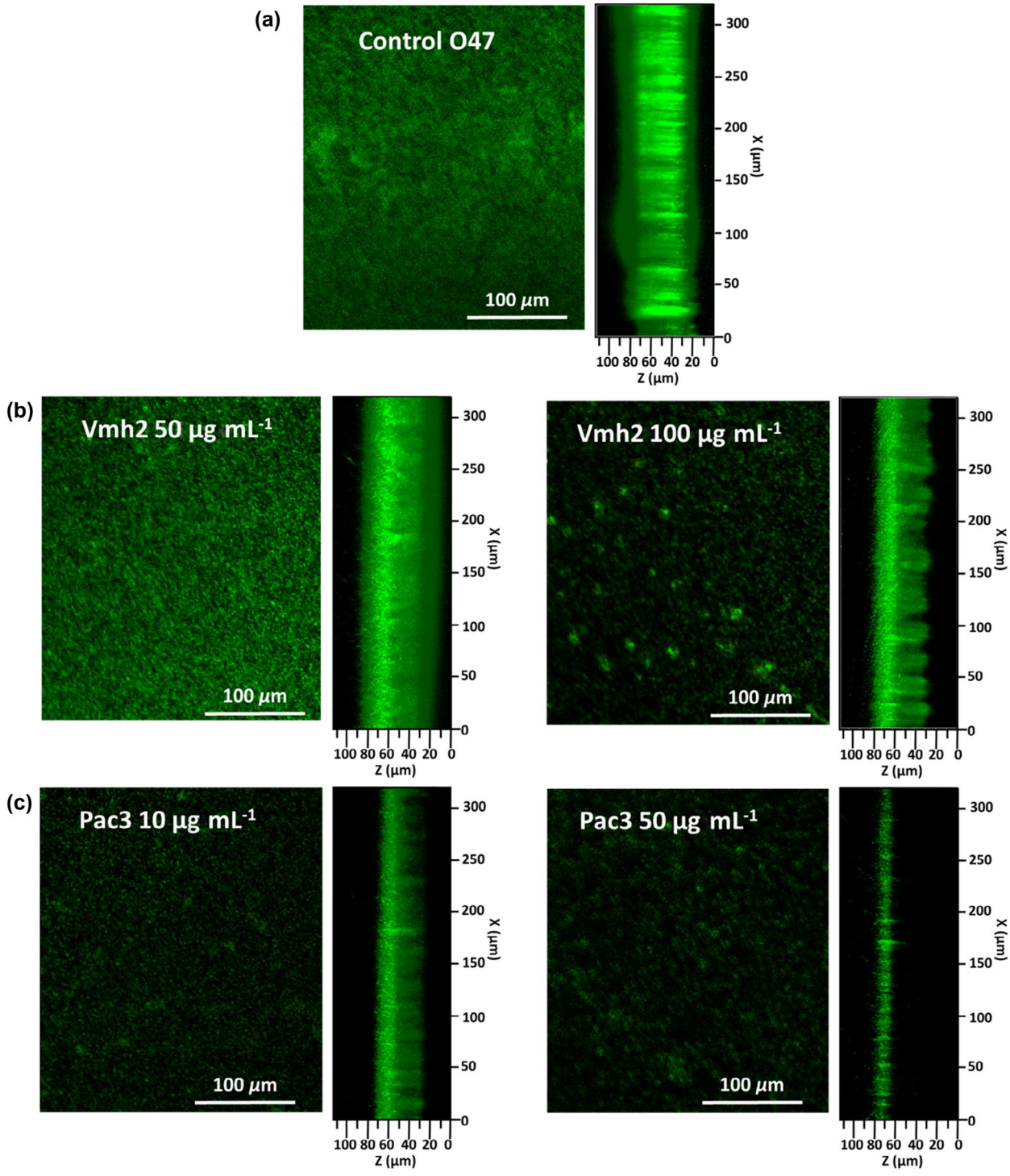

Figure 4. The effect of a hydrophobin coating on *S. epidermidis* O-47 biofilm structure and cell viability. (a) CLSM of *S. epidermidis* O-47 biofilm on an uncoated polystyrene well; (b) CLSM of *S. epidermidis* O-47 biofilm on Vmh2-coated polystyrene wells; and (c) CLSM of *S. epidermidis* O-47 biofilm on Pac3-coated polystyrene wells. The bacteria were grown in an eight-well chamber slide for 20 h and then stained with LIVE/DEAD reagents. The green fluorescence (SYTO9) indicates viable cells and the red fluorescence (PI) indicates dead cells. The Z-stack analysis of the *S. epidermidis* O-47 biofilm in all tested conditions is reported.

in comparison with that on untreated disks. Although to a different degree, both Pac3 and Vmh2 were able to prevent *S. epidermidis* biofilm formation on all the tested materials.

Discussion

In this paper, the effect of surface modification of abiotic materials on biofilm formation has been explored,

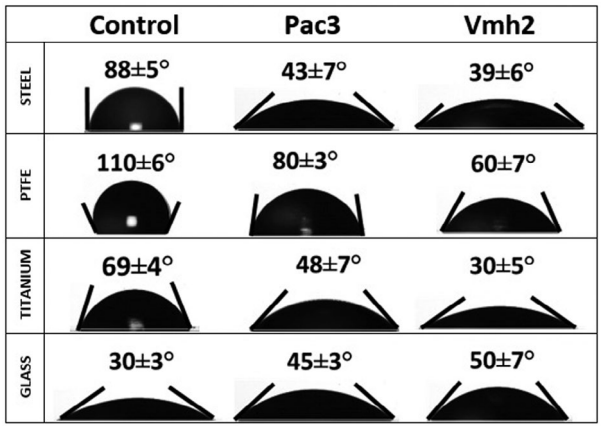

Figure 5. WCA analysis of different surfaces, upon coating with 50 μg ml^{-1} of Pac3 and 100 μg ml^{-1} of Vmh2. The data reported represent the means ± SD of independent WCA measurements on four drops.

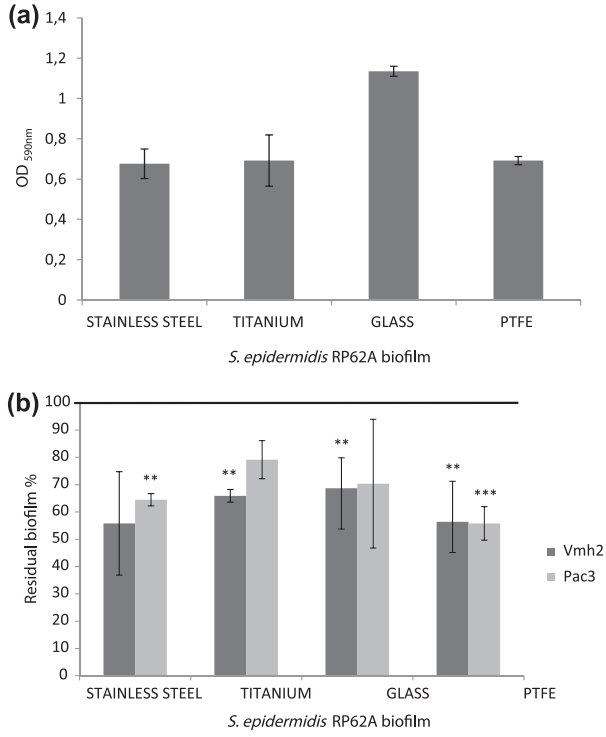

Figure 6. (a) Analysis of the capability of *S. epidermidis* RP62A to form a biofilm on different materials. Biofilm formation was performed in a CDC biofilm reactor. (b) The effect of a Vmh2 and a Pac3 coating on different materials on *S. epidermidis* RP62A biofilm formation in a CDC biofilm reactor. The data are reported as the percentage of residual biofilm. The black line represents the biofilm obtained on uncoated materials. Data reported represent the means ± SD of three measurements and the mean values were compared to the untreated control and considered significant when $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) according to the Student *t*-test.

studying the impact of hydrophobin coated surfaces on *S. epidermidis* biofilm formation. Indeed, coagulase-negative *S. epidermidis* has become a serious nosocomial pathogen, frequently causing infections associated with implanted foreign materials (Rupp and Fey 2001).

Although it is known that hydrophobins can coat different surfaces, changing their physico-chemical properties, the structural characteristics of the layers formed by Class I hydrophobins can differ, showing different morphologies (Lo et al. 2014). In order to obtain information on the specific structural morphologies of the Pac3 and Vmh2 layers, these layers were analyzed using the AFM technique. The images acquired (Figure 1) show that Pac3 formed the typical amyloid-like fibrils, while in the same conditions Vmh2 formed sponge-like structures. It is worth noting that the amyloid-like fibrils of Vmh2 had previously been observed in specific conditions (Houmadi et al. 2008, 2012). These results suggest that Pac3 has a greater propensity to form amyloid structures in comparison with Vmh2. Because of the structural differences between the layers formed, both proteins were used in the following analyses.

The first analysis was performed using three *S. epidermidis* strains, differing in biofilm structure and composition, and the two hydrophobins for the coating of the polystyrene surface. Depending on the strain tested, the anti-biofilm activity was more or less marked. Indeed, biofilm reduction in the O-47 strain in the presence of Pac3 coating was about 85%, while it was only 40% in case of Vmh2, at the same protein concentration (50 μg ml^{-1}). On the other hand, the effect of the two hydrophobins (at 50 μg ml^{-1}) on biofilm formation by the RP62A strain was quite similar. Therefore the differences in the anti-biofilm efficiency of the coating should be affected by both the specific layer and the strain used. Coating characteristics (eg morphology,) as well as differences in biofilm formation and structure in each strain can affect the reported activity.

The action of the hydrophobin surface coating on *S. epidermidis* biofilm formation was explored on different materials used for medical devices. Stainless steel and titanium are commonly used for orthopedic surgical instruments and joint replacement devices. A major use of PTFE is for the manufacture of prostheses adopted for the anterior cruciate ligament repair procedure. PTFE is also used in graft augmentation devices to protect biological grafts, and in microporous hydrophobic membranes used in products such as vented blood warmers, in-line suction filters and vented suction containers (Lauderdale et al. 2010). These studies, performed in a CDC biofilm reactor, revealed that glass was the most susceptible surface to *S. epidermidis* biofilm growth. Nevertheless, all the other tested materials were efficiently colonized by the bacterial cells.

S. epidermidis biofilm formation was always reduced when all the materials were coated with hydrophobins,

suggesting a similar molecular mechanism by the hydrophobin layers to inhibit biofilm formation.

The coating of all the studied materials with hydrophobins changed their hydrophobicity, either increasing or decreasing it, as revealed by the WCA modifications (Figure 5). Since all the coated materials became refractory to *S. epidermidis* colonization, this capacity is not related to surface wettability. In agreement, previous studies have demonstrated that surface hydrophobicity is not crucial for biofilm formation by *S. epidermidis* (Cerca et al. 2005). Therefore, the anti-biofilm action has to be related to other acquired features of the protein-coated surfaces.

As is well known, hydrophobins can be used to immobilize proteins on a surface, thus offering a non-covalent alternative to conventional surface modification methods (Qin, Hou, et al. 2007; De Stefano et al. 2009; Wang et al. 2010; Longobardi et al. 2015). Hence it is unlikely that the hydrophobin coating impairs *S. epidermidis* biofilm formation by reducing the affinity of the bacterial proteins involved in the attachment to abiotic surfaces.

The anti-biofilm effect could be related to the repulsion between the polysaccharides of the biofilm extracellular matrix and the surfaces. However, this hypothesis also has to be rejected for several reasons: (1) the hydrophobin-coated-materials prevent biofilm formation by *S. epidermidis* XX17, a strain which does not produce the main biofilm extracellular polysaccharide (Artini et al. 2013); (2) the step affected by the hydrophobin coating was the early phase of biofilm formation (Figure 3), while the extracellular matrix production is the hallmark of the following accumulation phase; and (3) it is well known that Vmh2 layers are able to bind glucose as well as oligosaccharides (Armenante et al. 2010; Della Ventura et al. 2016), and hence binding of polysaccharides to the layers of Vmh2, rather than their repulsion, would have been expected.

Extensive research has been focused on the role of eDNA, a structural component of the matrix in *S. epidermidis* biofilm formation (Izano et al. 2008; Das et al. 2010; Christner et al. 2012). eDNA is required for the initial attachment of *S. epidermidis* to surfaces, as well as for the following early phase of biofilm development (Biswas et al. 2006). A key role of eDNA in the initial adhesion has been demonstrated in genetically independent *S. epidermidis* backgrounds (Whitchurch 2002; Qin, Ou, et al. 2007). Thermodynamic analyses have shown that eDNA introduces advantageous acid–base interactions, between *S. epidermidis* bacterial cells and the abiotic surface (Das et al. 2010). In contrast, in *S. aureus* biofilm formation, eDNA does not have any impact on primary attachment, but instead it plays a critical role during the transition from the attachment phase to the accumulation phase (Moormeier et al. 2014). The influence of hydrophobin deposition on polystyrene surfaces in *S. aureus* biofilm formation has been preliminarily investigated. Neither the Pac3 nor Vmh2 coated surfaces have proven able to prevent *S. aureus* biofilm formation (data not shown), thus suggesting that the mechanism responsible for the anti-biofilm activity of the tested hydrophobin layers on *S. epidermidis* involves eDNA. A detailed study on the hydrophobin–eDNA interaction will be the subject of future investigations.

In conclusion, in this work novel properties of hydrophobin layers have been assessed, demonstrating their anti-biofilm activity with respect to *S. epidermidis*. This hydrophobin layer feature probably does not depend on interactions with bacterial proteins and polysaccharides but could depend on an interaction with matrix eDNA. These observations are interesting in view of the possible industrial applicative uses of hydrophobins in medical fields. Furthermore, new insights into the interaction mechanism between different bacterial species and abiotic surfaces have been provided.

Disclosure statement

No potential conflict of interest is reported by the authors.

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