Research Article

Cold-adapted bacterial extracts as source of anti-infective and antimicrobial compounds against *Staphylococcus aureus*

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Aim: The dramatic emergence of antibiotic resistance has directed the interest of research toward the discovery of novel antimicrobial molecules. In this context, cold-adapted marine bacteria living in polar regions represent an untapped reservoir of biodiversity endowed with an interesting chemical repertoire. The aim of this work was to identify new antimicrobials and/or antibiofilm molecules produced by cold-adapted bacteria. **Materials & methods:** Organic extracts obtained from polar marine bacteria were tested against *Staphylococcus aureus*. Most promising samples were subjected to suitable purification strategies. **Results:** Results obtained led to the identification of a novel lipopeptide able to effectively inhibit the biofilm formation of *S. aureus*. **Conclusion:** New lipopeptide may be potentially useful in a wide variety of biotechnological and medical applications.

In recent years, bacterial infections represent a serious challenge for human health due to the widespread diffusion of resistance to antibiotics, since more than 70% of pathogenic bacteria display resistance to at least one antibiotic commonly used in therapy [1]. This feature is often related to the ability to form biofilm [2], a phenotypic lifestyle that renders bacteria highly tolerant to exogenous stressors, including antibiotics or other biocides.

Staphylococci represent a prevalent cause of chronic infections associated to biofilm phenotype [3]; *Staphylococcus aureus* in particular, deploying a wide array of virulence factors, can acquire resistance to the majority of antibacterial compounds. This feature makes *S. aureus* a 'superbug' further reinforced by continuous emergence of new clones [4].

S. aureus can be frequently isolated as a commensal of the human body, colonizing districts of healthy individuals such as skin and mucous membranes, including nares and guts. *S. aureus* can be persistently isolated from the nasal cavity of 20% of healthy individuals, while 30% are intermittent carriers. This colonization represents an important reservoir of this pathogen, and significantly increases the chances of infections [4].

Within the past two decades, the continuous and rapid emergence of antibiotic resistance has directed the interest of research toward the discovery of novel antibiofilm compounds and antimicrobial molecules [5]. In the literature, there are several reports about the novel synthesis of biomaterials effective against *S. aureus* and able to combat drug resistance phenomenon [6].

Recently, many scientists are analyzing natural compounds extracted from plants and marine microorganisms, in order to identify new and effective antibiofilm molecules [7–10]. Indeed, the bioprospecting of natural environments has allowed to discover, produce and commercialize many new antibiotics [11] and offers novel key scaffolds for drug development [12].

Table 1. Strains used in this study.		
Strain	**Origin**	**Ref.**
P. haloplanktis TAE79	Antarctic seawater[†] (algae necrosized suspended in seawater)	Liège collection
P. haloplanktis TAE80	Antarctic seawater[†] (algae necrosized suspended in seawater)	Liège collection
Pseudomonas sp. TAD1S	Antarctic seawater[†]	Liège collection
Pseudomonas sp. TAD18	Antarctic seawater[†] (frozen algae)	Liège collection
Pseudomonas sp. TAA207	Antarctic seawater[†] (marine sediment)	Liège collection
Psychromonas arctica	Arctic seawater (Svalbard islands, Arctic)	[53]
P. haloplanktis TAB23	Antarctic seawater[†]	[54]
P. haloplanktis TAE56	Antarctic seawater[†] (algae necrosized suspended in seawater)	Liège collection
P. haloplanktis TAE57	Antarctic seawaterr[†] (algae necrosized suspended in seawater)	Liège collection
S. aureus 6538P	Clinical isolate, reference strain for antimicrobial tests	ATCC collection
S. aureus 20372	Clinical isolate	ATCC collection
S. aureus 25923	Clinical isolate, reference strain for antimicrobial tests	ATCC collection

[†]Isolated from Antarctic coastal seawater sample collected in the vicinity of the French Antarctic station Dumont d'Urville, Terre Adélie (66°40′ S; 140° 01′ E).

Marine microorganisms are considered an inexhaustible source for the discovery of new antimicrobial compounds, since they must survive in an environment characterized by climatic variations and compete with others for space and nutrition. Several biologically active compounds (such as antifouling, anticancer, antimicrobial) have been obtained from marine sources [13]. Some of them are already investigated for the treatment of various diseases [14–16].

In this context, cold-adapted marine bacteria deriving from polar regions represent an unexploited reservoir of biodiversity provided with an attracting chemical repertoire. Unusual survival strategies have been developed by bacteria living in prohibitive environmental conditions, according to an antagonistic behavior mandatory offering competitive advantages due to the limitation of the growth of other microorganisms [17]. When procuration of nutrients is limited or difficult, this behavior offers a growth advantage; in this view, Antarctic microorganisms could have developed peculiar characteristics, such as chemically complex metabolites, that guarantee survival in extreme habitats [18]. Some molecules obtained from these bacteria display antifouling, antimicrobial and other activities interesting for possible pharmaceutical applications [19]. We recently reported that *Pseudoalteromonas haloplanktis* TAC125, an Antarctic marine bacterium, produce and secrete many different compounds of biotechnological interest. These compounds include molecules that inhibit biofilm growth by *Staphylococcus epidermidis*, a saprophyte of the human skin acting as a pathogen after colonization of artificial medical devices [20–23], and antimicrobial compounds that inhibit the growth of *Burkholderia cepacia complex* (Bcc), organisms that pose a significant health risk to cystic fibrosis patients [24,25]. Furthermore, cell-free supernatants obtained from different bacterial cultures of polar marine bacteria showed interesting antibiofilm activities on *S. epidermidis, Pseudomonas aeruginosa* and *S. aureus* [26].

The aim of this paper was the identification of new antimicrobials and/or antibiofilm molecules produced by bacteria adapted to cold habitats. Intracellular and extracellular organic extracts obtained from polar marine bacteria, such as *Psychromonas, Pseudomonas* and *Pseudoalteromonas* genera, have been tested against *S. aureus*. Most promising samples were subjected to suitable purification strategies in order to identify molecules responsible for the sought biological activity.

Materials & methods
Bacterial strains & culture conditions
Bacterial strains used in this work are reported in Table 1. Polar bacteria were grown in flasks in synthetic medium GG (D-gluconic acid sodium 10 g/l, L-glutamic acid 10 g/l, NaCl 10 g/l; NH_4NO_3 1 g/l; $KH_2PO_4 \cdot 7H_2O$ 1 g/l; $MgSO_4 \cdot 7H_2O$ 200 mg/l; $FeSO_4 \cdot 7H_2O$ 5 mg/l; $CaCl_2 \cdot 2H_2O$ 5 mg/l) at 15°C under vigorous agitation (180 r.p.m) until stationary phase (72–90 h).

S. aureus was grown in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, UK) at 37°C. Culture broths were performed under vigorous agitation (180 rpm), while biofilm formation was assessed in static conditions.

Bacterial strains grown in BHI broth were kept at -80°C with glycerol at a final concentration of 15%.

Organic extract preparation

Polar bacterial cultures previously described were collected and subjected to a liquid–liquid extraction as an initial separation technique to process the cell-free supernatants and the cellular pellets, to obtain 'extracellular extracts' and 'intracellular extracts', respectively.

Cell-free supernatants and cell pellets were recovered by centrifugation at 6000 r.p.m 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 and cell-free supernatants were frozen at -80°C until use.

Liquid–liquid extraction was performed on cell pellets and cell-free supernatants separately, without adding cryoprotectants. In detail, cell pellets and supernatants were thawed, then cell pellets were resuspended in an opportune volume of sterile distilled water. Obtained samples were stirred with ethyl acetate in a volume ratio of 2:1 (assay percent range ≥ 99.5%) (Sigma-Aldrich, St. Louis, MO, US) and mixed at 1% with acid formic (assay percent range = 90%; JT Baker, Munich, Germany). Each solution was stirred at least for 30 min and then centrifuged at 3000 r.p.m 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 organic extracts were aliquoted in 4 mg dry-weight samples and stored at -20°C.

Fractionation of the intracellular extract of TAA207 & TAD1S

The intracellular extracts (named IN_) from *Pseudomonas* sp. TAA 207 (IN_TAA207, 100 mg) and from *Pseudomonas* sp. TAD1S (IN_TAD1S, 113 mg) were both fractionated on silica gel columns. The columns (30 ml, 50 × 0.7 cm for IN_TAA207 and 49 ml, 32 × 0.7 cm for IN_TAD1S, 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 IN_TAD1S, a further elution with $CHCl_3:CH_3OH:H_2O$ 6/4/0.5 v/v/v was performed.

The fractions resulted to be active against *S. aureus* (IN_ TAA207: fraction 3; and IN_TAD1S: fractions 5-8-11-20, respectively) were acetylated and analyzed by GC–MS technique. In detail, fraction 3 from IN_ TAA207 was analyzed on an Agilent Technologies gas chromatograph 6850A equipped with a mass selective detector 5973N and a Zebron ZB-5 capillary column (Phenomenex, 30 m × 0.25 mm i.d., flow rate 1 ml/min, He as carrier gas); fractions 5-8-11-20 from IN_TAD1S were analyzed on a Agilent Technologies gas chromatograph 7820A equipped with a mass selective detector 5977Band a HP-5 capillary column (Agilent, 30 m × 0.25 mm i.d., flow rate 1 ml/min, He as carrier gas). All derivatives were analyzed 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.

The same purification procedure was used for EX_TAD1S (70 mg). The silica gel column (46 ml, 30 × 0.7 cm) was eluted with the same solvent mixtures used for IN_TAD1S. Fraction 15, active against *S. aureus* USA300, was analyzed by ^1H NMR spectrum performed by using a Bruker Avance-DRX 600 MHz spectrometer equipped with a cryoprobe. The spectrum was acquired at 298 K in $CDCl_3:CD_3OD$ 3:1.

Minimal inhibitory concentration

The antimicrobial activity of each extract was evaluated. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of antimicrobial that yields at least 99.9% reduction (i.e., three log-units) of bacterial growth compared with the untreated bacteria as reported by Clinical & Laboratory Standards Institute (CLSI) and National Committee for Clinical Laboratory Standards (NCCLS) methods. MIC was determined in duplicate by twofold serial dilution (CLSI, 2017), as reported [27].

The wells of a sterile 48-well flat-bottomed polystyrene plate were filled with 400 μl of 1/100 diluted overnight bacterial cultures grown in BHI. Each extract was tested starting from a concentration of 4 mg/ml, as reported in the literature [28]. After overnight incubation at 37°C, the antimicrobial activity was optically evaluated comparing treated and untreated samples.

Biofilm formation by *S. aureus*

Biofilm formation by *S. aureus* was evaluated in the presence of organic extracts. Quantification of *in vitro* biofilm production was based on a method previously reported [29]. Briefly, the wells of a sterile 96-well flat-bottomed polystyrene plate were filled with 100 μl of BHI broth. 1/100 dilution of overnight bacterial cultures was added

into each well. Dried extracts were first dissolved in DMSO at an opportune concentration, then diluted in BHI before use.

The first row of the multiwell contained the untreated bacteria, while each of the remaining rows contained serial dilutions of the organic extract, starting from the second row.

After the inoculation, the plates were incubated aerobically for 20 h at 37°C in static condition. Biofilm formation was measured by crystal violet staining. After treatment, culture broths were gently removed; each well was washed three-times with water and patted dry on a piece of paper towel in an inverted position. Each well was stained with 0.1% crystal violet (incubation at room temperature for 15 min). To remove the excess of dye, each well was washed three-times with water, and thoroughly dried. The remaining dye, bound to biofilm, was solubilized with 80% (v/v) ethanol and 20% (v/v) glacial acetic acid. After 30 min of incubation at room temperature, (optical density) OD at 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 six replicates.

Statistics & reproducibility of results

Data reported were statistically validated using Student's *t*-test comparing the 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 of less than 0.05 was considered significant.

Results

Setup of two libraries from polar bacterial cultures

A selection of nine polar marine bacteria (Table 1) were grown, as described in the Materials & methods section. The synthetic minimal medium named D-gluconic and L-glutamic acid medium (GG) [25] was chosen to minimize the potential interference of culture medium on the organic extraction step due to reduction of the sample complexity, and to exploit the biosynthetic potential of bacterial metabolism. Furthermore, the choice of a minimum medium should promote in the bacterial cell the activation of alternative metabolic pathway that could maximize the production of secondary metabolites. The whole bacterial cultures were then recovered and separately extracted (cell pellet and supernatant) by using ethyl acetate. It is often adopted for the downstream recovery of fermentation products such as antibiotics or secondary metabolite.

In this way, for each bacterial strain, we obtained an intracellular extract (IN_*StrainName*) and an extracellular one (EX_ *StrainName*).

Screening of antimicrobial activities of extracts against *S. aureus*

Intracellular and extracellular extracts were analyzed for their antimicrobial activity against *S. aureus* 6538P. The MIC of extracts is reported in Table 2. A discrete number of selected extracts showed antimicrobial activity against *S. aureus* 6538P. In detail, IN_TAB23, EX_TAE56 and EX_TAE57 showed antimicrobial activity at a concentration higher than 1 mg/ml.

Screening of antibiofilm activity of intracellular extracts against *S. aureus*

Intracellular extracts were also screened for their ability to inhibit biofilm formation of *S. aureus* 6538P. Several extracts were able to inhibit biofilm formation (Figure 1). Considering that IN_TAB23 displayed an antimicrobial activity at a concentration of 1 mg/ml (Table 2), it was tested for antibiofilm activity at a concentration of 0.5 mg/ml (1/2 MIC value). Results obtained showed that this extract also possessed an antibiofilm activity at sub-MIC concentration. Best results were obtained with IN_TAA207 and IN_TAD1S extracts and, for this reason, they were selected for further analyses.

Experiments were performed on three different *S. aureus* strains, characterized by qualitative and quantitative differences in biofilm composition [29], using serial dilutions of each extract, starting from a concentration of 4 mg/ml (Figure 2). Figure 2A describes the activity of IN_TAA207 on biofilm formation of *S. aureus* 6538P, 20372 and 25923 strains. A strong inhibitory effect was confirmed for all tested strains, despite their differences in biofilm composition. Furthermore, inhibition of biofilm formation is clearly dose-dependent, starting from a reduction of about 40% at a concentration of 0.5 mg/ml.

As shown in Figure 2B, IN_TAD1S extract reduced biofilm production of all three tested *S. aureus* strains, with different extent. However, in these experiments, a clear dose-dependent effect was not observed.

Table 2. Minimal inhibitory concentration of organic extracts from bacterial supernatants and whole cells on *S. aureus* 6538P.

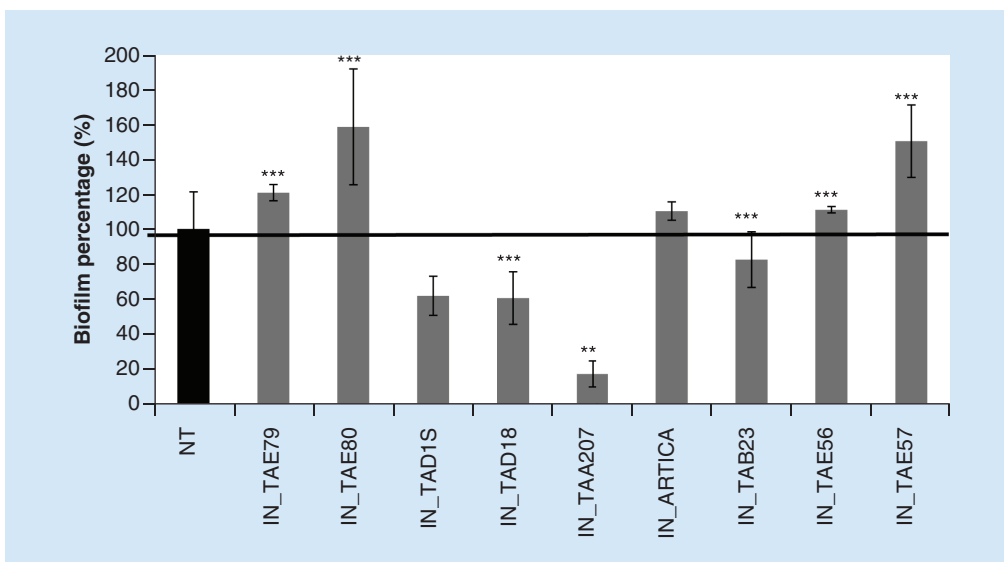| Extract	*S. aureus* 6538P
Intracellular extracts	
IN_TAE79	>4 mg/ml
IN_TAE80	>4 mg/ml
IN_TAD1S	>4 mg/ml
IN_TAD18	>4 mg/ml
IN_TAA207	>4 mg/ml
IN_ARTICA	>4 mg/ml
IN_TAB23	1 mg/ml
IN_TAE56	>4 mg/ml
IN_TAE57	>4 mg/ml
Supernatant extracts	
EX_TAE79	>4 mg/ml
EX_TAE80	>4 mg/ml
EX_TAD1S	>4 mg/ml
EX_TAD18	>4 mg/ml
EX_TAA207	>4 mg/ml
EX_ARTICA	>4 mg/ml
EX_TAB23	>4 mg/ml
EX_TAE56	1 mg/ml
EX_TAE57	1 mg/ml

Figure 1. Intracellular extracts from cold-adapted bacteria on *S. aureus* 6538P biofilm formation. Data are reported as the percentage of residual biofilm after the treatment. Each data point represents the mean ± SD of five independent samples. Differences in mean absorbance were compared with the untreated control (NT, black bar) and considered significant when p < 0.05 according to Student's *t*-test.
*p < 0.05; **p < 0.01; ***p < 0.001.
NT: Not treated; SD: Standard deviation.

Fractionation of intracellular extracts from *Pseudomonas* sp. TAA207 & from *Pseudomonas* sp. TAD1S

IN_TAA207 and IN_TAD1S were fractionated on a silica gel column as reported in the Methods section (Figure 3). The fractions obtained from IN_TAA207 were tested, at a concentration of 200 μg/ml, to evaluate the antibiofilm
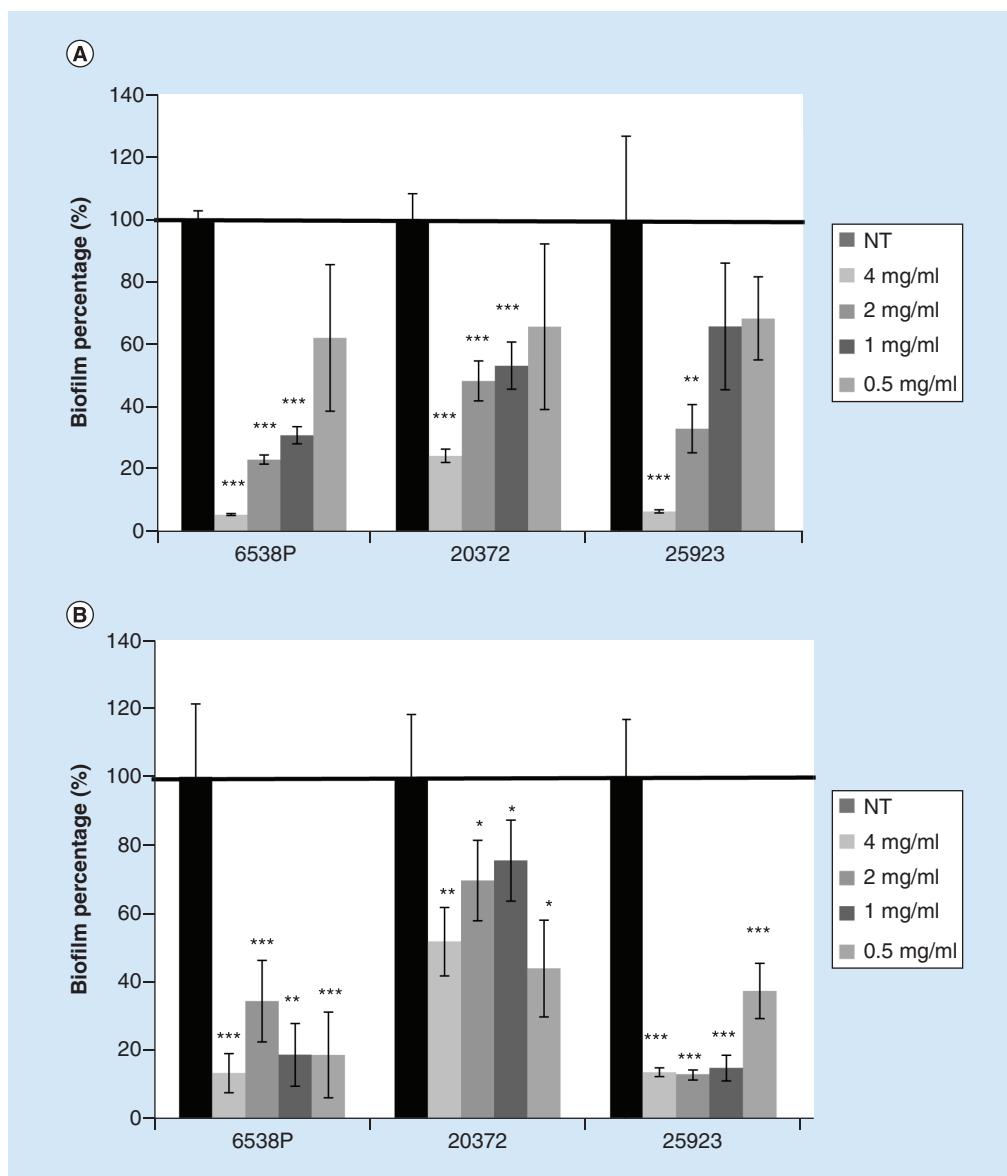
Figure 2.   IN_TAA207 (A) and IN_TAD1S (B) extracts on biofilm formation of different *S. aureus* strains. Serial dilutions of each extract starting from 4 mg/ml were evaluated. Data are reported as the percentage of residual biofilm. Each data point represents the mean ± SD of three independent samples. Differences in mean absorbance were compared with the untreated control (NT, black bar) and considered significant when p < 0.05 according to Student's *t*-test.
*p < 0.05; **p < 0.01; ***p < 0.001.
NT: Not-treated; SD: Standard deviation.

activity on *S. aureus* strain 6538P (Figure 3A). Results showed strong antibiofilm activity in fraction 3, eluted with only chloroform. This sample was analyzed by GC–MS revealing the presence of saturated and unsaturated long-chain fatty acids as follows: C14:0, C14:1, C16:0, C16:1, C18:1, and a very low amount of C18:0 (Figure 3B).

Antibiofilm activity of fractions derived from chromatographic separation of IN_TAD1S was also evaluated, as reported in Figure 3C. In this case, different fractions were able to inhibit biofilm formation of *S. aureus* 6538P. The strongest effect was observed for fractions 5, 8, 11 (eluted with chloroform) and 20 (eluted with the chloroform/methanol 99:1). Moreover, for these fractions, an antibacterial activity has been observed.

To obtain more information about the chemical composition, each fraction was analyzed by GC–MS. Also, in this case, chromatograms revealed the presence of saturated and unsaturated long-chain fatty acids, with slight differences in the relative amount of each chemical species (Supplementary Figure 1). A comparison between
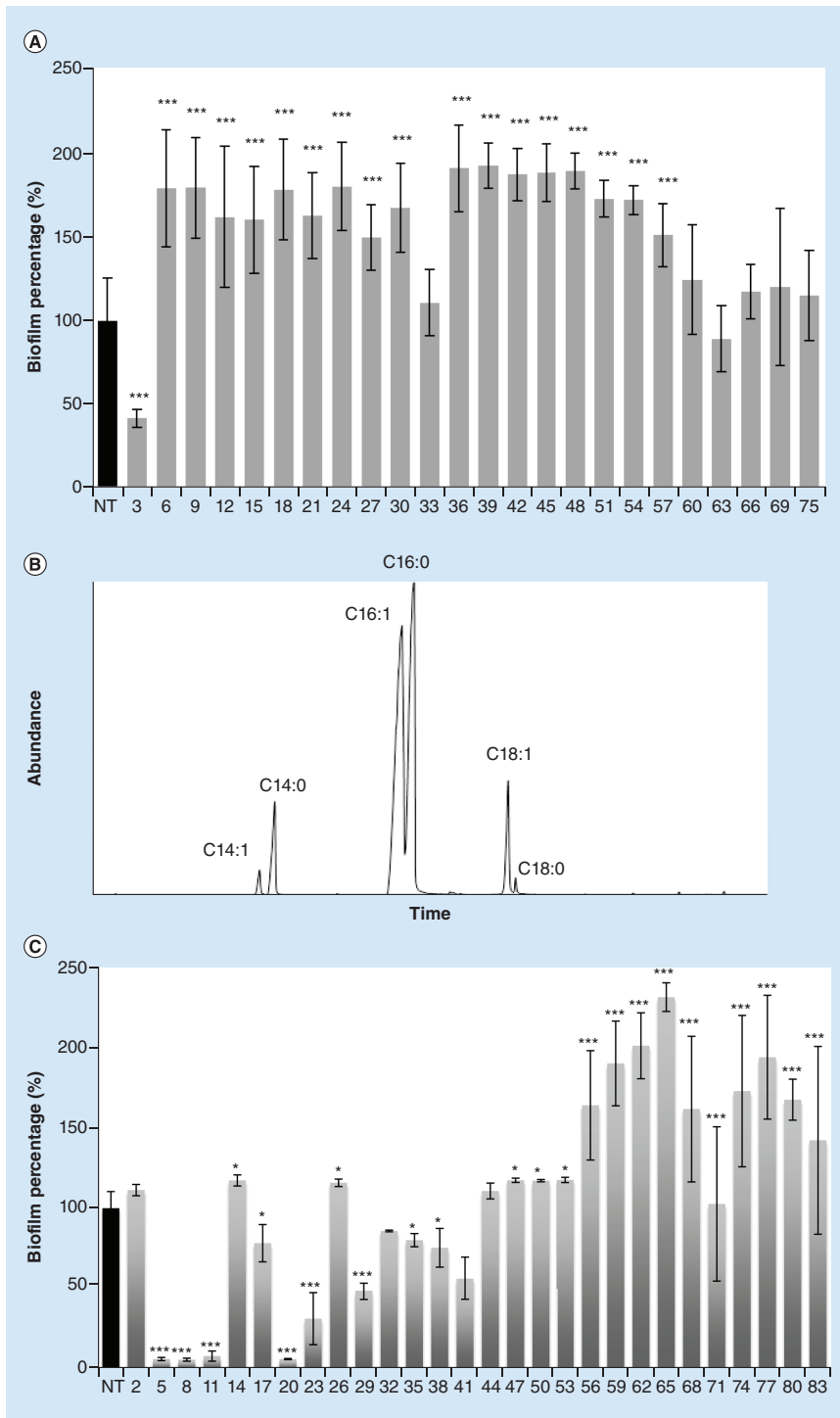
Figure 3.   Chromatographic fractions from IN_TAA207 (A) and IN_TAD1S (C) on biofilm formation of *S. aureus* 6538P.
Chromatographic profile of fraction 3 from IN_TAA207 **(B)**. Each fraction was used at a concentration of 250 μg/ml.
Data are reported as the percentage of residual biofilm. Each data point represents the mean ± SD of three different
samples. Biofilm formation was considered unaffected in the range of 90–100%. Differences in mean absorbance
were compared with the untreated control and considered significant when $p < 0.05$ according to Student's *t*-test.
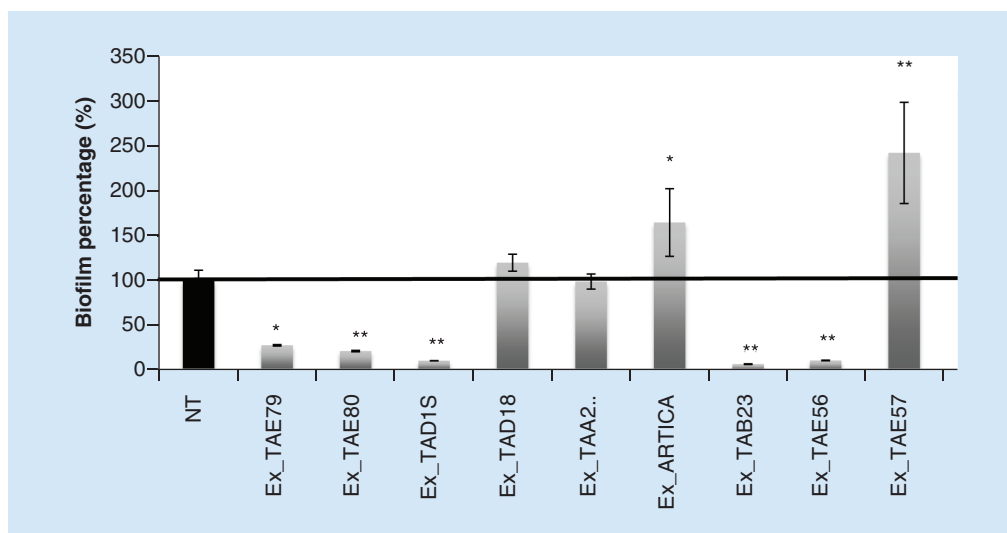*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
SD: Standard deviation.

Figure 4.   Extracellular extracts from cold-adapted bacteria on *S. aureus* 6538P biofilm formation. Data are reported as the percentage of residual biofilm after the treatment. Each data point represents the mean ± SD of five independent samples. Differences in mean absorbance were compared with the untreated control (NT, black bar) and considered significant when p < 0.05 according to Student's *t*-test.
*p < 0.05; **p < 0.01; ***p < 0.001.
NT: Not-treated; SD: Standard deviation.

IN_TAA207 and IN_TAD1S composition profiles revealed the presence in IN_TAD1S extract of fatty acids with an odd number of carbon atoms (i.e., C17:1; Supplementary Figure 1).

Screening of antibiofilm activity of extracellular organic extracts against *S. aureus*

Figure 4 shows the results obtained on *S. aureus* 6538P biofilm formation in the presence of different extracellular extracts from polar bacteria. Several extracts induced a strong reduction in biofilm formation by *S. aureus* 6538P. EX_TAE56 and EX_TAE57 were tested at a concentration of 0.5 mg/ml (1/2 MIC value), considering their antimicrobial activity reported in Table 2. Active extracts were selected for further analysis and tested on biofilm formation of three different *S. aureus* strains (65385P, 20372 and 25923). Furthermore, an assessment of the activity at different concentrations suggested a dose-dependent effect (Figure 5). Best inhibition in biofilm formation was obtained using EX_TAD1S on all tested strains (Figure 5C), indeed the percentage of residual biofilm was lower than 40%, even at a lower concentration of the extract (0.5 mg/ml). For this reason, EX_TAD1S was chosen for further analysis.

Fractionation of extracellular extract from *Pseudomonas* sp. TAD1S

EX_TAD1S was analyzed by ^{1}H NMR. This latter suggests the presence of a complex mixture of compounds (data not shown). To isolate the active molecule/s from EX_TAD1S, a preliminary purification step on a silica gel column was carried out, as reported in the Materials & methods section. The obtained fractions were tested to evaluate the antibiofilm activity on *S. aureus* (Figure 6A). The results clearly indicated the presence of the active molecule/s in fractions 15 and 17 (eluted with chloroform/methanol 8:2). The ^{1}H NMR spectra showed a profile almost identical for these two fractions. The analysis of the spectrum of fraction 15, reported in Figure 6B, suggested the presence of hydrocarbon chains, as indicated by the signals in the range of δ 0.5–1.5 ppm. In addition, signals appearing between δ 6.4 and 8.8 ppm strongly supported N–H protons (Figure 6B) that together with signals around δ 4 ppm, indicated a peptide backbone. All together, these signals indicated the lipopeptide nature of the active compound [30–32]. The GC–MS analysis of products obtained after the sample methanolysis followed by acetylation revealed the presence of the following amino acids: serine, valine, leucine and glutamic acid. This aminoacidic composition suggests a structure like those usually found for lipopeptides isolated from *Bacillus* and *Pseudomonas* species [33]. In addition, GC–MS analysis suggested that the lipopeptide was constituted by various isoforms, due to the difference in the length of the fatty acids, since it revealed a mixture of C15:0, C16:0, C16:1, C17:0, C18:0 and C18:1. Finally, the absence in the GC–MS chromatogram of peaks attributable to β-hydroxylated fatty acids
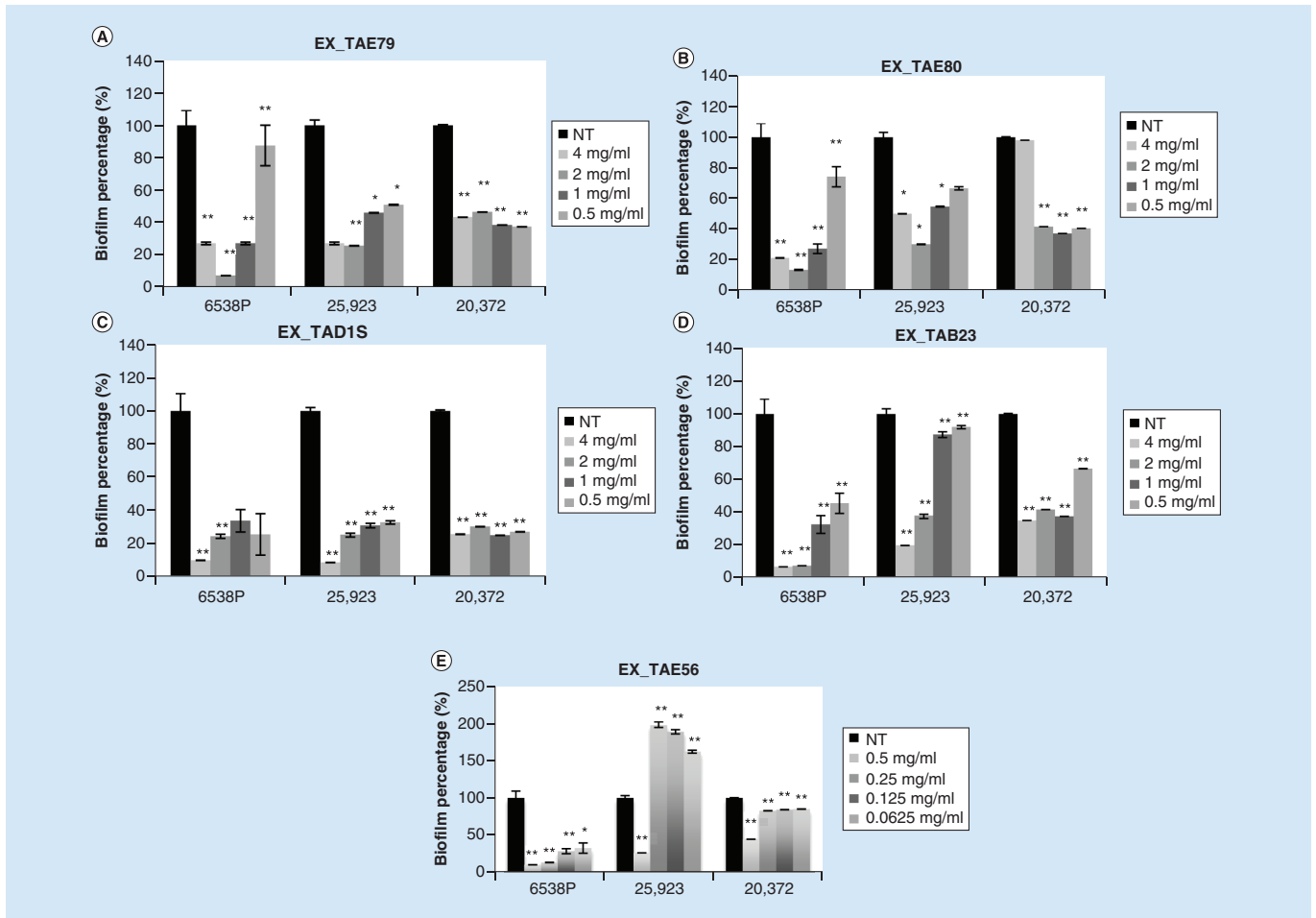
Figure 5.   Selected extracellular extracts on biofilm formation of different *S. aureus* strains. Biofilm formation was evaluated after incubation of serial dilutions of extracts starting from 4 mg/ml (EX_TAE56 was tested starting from $\frac{1}{2}$ MIC value corresponding to 1 mg/ml). Data are reported as the percentage of residual biofilm. Each data point represents the mean $\pm$ SD of three independent samples. Differences in mean absorbance were compared with the untreated control (NT, black bar) and considered significant when $p < 0.05$ according to Student *t*-test.
*p < 0.05; **p < 0.01; ***p < 0.001.
NT: Not-treated; SD: Standard deviation.

suggested a cyclic structure with a terminal amide-linked fatty acid [34]. We also evaluated the antimicrobial activity of this fraction on *S. aureus* 6538P that was found at a concentration of 400 μg/ml.

Discussion

The investigation of new unexplored habitats and uncommon environments has become an important source for the discovery of novel bacterial metabolites with antimicrobial activity [35]. Since the antimicrobial activity of microorganisms living in extreme environments has not been explored as widely as for mesophilic microorganisms, the traditional approach of isolating and cultivating new microorganisms from underexplored habitats can be productive.

In this paper, polar marine bacteria belonging to *Pseudoalteromonas, Pseudomonas* and *Psychromonas* genera were grown in a synthetic medium and organic extracts of intracellular and from extracellular contents were analyzed to identify novel antibiofilm/antimicrobial molecules. The MIC evaluation against *S. aureus* 6538P revealed that the samples did not possess a relevant antimicrobial activity, while some of them strongly prevented biofilm formation by *S. aureus* 6538P.

IN_TAA207 and IN_TAD1S revealed a similar antibiofilm activity but were both selected because a preliminary analysis indicated that their chemical composition was different (data not shown). The samples were tested against
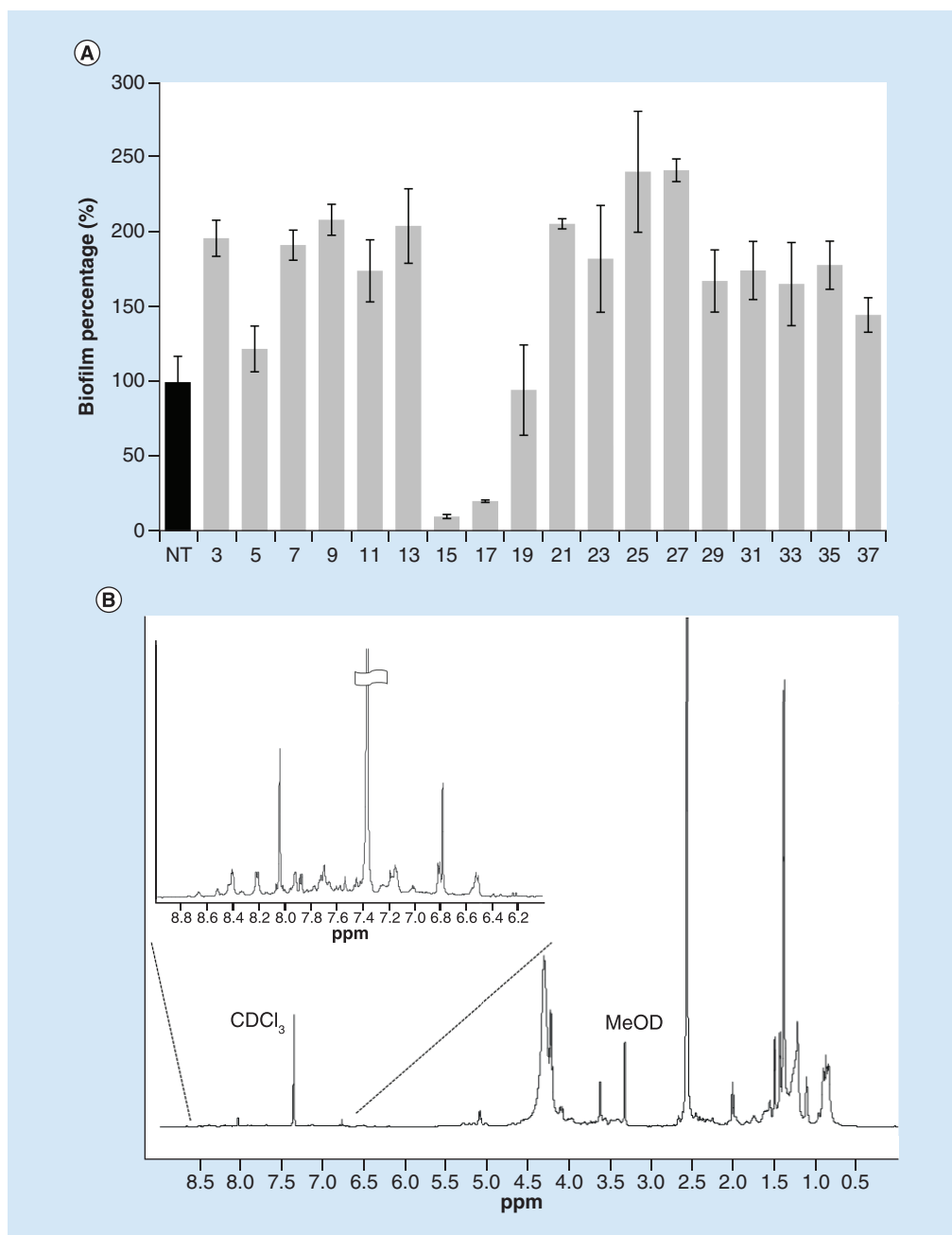
Figure 6.   Chromatographic fractions from EX_TAD1S against *S. aureus* 6538P (A) and ¹H NMR spectrum of the active fraction (B). Each fraction was used at a concentration of 250 μg/ml. Data are reported as the percentage of residual biofilm. Each data point represents the mean ± SD of three different samples. Biofilm formation was considered unaffected in the range of 90–100%. Differences in mean absorbance were compared with the untreated control and considered significant when $p < 0.05$ according to Student's *t*-test. **(B)** ¹H NMR spectrum of the active fraction 15 from EX_TAD1S. Spectrum was recorded in $CDCl_3:CD_3OD$ at 600 MHz and 298 K.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
SD: Standard deviation.

three different *S. aureus* strains: ATCC 6538P and ATCC 25923 are both reference strains for antimicrobial testing, while ATCC 20372 is a clinical isolate. These strains show a different ability to form biofilm: ATCC 25923 is classified as a strong biofilm producer, ATCC 6538P is a medium/strong biofilm producer and ATCC 20372 is considered a medium/weak biofilm producer [36]. IN_TAA207 and IN_TAD1S were both able to inhibit biofilm formation of all tested *S. aureus* strains, and the effect of IN_TAA207 was clearly dose dependent, whereas

the activity of IN_TAD1S was independent of the extract concentration. The different chemical composition of IN_TAA207 and IN_TAD1S extracts and the different features of the three tested *S. aureus* strains could explain the reported results. Both extracts were fractionated, and the resulting fractions were tested to assess their antibiofilm activity. The chromatographic fraction 3, obtained from IN_TAA207 extract, and chromatographic fractions 5, 8, 11 and 20 from IN_TAD1S extract showed the best antibiofilm activity. Moreover, all fractions from IN_TAD1S with antibiofilm action showed antimicrobial activity. All these fractions contained saturated and unsaturated long-chain fatty acids. Free fatty acids are ubiquitous on the surface of human skin and represent the predominant components in human sebum [37]. Particularly, medium- to long-chain fatty acids (C8 to C18) display antibacterial activity against a broad range of Gram-positive bacteria and are considered responsible for at least part of the direct antimicrobial activity of the skin surface against pathogen colonization and infection [38]. The antibacterial efficacy of fatty acids stems from their membrane-destabilizing activity that causes increased cell permeability and cell lysis.

Some of the identified fatty acids were common to different active fractions; in detail, either fraction 3 from IN_TAA207 and fractions 5, 8, 11 from IN_TAD1S contained C16:1 (palmitoleic), C16:0 and C18:1 (oleic acid). For these fatty acids, the antibacterial activity against *S. aureus* was previously observed [39–41]. Two odd long fatty acids, C17:1 and C19:1, were identified in fractions 5, 8, 11 and 20 from IN_TAD1S. Although several bacteria produce odd-numbered fatty acid [42,43], to the best of our knowledge, no information about their possible antimicrobial activity against *S. aureus* was previously recorded. Future studies will be aimed to clarify if these molecules contribute to the antimicrobial activity of IN_TAD1S fractions and if they can be considered responsible for the differences in the activity of IN_TAD1S and IN_TAA207 extracts. Anyway, the reported antibiofilm activity of IN_TAD1S and IN_TAA207 was found at sub-MIC concentrations.

The screening of polar extracellular extracts revealed that EX_TAE79, EX_TAE80 EX_TAB23, EX_TAD1S and EX_TAE56 were able to affect *S. aureus* biofilm formation. The attention was focused on EX_TAD1S since this sample efficiently impairs biofilm formation in all three tested strains, with a percentage of residual biofilm lower than 40% as compared with the untreated sample. Also, in this case, the extract was fractionated, and the corresponding fractions were analyzed. Fractions 15 and 17 displayed a high level of activity against *S. aureus* biofilm (Figure 6A). The ^{1}H NMR spectrum of fraction 15 (Figure 6B) indicated the presence of N-H (6.4–8.6 ppm) and C-H (4.5–5.5 ppm) signals that, together with signals of a long aliphatic chain (0.5–1.5 ppm) suggested the lipopeptide nature of the active compound [30–32]. The GC–MS analysis revealed the presence of serine, leucine, valine and glutamic acid, an amino acid composition agreed with the compositions obtained for other lipopeptides [30,31], moreover this analysis suggests a cyclic structure of this lipopeptide.

Lipopeptides are produced by several bacterial species; they are amphiphilic molecules consisting of short linear chains or cyclic structures of amino acids, linked to a fatty acid via ester or amide bonds or both. They are proved to act as antibiotics, antiadhesives, antitumor compounds and foaming agents [44–48]. Very few examples of lipopeptides isolated from cold-adapted bacteria were reported. *Pseudomonas fluorescens* BD5, a bacterium isolated from Archipelago of Svalbard, produces a cyclic lipopeptide named pseudofactin [49] endowed with a very good emulsification activity and with antiadhesive activity against several pathogenic microorganisms (*Escherichia coli*, *Enterococcus faecalis*, *Enterococcus hirae*, *S. epidermidis*, *Proteus mirabilis* and *Candida albicans*) [49]. Another cold-adapted bacterium able to produce lipopeptides is *Bacillus amyloliquefaciens* Pc3 that was isolated from Antarctic seawater [50].

Although in the literature few publications describe lipopeptides from cold-adapted bacteria, many reports describe the ability of lipopeptides to kill pathogenic bacteria. Interestingly, several lipopeptides are active against *S. aureus*. Some examples are Daptomycin, a cyclic lipopeptide, that was approved as an antibiotic by the US FDA in 2003 for the treatment of complicated skin [51]; brevibacillin, a lipopeptide produced by a strain of *Brevibacillus laterosporus* showing antimicrobial activity against *S. aureus* [52].

Conclusion

The cyclic lipopeptide isolated from *Pseudomonas* sp. TAD1S does not display antimicrobial activity on *S. aureus* (MIC resulted to be 400 µg/ml) but it resulted to be an effective antibiofilm molecule able to strongly reduce the *S. aureus* biofilm formation. Furthermore, preliminary results indicate that it also works as a biosurfactant (Supplementary Figure 2).

Future perspective

The new cyclic lipopeptide isolated from Antarctic bacterium *Pseudomonas* sp. TAD1S may be potentially useful in a wide variety of biotechnological and medical applications. Further studies are needed to better define its chemical structure and to explore its biological activity and applicability as an anti-infective agent.

Summary points

Among bacteria, staphylococci are recognized as the most frequent causes of biofilm-associated infections
- *Staphylococcus aureus* is often present asymptomatically on districts of the human body.
- *S. aureus* possesses a collection of virulence factors and displays the ability to acquire resistance to most antibiotics.
- The antibiotic resistance of *S. aureus* is related, in most cases, to its ability to develop a protective architecture called biofilm.

Unexplored habitats & uncommon environments can be considered an important source for the discovery of novel bacterial metabolites with antimicrobial activity
- Marine microorganisms are considered potential sources for the discovery of new metabolically active molecules.
- Some of these bioactive molecules have already been selected for the treatment of various diseases and many of them are under clinical investigations.
- Cold-adapted marine bacteria deriving from polar regions represent an untapped reservoir of biodiversity endowed with an interesting chemical repertoire.
- Intracellular and extracellular organic extracts obtained from cultures of polar marine bacteria were tested against *S. aureus*.

Conclusion
- Identification of a lipopeptide able to strongly reduce the *S. aureus* biofilm formation with antimicrobial activity at high concentration.
- The new cyclic lipopeptide isolated from Antarctic bacterium *Pseudomonas* sp. TAD1S may be potentially useful in a wide variety of biotechnological and medical applications.
- Our findings indicate that the lipopeptide also works as a biosurfactant.

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