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Antimicrobial activity of *Pseudoalteromonas* strains isolated from the Ross Sea (Antarctica) versus Cystic Fibrosis opportunistic pathogens

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Abstract In Antarctica, the selective pressure may have led to the evolution of novel capabilities by indigenous organisms, including microorganisms, to achieve competitive advantages. In this work, the ability of thirteen Antarctic *Pseudoalteromonas* isolates from different sources (sponges, seawater and sediments) to synthesize antimicrobial compounds was analysed. The antibacterial activity was tested against Cystic Fibrosis opportunistic pathogens belonging to the *Burkholderia cepacia* complex (Bcc). Data obtained revealed that all the *Pseudoalteromonas* strains synthesize a plethora of microbial volatile organic compounds (mVOCs) and diffusible molecules that strongly interfere with the growth of Bcc bacteria and that this synthesis may be influenced by the growth media essentially in terms of amount of each mVOC. The finding that mVOCs profiles can be obtained from bacteria belonging to very different taxa strongly suggests that the synthesis of such compounds might have a great relevance from an evolutionary and/or ecological viewpoint. Since these mVOCs are able to completely inhibit the growth of Bcc bacteria, thus exhibiting an antibacterial activity, it is possible that such compounds might represent one of the forces driving the structuring of bacterial communities inhabiting the same ecological niche.

Keywords Antarctic strains · *Pseudoalteromonas* · *Burkholderia cepacia* complex · mVOCs · Cystic Fibrosis

Isabel Maida and Emanuele Bosi contributed equally to this study.

This manuscript is dedicated to Luigi Michaud.

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Introduction

The Antarctic continent is among the coldest and most hostile areas of Earth. Selective pressure in such harsh environment may have led to the evolution of novel capabilities by indigenous organisms, including microorganisms, to achieve competitive advantages. The microbial community is likely to contain unusual and phylogenetically divergent microorganisms with unique adaptations to their habitats (Vincent, 2000). In addition to cellular modifications, antagonistic features may contribute to the adaptation of Antarctic bacteria to permanently low temperatures by reducing the presence of competitive microorganisms (Lo Giudice et al., 2007a). Previous systematic analyses carried out on marine Antarctic isolates (from seawater and sponges) highlighted the existence of a complex net of inter-specific antagonistic interactions among bacteria colonising the same habitat (Lo Giudice et al., 2007a; Mangano et al., 2009) and likely acting as an effective controller of microbial populations inhabiting the same ecological niche. These results were highly encouraging to further explore the ability of cold-adapted marine Antarctic bacteria to produce novel, and still unexploited secondary metabolites that might act as antibiotics, which in turn, may interfere with the growth of other bacteria inhabiting the same niche.

It is a public perception that the observed upsurge of pathogen resistance to all available antibiotics needs to be counteracted by the discovery of novel effective molecules. Among the clinically used antibiotics, over two-thirds have been discovered from natural sources or are the semi-synthetic derivatives of natural antibiotics (Newman & Cragg, 2004; Lam, 2007; Newman & Cragg, 2012). Up to now, the dominant effort to discover new natural antibiotics has involved the terrestrial environments, whereas relatively little attention has been paid to other habitats. For example, marine bacteria have attracted the attention of researchers because of their ability to produce bioactive molecules as secondary metabolite compounds that are able to inhibit the growth of many bacteria and have a wide range of pharmaceutical and biotechnological applications (Bull & Stach, 2007; Blunt et al., 2008). Recently, the inhibitory power of bacteria isolated from Antarctic sponges, water and sediment was demonstrated (Rojas et al., 2009; Papaleo et al., 2012, 2013; Maida et al., 2014), and among these bacteria, the genus *Pseudoalteromonas* has been shown to be a producer of antimicrobial compounds (Bowman, 2007). Representatives of this genus are widespread in marine environment and were also isolated from Polar regions, either from water samples or associated to biotic surfaces (Lo Giudice et al., 2012; Papaleo et al., 2012). Probably, the production of molecules active against different bacteria confers some advantages to *Pseudoalteromonas* members in the competition for nutrients and gives them the opportunity to persist on marine surfaces (Yu et al., 2013). In this respect, the *Pseudoalteromonas*, Actinobacteria and α-Proteobacteria strains isolated from sponges and endowed with antimicrobial activity are particularly abundant (Thomas et al., 2010; Papaleo et al., 2013). They also displayed inhibition properties against terrestrial microorganisms, including some human pathogens, such as those belonging to the *Burkholderia cepacia* complex (Bcc) (Ireland et al., 2000; Lo Giudice et al., 2007b; Rojas et al., 2009), which is a heterogeneous group of bacteria, occupying different ecological niches, such as soil, rhizosphere and/or water. Besides, members of Bcc interact with eukaryotic organisms, including humans, and are able to infect immune-compromised patients, such as those affected by Cystic Fibrosis (CF) (Mahenthiralingam et al., 2008). Infections by Bcc are particularly recalcitrant to antibiotic treatment, also due to the presence of several multidrug efflux pumps, which makes these strains multi-drug-resistant bacteria (MDR) (Perrin et al., 2010, 2013). This indicates the need for more research into the discovery and rational design of new and more efficient antibacterial drugs in fighting Bcc infections in CF patients. Quite interestingly, Bcc strains appeared to be specifically inhibited by Antarctic bacteria belonging to different genera like *Gillisia*, *Psychrobacter* and especially *Pseudoalteromonas* (Papaleo et al., 2012, 2013; Maida et al., 2014). The inhibitory activity very likely relies also in the production of microbial Volatile Organic Compounds (mVOCs) (Romoli et al., 2011, 2014), chemical signals that can be involved in the volatile-based interactions between individuals of the same/different species. VOCs produced by a given bacterial

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species can have multiple effects on other microbes and organisms and can be used for defence, environmental monitoring and nutrient acquisition, thus assuming a crucial importance in VOC-mediated cross-talk between species (Bennet et al., 2012). Head space solid-phase micro extraction coupled with gas chromatography–mass spectrometry (HS–SPME–GC–MS) analysis performed under aerobic conditions revealed that some of these bacteria, belonging to the genera *Pseudoalteromonas* and *Psychrobacter*, synthesize a mixture of no less than 30 different compounds that might be responsible for the inhibition of the growth of Bcc bacteria (Romoli et al., 2011, 2014). It is known that the mVOCs qualitative and quantitative composition might depend on the growth conditions of microorganisms, especially in terms of temperature, oxygen availability, pH, carbon sources availability and growth phase. Furthermore, it seems that the mVOCs spectra are species specific (Kai et al., 2007; Lemfack et al., 2014). However, it was not clear whether Antarctic strains belonging to the same genus/species express a similar pattern of volatile and diffusible organic compounds, information that could be instrumental for identifying the genes and/or the metabolic pathways involved in their biosynthesis. Therefore, the aim of this work was to further investigate on the ability of a selection of 13 Antarctic *Pseudoalteromonas* strains from different sources (water column, sediment, sponge tissue) to produce mVOCs and diffusible molecules under different growth conditions in terms of antagonistic efficiency against Bcc bacteria.

Materials and methods

Bacterial strains and growth conditions

Bacterial strains used in this work are listed in Table 1. In particular, strains S8-8, S8-38, TB13, TB25, TB41, TB51, TB64 and AC163 belong to the Italian Collection of Antarctic Bacteria of the National Antarctic Museum (CIBAN-MNA). Antarctic *Pseudoalteromonas* strains were grown at 21°C for 4 days on three different media, which are usually used to grow Antarctic bacteria: TYP (containing per litre of distilled water: tryptone 16 g, yeast extract 16 g, sodium chloride 10 g and technical agar 16 g; OXOID), Plate Count Agar (PCA; containing per litre of distilled water: tryptone 5 g, yeast extract 2.5 g, glucose 1 g, sodium chloride 24 g and technical agar 16 g; OXOID) and Marine Agar (MA; containing per litre of distilled water: sodium chloride 19.4 g, magnesium chloride 8.8 g, bacteriological peptone 5 g, sodium sulphate 3.24 g, calcium chloride 1.8 g, yeast extract 1 g, potassium chloride 0.55 g, sodium bicarbonate 0.16 g, ferric citrate 0.1 g, potassium bromide 0.08 g, strontium chloride 0.034 g, boric acid 0.022 g, disodium phosphate 0.008 g, sodium silicate 0.004 g, sodium fluoride 0.0024 g, ammonium nitrate 0.0016 g, bacteriological agar 15 g; CONDA Pronadisa).

Inhibitory activity

Antibacterial activity was determined using the cross-streak method. Tester and target strains were grown on different media without any physical contact using Petri dishes with a central septum separating two hemi-cycles (Papaleo et al., 2013), thus creating a physical separation of media on which tester and target strains were grown. Antarctic (tester) strains were pre-grown on MA for 4 days at 21°C, then streaked across one-half of an agar plate containing either PCA, TYP or MA and incubated at 21°C for 4 days (this time was enough for the Antarctic cells to growth and fill the hemi-cycle in which they were plated). The three media (PCA, TYP and MA) display a quite different composition; indeed, MA is the medium currently used for marine bacteria because of its abundance of different salts, whereas the PCA and TYP are two rich media differing in their composition. The experiments on PCA were also carried out using Petri dishes without the central septum in order to check whether the absence of a physical barrier might allow the flow of non-volatile antimicrobial compounds from the tester towards the target strains also. Bcc (target) strains were perpendicularly streaked to the initial streak and plates were further incubated at 21°C for 2 days and at 37°C for two additional days. The experiments were conducted in parallel with a positive control to verify the viability of Bcc cells.

Heatmap and cluster analysis

The results from the cross-streak inhibition assay were organized in the form of an *inhibition matrix*. In this matrix, each row represents an Antarctic tester strain grown in a given medium, while each column stands for a Bcc target strain. Hence, the *ij*-th entry of the inhibition matrix corresponds to the inhibition

Table 1 List of bacterial strains used in this work

Strain	Species	Origin	Strain	Species	Origin	
FCF 1	*B. cepacia*	CF	MCI 7	*B. ambifaria*	Env	
FCF 3	*B. cepacia*	CF	LMG 19467	*B. ambifaria*	CF	
LMG 17588	*B. multivorans*	Env	LMG 19182	*B. ambifaria*	Env	
FCF 16	*B. cenocepacia* (III A)	CF	LMG 16670	*B. anthina*	Env	
J2315	*B. cenocepacia* (III A)	CF	FCF 43	*B. pyrrocinia*	CF	
FCF 18	*B. cenocepacia* (III B)	CF	LSED 4	*B. lata*	CF	
FCF 20	*B. cenocepacia* (III B)	CF	LMG 24064	*B. latens*	CF	
FCF 23	*B. cenocepacia* (III B)	CF	LMG 24065	*B. diffusa*	CF	
FCF 24	*B. cenocepacia* (III B)	CF	LMG 23361	*B. contaminans*	AI	
FCF 27	*B. cenocepacia* (III B)	CF	LMG 24067	*B. seminalis*	CF	
FCF 29	*B. cenocepacia* (III B)	CF	LMG 24068	*B. metallica*	CF	
FCF 30	*B. cenocepacia* (III B)	CF	LMG 24066	*B. arboris*	Env	
LMG 16654	*B. cenocepacia* (III B)	CF	LMG 24263	*B. ubonensis*	NI	
C5424	*B. cenocepacia* (III B)	CF	S8-8	*Pseudoalteromonas* sp.	Sediments	Ross Sea
CEP 511	*B. cenocepacia* (III B)	CF	S8-38			
MVPC 1/16	*B. cenocepacia* (III B)	Env	TB41		Sponge *A. joubini*	
MVPC 1/73	*B. cenocepacia* (III B)	Env	TB51			
LMG 19230	*B. cenocepacia* (III C)	Env	TB64			
LMG 19240	*B. cenocepacia* (III C)	Env	TB13		Sponge *L. nobilis*	
FCF 38	*B. cenocepacia* (III D)	CF	TB25			
LMG 21462	*B. cenocepacia* (III D)	CF	AC163		Sponge *H. verrucosa*	
FCF 41	*B. stabilis*	CF	TAB23		Water column	
FCF 42	*B. vietnamiensis*	CF	TAE56			
TVV 75	*B. vietnamiensis*	Env	TAE79			
LMG 18941	*B. dolosa*	CF	TAE80			
LMG 18942	*B. dolosa*	CF	TAC125	*P. haloplanktis*		
LMG 18943	*B. dolosa*	CF				

CF strain isolated from Cystic Fibrosis patient, *Env* environmental strain, *AI* animal infection, *NI* nosocomial infection

mediated by a given tester strain, grown in a specific medium (*i*-th row), against the target strain corresponding to the *j*-th column.

The inhibition values reflect four different inhibition levels (ranging from 0 to 3) observed during the cross-streak experiments (complete, strong, weak and absence of inhibition). For computing Euclidean distance between column and row vectors, the levels were treated as numeric integer values (in order: 3, 2, 1, 0).

The inhibition matrix was graphically represented as a heatmap with a colour key code indicating the different inhibition levels. The heatmap columns were clustered by computing the Euclidean distance and applying the complete-linkage hierarchical clustering algorithm implemented in R (Murtagh, 1985).

To measure the inhibitory power of a tester strain in a given growth medium an inhibition score was computed for each strain as the sum of the entries of each row (numeric integer values reflecting the inhibitory efficiency) from the inhibition matrix. This score can also be computed for the target strain, to measure their sensibility, by summing up those entries of the columns corresponding to a single growth medium.

To test the possible effect of the tester/target strain origins on the inhibition patterns, a non-parametric multivariate analysis, namely the permutational MANOVA (Anderson, 2001), was carried out using the implementation provided by the R package vegan (Oksanen et al., 2013).

Target strain			Growth				C^-
Name	Species	Origin	MA	PCA	TYP	C^+	
FCF 1	*B. cepacia* (I)	CF	−	−	−	−	+
FCF 3	*B. cepacia* (I)	CF	−	−	−	−	+
LMG 17588	*B. multivorans* (II)	Env	−	−	−	−	+
FCF 16	*B. cenocepacia* (III A)	CF	−	−	−	−	+
J2315	*B. cenocepacia* (III A)	CF	−	−	−	−	+
FCF 18	*B. cenocepacia* (III B)	CF	−	−	−	−	+
FCF 20	*B. cenocepacia* (III B)	CF	−	−	−	−	+
FCF 23	*B. cenocepacia* (III B)	CF	−	−	−	−	+
FCF 24	*B. cenocepacia* (III B)	CF	−	−	−	−	+
FCF 27	*B. cenocepacia* (III B)	CF	−	−	−	−	+
FCF 29	*B. cenocepacia* (III B)	CF	−	−	−	−	+
FCF 30	*B. cenocepacia* (III B)	CF	−	−	−	−	+
LMG 16654	*B. cenocepacia* (III B)	CF	−	−	−	−	+
C5424	*B. cenocepacia* (III B)	CF	−	−	−	−	+
CEP 511	*B. cenocepacia* (III B)	CF	−	−	−	−	+
MVPC 1/16	*B. cenocepacia* (III B)	Env	−	−	−	−	+
MVPC 1/73	*B. cenocepacia* (III B)	Env	+	±	−	−	+
LMG 19230	*B. cenocepacia* (III C)	Env	−	−	−	−	+
LMG 19240	*B. cenocepacia* (III C)	Env	−	−	−	−	+
FCF 38	*B. cenocepacia* (III D)	CF	−	−	−	−	+
LMG 21462	*B. cenocepacia* (III D)	CF	+	+	+	−	+
FCF 41	*B. stabilis* (IV)	CF	±	+	±	−	+
FCF 42	*B. vietnamiensis* (V)	CF	−	−	−	−	+
TVV 75	*B. vietnamiensis* (V)	Env	−	−	−	−	+
LMG 18941	*B. dolosa* (VI)	CF	−	−	−	−	+
LMG 18942	*B. dolosa* (VI)	CF	−	−	−	−	+
LMG 18943	*B. dolosa* (VI)	CF	±	−	−	−	+
MCI 7	*B. ambifaria* (VII)	Env	−	−	−	−	+
LMG 19467	*B. ambifaria* (VII)	CF	−	−	−	−	+
LMG 19182	*B. ambifaria* (VII)	Env	−	−	−	−	+
LMG 16670	*B. anthina* (VIII)	Env	−	−	−	−	+
FCF 43	*B. pyrrocinia* (IX)	CF	−	−	+	−	+
LSED 4	*B. lata*	CF	−	−	−	−	+
LMG 24064	*B. latens*	CF	−	−	−	−	+
LMG 24065	*B. diffusa*	CF	±	−	−	−	+
LMG 23361	*B. contaminans*	AI	±	−	−	−	+
LMG 24067	*B. seminalis*	CF	−	−	−	−	+
LMG 24068	*B. metallica*	CF	+	±	−	−	+
LMG 24066	*B. arboris*	Env	−	−	−	−	+
LMG 24263	*B. ubonensis*	NI	−	−	−	−	+

Bcc bacteria were streaked onto PCA medium

CF strain isolated from Cystic Fibrosis patient, *Env* environmental strain, *AI* animal infection, *NI* nosocomial infection, C^+ Petri dishes without septum, C^- Bcc strain grown in the absence of tester strain

+ Growth, − no growth, ± reduced growth

Results

Inhibition of *Burkholderia cepacia* complex strains growth by Antarctic *Pseudoalteromonas* isolates as a result of mVOCs production

The influence of the growth medium on the ability of thirteen *Pseudoalteromonas* strains to produce different mVOCs was tested by looking at their capacity to inhibit the growth of the 40 Bcc strains listed in Table 1 by cross-streak experiments carried out using Petri dishes with a central septum. *Pseudoalteromonas* strains were grown on PCA, MA or TYP; the Bcc (target) strains were grown on PCA medium. Simultaneously, an experiment was carried out growing both tester and target strains in Petri dishes containing PCA medium without the septum.

Data obtained concerning the entire panel of Antarctic strains are included in Supplementary Material 1. The results for the *Pseudoalteromonas* strain TB64 are reported in Table 2 and Fig. 1 whose analysis revealed that this strain exhibited a different pattern of Bcc inhibition depending on the growth medium. Indeed, even though the growth of the large majority of Bcc strains was inhibited by the presence of TB64, some of them were inhibited only when strain TB64 was grown in a given medium. The discovery that Bcc strains were inhibited in the presence of a physical barrier between the two growth media suggested that the antimicrobial ability exhibited by strain TB64 was due to the synthesis of mVOCs. Also, the finding that, in the absence of the septum, the entire panel of Bcc strains was completely inhibited strongly suggests the presence of non-volatile compounds playing an inhibitory role.

In order to gain a deeper view of the entire set of results obtained, data from cross-streak inhibition assays have been organized in the form of a matrix that in turn can be represented as a heatmap (Fig. 2). In this representation, each of the 13 rows corresponds to a *Pseudoalteromonas* strain, whereas the 160 columns represent the degree of growth for each of the 40 Bcc strains in the four different growth conditions (see above). The analysis of data reported in Fig. 2 revealed that

i. All the *Pseudoalteromonas* Antarctic strains were able to inhibit the growth of Bcc members in the presence of a physical barrier.
ii. In some cases different Antarctic strains (see for instance strains TAC125 and TAE80) exhibited a different inhibitory pattern suggesting that they might synthesize different antimicrobial compounds and/or different quantities of the same compound(s).
iii. The highest and the lowest degree of inhibition was detected when *Pseudoalteromonas* strains were grown in TYP and MA, respectively.
iv. Concerning the experiments performed using the PCA medium in Petri dishes with and without a septum, data obtained revealed an increased number of inhibited Bcc strains in the absence of the septum.
v. Hierarchical clustering of Antarctic strains on the basis of their relative ability to inhibit the growth of Bcc bacteria revealed that there is no correlation with the phylogenetic relationships existing among them. Indeed, the branching order of the phylogenetic tree constructed using a concatenated amino acid sequence of 2,128 proteins of the *core* genome of the 13 *Pseudoalteromonas* strains (Bosi, personal communication) displays a topology quite different from that of the dendrogram in Fig. 1. The same analysis also showed that there is no apparent correlation between the inhibitory efficiency and the source of each strain. This was tested using a non-parametrical statistical test (see "Materials and methods" section) under the hypothesis that the variance of the inhibition matrix could be explained by the isolation site of the tester strain (water column, sediments, sponges *A. joubini*, *L. nobilis*, *H. verrucosa*), which proved to be non-significant ($P = 0.19$).

Data from cross-streaking experiments were also arranged in a different heatmap, where the diverse sensitivity of each Bcc strain towards the "Antarctic" antimicrobials is highlighted (Fig. 3). The analysis of Fig. 3 revealed that the 40 Bcc strains exhibited a wide range of sensitivity to antimicrobial compounds synthesized by *Pseudoalteromonas* strains, with some members inhibited by the vast majority of Antarctic strains, independently from the growth medium used (see for instance *B. anthina* LMG16670) and other members much less susceptible to the antagonistic

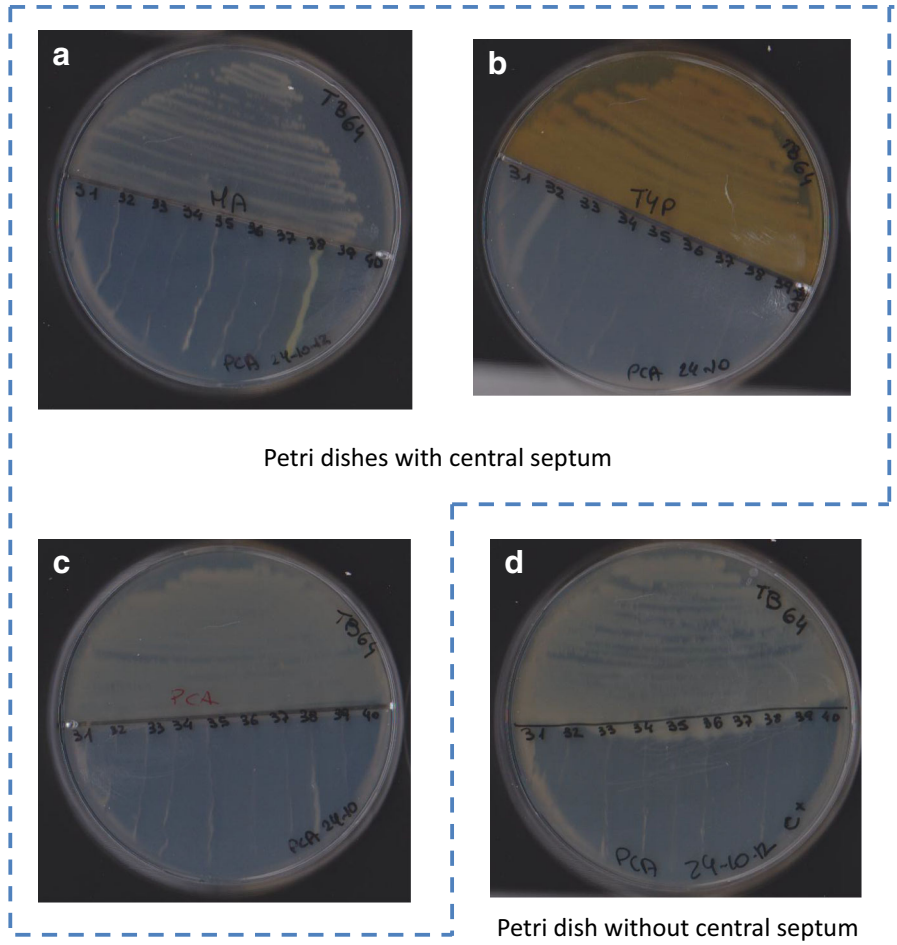

Fig. 1 Example of results obtained from the cross-streak experiment performed on the *Pseudoalteromonas* sp. TB64 strain. The different used media for the tester strains showed in the different sections are in **a** MA, in **b** TYP, in **c** and **d** PCA. The target strains are 31: *B. anthina*; LMG 16670; 32: *B. pyrrocinia* FCF 43; 33: *B. lata* LSED 4; 34: *B. latens* LMG 24064; 35: *B. diffusa* LMG 24065; 36: *B. contaminans* LMG 23361; 37: *B. seminalis* LMG 24067; 38: *B. metallica* LMG 24068; 39: *B. arboris* LMG 24066; 40: *B. ubonensis* LMG 24263

action of *Pseudoalteromonas* (i.e. *B. metallica* LMG24068).

Differential inhibitory activity of *Pseudoalteromonas* strains and sensitivity of Bcc strains to *Pseudoalteromonas*

We further analysed the differential inhibitory activity of *Pseudoalteromonas* strains by calculating the inhibition scores for each strain.

Data obtained (Fig. 4) revealed that most of *Pseudoalteromonas* strains exhibited very similar antagonistic activity versus Bcc members, except for strains TAE80 and TAE56 that showed a reduced ability to inhibit the growth of Bcc bacteria. This was mainly attributed to the growth of these strains on MA and PCA in Petri dishes with a septum. When grown on TYP or PCA in Petri dishes without a septum, the inhibitory activity was comparable to that of the other *Pseudoalteromonas* strains.

Overall, the highest degree of antagonistic effect versus Bcc members was obtained when Antarctic strains were grown either on TYP or PCA without a septum.

The different sensitivity of Bcc strains to antimicrobial compounds synthesized by Antarctic *Pseudoalteromonas* spp. was analysed by computing the inhibition score of each strain.

Data obtained (Fig. 5) revealed that all Bcc strains tested were inhibited by mVOCs produced by *Pseudoalteromonas* strains at a different extent. The most resistant strain is *B. metallica* LMG24068. Overall, data from Fig. 5 suggested that the highest sensitivity to the presence of *Pseudoalteromonas* was exhibited when Antarctic bacteria were grown on TYP or PCA (without septum). The sensitivity to antimicrobial compounds of *Burkholderia* strains was

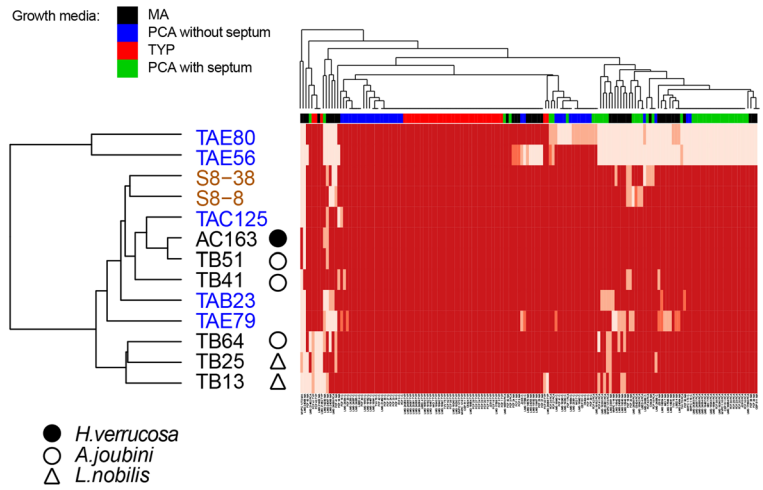

Fig. 2 Heatmap showing the inhibitory pattern of *Pseudoalteromonas* Antarctic strains grown on different media versus Bcc bacteria and clustering of *Pseudoalteromonas* strains on the basis of similarity of their inhibitory patterns. *Pseudoalteromonas* strains in *brown*, *light blue* and *black* were isolated from sediments, seawater and sponges, respectively

tested in relation to their origin (clinical or environmental) and taxonomical position, that is, if strains belonging to a same species have a similar response to the antimicrobial compounds. These two hypotheses were tested using the permutational MANOVA, which revealed that there was no significant correlation between the origin of the strains and inhibition pattern ($P = 0.17$), while a slightly significant relation between species and inhibition response ($P = 0.04$) was disclosed.

Influence of the Bcc growth media on the susceptibility of Bcc strains to "Antarctic" antimicrobials

To check the possibility that the sensitivity of Bcc strains to "Antarctic" drugs might also be affected by the growth medium of the target strains, we performed an additional experiment using two bacterial subsets in cross-streaking experiments: two out of 13 Antarctic (TB41 and TAC125) and seven out of 40 Bcc strains [representative of seven different species with either environmental (Env), animal infections (AI) or clinical origin (CF) [LMG18943 *B. dolosa* (CF), LMG19182 *B. ambifaria* (Env), LMG24064, *B. latens* (CF), LMG24065 *B. diffusa* (CF), LMG23361 *B. contaminans* (AI), LMG24067 *B. seminalis* (CF), LMG24068 *B. metallica* (CF)].

The resulting combination of different media was: PCA, TYP and MA for the tester and PCA or TYP for the target. Moreover, to establish if the possible inhibition is due to the production of mVOCs or to a combination of diffusible and volatile molecules, we used Petri dishes with or without a central septum with MA, TYP or PCA. Data obtained are shown in Table 3 and schematically represented in Fig. 6, whose analysis revealed that

i. The two Antarctic strains exhibited the same pattern of inhibition of Bcc strains growth.
ii. The growth of all the seven target strains was inhibited by both Antarctic strains when Petri dishes without the central septum were used, independently from the growth medium used.
iii. Different results were obtained when using the Petri dishes with a central septum. Indeed, the growth of the seven Bcc strains grown on PCA was completely inhibited by the two Antarctic strains (independently from the growth medium used for the latter). However, when the target strains were streaked on TYP, their growth was inhibited only when the tester strains were grown in TYP.

Discussion

It is widely accepted that bacteria are able to emit an unexpectedly high number of microbial Volatile Organic Compounds (mVOCs). Metabolically speaking, mVOCs can be alternatively end-products of secondary metabolism or simply waste materials from other pathways (Kai et al., 2007; Lemfack et al., 2014). In any case, their release has ecological consequences as they may modify populations and communities when the producer interacts with other organisms.

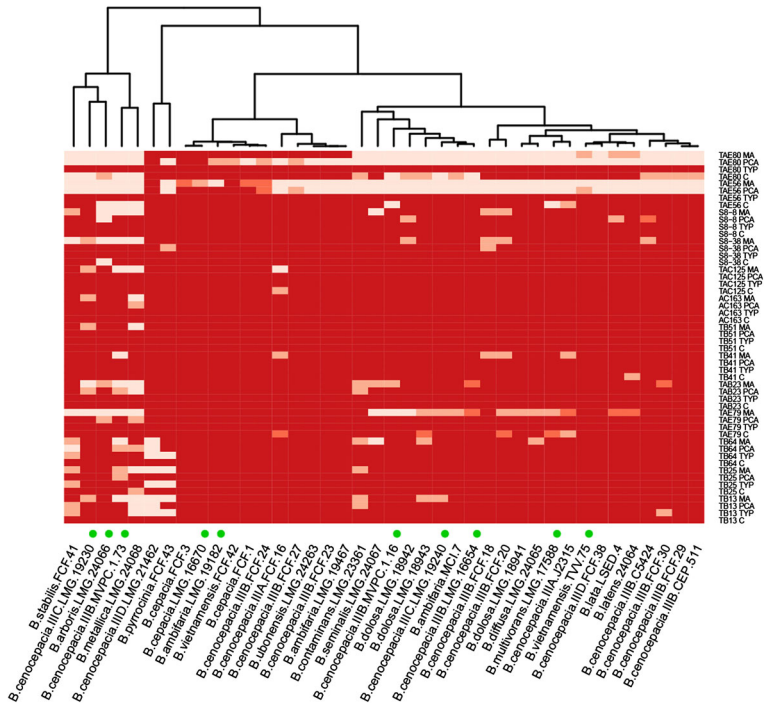

Fig. 3 Heatmap showing the sensitivity of Bcc bacteria to the inhibitory activity of *Pseudoalteromonas* Antarctic strains grown on different media and clustering of Bcc strains on the basis of the similarity of their sensitivity patterns. Bcc environmental strains are marked with a *green dot*

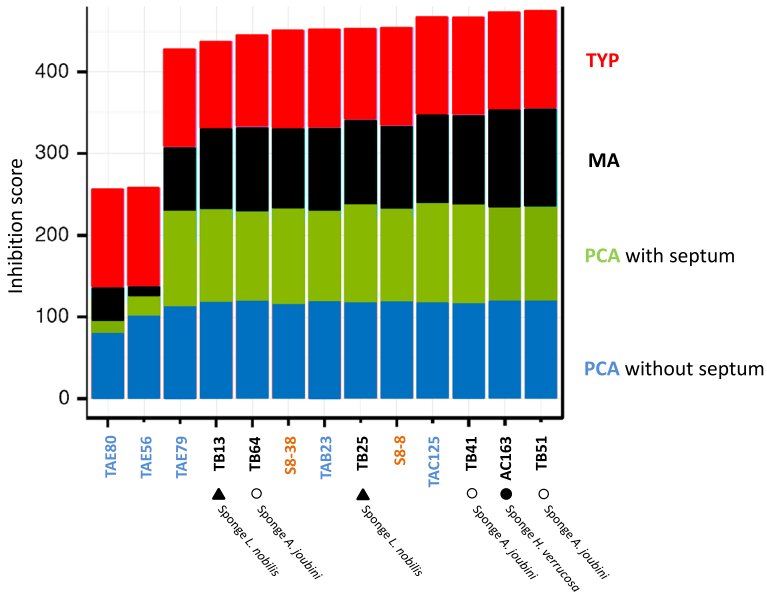

Fig. 4 Inhibition score of *Pseudoalteromonas* Antarctic strains. Strains in *brown*, *light blue* and *black* were isolated from sediments, seawater and sponges, respectively

Antarctic marine bacteria do not constitute an exception to this general behaviour, and over the last years, we reported the VOCs production by Antarctic isolates belonging to different genera, i.e. *Psychrobacter*, *Gillisia*, *Arthrobacter* and *Pseudoalteromonas* (Papaleo et al., 2013; Maida et al., 2014; Orlandini et al., 2014). Moreover, previous data demonstrated that mVOCs produced by Antarctic isolates are able to inhibit the growth of Bcc strains (Romoli et al., 2011; Papaleo et al., 2012, 2013; Romoli et al., 2014). In agreement with the idea that mVOCs profiles produced by microorganisms might depend on cultivation conditions (Sunesson et al., 1997; Lemfack et al., 2014), the analysis of volatile chemicals produced by some

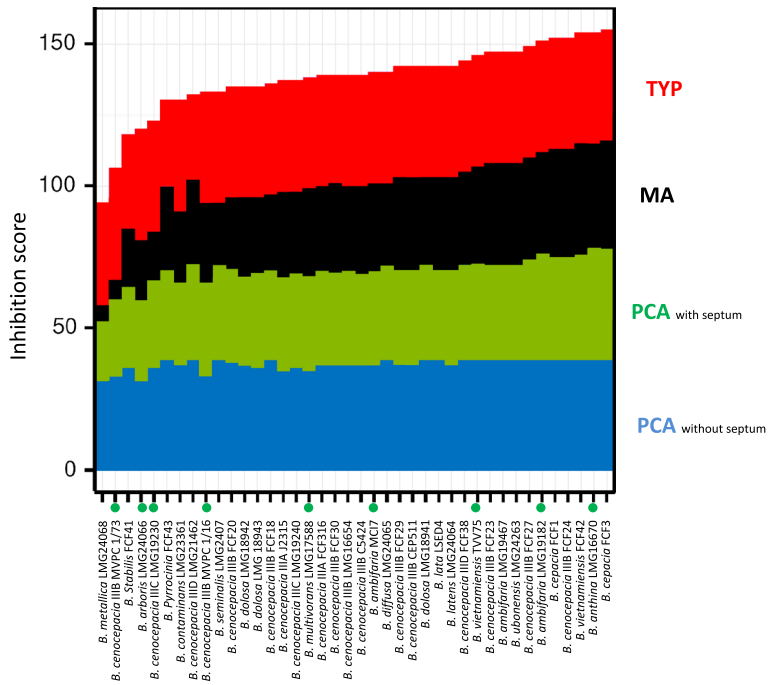

Fig. 5 Inhibition score of *Burkholderia cepacia* complex strains by *Pseudoalteromonas* Antarctic strains grown on different media. Bcc environmental strains are marked with a *green dot*

selected Antarctic strains was carried out by HS–SPME–GC–MS experiments, and data obtained revealed that Antarctic strains belonging to different genera share similar mVOCs production profiles and that the same mVOCs are synthesized under different growth conditions (MA, TYP, PCA) but at different relative concentrations. This observed "chemical relatedness" among bacteria otherwise not closely related (as the investigated strains do affiliate to quite diverse taxonomic groups) could be regarded as a sort of co-evolution or co-adaptation of individuals sharing the same environment.

In the present paper, we focused our attention on a collection of *Pseudoalteromonas* strains, isolated from different Antarctic marine habitats and whose genome was sequenced. In particular, we investigated their ability to produce different volatile and non-volatile organic compounds with inhibitory potential against 40 Bcc members, representative of 17 species from clinical or environmental sources. All the analysed *Pseudoalteromonas* strains were able to inhibit Bcc strains, although at different extent. The cross-streaking experiments performed using Petri dishes with a septum revealed that most of the antagonistic activity relies on the synthesis of mVOCs, in agreement with HS–SPME–GC mass spectrometry analysis previously reported for few of them (Papaleo et al., 2013; Romoli et al., 2014). Furthermore, results reported in Figs. 1 and 2 support the idea that (at least) some tested *Pseudoalteromonas* strains might synthesize also non-volatile compounds, which diffuse through the solid growth medium and can inhibit the growth of Bcc strains. This is in agreement with the outcomes of a genome search analysis of the tested thirteen *Pseudoalteromonas* strains (Bosi, personal communication), which revealed that they harbour genes involved in the biosynthesis of secondary metabolites that may act as antimicrobials (e.g. polyketides, bacteriocins and siderophores). However, we cannot a priori exclude the possibility that the increased inhibitory efficiency by Antarctic strains shown in Figs. 1 and 2 might be due to the same mVOCs embedded in the growth medium and in equilibrium with the volatile phase.

Moreover, the synthesis of the mVOCs is strongly dependent on the growth medium composition, as the supplement of different growth substrates may modify significantly the metabolic fluxes and eventually the end-products released. Indeed, the highest and the lowest degree of inhibition was detected when *Pseudoalteromonas* strains were grown in TYP and MA, respectively, in agreement with previous data reported

Table 3 Growth of seven Bcc strains in cross-streaking experiments using the *Pseudoalteromonas* Antarctic strains TAC125 andTB41 grown in MA, PCA, or TYP medium

Bcc growth medium			PCA						TYP						
			Antarctic strain grown in Petri dishes												
Bcc strain	Species	Origin	With septum			Without septum			With septum			Without septum			
			MA	PCA	TYP	MA	PCA	TYP	MA	PCA	TYP	MA	PCA	TYP	
LMG19182	*B. ambifaria*	Env	−	−	−	−	−	−	−	±	−	−	−	−	*P. haloplanktis* TAC125
LMG23361	*B. contaminans*	AI	−	−	−	−	−	−	+	+	−	−	−	−	
LMG24065	*B. diffusa*	CF	−	−	−	−	−	−	+	+	−	−	−	−	
LMG18943	*B. dolosa*	CF	−	−	−	−	−	−	+	+	−	−	−	−	
LMG24064	*B. latens*	CF	−	−	−	−	−	−	+	+	−	−	−	−	
LMG24068	*B. metallica*	CF	−	−	−	−	−	−	+	+	−	−	−	−	
LMG24067	*B. seminalis*	CF	−	−	−	−	−	−	+	+	−	−	−	−	
LMG19182	*B. ambifaria*	Env	−	−	−	−	−	−	−	+	−	−	−	−	*Pseudoalteromonas* sp. TB41
LMG23361	*B. contaminans*	AI	−	−	−	−	−	−	+	+	−	−	−	−	
LMG24065	*B. diffusa*	CF	−	−	−	−	−	−	+	+	−	−	−	−	
LMG18943	*B. dolosa*	CF	−	−	−	−	−	−	+	+	−	−	−	−	
LMG24064	*B. latens*	CF	−	−	−	−	−	−	+	+	−	−	−	−	
LMG24068	*B. metallica*	CF	−	−	−	−	−	−	+	+	−	−	−	−	
LMG24067	*B. seminalis*	CF	−	−	−	−	−	−	+	+	−	−	−	−	

Bcc bacteria were streaked onto PCA and TYP media

CF strain isolated from Cystic Fibrosis patient, *Env* environmental strain, *AI* animal infection

+ Growth, − no growth, ± reduced growth

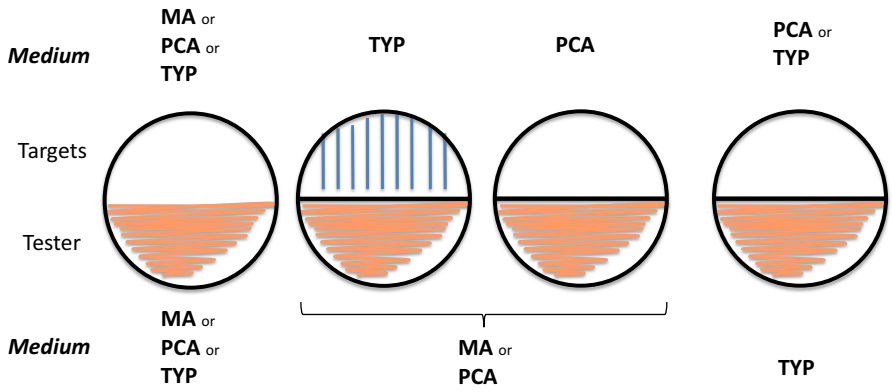

Fig. 6 Schematic representation of results obtained in cross-streaking experiments performed using the Antarctic strains TB41 and TAC125 grown in MA, PCA or TYP and seven target Bcc strains grown in TYP or PCA

on a few number of *Pseudoalteromonas* and *Psychrobacter* strains (Papaleo et al., 2012). Interestingly, opposite results were obtained with the Antarctic *Gillisia* sp. CAL575 strain (Maida et al., 2014). The reason of this finding remains unclear.

Moreover, data obtained revealed that there was no apparent correlation between the source of each Antarctic strain and their inhibitory efficiency as well as their mVOCs profiles. Indeed, strains isolated from different ecological niches (sediment, water column, sponge tissue) were intermixed each other. Besides, it has been also shown that the synthesis of mVOCs was constitutive and not induced by the presence of target strains (Romoli et al., 2011, 2014). These findings are quite interesting from an ecological and evolutionary viewpoint, since they may suggest that the synthesis of such mVOCs is a common feature for the Antarctic strains, whose biological significance is still unknown and a better ecological understanding of this interesting phenomenon deserves a more in depth analysis. However, it cannot be a priori excluded that the synthesis of such antibacterial compounds might be (at least in part) responsible for the structuring of bacterial communities inhabiting the same ecological niche. This is in agreement with previous findings of antagonistic interactions between Antarctic bacteria strains inhabiting different sponges (Lo Giudice et al., 2007a; Mangano et al., 2009). Indeed, it is becoming more and more evident that antagonistic interactions represent one of the major forces that can drive the structuring of microbial communities inside the same (micro) habitat.

If the observation that the ability of synthesize the same mVOCs is shared by Antarctic bacteria belonging to the same or to different species is correct, it follows that the metabolic pathways responsible for their synthesis should be shared by all of them. If these were true, the relative encoding genes would belong to their *core* genome. A previous analysis performed on the *core* genome of four Antarctic strains (two of which affiliated to *Psychrobacter* and *Pseudoalteromonas* TB41 and TAC125) revealed that all of them shared eleven genes (belonging to 8 different COG classes) involved in the biosynthesis of secondary metabolites, which could be likely involved in the synthesis of antimicrobial compounds (Papaleo et al., 2012).

In order to check the presence of such genes in the other eleven genomes, a genomic comparative analysis was performed revealing that the *core* genome is composed of 2478 genes, while 3917 and 3345 genes represent the accessory and the unique genomes, respectively. However, the analysis of the *core* genome revealed that a very low number of genes (28, <0.28%) shared by the thirteen *Pseudoalteromonas* strains belong to the COG functional family Q (secondary metabolite biosynthesis, transport and catabolism). A similar number of genes belonging to the Q family are embedded in the accessory and unique genomes (28 and 29 genes, respectively) (Table 4). The 28 Q family genes of the *core* genome are split into 16 COGs. As it might be expected, genes coding for polyketide synthases (PKS) or non-ribosomal peptide synthetases (NRPS) are not shared by the thirteen strains, indeed, they belong to the unique genome. It is quite interesting that the eight COGs shared by the two *Psychrobacter* and the two *Pseudoalteromonas* strains mentioned above are embedded in the *Pseudoalteromonas core* genome although their involvement in metabolic pathways

Table 4 List of the twenty-eight genes belonging to the *core* genome of the Antarctic *Pseudoalteromonas* strains analysed in this work and putatively involved in the biosynthesis of secondary metabolites (eleven Cluster of Orthologous Genes, COG)

COG	Gene ID	COG class
COG0179 2-keto-4-pentenoate hydratase/2-oxohepta-3-ene-1,7-dioic acid hydratase (catechol pathway)	gi|1410019410|ref|YP_54002|[ctg281_41] (437 letters)	Q
	gi|1410020033|ref|YP_54625|[ctg320_2] (204 letters)	Q
COG0412 dienelactone hydrolase and related enzymes	gi|1410018710|ref|YP_53302|[ctg235_24] (244 letters)	Q
	gi|1410017521|ref|YP_52113|[ctg138_5] (181 letters)	Q
COG0500 SAM-dependent methyltransferases	gi|1409986196|ref|YP_20788|[ctg74_20] (273 letters)	QR
	gi|1410017372|ref|YP_51964|[ctg127_12] (322 letters)	QR
	gi|1410000978|ref|YP_35570|[ctg243_2] (241 letters)	QR
	gi|1410019070|ref|YP_53662|[ctg256_31] (298 letters)	QR
	gi|1410019727|ref|YP_54319|[ctg303_4] (258 letters)	QR
	gi|1410019613|ref|YP_54205|[ctg296_5] (218 letters)	QR
	gi|1410016647|ref|YP_51239|[ctg69_7] (251 letters)	QR
COG0767 ABC-type transport system involved in resistance to organic solvents, permease component	gi|1410019869|ref|YP_54461|[ctg310_32] (259 letters)	Q
COG1127 ABC-type transport system involved in resistance to organic solvents, ATPase component	gi|1410019870|ref|YP_54462|[ctg310_33] (275 letters)	Q
COG1228 imidazolonepropionase and related amidohydrolases	gi|999991682|ref|YP_1682|[ctg155_8] (165 letters)	Q
	gi|1409987156|ref|YP_21748|[ctg127_8] (413 letters)	Q
	gi|1409998757|ref|YP_33349|[ctg90_2] (251 letters)	Q
COG1335 amidases related to nicotinamidase	gi|1410020496|ref|YP_55088|[ctg347_3] (184 letters)	Q
COG2050 uncharacterized protein, possibly involved in aromatic compounds catabolism	gi|1410018553|ref|YP_53145|[ctg220_15] (146 letters)	Q
	gi|1410020095|ref|YP_54687|[ctg324_2] (151 letters)	Q
COG2132 putative multicopper oxidases	gi|1409998250|ref|YP_32842|[ctg53_9] (624 letters)	Q
COG2761 predicted dithiol-disulphide isomerase involved in polyketide biosynthesis	gi|1409987771|ref|YP_22363|[ctg160_29] (220 letters)	Q
COG2854 ABC-type transport system involved in resistance to organic solvents, auxiliary component	gi|1410019867|ref|YP_54459|[ctg310_30] (226 letters)	Q
	gi|1410017209|ref|YP_51801|[ctg110_4] (197 letters)	Q
COG3127 predicted ABC-type transport system involved in lysophospholipase L1 biosynthesis, permease component	gi|1409995140|ref|YP_29732|[ctg148_39] (833 letters)	Q
COG3135 uncharacterized protein involved in benzoate metabolism	gi|1410019220|ref|YP_53812|[ctg270_2] (385 letters)	Q
COG3155 uncharacterized protein involved in an early stage of isoprenoid biosynthesis	gi|1409987813|ref|YP_22405|[ctg165_4] (215 letters)	Q
COG3508 Homogentisate 1,2-dioxygenase	gi|1409987745|ref|YP_22337|[ctg160_3] (433 letters)	Q
COG4181 predicted ABC-type transport system involved in lysophospholipase L1 biosynthesis, ATPase component	gi|1409995139|ref|YP_29731|[ctg148_38] (237 letters)	Q

COGs in bold are those shared with Antarctic *Psychrobacter* strains (Papaleo et al., 2013)

responsible for mVOCs synthesis is still an open question and needs to be addressed by molecular approaches.

Concerning the different ability of bacteria belonging to different genera to inhibit the growth of Bcc strains, it can be argued that in spite of the strong similarity between the mVOCs profile obtained from *Gillisia* sp. CAL575 (Maida et al., 2014) and Antarctic *Pseudoalteromonas* and *Psychrobacter* strains (Papaleo et al., 2012), in our opinion, the relative different concentrations of mVOCs might influence their respective inhibitory efficiency. Thus, it is possible that a combination and the relative concentration of different mVOCs, rather than a single mVOC, might be responsible for the growth

inhibition of Bcc strains, and that their concentration may vary depending on the cultivation conditions.

As far as the Bcc molecular target(s) of the antimicrobial compounds synthesized by Antarctic *Pseudoalteromonas* is concerned, both their nature and number are still unknown. However, we were not able to isolate Bcc mutant resistant to mVOCs produced by Antarctic bacteria, which might suggest that the antimicrobial compounds might be directed towards different molecular targets. However, also the Bcc strain sensitivity to Antarctic mVOCs is influenced by the medium on which Bcc are grown (Fig. 6; Table 3) and/or by the growth medium of the target strains. Furthermore, apparently also the sensitivity to the antimicrobial compounds produced by *Pseudoalteromonas* is not related to the origin (clinical or environmental) of Bcc members taxonomical position; however, a slightly significant relation between Bcc species and inhibition response exists. These results may suggest that "Antarctic drug" targets may be involved (at least some) relevant housekeeping functions.

Conclusions

The ability of Antarctic *Pseudoalteromonas* isolates from different ecological niches in the Ross Sea (i.e. sediment, water column, sponge tissue) to produce mVOCs and diffusible molecules with inhibitory activity against Bcc CF opportunistic pathogens was analysed. Data obtained demonstrated that all Antarctic strains used in this work are able to synthesize mVOCs exhibiting an antimicrobial activity that is also dependent on the composition of the medium used for the growth of Antarctic isolates. Moreover, it is quite possible that both volatile and non-volatile compounds might be responsible for the growth inhibition of Bcc strains. The statistical analysis showed no correlation between the different ecological sources of the *Pseudoalteromonas* strains and their inhibitory power, as well as the sensitivity to the antimicrobial compounds produced by *Pseudoalteromonas* appeared to be not related to origin (clinical or environmental) of Bcc members, whereas their taxonomical position might have an effect. These findings suggest that the synthesis of molecules with inhibitory activity might be a common feature of Antarctic strains and such molecules might be involved in structuring bacterial populations sharing the same habitat. Moreover, Antarctic strains belonging to phylogenetically different species display similar inhibitory pattern, suggesting that common metabolic pathways responsible for the production of these molecules might exist. Hence, the different pattern of inhibition might be due to the different plenty of production, instead of the production of molecules with different features. This plethora of substances might act versus different molecular target. Moreover, we have demonstrated the importance to better investigate the inhibitory power of Antarctic strains either from a clinical point of view, for a possible exploitation of these strains as a source of novel antibacterial molecules, or from an ecological point of view, to better understand the involvement of mVOCs in structuring Antarctic bacterial communities.

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Conflict of interest The authors declare that they have no conflict of interest.

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