RESEARCH PAPER

New Biotechnology • Volume 30, Number 6 • September 2013

ELSEVIER

Research Paper

Bioactive volatile organic compounds from Antarctic (sponges) bacteria

Maria Cristiana Papaleo[1,8], Riccardo Romoli[7,8], Gianluca Bartolucci[2], Isabel Maida[1], Elena Perrin[1], Marco Fondi[1], Valerio Orlandini[1], Alessio Mengoni[1], Giovanni Emiliani[1,6], Maria Luisa Tutino[3], Ermenegilda Parrilli[3], Donatella de Pascale[4], Luigi Michaud[5], Angelina Lo Giudice[5] and Renato Fani[1,]

[1] Lab. of Microbial and Molecular Evolution, Dept of Biology, University of Florence, Italy
[2] Department of Neurosciences, Psychology, Drug Research and Child Health, Section of Pharmaceutical and Nutraceutical Sciences, University of Florence, Italy
[3] Dept of Chemical Sciences, University of Naples Federico II, Naples, Italy
[4] Institute of Protein Biochemistry, National Research Council, Naples, Italy
[5] Dept of Biological and Environmental Sciences, University of Messina, Messina, Italy
[6] Trees and Timber Institute, National Research Council, Florence, Italy
[7] Mass Spectrometry Center (CISM), University of Florence, Italy

Antarctic bacteria represent a reservoir of unexplored biodiversity, which, in turn, might be correlated to the synthesis of still undescribed bioactive molecules, such as antibiotics. In this work we have further characterized a panel of four marine Antarctic bacteria able to inhibit the growth of human opportunistic multiresistant pathogenic bacteria belonging to the *Burkholderia cepacia* complex (responsible for the 'cepacia' syndrome in Cystic Fibrosis patients) through the production of a set of microbial Volatile Organic Compounds (mVOCs). A list of 30 different mVOCs synthesized under aerobic conditions by Antarctic bacteria was identified by GC-SPME analysis. Cross-streaking experiments suggested that Antarctic bacteria might also synthesize non-volatile molecules able to enhance the anti-*Burkholderia* activity. The biosynthesis of such a mixture of mVOCs was very probably influenced by both the presence/absence of oxygen and the composition of media used to grow the Antarctic strains. The antimicrobial activity exhibited by Antarctic strains also appeared to be more related to their taxonomical position rather than to the sampling site. Different Bcc bacteria were differently sensitive to the 'Antarctic' mVOCs and this was apparently related neither to the taxonomical position of the different strains nor to their source. The genome sequence of three new Antarctic strains was determined revealing that only *P. atlantica* TB41 possesses some genes belonging to the *nrps–pks* cluster. The comparative genomic analysis performed on the genome of the four strains also revealed the presence of a few genes belonging to the *core* genome and involved in the secondary metabolites biosynthesis. Data obtained suggest that the antimicrobial activity exhibited by Antarctic bacteria might rely on a (complex) mixture of mVOCs whose relative concentration may vary depending on the growth conditions. Besides, it is also possible that the biosynthesis of these compounds might occur through still unknown metabolic pathways.

Corresponding author: Fani, R. (renato.fani@unifi.it)
[8] Equal contributors.

1871-6784/$ - see front matter http://dx.doi.org/10.1016/j.nbt.2013.03.011

Introduction

The exploitation of the biodiversity still remains the main road in the bioprospecting efforts and many endeavours are focused on microorganisms, which are able to thrive in harsh conditions like in Antarctic environments. Hence, Antarctic habitats represent a fascinating resource of biodiversity, and therefore of new bioproducts, such as enzymes, proteins, secondary metabolites, and bioactive compounds, because several evident extremophilic adaptations are simultaneously present and the general life conditions are reflected on molecules.

Diversity of living organisms at low temperatures is rich, assorted and widespread, and organisms belonging to three domains of life are all well represented. Psychrophilic and psychrotolerant bacteria have been found to not only tolerate, but also proliferate under the harsh conditions of enduringly low-temperatures. In fact, for some of them, this environment is not only optimal, but also compulsory for sustained cell propagation, and in some cases the increasing of temperature (e.g. >12°C) may have a deleterious effect.

Undoubtedly, adaptations to low temperatures seems to occur at different levels (such as cellular membrane adaptation, proteins and the entire physiological organisation) and let psychrophilic microorganisms to overcome crucial obstacles connected to life in the cold [1,2].

Antarctic bacteria represent a reservoir of undiscovered biodiversity, because they achieve their ecological success coping with very harsh conditions. Their success may be due to the synthesis of a broad range of compounds potentially valuable in many pharmaceutical applications [3].

Bioprospecting is the systematic exploration of biodiversity to discovery new enzymes, genes, microorganisms and bioactive compounds, and the exploration of extremophilic environments, that is Antarctic environments, may represent an added value for discovering new drugs, such as new antibiotic, because the Antarctic biodiversity is almost unexplored.

Many bacteria, both from marine and terrestrial environments, are endowed with the ability to synthesize several infochemicals, some of which are volatile compounds, that is microbial volatile organic compounds (mVOCs) [4,5], formed via primary and/or secondary metabolic pathways. The function of these volatile compounds is not completely clear. It has been proposed that they could represent waste material or the end-products of detoxification pathways.

Generally, the best known secondary metabolites, antibiotics, toxins and dyes [6] are significant for the sustainment of bacterial populations in ecological niches and for the cooperative development of a community of different organisms; they can support the selective advantage of some community member(s) and survival during evolution. Due to their volatility, these compounds may travel large distances in the structurally heterogeneous environment of the ecological system, a major advantage for a component of an interactive system. Because microbial activity is greatly influenced by environmental factors, production of these volatiles will be affected by the dynamics of the whole ecosystem [7].

The different mVOCs can belong to different chemical classes such as alcohols, esters, hydrocarbons, terpenes, ketones, sulphur-containing compounds and carboxylic acids.

Recently, it has been demonstrated by the cross-streaking methodology that Antarctic bacteria isolated from three different sponge species (*Haliclonissa verrucosa*, *Anoxycalyx joubini* and *Lissodendoryx nobilis*) are able to inhibit the growth of *Burkholderia cepacia* complex (Bcc) strains [8]. The *B. cepacia* complex includes 17 closely related species that inhabit different environmental niches. Most Bcc species are also opportunistic human pathogens, being particularly problematic for immuno-compromised individuals, including Cystic Fibrosis (CF) patients. The CF airway microbiota includes chronic opportunistic pathogens and a variety of organisms derived mostly from the normal microbiota of the upper respiratory tract. Among them, the group of Bcc is of major concern within the CF community. This emergent pathogen might cause the 'cepacia syndrome', characterized by high fever, severe progressive respiratory failure, leucocytosis and elevated erythrocyte sedimentation rate. The main problem in managing Bcc-infected patients is the resistance of these bacteria to various antimicrobials and lack of newer effective antibiotics. Indeed, they are intrinsically resistant to antimicrobial agents such as aminoglycosides, first- and second-generation cephalosporins, antipseudomonal penicillins and polymyxins. Bcc bacteria often develop resistance to β-lactams due to presence of inducible chromosomal β-lactamases and altered penicillin-binding proteins. The intrinsic resistance to antibiotics mainly depends on the constitutive or inducible expression of active efflux systems [9,10]. This is particularly true for multidrug efflux pumps allowing bacterial cells to extrude a wide range of different substrates, including antibiotics. Thus, because Bcc bacteria are resistant to a wide range of antibiotics, the search of new drugs and antibiotic is of particularly challenging [11]. In principle, different approaches to the treatment of Bcc infection (and, more generally, to infection diseases) can be used. One of them relies in the modification of the pre-existing classes of previous successful antibiotics; another one is the identification of inhibitors that are effective against previously unexplored essential bacterial molecular targets. The last one is the identification and characterization of new natural antimicrobial compounds from unusual environmental sources that very probably represent the most promising reservoirs of novel antibiotics. In this context our previous finding demonstrating that the inhibition of the growth of Bcc bacteria occurred through the synthesis of mVOCs by Antarctic bacteria, is very promising. These data were confirmed by solid phase micro extraction coupled with gas chromatography–mass spectrometry (HS-SPME-GC–MS), which allowed to identify a panel of (at least) 128 different compounds, some of which might have been responsible for the Bcc growth inhibition [12].

However, these volatile profiles were obtained under anaerobic conditions, that is in normal HS sampling; because the Antarctic bacteria used in the previous study are aerobic, these conditions might have probably caused some abiotic stresses modifying the composition of the volatile profile. So it was not possible to define the actual nature of the antimicrobial compound(s), possibly inhibiting the growth of Bcc bacteria. For this reason we have very recently set up a method allowing detecting the mVOCs produced by Antarctic bacteria under aerobic conditions and in cross-streaking conditions [13]. The experiments were carried out using the *Pseudoalteromonas atlantica* TB41 strain and revealed that the number and the molecular nature of the mVOCs produced

were different from those obtained in anaerobic conditions [12]. This is in agreement with previous data showing that, generally, mVOCs profiles produced by microorganisms are consistent, related to cultural conditions, environments and inputs, and therefore to population and function dynamics. Indeed, different environmental parameters might (strongly) influence the mVOCs production [14]. Other environmental factors such as pH of the substrate, light and levels of CO_2 or O_2 probably also influence the mVOC pattern. The substrate composition has a great influence on both qualitative and quantitative production of volatile metabolites [14].

Herein, we report our study aimed to investigate the volatile profile of three different Antarctic bacteria, that is *Pseudoalteromonas haloplanktis* TAC125 [15], *Psychrobacter* sp. TB67 and TB47 strains under aerobic conditions and to check whether the growth on different growth media might influence their ability to synthesize mVOCs and inhibit the growth of Bcc bacteria. In addition to this, data obtained were compared with those previously reported on *P. atlantica* strain TB41 to unveil whether strains belonging to different species of the same genus or to different genera might produce different mVOCs. Lastly, we determined the nucleotide sequence of the genome of three Antarctic strains to identify genes that are probably responsible for the biosynthesis of antimicrobial compounds.

Materials and methods

Antarctic strains and growth conditions

The Antarctic bacteria analyzed in this work are listed in Table 1, whereas the target strains belonging to a panel of Bcc bacteria are listed in Table 2.

Antarctic strains were grown in PCA, TYP or Marine Agar (MA) at 20°C; Bcc bacteria were grown on PCA at either 20°C or 37°C [8].

Cross streaking

Cross-streaking experiments were carried out as previously described in Papaleo *et al.* [8] by using Petri dishes with a septum separating two hemi-cycles, to permit the growth of the tester and target strains on different media without any physical contact. Data were statistically evaluated performing a principal component analysis (PCA) an UPGMA clustering with Past software [16].

Sequencing and analysis of Antarctic bacteria genome

Genomic DNA from Antarctic strains was purified as described by Papaleo *et al.* [17]. Bacterial genomes were sequenced using Illumina HiSeq2000. The resulting reads were trimmed with SolexaQA [18] before the assembly step.

Genomes were assembled using ABySS 1.3.4. For each strain, several assemblages were performed, with *k*-mer values between 50 and 80 [18]. An utility written in Ruby and C++ was used to automatically choose the best assembly. The choice is primarily based on the ratio between assembled nucleotides and the number of contigs. Finally, the same software performs an ORF prediction of the chosen assembly using Prodigal. The pangenomes were obtained with a Perl script called Parapipe, which uses Multiparanoid and automatically performs paired comparisons within each possible couple of genomes among the provided ones [19–21].

TABLE 1
List of Antarctic strains used in this work

Genus	Strain	Origin
Pseudoalteromonas	TAC125	Water column
	TB41	*A. joubini*
Psychrobacter	TB47	*A. joubini*
	TB67	

TABLE 2
List of Bcc bacteria used as targets strains in this work

Species	Strain	Origin
B. cepacia	FCF 1	CF
	FCF 3	
B. multivorans	LMG 17588	Environmental
B. cenocepacia	FCF 16	CF
	J2315	
	FCF 18	
	FCF 20	
	FCF 23	
	FCF 24	
	FCF 27	
	FCF 29	
	FCF 30	
	LMG 16654	
	C5424	
	CEP511	
	MVPC 1/16	Environmental
	MVPC 1/73	
	LMG 19230	
	LMG 19240	
	FCF 38	CF
	LMG 21462	
B. stabilis	FCF 41	
B. vietnamiensis	FCF 42	
B. vietnamiensis	TVV 75	Environmental
B. dolosa	LMG 18941	CF
B. dolosa	LMG 18942	
B. dolosa	LMG 18943	
B. ambifaria	MCI 7	Environmental
B. ambifaria	LMG 19467	CF
B. ambifaria	LMG 19182	Environmental
B. anthina	LMG 16670	
B. pyrrocinia	FCF 43	CF
B. lata	LSED 4	CF
B. latens	LMG 24064	
B. diffusa	LMG 24065	
B. contaminans	LMG 23361	Environmental
B. seminalis	LMG 24067	CF
B. metallica	LMG 24068	
B. arboris	LMG 24066	Environmental
B. ubonensis	LMG 24263	Nosocomial infection

Abbreviation: CF, strain isolated from Cystic Fibrosis patient.

Sample preparation for the determination of microbial volatile organic compounds (mVOCs) under aerobic conditions

The mVOC production by Antarctic strains was tested both alone and in the presence of *B. cenocepacia* LMG 16654 strain (i.e. under

cross-streak conditions). Also the *B. cenocepacia* LMG 16654 strain mVOC profile was analyzed, according to Romoli *et al.* [12,13]. Briefly, 9 ml of PCA medium were added to each pre-sterilized 20 ml HS vial with aluminum crimp caps (Gerstel, Mülheim an der Ruhr, Germany). For the analysis in the absence of *B. cenocepacia* LMG16654 cells, Antarctic strains were streaked on the surface of PCA medium and the vial immediately crimped, whereas to maintain aerobic conditions, 2 needles 18GX11/2 (40 length, 1.4 ϕ, BD Microlance TM) were inserted in the caps to allow the go through of air and cut just at the top vial septum. All vials were incubated at room temperature (20 ± 1°C) and the mVOC production were evaluated every 24 for six days consecutively. The same experiment was also carried out for the *B. cenocepacia* LMG16654 strain. The variability over the results recorded among the different days of analysis was checked finding no significant difference among the days (by linear discriminant analysis, LDA) [13].

The evaluation of mVOCs production in the presence of *B. cenocepacia* strain LMG16654, that is under cross-streak conditions, was carried out as above, except that Antarctic strain was streaked across one-third of the surface of PCA medium of each 20 ml HS vial, which was crimped with two needles inserted in the caps. All vials were incubated at room temperature (20 ±1 °C) and the mVOC production was checked every 24 for four days consecutively. To the fourth day, the vials were quickly opened and *B. cenocepacia* strain LMG16654 was streaked perpendicular to the initial streak and vials were further crimped in the previous conditions and incubated at room temperature (20 ±1 °C) and the mVOC production checked after 24 hours.

Solid phase micro extraction GC–MS analysis

The volatile compounds profile was obtained by Solid Phase Micro Extraction (SPME) GC–MS technique according to methodology recently described [12,13], using an Agilent 7890 GC-chromatograph (Agilent, Palo Alto, CA, USA) equipped with a 5975C MSD with EI ionization and a three-phase DVB/Carboxen/PDMS 75 SPME fiber (Supelco, Bellafonte, PA, USA), according to [12,19,20].

After acquisition of chromatographic raw data, a tentative identification of volatile compounds was made by matching EI deconvoluted mass spectra, obtained using NIST-AMDIS (Automated Mass Spectral Deconvolution and Identification System) (v.2.68) software, against NIST 08 and Wiley 07 spectral libraries. The NIST-MS-Search program was used for mass spectra comparison.

PCA and LDA were applied to evaluate the relationships among variables and identifying the possible differences among the microorganisms by their volatile profile [21,22]. The most significant compounds were identified by the support vector machine-recursive feature elimination (SVM-RFE) method. A SVM classifier was constructed using all the input variables; the classification accuracy rate and the squared coefficient of the weight of each feature was computed. Than the features with the smallest squared coefficient of the weight were deleted from the feature set. This procedure was repeated until the feature set was empty [23].

All statistical analyses were performed by means of R [24] version 2.15.2 software, with Ubuntu 10.04 (lucid), kernel Linux 2.6.32-45-amd64, a 2.20 GHz CoreDuo CPU and 4 GiB memory.

Results and discussion

Cross-streaking experiments using different growth media

In a previous work [8] we checked the ability of a panel of 132 Antarctic sponge-associated bacteria to antagonize the growth of (opportunistic) human pathogenic bacteria through cross-streak experiments. Data obtained revealed that most of the tester strains were able to specifically inhibit the growth of a very large fraction of Bcc strains, very probably through the synthesis of mVOCs. However, these experiments were carried out growing the tester and the target strain on the same growth medium (PCA), whose composition allowed the growth of both of them.

To check the influence of the growth medium on the ability of Antarctic bacteria to inhibit the growth of Bcc target strains, cross-streaking experiments were carried out using Petri dishes with a septum to permit the growth of tester and target strains on different media. In these assays, four Antarctic tester strains (i.e. *P. atlantica* TB41, *P. haloplanktis* TAC125, and *Psychrobacter* sp. TB47 and TB67) were used. These four strains were chosen because they exhibited different patterns of inhibition of Bcc strains [8]; besides, they belong to different species and/or genera. Accordingly, it should be possible to correlate the mVOCs profile to the taxonomical position of each strain and/or to the inhibition pattern. The four strains were grown on three different media, that is MA, TYP and PCA. The panel of target strains consisted of 40 Bcc strains representative of all the known 17 species and with either clinical or environmental origin (Table 2). Bcc strains were grown on PCA medium. Besides, and additional cross-streaking experiment was performed using PCA plates without septum, to evaluate whether the absence of a physical barrier to the flow of molecules from Antarctic tester strains might enhance the inhibitory effect (due to the synthesis of additional non-volatile antimicrobial compounds). The experiments were performed as described in Materials and Methods and data obtained are reported in Table 3. Results revealed that:

(i) The two *Pseudoalteromonas* strains (TB41 and TAC125) exhibited a very similar pattern of inhibition and were able to inhibit the growth of all or most of the Bcc strains (depending on the medium on which they grew).

(ii) The two *Psychrobacter* strains (TB47 and TB67) exhibited different patterns of inhibition of Bcc growth, in spite of the fact they shared the very same RAPD (Random Amplified Polymorphic DNA) profile [8]. However, the diversity of the two strains also at the genome level was indeed confirmed by the comparative analysis of their entire genome (see below).

(iii) Overall, the two *Pseudoalteromonas* strains (TAC125 and TB41) were more effective than the two *Psychrobacter* strains (TB47 and TB67) in inhibiting the growth of Bcc strains, independently from the growth medium. These data suggest that the different inhibitory effect exhibited by the four Antarctic strains effect might be mostly attributed to the taxonomical position rather than to their isolation site (sponge and seawater). Indeed, three of these strains, that is *P. atlantica* TB41 and *Psychrobacter* sp. TB47 and TB67, were isolated from the same Antarctic *A. joubini* sponge [8], whereas *P. haloplanktis* TAC125 was isolated from seawater column. However, more data are needed to provide a statistically grounded inference on the importance of taxon

TABLE 3

Growth of (opportunistic) pathogens belonging to the *B. cepacia* complex in the presence of Antarctic *Pseudoalteromonas* and *Psychrobacter* strains

Strain	Species	Origin	Growth in the presence of *P. haloplanktis* TAC125 grown on:			
			TYP	PCA*	PCA	MA
FCF1	*B. cepacia*	CF	–	–	–	–
FCF3		CF	–	–	–	–
LMG17588	*B. multivorans*	ENV	–	–	–	–
FCF16	*B. cenocepacia* IIIA	CF	–	+/–	–	+
J2315		CF	–	–	–	–
FCF18	*B. cenocepacia* IIIB	CF	–	–	–	–
FCF20		CF	–	–	–	–
FCF23		CF	–	–	–	–
FCF24		CF	–	–	–	–
FCF27		CF	–	–	–	–
FCF29		CF	–	–	–	–
FCF30		CF	–	–	–	–
LMG16654		CF	–	–	–	–
C5424		CF	–	–	–	–
CEP511		CF	–	–	–	–
MVPC1/16		ENV	–	–	–	–
MVPC1/73		ENV	–	–	–	+
LMG19230	*B. cenocepacia* IIIC	ENV	–	–	–	+/–
LMG19240		ENV	–	–	–	–
FCF38	*B. cenocepacia* IIID	CF	–	–	–	–
LMG21462		CF	–	–	–	–
FCF41	*B. stabilis*	CF	–	–	–	–
FCF42	*B. vietnamiensis*	CF	–	–	–	–
TVV75		ENV	–	–	–	–
LMG18941	*B. dolosa*	CF	–	–	–	–
LMG18942		CF	–	–	–	–
LMG18943		CF	–	–	–	–
MCI7	*B. ambifaria*	ENV	–	–	–	–
LMG19467		CF	–	–	–	–
LMG19182		ENV	–	–	–	–
LMG16670	*B. anthina*	ENV	–	–	–	–
FCF43	*B. pyrrocinia*	CF	–	–	–	–
LSED4	*B. lata*	CF	–	–	–	–
LMG24064	*B. latens*	CF	–	–	–	–
LMG24065	*B. diffusa*	CF	–	–	–	–
LMG23361	*B. contaminans*	AI	–	–	–	–
LMG24067	*B. seminalis*	CF	–	–	–	–
LMG24068	*B. metallica*	CF	–	–	–	+
LMG24066	*B. arboris*	ENV	–	–	–	–
LMG24263	*B. ubonensis*	NI	–	–	–	–
Strain	**Species**	**Origin**	**Growth in the presence of *P. atlantica* TB41 grown on:**			
			TYP	**PCA***	**PCA**	**MA**
FCF1	*B. cepacia*	CF	–	–	–	–
FCF3		CF	–	–	–	–
LMG17588	*B. multivorans*	ENV	–	–	–	–
FCF16	*B. cenocepacia* IIIA	CF	–	–	–	+/–
J2315		CF	–	–	–	+/–
FCF18	*B. cenocepacia* IIIB	CF	–	–	–	+/–
FCF20		CF	–	–	–	+/–
FCF23		CF	–	–	–	–
FCF24		CF	–	–	–	–
FCF27		CF	–	–	–	–
FCF29		CF	–	–	–	–

TABLE 3 (*Continued*)

Strain	Species	Origin	Growth in the presence of *P. atlantica* TB41 grown on:			
			TYP	PCA*	PCA	MA
FCF30		CF	–	–	–	–
LMG16654		CF	–	–	–	–
C5424		CF	–	–	–	–
CEP511		CF	–	–	–	–
MVPC1/16		ENV	–	–	–	–
MVPC1/73		ENV	–	–	–	+
LMG19230	*B. cenocepacia* IIIC	ENV	–	–	–	–
LMG19240		ENV	–	–	–	–
FCF38	*B. cenocepacia* IIID	CF	–	–	–	–
LMG21462		CF	–	–	–	–
FCF41	*B. stabilis*	CF	–	–	–	–
FCF42	*B. vietnamiensis*	CF	–	–	–	–
TVV75		ENV	–	–	–	–
LMG18941	*B. dolosa*	CF	–	–	–	–
LMG18942		CF	–	–	–	–
LMG18943		CF	–	–	–	–
MCI7	*B. ambifaria*	ENV	–	–	–	–
LMG19467		CF	–	–	–	–
LMG19182		ENV	–	–	–	–
LMG16670	*B. anthina*	ENV	–	–	–	–
FCF43	*B. pyrrocinia*	CF	–	–	–	–
LSED4	*B. lata*	CF	–	–	–	–
LMG24064	*B. latens*	CF	–	+/–	–	–
LMG24065	*B. diffusa*	CF	–	–	–	–
LMG23361	*B. contaminans*	AI	–	–	–	–
LMG24067	*B. seminalis*	CF	–	–	–	–
LMG24068	*B. metallica*	CF	–	–	–	–
LMG24066	*B. arboris*	ENV	–	–	–	–
LMG24263	*B. ubonensis*	NI	–	–	–	–
Strain	**Species**	**Origin**	**Growth in the presence of *Psychrobacter* sp. TB47 grown on:**			
			TYP	**PCA***	**PCA**	**MA**
FCF1	*B. cepacia*	CF	–	–	–	–
FCF3		CF	–	–	–	–
LMG17588	*B. multivorans*	ENV	–	+	–	+
FCF16	*B. cenocepacia* IIIA	CF	–	–	–	+
J2315		CF	–	–	+	+
FCF18	*B. cenocepacia* IIIB	CF	–	+	+	+
FCF20		CF	–	–	+	+
FCF23		CF	–	–	+/–	+
FCF24		CF	–	–	+/–	+
FCF27		CF	–	–	+	+
FCF29		CF	–	+	+	+
FCF30		CF	–	+	+	+
LMG16654		CF	–	+	+	+
C5424		CF	–	+	+/–	+
CEP511		CF	–	+/–	+/–	+
MVPC1/16		ENV	+/–	+	+	+
MVPC1717		ENV	–	+	+	+
LMG19230	*B. cenocepacia* IIIC	ENV	+/–	+	+	+
LMG19240		ENV	–	+/–	–	–
FCF38	*B. cenocepacia* IIID	CF	+/–	+	+	+
LMG21462		CF	–	–	–	–
FCF41	*B. stabilis*	CF	–	+/–	+	+
FCF42	*B. vietnamiensis*	CF	–	+	–	–
TVV75		ENV	–	+	–	+

TABLE 3 (*Continued*)

Strain	Species	Origin	Growth in the presence of *Psychrobacter* sp. TB47 grown on:			
			TYP	PCA*	PCA	MA
LMG18941	*B. dolosa*	CF	–	+	–	+
LMG18942		CF	–	+	+/–	+
LMG18943		CF	+	+	+	+
MCI7	*B. ambifaria*	ENV	–	+	+/–	+
LMG19467		CF	+	+	+	+
LMG19182		ENV	–	–	–	+
LMG16670	*B. anthina*	ENV	–	–	+/–	+
FCF43	*B. pyrrocinia*	CF	–	+	+	+
LSED4	*B. lata*	CF	–	–	+	+
LMG24064	*B. latens*	CF	–	–	+	+
LMG24065	*B. diffusa*	CF	–	+	+	+
LMG23361	*B. contaminans*	AI	–	+	+	+
LMG24067	*B. seminalis*	CF	–	+	+	+
LMG24068	*B. metallica*	CF	+/–	+/–	+	+
LMG24066	*B. arboris*	ENV	+/–	+/–	+	+
LMG24263	*B. ubonensis*	NI	–	–	+	+
Strain	**Species**	**Origin**	**Growth in the presence of *Psychrobacter* sp. TB67 grown on:**			
			TYP	**PCA***	**PCA**	**MA**
FCF1	*B. cepacia*	CF	–	–	–	–
FCF3		CF	–	–	–	–
LMG17588	*B. multivorans*	ENV	–	+	+	+
FCF16	*B. cenocepacia* IIIA	CF	–	–	+/–	+/–
J2315		CF	–	+	+	+
FCF18	*B. cenocepacia* IIIB	CF	–	+	+	+
FCF20		CF	–	+	+	+
FCF23		CF	–	+	+/–	+
FCF24		CF	–	–	+/–	+
FCF27		CF	–	+	+	+
FCF29		CF	–	+	+	+
FCF30		CF	–	–	+	+
LMG16654		CF	–	–	+	+
C5424		CF	–	–	+	+
CEP511		CF	–	–	+/–	+
MVPC1/16		ENV	–	+	+	+
MVPC1717		ENV	–	+	+	+
LMG19230	*B. cenocepacia* IIIC	ENV	–	+	+	+
LMG19240		ENV	–	+	–	–
FCF38	*B. cenocepacia* IIID	CF	–	–	+	+
LMG21462		CF	–	–	–	–
FCF41	*B. stabilis*	CF	–	–	+	+
FCF42	*B. vietnamiensis*	CF	–	–	–	–
TVV75		ENV	–	–	+/–	+
LMG18941	*B. dolosa*	CF	–	–	+	+
LMG18942		CF	–	–	+	+
LMG18943		CF	–	–	+/–	+
MCI7	*B. ambifaria*	ENV	–	–	+/–	+
LMG19467		CF	–	–	+/–	+
LMG19182		ENV	–	–	+/–	+
LMG16670	*B. anthina*	ENV	–	–	–	+
FCF43	*B. pyrrocinia*	CF	–	–	+/–	+
LSED4	*B. lata*	CF	–	–	+/–	+
LMG24064	*B. latens*	CF	–	–	+/–	+
LMG24065	*B. diffusa*	CF	–	–	+/–	+
LMG23361	*B. contaminans*	AI	–	–	+/–	+

TABLE 3 (*Continued*)

Strain	Species	Origin	Growth in the presence of *Psychrobacter* sp. TB67 grown on:			
			TYP	PCA*	PCA	MA
LMG24067	*B. seminalis*	CF	–	–	+/–	+
LMG24068	*B. metallica*	CF	–	–	+/–	+
LMG24066	*B. arboris*	ENV	–	–	+/–	+
LMG24263	*B. ubonensis*	NI	–	–	+/–	+

Symbols: *, PCA plates without septum; + growth; +/– reduced growth; – no growth.
Abbreviations: CF, ENV, AI, NI are Cystic fibrosis patient, environmental, animal infection and nosocomial infection, respectively.

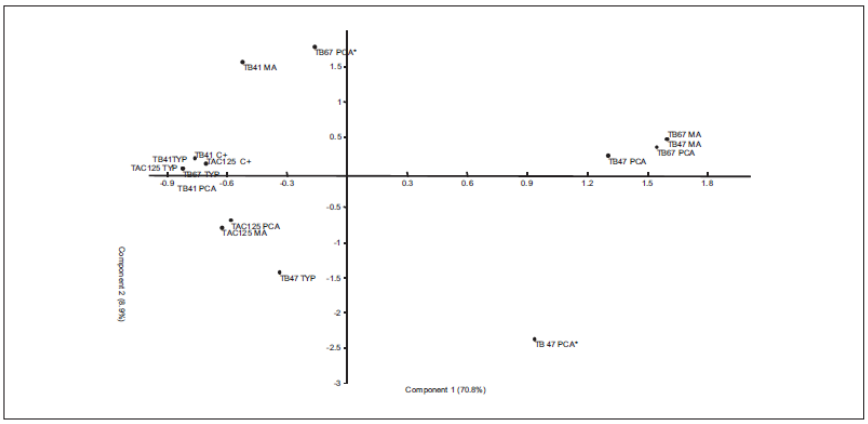


FIGURE 1

Principal component analysis of inhibitory patterns of *P. atlantica* TB41 and *P. haloplanktis* TAC125, and *Psychrobacter* sp. TB47 and TB67 strains versus a panel of 40 Bcc strains. Percentages indicate the variance explained by the first two components.

compared to isolation site in determining the inhibitory activity.

(iv) Concerning the effect of the different growth media of Antarctic bacteria on the inhibition of Bcc strains, data from Table 3 clearly revealed that, in the case of the two *Psychrobacter* strains (TB47 and TB67), the inhibition pattern was depending on the growth medium. Indeed, when the two strains were grown on TYP they were able to inhibit most (or all) the Bcc bacteria. The inhibitory effect was strongly reduced when the two *Psychrobacter* strains were grown either PCA or MA (with only a limited number, ranging from 5 to 10, of Bcc strains inhibited). A PCA on inhibitory patterns (Fig. 1) confirmed these findings, showing the presence of two main groups of activities, mainly separating (on the first principal component) the *Pseudoalteromonas* strains TAC125 and TB41 from *Psychrobacter* strains TB47 and TB67.

(v) The comparative analysis of data obtained from cross-streaking experiments performed on PCA medium using Petri dishes with and without septum, revealed that the absence of the septum allowed the two *Psychrobacter* strains (TB47 and TB67) to inhibit a much larger panel of Bcc strains. This finding might be explained by assuming that the two strains are able to synthesize *also* non-volatile antimicrobial compounds that were able to inhibit the growth of Bcc bacteria only in the absence of the physical barrier represented by the septum. However, we cannot a priori exclude the possibility that stronger inhibitory effect might be due to a higher concentration of mVOCs produced by the two strains embedding the growth medium of Bcc strains.

(vi) Concerning the sensitivity of Bcc strains towards the antimicrobial mVOCs produced by the Antarctic strains, a clustering of different sensitivity was observed (Fig. 2); notably, there was not a strong difference of sensitivity to the 'Antarctic' mVOCs between environmental and clinical strains, even though a slightly stronger inhibitory effect was observed on clinical isolates. Even though we cannot *a priori* exclude the possibility of a bias in the sampling (the number of environmental strains included in the panel is much lower than the clinical ones), these data might also reflect a biological issue, whose significance is still unknown.

Solid phase micro extraction GC–MS analysis

To try to identify the mVOCs produced by the Antarctic bacteria the SPME technique was used. The analysis was performed in

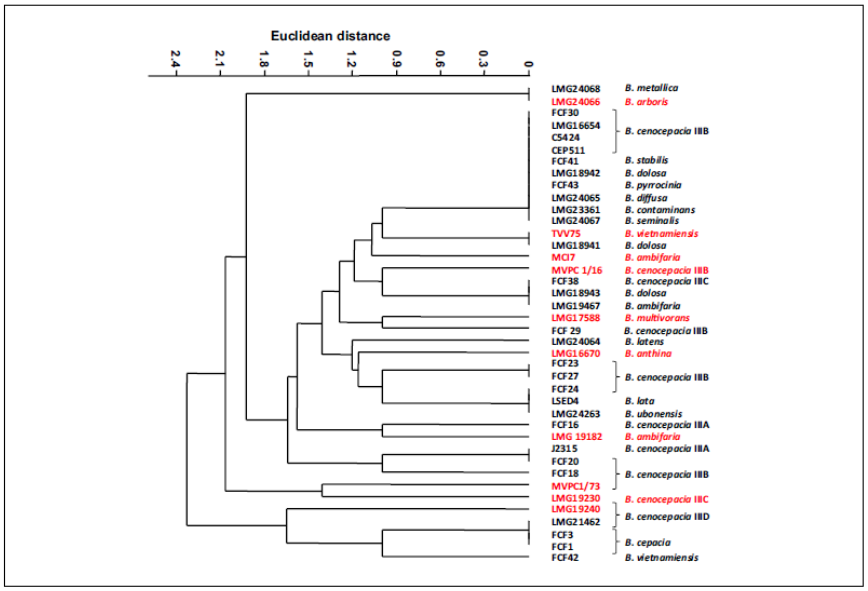


FIGURE 2

Cluster analysis (UPGMA) of 40 Bcc strains with respect to their pattern of inhibition by *P. atlantica* TB41, *P. haloplanktis* TAC125, and *Psychrobacter* sp. TB47 and TB67 strains. Strains in red are those with an environmental origin.

triplicate on *Psychrobacter* sp. TB47 and TB67, and *P. haloplankstis* TAC125 strains. The mVOC profile were obtained growing under the aerobic conditions (recently described by Romoli *et al.* [13]) the Antarctic strains either alone or in cross-streak conditions (using the *B. cenocepacia* LMG16654 as target strain) (i) to correlate the mVOC profile to the inhibition of Bcc strains growth, (ii) to check whether the mVOC production by Antarctic bacteria might be induced and/or modified by the presence of the target strain. All experiments were carried out growing the target and tester strains in PCA medium. The production of mVOCs by the target strain *B. cenocepacia* LMG16654 grown alone in PCA medium was also checked. Data obtained are reported in Fig. 3 and were compared with those previously obtained under anaerobic conditions [12] and with data coming from *P. atlantica* TB41 [13]. The analysis of the chromatograms revealed that:

1. mVOC profiles from the three Antarctic strains were very similar between them and to that exhibited by *P. atlantica* TB41 [13].
2. The profiles obtained under aerobic conditions appeared to be very different from those previously obtained in anaerobic conditions (Fig. 3). This is in agreement with recently findings by Romoli *et al.* [13] who showed that the production of mVOCs under aerobic conditions gave different volatolomic profiles in respect to those obtained previously [10] under anaerobic conditions.
3. The *B. cenocepacia* LMG16654 strain also produced a mVOC profile belonging to a different cluster than that of Antarctic strains (see below).
4. The growth of *B. cenocepacia* LMG16654 strain in the presence of the Antarctic bacteria was inhibited, according to previous findings [8,13].

The mVOC data obtained were then analyzed by a SVM-RFE method and a list of 30 different compounds that characterized the samples classification was extracted (Table 3). It can be noticed that some of these compounds are sulfur-containing ones.

To check whether Antarctic bacteria might be distinguished between them and/or from *B. cenocepacia* strain LMG16654, a hierarchical cluster analysis was performed using AMDIS to clean-up the raw data and extracting the mass spectra of components present also at low-level concentrations, but potentially informative. The amount of each component in the mVOC profile was expressed as relative concentration (area of single components versus the sum of total area) and not as absolute quantification. Indeed, the thirty components listed in Table 4 might be either present or not in the mVOC profiles, or expressed at different relative concentration in the different samples. Therefore, to

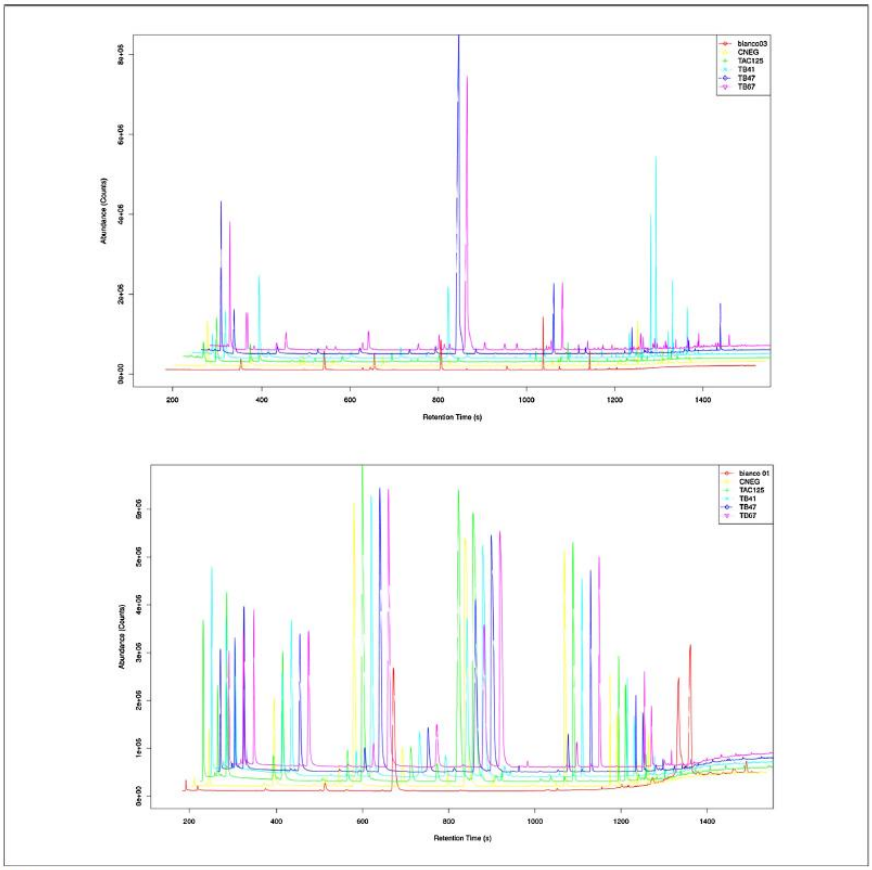


FIGURE 3

Total ion current of chromatogram of Antarctic bacteria in anaerobic (top) and aerobic (bottom) conditions.

extract the useful information both AMDIS and a SVM for recursive features elimination were used. Data obtained are reported in Fig. 4 where Antarctic Bacteria are clearly separated from blank and *B. cenocepacia* strain LMG16654. Moreover, *Pseudoalteromonas* strains TB41 and TAC125 clustered together and were separated from *Psychrobacter* spp. TB47 and TB67 samples, which, in turn, grouped together. It is worth of noticing that the mVOC profiles of the same strain grown alone or in the presence of the target strain *B. cenocepacia* LMG16654 (i.e. under cross-streak conditions) were clustered together. This revealed that the mVOC profiles obtained from Antarctic bacteria cells alone or in cross-streaking conditions were the same. This finding suggest that the *presence* of *B. cenocepacia* LMG16654 cells added in the cross-streaking conditions (and whose growth was inhibited) neither interferes with the production of mVOCs nor induces the synthesis of different mVOCs, in agreement with very recent data obtained from *P. atlantica* TB41 [13]. The *B. cenocepacia* strain LMG16654 mVOCs samples clustered together in a group clearly separated from those of Antarctic bacteria, as expected on the basis of the different profile exhibited by the target strain. Because in cross-

TABLE 4

List of compounds from Antarctic strains analysis, obtained excluding compounds present in both negative controls (i.e. in vials containing only PCA medium) and blank samples (i.e. empty vials)

	Compound		Compound
1	Carbon dioxide	16	2-Pentanone, 4-methyl-
2	2-Butene	17	Ethanethionic acid, S-methyl estere
3	2-Butene, 2 methyl-	18	Disulfide, dimethyl
4	1,3-Butadiene, 2 methyl-	19	1-Propanol, 2-methyl
5	Methanethiol	20	1-Butanol, 3-methyl
6	Carbon disulfide	21	S-Methylpropanethioate
7	Dimethyl sulfide	22	1-Butanol
8	Furan	23	1-Butanol, 3-methyl-
9	Acetone	24	S-Methyl, 3methylbutanethioate
10	Dimethyl selenite	25	3-Buten-1-ol, 3-methyl
11	Furan, 3-methyl-	26	Dimethyl trisulfide
12	Butanal	27	Oxime-, methoxy-phenyl-
13	2-Butanone	28	Acetophenone
14	2-Propanol	29	Disulfide, methyl-methylthio-methyl
15	2-Butanone, 3 methyl-	30	Phenylethyl Alcohol

streaking conditions the growth of *B. cenocepacia* LMG16654 cells was inhibited by the presence of Antarctic bacteria, this suggests that the mVOCs produced might be responsible for the growth inhibition.

The hierarchical cluster analysis splitting the Antarctic bacteria into two clusters, one embedding the *Pseudoalteromonas* samples and the other one including the *Psychrobacter* strains is in agreement with the cross-streaking experiments revealing that strains belonging to the same genus shared a (very) similar inhibition pattern, which, in turn, is different from that exhibited from strains belonging to the other group. Because the mVOCs produced by the four strains appeared to be the same, the different clustering and inhibition patterns might be attributed to the different (relative) concentration of one or (more probably) more mVOCs produced by the different strains. If this is confirmed, the different inhibition pattern might rely on a (complex?) combination of more mVOC with a different relative concentration.

Sequencing and preliminary analysis of P. atlantica *TB41 and* Psychrobacter *sp. TB47 and TB67 genomes*

Data reported in the previous sections suggested that the four Antarctic strains analyzed were able to inhibit the growth of the CF opportunistic pathogens belonging to the Bcc. By assuming that inhibition of these target strains might be due (at least) to some of

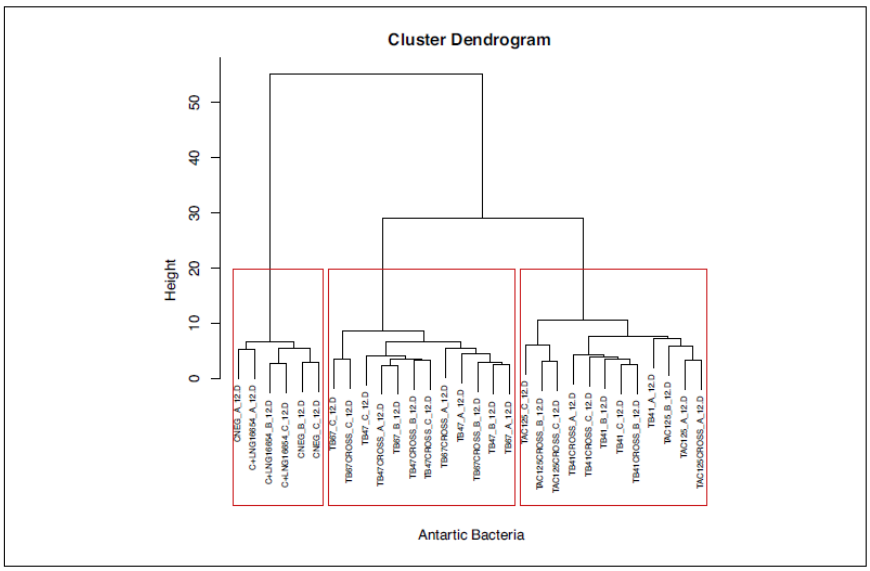


FIGURE 4

Hierarchical cluster analysis using Euclidean distances.

TABLE 5

Summary of the main features of the genomes sequenced from four Antarctic bacterial strains analyzed in this work

Strain	Taxonomy	Origin	Number of contigs	Assembled bp	%GC	ORFs
TAC125	*Pseudoalteromonas haloplanktis*	Seawater column	187	3,905,389	40.0	3523
TB41	*Pseudoalteromonas atlantica*	*A. joubini*	156	4,638,294	40.3	4222
TB47	*Psychrobacter* sp.	*A. joubini*	278	3,560,746	42.4	3221
TB67	*Psychrobacter* sp.	*A. joubini*	121	3,490,713	41.7	3060

the mVOCs produced by the four strains (and maybe to other non/volatile antimicrobial compounds), it could be possible that the genes responsible for the synthesis of such compounds might be shared by the four strains, and should belong to their common '*core*' genome.

With this aim, we determined the genome sequence of the three Antarctic strains, that is *P. atlantica* TB41 and *Psychrobacter* sp. TB47 and TB67. We also re-sequenced the genome of *P. haloplanktis* TAC125. The features of the four genomes are shown in Table 5; data obtained revealed that the genome of the two *Psychrobacter* strains (TB47 and TB67) possessed similar features, as it might be expected on the basis of their identical RAPD profiles (8). The two *Pseudoalteromonas* strains (TB41 and TAC125) are more divergent in terms of genome features and this might be also expected, because the two strains very probably are affiliated to two different species.

Because the cross-streaking experiments performed with the two *Psychrobacter* strains revealed an increased inhibitory effect when the assays were performed in Petri dishes without septum, it is reasonable to hypothesize that these two strains might synthesize non-volatile antimicrobial compounds. For this reason, the genome sequence of the two *Psychrobacter* strains was analyzed using the antiSMASH program [25], which allows the identification, annotation and analysis of gene clusters involved in the biosynthesis of secondary metabolites, which, in turn, might act as antimicrobial compounds. Data obtained revealed that, apparently, the two *Psychrobacter* sp. strains (TB47 and TB67) did not harbor any gene belonging to clusters involved in the biosynthesis of secondary metabolites. Therefore, it could be possible that the non-volatile antimicrobial compounds produced by *Psychrobacter* might be synthesized through still unknown and/or unusual biosynthetic pathways. The same search with antiSMASH was also carried out on the genome of the two *Pseudoalteromonas* strains (TB41 and TAC125). The scanning of the *P. atlantica* TB41 genome revealed the presence of a non-ribosomal peptide synthetases (*nrps*) and polyketide synthetases (*pks*) hybrid cluster. On the contrary, none of these genes were disclosed in the *P. haloplanktis* TAC125 genome. This is in full agreement with previous experimental data showing that *nrps-pks* genes were amplified via PCR only from TB41 genome [8].

In addition to this, if we agree with the previous assumption, that is the possibility that the genetic determinants responsible for the synthesis of antimicrobial compounds are shared by Antarctic bacteria, this implies that they might belong to the set of genes shared by the four genomes (the *core* genome). For this reason we performed a comparative analysis of the four genomes to identify the *core* and the *accessory* genomes. Data obtained are shown in Fig. 5, whose analysis revealed that:

(i) The *core* genome was represented by a set of 1174 genes (the entire list of these genes in reported in Additional file 1).

(ii) The number of unique genes (genes specific for a given strain) was much higher for the *Pseudoalteromonas* strains than that exhibited by *Psychrobacter* strains. This might be expected, because the two *Pseudoalteromonas* strains belong to two different species, whereas strains TB47 and TB67 very probably belong to the same *Psychrobacter* species.

(iii) In spite of their identity of RAPD profiles [8], the two *Psychrobacter* strains are clearly separated, even though they share a large proportion of genes.

We also characterized the genes belonging to the *core* and accessory genomes according to the Clusters of Orthologous Groups (COG) functional categories; data obtained are shown in Table 6 and Fig. 6. The analysis of these data revealed that, as expected, a large fraction of *core* genome genes are involved in the flux of genetic information, in energy production and conversion, as well as carbohydrate, amino acid, nucleotide, coenzyme and lipid transport and metabolism. An important

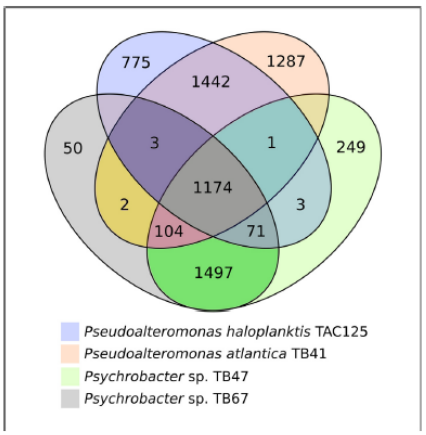


FIGURE 5

Schematic representation of the *core* and accessory genome of four Antarctic bacterial strains. The (slight) discrepancy between the actual number of ORFs of each genome (see Table 5) and the value that can be retrieved from the Venn diagram of the pangenome is due to the fact that the software used to obtain the pangenome groups the clusters of orthologous proteins and counts them as one.

TABLE 6

Classification, according to the Clusters of Orthologous Groups (COG) categories, of the genes belonging to the *core* or the accessory genomes of the *Pseudoalteromonas* and *Psychrobacter* strains analyzed in this work

COG functional category	Description	Accessory genome		Core genome	
		No. of genes	%	No. of genes	%
J	Translation, ribosomal structure and biogenesis	60	1.09	135	11.49
A	RNA processing and modification	0	0.00	1	0.08
K	Transcription	224	4.08	45	3.83
L	Replication, recombination and repair	324	5.90	71	6.04
B	Chromatin structure and dynamics	2	0.03	1	0.08
D	Cell cycle control, cell division, chromosome partitioning	22	0.40	17	1.44
Y	Nuclear structure	0	0.00	0	0.00
V	Defense mechanisms	72	1.31	20	1.70
T	Signal transduction mechanisms	220	4.01	36	3.06
M	Cell wall/membrane/envelope biogenesis	186	3.39	81	6.89
N	Cell motility	105	1.91	18	1.53
Z	Cytoskeleton	0	0.00	0	0.00
W	Extracellular structures	0	0.00	0	0.00
U	Intracellular trafficking, secretion, and vesicular transport	36	0.65	21	1.78
O	Post-translational modification, protein turnover, chaperones	114	2.07	73	6.21
C	Energy production and conversion	155	2.82	99	8.43
G	Carbohydrate transport and metabolism	137	2.49	30	2.55
E	Amino acid transport and metabolism	245	4.46	105	8.94
F	Nucleotide transport and metabolism	43	0.78	41	3.49
H	Coenzyme transport and metabolism	87	1.58	65	5.53
I	Lipid transport and metabolism	157	2.86	56	4.77
P	Inorganic ion transport and metabolism	203	3.70	44	3.74
Q	Secondary metabolites biosynthesis, transport and catabolism	59	1.00	11	0.93
R	General function prediction only	362	6.60	106	9.02
S	Function unknown	400	7.29	73	6.21
X	No functional class found	2271	41.41	25	2.12

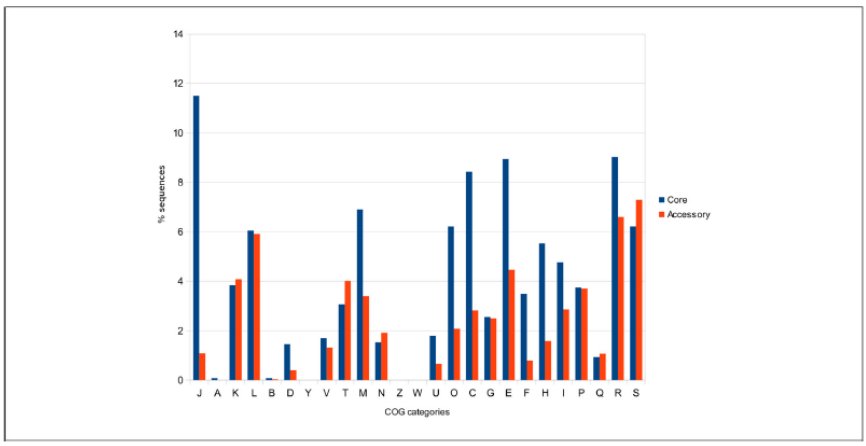


FIGURE 6

Classification, according to the Clusters of Orthologous Groups (COG) categories, of the genes belonging to the *core* and the *accessory* genomes of four Antarctic bacterial strains analyzed in this work.

TABLE 7

List of the eleven genes belonging to the core genome of Antarctic strains TAC125, TB41, TB47 and TB67 and putatively involved in the biosynthesis of secondary metabolites

COG2761 Predicted dithiol-disulfide isomerase involved in polyketide biosynthesis
COG1335 Amidases related to nicotinamidase
COG2132 Putative multicopper oxidases
COG0179 2-keto-4-pentenoate hydratase/2-oxohepta-3-ene-1,7-dioic acid hydratase (catechol pathway)
COG4181 Predicted ABC-type transport system involved in lysophospholipase L1 biosynthesis, ATPase component
COG3127 Predicted ABC-type transport system involved in lysophospholipase L1 biosynthesis, permease component
COG1228 Imidazolonepropionase and related amidohydrolases
COG0500 SAM-dependent methyltransferases
COG0500 SAM-dependent methyltransferases
COG0500 SAM-dependent methyltransferases
COG0500 SAM-dependent methyltransferases

fraction of genes (204 genes) belong to classes R, S and X, that is those embedding generic or unknown COGs (Fig. 6).

A very low number of genes (11, less than 1%) shared by the four genomes are involved in secondary metabolites biosynthesis, transport and catabolism. These genes are listed in Table 7.

Conclusions

In this work we have further characterized a panel of four Antarctic bacteria able to inhibit the growth of opportunistic pathogens belonging to the Bcc through the production of a set of mVOCs. Even though the biological significance of such production is still unclear, these compounds are synthesized under aerobic conditions by Antarctic bacteria. A list of 30 different mVOCs was identified by GC-SPME analysis. The cross-streaking experiments performed with Petri dishes without a septum also suggested that non-volatile molecules with an anti-*Burkholderia* activity might be synthesized by Antarctic bacteria. The biosynthesis of such a mixture of mVOCs was very probably influenced by both the presence/absence of oxygen and the media used to grow the Antarctic strains. The antimicrobial activity exhibited by Antarctic strains also appeared to be more related to their taxonomical position rather than to the sampling site. The sensitivity of Bcc bacteria versus the 'Antarctic' mVOCs is apparently related neither to the taxonomical position of the different strains nor to their source, even though a slightly stronger inhibitory effect might be observed for clinical isolates. Concerning the molecular basis of the antibacterial molecules production the genome analysis of the four Antarctic strains revealed that only *P. atlantica* TB41 possessed some genes belonging to the *nrps–pks* cluster, similarly to the *Arthrobacter* sp. TB23 strain isolated from *Haliclonissa verrucosa* Antarctic sponge whose genome sequence has been recently reported [26]. The analysis of the TB23 strain genome revealed the presence of three gene clusters including a type III PKS, a NRPS gene, and terpene biosynthetic genes, respectively, which might be involved in the biosynthesis of antimicrobial compounds targeted towards Bcc bacteria. The comparative genomic analysis performed on the genome of the four strains also revealed the presence of a few genes belonging to the *core* genome and involved in the secondary metabolites biosynthesis. However, the role of these genes in the biosynthesis of mVOCs is still unclear. In our opinion, it is possible that the antimicrobial activity exhibited by Antarctic bacteria might be rely on a (complex) mixture of mVOCs whose relative concentration may vary depending on the growth conditions. Besides, it is also possible that the biosynthesis of these compounds occurs through unknown metabolic pathways. By contrast, it is not still clear if these compounds are targeted towards a single molecular target of Bcc cells or to a plethora of them. However, even though different Bcc strains were differently sensitive to the 'Antarctic' mVOCs, it is plausible that Bcc bacteria may share at least some of the genetic determinants responsible for the synthesis of molecule(s) targeted by Antarctic bacteria. Thus, it is quite possible that the *core* genome of Bcc strains contains the genes responsible for the synthesis of such targets. Sequencing of the genome of several Bcc strains is in progress and will allow to shed some light on this issue. Finally, data obtained confirmed that the mVOCs synthesized by Antarctic bacteria may act as new natural antimicrobial compounds that might be exploited in the control of infections in CF patients by significant multidrug-resistant bacterial pathogens.

Acknowledgements

This work was supported by Italian Cystic Fibrosis Research Foundation (FFC project 12#2011) and Ente Cassa di Risparmio (Grant 2008.1103). Valerio Orlandini and Marco Fondi are financially supported by an Italian Cystic Fibrosis Research Foundation fellowship and a FEMS Advanced Fellowship (FAF2012), respectively. We thank the EU KBBE Project Pharmasea 2012–2016. We are also very grateful to two anonymous referees for their helpful comments and suggestions in improving the manuscript. *P. atlantica* TB41, and *Psychrobacter* spp. TB47 and TB67 belong to the Italian Collection of Antarctic Bacteria of the National Antarctic Museum (CIBAN-MNA).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nbt.2013.03.011.

References

[1] de Pascale D. The microbial diversity of Polar environments is a fertile ground for bioprospecting. Marine Genom 2012;8:15–22.

[2] Bej S. Polar microbiology. In: The Ecology, Biodiversity and Bioremediation Potential of Microorganisms in Extremely Cold Environments. Boca Raton, FL: CRC Press; 2010.

[3] Baker JT. Natural product drug discovery: new perspective on international collaboration. J Nat Prod 1995;58:1325–57.

[4] Minerdi D. Volatile organic compounds: a potential direct long-distance mechanism for antagonistic action of *Fusarium oxysporum* strain MSA 35. Environ Microbiol 2009;11:844–54.

[5] Vining LC. Functions of secondary metabolites. Annu Rev Microbiol 1990; 44:395–427.
[6] Bentley B. Biosynthesis of secondary metabolites in berry. In: Physiology of Industrial Fungi; 1998. pp. 161–183.
[7] Ireland CM. In: Martin RE, Carter EP, Davis LM, editors. Bioactive Compounds from the Sea. Technomic Publishing; 2000.
[8] Papaleo MC. Sponge-associated microbial Antarctic communities exhibiting antimicrobial activity against *Burkholderia cepacia* complex bacteria. Biotechnol Adv 2012;30:272–93.
[9] Nikaido H. Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. Semin Cell Dev Biol 2001;12:215–23.
[10] Ryan BM. Efflux in bacteria: what do we really know about it. Expert Opin Investig Drugs 2001;10:1409–22.
[11] Tegos G. Dissecting novel virulent determinants in the *Burkholderia cepacia* complex. Virulence 2012;3:1–4.
[12] Romoli R. Characterization of the volatile profile of antarctic bacteria by using solid-phase microextraction-gas chromatography–mass spectrometry. J Mass Spectrom 2011;46:1051–9.
[13] Romoli, R. Characterization of HS-SPME-GC–MS volatolomic profile of antarctic bacteria. *Metabolomics* (submitted for publication)
[14] Sunesson AL. Influence of temperature, oxygen and carbon dioxide levels on the production of volatile metabolites from *Streptomyces albidoflavus* cultivated on gypsum board and tryptone glucose extract agar. Ann Occup Hygiene 1997; 41:393–413.
[15] Medigue C. Coping with cold: the genome of the versatile marine Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125. Genome Res 2005;15:1325–35.
[16] Hammer Ø. PAST: paleontological statistics software package for education and data analysis. Palaeontol Electron 2001;41:9.
[17] Papaleo MC. Identification of species of the *Burkholderia cepacia* complex by sequence analysis of the *hisA* gene. J Med Microbiol 2010;59:1163–70.
[18] Cock PJA. The Sanger FASTQ file format for sequences with quality scores, and the Solexa/Illumina FASTQ variants. Nucleic Acids Res 2010;38:1767–71.
[19] Simpson JT. ABySS: a parallel assembler for short read sequence data. Genome Res 2009;19:1117–23.
[20] Hyatt D. Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics 2012;8:119.
[21] Alexeyenko A. Automatic clustering of orthologs and in paralogs shared by multiple proteomes. Bioinformatics 2006;22:e9–15.
[22] Bicchi C. Reliability of fibers in solid-phase microextraction for routine analysis of the headspace of aromatic and medicinal plants. J Chromatogr A 2007; 1152:138–49.
[23] Guyon I. Gene selection for cancer classification using support vector machines. Mach Learn 2002;46:389–422.
[24] R Development Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2012 , ISBN: 3-900051-07-0.
[25] Medema MH. antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. Nucleic Acids Res 2011;39:W339–46.
[26] Fondi M. The draft genome of the VOCs-producing Antarctic bacterium *Arthrobacter* sp. TB23 able to inhibit Cystic Fibrosis pathogens belonging to the *Burkholderia cepacia* complex. J Bacteriol 2012;194:6334–5.

Extremophiles
DOI 10.1007/s00792-013-0590-0

ORIGINAL PAPER

Phenotypic and genomic characterization of the Antarctic bacterium *Gillisia* sp. CAL575, a producer of antimicrobial compounds

Isabel Maida · Marco Fondi · Maria Cristiana Papaleo · Elena Perrin · Valerio Orlandini · Giovanni Emiliani · Donatella de Pascale · Ermenegilda Parrilli · Maria Luisa Tutino · Luigi Michaud · Angelina Lo Giudice · Riccardo Romoli · Gianluca Bartolucci · Renato Fani

Received: 15 March 2013 / Accepted: 26 September 2013

Abstract Microorganisms from Antarctica have evolved particular strategies to cope with cold. Moreover, they have been recently reported as producers of antimicrobial compounds, which inhibit the growth of other bacteria. In this work we characterized from different viewpoints the *Gillisia* sp. CAL575 strain, a psychrotrophic bacterium that produces microbial volatile organic compounds involved in the growth inhibition of *Burkholderia cepacia* complex members. Sequencing and analysis of the whole genome of *Gillisia* sp. CAL575 revealed that it includes genes that are involved in secondary metabolite production, adaptation to cold conditions, and different metabolic pathways for the production of energy. All these features make *Gillisia* sp. CAL575 a possible tool for biotechnology.

Communicated by L. Huang.

Electronic supplementary material The online version of this article (doi:10.1007/s00792-013-0590-0) contains supplementary material, which is available to authorized users.

I. Maida · M. Fondi · M. C. Papaleo · E. Perrin · V. Orlandini · R. Fani (✉)
Laboratory of Microbial and Molecular Evolution, Department of Biology, University of Florence, Via Madonna del Piano 6, 50019 Sesto Fiorentino (Florence), Italy
e-mail: renato.fani@unifi.it

G. Emiliani
Trees and Timber Institute, National Research Council, via Madonna del Piano 10, 50019 Sesto Fiorentino (Florence), Italy

D. de Pascale
Institute of Protein Biochemistry, National Research Council, Via P. Castellino, 111, 80134 Naples, Italy

E. Parrilli · M. L. Tutino
Department of Chemical Sciences, University of Naples Federico II, Naples, Italy

Keywords Antarctic bacterium · *Gillisia* sp. CAL575 · Cystic fibrosis · *Burkholderia cepacia* complex · VOCs

Introduction

The vast majority of Earth' surface is covered by water and >90 % of its surface experiences yearly temperatures <15 °C. Moving toward polar latitudes, average water temperature approaches the freezing point, and organisms colonizing these habitats are expected to have evolved strategies to cope with low temperatures. In particular, cold-adapted microorganisms survive and proliferate at permanently low temperatures thanks to a variety of structural and functional adaptations (Casanueva et al. 2010).

The exploitation of bacterial biodiversity is still the main road toward the discovery of novel bioactive

E. Parrilli · M. L. Tutino
School of Biotechnological Sciences, University of Naples Federico II, Complesso Universitario M. S. Angelo, Via Cintia, 80126 Naples, Italy

L. Michaud · A. Lo Giudice
Department of Biological and Environmental Sciences (DiSBA-CIBAN), University of Messina, Viale Ferdinando Stagno d'Alcontrès 31, 98166 Messina, Italy

R. Romoli
Mass Spectrometry Center (CISM), Polo Scientifico, Via U. Schiff 6, 50019 Sesto Fiorentino (FI), Italy

G. Bartolucci
Department of Neurosciences, Psychology, Drug Research and Child Health, Section of Pharmaceutical and Nutraceutical Sciences, University of Florence, Via U. Schiff, 6, 50019 Sesto Fiorentino (FI), Italy

Published online: 23 October 2013

compounds. In particular, microorganisms from marine sources continue to yield novel compounds with a broad array of bioactivity. These compounds seemed to be promising in treating cancer, pain, inflammation, allergies, and bacterial or viral infections (Newman and Cragg 2004). Antarctic microorganisms can synthesize, likely as a result of environmental stresses (Baker et al. 1995), a broad range of potentially valuable natural compounds, mainly soluble secondary metabolites, many of which can be volatile. Therefore, bacteria from Antarctica represent an untapped reservoir of biodiversity, and only few studies have been performed on the antimicrobial activity of isolates from Antarctic soils (O'Brien et al. 2004), seawater and sediments (Lo Giudice et al. 2007a; Lo Giudice et al. 2007b; Papaleo et al. 2012). We have recently reported (Papaleo et al. 2012) the characterization of 132 sponge-associated Antarctic strains, which have been proved to display (although to different extents) an antimicrobial activity against *Burkholderia cepacia* complex (Bcc) bacteria, a wide group of opportunistic pathogens that can colonize the lung of Cystic Fibrosis (CF) patients. The inhibitory activity very likely relies on the production of microbial Volatile Organic Compounds (mVOCs) by Antarctic bacteria (Romoli et al. 2011). 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 (at least) 30 different compounds that might be responsible for the inhibition of the growth of Bcc bacteria (Romoli et al. 2013, Papaleo et al. 2013). Moreover, it has been also demonstrated that the synthesis of mVOCs by Antarctic bacteria is strongly dependent on the growth media composition (Papaleo et al. 2013).

Among the isolates characterized by Papaleo and coworkers (2012), five *Gillisia* representatives were also identified, all coming from one of the studied sponges, *Haliclonissa verrucosa*. The genus *Gillisia* includes Gram-negative, rod-shaped cells and belongs to the family *Flavobacteriaceae*, whose representatives constitute a considerable fraction of ocean bacterioplankton (Ivanova et al. 2004). Members of the family *Flavobacteriaceae* play significant roles in aquatic ecosystems since investigations highlighted that marine genera are responsible for a major fraction of organic matter remineralisation in the oceans (Bowman et al. 2000; Kirchman 2002). Moreover, strains belonging to the genus *Gillisia* are strictly aerobic, moderately halotolerant, psychrophilic and chemoheterotrophic. They have been reported to produce yellow pigments and gliding motility is generally not detected. To date, the genus includes six species whose strains have been isolated from Antarctica (microbial mats), Japan (seawater) and USA (associated to a marine sponge) (Nam et al. 2012). The *Gillisia* strains analyzed by Papaleo et al. (2012) showed an inhibitory activity lower than that exhibited by other Antarctic bacteria belonging to different genera and isolated from either the same or different sponge(s). In addition to this, it was not completely clear whether the inhibitory activity was linked to the synthesis of mVOCs. Therefore, in this work we have characterized the *Gillisia* sp. CAL575 strain, one of the five representatives that was isolated from the sponge *H. verrucosa*, using a combination of different techniques, including genomics, phenotypic characterization and analysis of mVOCs, in order to (1) determine whether the inhibitory activity was dependent on the growth media, (2) whether it might be attributed to the synthesis of mVOCs, (3) or whether it is linked to non-volatile molecules, (4) identify genes/metabolic pathways involved in the biosynthesis of antimicrobial compounds, (5) try to shed some light on the mechanism(s) of inhibition of Bcc strains growth, and lastly, (6) determine the whole genome sequence of this strain and carry out the comparative genomic analysis, which might also help in elucidating the mechanisms of adaptation to low temperatures.

Results and discussion

Phenotypic characterization and phylogenetic affiliation of *Gillisia* sp. CAL575 strain

The phenotypic characterization of *Gillisia* sp. CAL575 was carried out as described in the "Materials and methods" section. Data obtained revealed that CAL575 cells are Gram-negative, strictly aerobic with an oxidative metabolism, occur as asporogenic rods, and are non-motile. Colonies are circular, convex, and shiny with entire edges, 2–4 mm in diameter on marine agar 2216. It produces yellow non-diffusible pigments. Growth occurs between pH 6 and 9 (pH 7–8 optimum) and between 4 and 30 °C (with an optimum range between 10 and 15 °C), but not at 37 °C or higher temperatures. The strain requires NaCl (7–13 %; 9–11 % optimum) for growth and does not grow on TSA and TCBS. It is not able to hydrolyze agar, chitin, starch and tween 80, whereas aesculin and gelatin are degraded. Acids from carbohydrates included in the API20E system are not produced. The following substances: glucose, arabinose, mannose, mannitol, *N*-acetyl-glucosamine, maltose, gluconate, caprate, adipate, malate, citrate and phenyl-acetate were not utilized by CAL575 strain as the sole carbon and energy source. Nitrate reduction is negative. H_2S, indole and acetoin (Voges–Proskauer reaction) are not produced. It was positive for oxidase, catalase and β-galactosidase and negative for urease, ornithine and lysine decarboxylase, arginine

dihydrolase and tryptophan deaminase activities. The strain was also tested for its ability to grow in the presence of different antibiotics and it was found to be sensitive to ampicillin (25 µg), chloramphenicol (30 µg), penicillin G (10 µg) and tetracycline (30 µg), whereas it was resistant to nalidixic acid (30 µg), O/129 (10 µg), polymyxin B (30 µg) and tobramycin (10 µg). The similarities in phenotypic characteristics support the inclusion of the strain studied in the genus *Gillisia*. However, strain CAL575 clearly differed from strains of *G. limnaea* and *G. mitskevichiae* by the NaCl requirement for growth, utilization of glucose and the ability to hydrolyze Tween 80 (Supplementary Material 1).

Previous phylogenetic data, based on the sequencing and analysis of the 16S rRNA gene partial sequence, affiliated the strain CAL575 to the genus *Gillisia* (Papaleo et al. 2012). In order to obtain a more precise affiliation, the complete 16S rRNA gene (1,519 bp) sequence was recovered from the contigs (see below) and was aligned to the most similar ones retrieved from databases. The phylogenetic tree constructed using this alignment revealed that this strain is embedded into the *Flavobacteriaceae* family, joining the genus *Gillisia* (Supplementary Material 2). Then, we aligned this sequence with all the *Gillisia* 16S rRNA gene sequences available in the RDP (Cole et al. 2009). The alignment was then used to construct the phylogenetic tree shown in Fig. 1, whose analysis revealed that the strain CAL575 is closely related to the *G. mitskevichiae* species (Lee et al. 2006; Nedashkovskaya et al. 2005). The value of 16S rRNA gene sequence identity (99.6 %) shared by *Gillisia* sp. CAL575 and *G. mitskevichiae* AY576655 strongly suggests that the two strains might belong to the same species, even though the correct assignment of *Gillisia* sp. CAL575 to a given species would require additional tests.

Sequencing and assembly of *Gillisia* sp. CAL575 genome

In order to further characterize (at the genomic level) the *Gillisia* sp. CAL575 strain, the nucleotide sequence of its genome was obtained using the Illumina paired-end sequencing technique. Twenty millions of reads were obtained and their mean length after the trimming was of 74 bp. The assembly performed using the Ray software (see "Materials and methods"), resulted in 56 contigs with a length ranging from 130 to 735,795 bp, with an average length of about 6,500 bp (and a median of almost 7,000 bp). The total amount of assembled sequences corresponded to 3,647,045 bp.

Gillisia sp. CAL575 genome general features

The assembled base pairs exhibited a 33.89 % G+C content, a value that is within the range of G+C content observed for the other members of the genus *Gillisia* (32–38 %) (Lee et al. 2006). Genome annotation using the RAST annotation system (Aziz et al. 2008) ("Materials and methods") allowed prediction of 3,273 ORFs. A hypothetical function was assigned to most of them (2003, 61.2 %) and 42 RNAs genes (including both tRNAs and rRNAs) were found (Table 1; Supplementary Material 3).

Moreover, using the RAST server it was possible to identify genes that are not in a subsystem (i.e. a set of functional roles that together implement a specific biological process or structural complex). Frequently, subsystems represent the collection of functional processes that make up a metabolic pathway, a complex (e.g., the ribosome), or a class of proteins (Aziz et al. 2008). Thus, it was possible to classify a total of 1,277 genes as belonging to a particular subsystem (Fig. 2) (49 of them have a hypothetical function) and 2,046 genes that are not in subsystems (1,221 of them have a hypothetical function).

The *Gillisia* pangenome

The genomic data obtained were used, together with the sequences of the two *Gillisia* genomes available on NCBI GenBank as of January 2013 (namely, *Gillisia limnaea* DSM 15749 and *Gillisia* sp. CBA3202), allowed to determine the pangenome of these strains. Data obtained

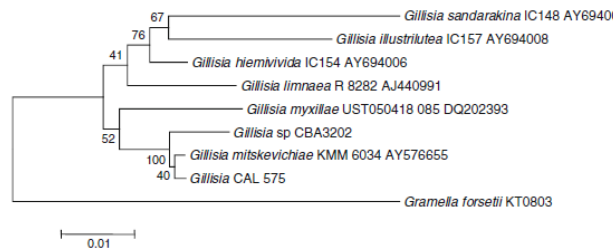


Fig. 1 Phylogenetic tree constructed using the 16S rRNA gene sequences representative of all the known *Gillisia* species

revealed that 1843 genes are shared by the three strains, and that *Gillisia limnaea* and *Gillisia* sp. CAL575 share a much higher number of genes (525) between them than with *Gillisia* sp. CBA3202 (117 and 191, respectively) (Fig. 3). This might be expected since the former two strains share the same Antarctic environment, whereas the latter is typical of temperate marine areas. Given the different environments these strains live in, it was interesting to characterize the function of the genes of the *core* genome and of each part of the accessory ones. This characterization was carried out according to the COG (Cluster of Orthologous Groups) functional categories (Tatusov et al. 2003). Figure 4 reports the histogram summarizing the results of this analysis, with each bar indicating an intersection of the Venn diagram of Fig. 3. It can be noted that most of the genes shared (almost 20 %, a large percentage considering that the vast majority of genes were not characterized and therefore excluded from these observations) by the two Antarctic strains only belongs to the categories C (energy production, 7 %) and E (amino acid transport and metabolism, 12 %). These sets might contain part of the genes involved in the adaptation to this extreme environment.

Table 1 General features of the *Gillisia* sp. CAL575 genome

Attribute	Value
Assembled base pairs (bp)	3,647,045
Number of contigs	56
Mean of contigs length (bp)	6,500
G + C content (%)	33.89
Number of genes	3,315
Number of 5S RNA genes	2
Number of 16S RNA genes	1
Number of 23S RNA genes	1
Number of tRNA genes	38
Number of coding sequences	3,273
Genes with predicted function	2,003

General metabolism

The analysis performed revealed the absence in the *Gillisia* sp. CAL575 genome of genes involved in photosynthesis or genes related to motility and chemotaxis (Fig. 2). These data are in agreement with the phenotypic characterization (see above) and with the notion that the species *G. mitskevichiae* (closely related to the strain CAL575) is described as devoid of gliding and flagellar motility (Nedashkovskaya et al. 2005). The *Gillisia* sp. CAL575 genome also harbors genes related to mobile genetic elements (MGE), including one phage intron, one putative mobilization protein and six conjugative transposons. Preliminary data on the plasmid content have shown that this strain does not

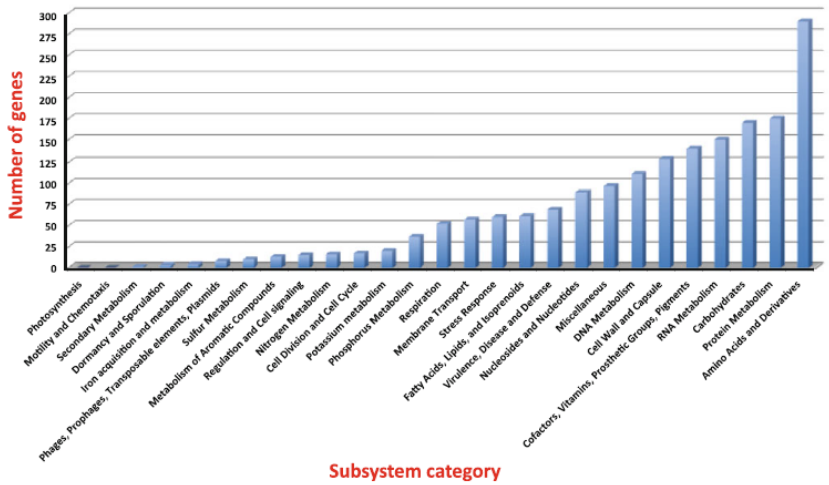


Fig. 2 Distribution of genes involved in the subsytems. The order is from the smallest to the larger number of genes

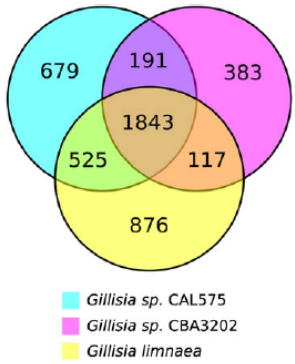


Fig. 3 Venn diagram representing the pangenome of the three *Gillisia* strains analyzed in this work

possess plasmid molecules in its cytoplasm (Papaleo et al. 2012), but the methodology adopted for this analysis did not permit the isolation of plasmids whose size is higher than 40 kb. Therefore, we cannot exclude the possibility that a large plasmid might be present and that the MGE retrieved in this genome might be localized on large plasmid molecules.

Sixty genes for the response to the stress were found, with half of them (i.e. 33) related to oxidative stress. Among the remaining 27 genes, 13 of them encode heat shock proteins, 3 are induced in response to temperature downshifts (cold shock, 2 *cspA* and 1 *cspG*), enabling cells to adapt to cold temperatures, the other 11 genes encode proteins related to periplasmic response, detoxification and generic stress response. In this context, it is interesting to note that in the subsystem related to protein metabolism including 175 members, the two heat shock protein 60 family chaperones GroES and GroEL are also present. These molecular chaperones may help the *Gillisia* sp. CAL575 strain to survive in cold environments (Rodrigues and Tiedje 2008). In all the other subsystem categories, there are a variable number of genes and the group that includes most of them is the one that includes the amino acid metabolism in general.

Genes involved in cold adaptation, salt adaptation, osmotic shock protection, energy production and in ROS response

Gillisia sp. CAL575 possesses the ability to grow at a temperature ranging between 4 and 30 °C. For this reason we explored its genome for the presence of those genes involved in cold adaptation identified by Medigue and colleagues (2005) in the genome of the psychrophilic Antarctic bacterium *P. haloplanktis* TAC125. Moreover, we extended this analysis to the two genomes available in database, belonging to the genus *Gillisia*, i.e. *G. limnaea* DSM 15749 that was isolated from a microbial mat in Lake Fryxell (Antarctica) (Riedel et al. 2012) and *Gillisia* sp. CBA3202 that was isolated from sand of the seashore on Jeju Island, Republic of Korea (Nam et al. 2012). Data

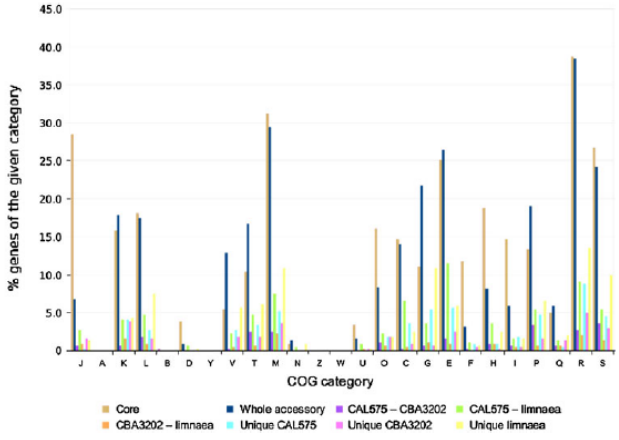


Fig. 4 Functional characterization, according to the COG database, of genes found in the pangenome of the three *Gillisia* strains taken into account. Genes belonging to the core and to the accessory genomes are split into two different *bars*. Genes embedded in the accessory genome are further split according to the exact subset they belong to

obtained are reported in Supplemental Material 4 and showed that most of the 30 specific genes searched were shared by these genomes, suggesting that the cold adaptation eventually conferred by these functions evolved early during the *Gillisia* genus evolution. However, it is worth of mentioning that the genomes of Antarctic *Gillisia* strains do share a larger number of genes. This observation opens the fascinating possibility that other genes (out of those listed in Supplemental Material 4) may be responsible of the cold lifestyle of *Gillisia* sp. CAL575 and *G. limnaea* DSM 15749.

The *Gillisia* sp. CAL575 cells can grow in media containing NaCl (7–13 %; 9–11 % optimum); for this reason we searched for the presence of genetic systems that would account for controlling cell osmolarity (Supplemental Material 4). The absence of any choline dehydrogenase encoding gene ruled out the possibility for the *Gillisia* strains to synthesize glycine and betaine, an extremely efficient osmoprotectant (Felitsk et al. 2004). On the contrary, the presence of GOGAT glutamate synthase suggests the possibility that glutamate, as potassium glutamate, may represent the main cellular osmobalancer, the most common response of bacteria to increased osmolarity (Lee and Gralla 2004).

The solubility of gasses increases rapidly at low temperature. This is the case of dioxygen, which is a very reactive molecule. We expected that the proteome would comprise a vast arsenal of enzymes active against H_2O_2 and superoxide. Thus, we searched for the genes involved in the response to the reactive oxygen species (ROS) in these genomes. Data reported in Table 3 showed that there are differences in the gene patterns involved in the response to ROS among the three genomes; indeed, most of these genes are present in the genome of the two Antarctic strains but only 3 genes were found in the genome of *Gillisia* sp. CBA3202. Thus, the two Antarctic *Gillisia* strains might be adapted to protection against ROS under cold conditions.

Finally, starting from the phenotypic characterization of *Gillisia* sp. CAL575 substrate profile, we checked the presence in the three genomes for genes involved in energy production, i.e. those coding for the enzymes involved in glycolysis or for the alternative Entner–Doudoroff pathway, that is an oxidative shunt allowing bacteria to produce energy from gluconate catabolism (Ponce et al. 2005). Data obtained are reported as Supplemental Material 5 and revealed that just one gene involved in glycolysis was absent in the genomes of *Gillisia* sp. CAL575 and *G. limnaea* DSM 15749, whereas in *Gillisia* sp. CBA3202 genome two genes are missing. Concerning the Entner–Doudoroff pathway, all the four genes encoding the relative enzymes are absent in *Gillisia* sp. CBA3202, while two of them are harbored by the Antarctic strains genomes. These data are in further agreement with the reported inability of *Gillisia* sp. CAL575 to grow on glucose or gluconate as the sole carbon source, and suggest that genus *Gillisia* might have evolved to colonize environments in which peptides or amino acids represent the main carbon and energy sources. Evaluating the genetic determinants for the anaerobic respiration, the genome of the three *Gillisia* strains are devoid of most of genes involved in nitrate and fumarate reduction, in line with the experimental observation that *Gillisia* sp. CAL575 cannot grow under anaerobic conditions.

Genes involved in secondary metabolism

It is known that secondary metabolites can act as antimicrobial molecules and, in some cases, they are synthesized by enzymes encoded by gene clusters, like polyketide synthase genes (*pks*) (Staunton and Weissman 2001). We have previously checked for the presence of *pks* type I genes in the genome of CAL575 through PCR amplification using primers targeted toward *pks* gene, but no amplicon was obtained under the experimental conditions used (Papaleo et al. 2012). The availability of the draft genome of this strain allowed to perform a deeper analysis on the genome of *Gillisia* sp. CAL575, in order to check for the presence of such gene cluster(s). According to experimental data, we did not find genes encoding class I *pks*. However, two gene clusters (each of which consisting of about 20 genes) involved in the production of *pks* type III and terpene, respectively, were detected. The use of Antismash software (Medema et al. 2011) allowed identifying putative orthologs of the terpene gene cluster in *Gillisia limnaea* DSM 1749 and in *Gramella forsetii* KT 08023; conversely the *pks* type III cluster exhibited orthologs in *G. forsetii* KT 08023 and *Robiginitalea biformata* HTCC250 (Supplementary Material 6).

It has been reported that other Antarctic strains belonging to the genera *Arthrobacter* (Orlandini et al. 2013), *Psychrobacter* and *Pseudoalteromonas* (Papaleo et al. 2013) harbor a (very) limited number of genes involved in the production of secondary metabolites, different from that found in the *Gillisia* genomes analyzed in this work. Thus, it is quite possible that the antimicrobial activity exhibited by members of different Antarctic bacterial genera might rely on the production of mVOCs.

Inhibition of *Burkholderia cepacia* complex strains growth by *Gillisia* sp. CAL575

Preliminary data demonstrated that *Gillisia* sp. CAL575 was able to inhibit the growth of some Bcc strains. Moreover, this inhibition was attributed (at least partly) to the production of mVOCs, as shown for other sponge-associated Antarctic bacteria (Papaleo et al. 2012). Since it

Table 2 Growth of 38 strains belonging to the *Burkholderia cepacia* complex in the presence of the Antarctic bacterium *Gillisia* sp. CAL575 grown in three different media (PCA, MA, TYP)

Strain	Species	Origin	PCA	MA	TYP
FCF 1	*B. cepacia* (*I*)	CF	−	−	−
FCF 3	*B. cepacia* (*I*)	CF	−	−	−
LMG 17588	*B. multivorans* (*II*)	Environmental	+	−	+
FCF 16	*B. cenocepacia* (*IIIA*)	CF	−	−	−
J2315	*B. cenocepacia* (*IIIA*)	CF	−	−	+
FCF 18	*B. cenocepacia* (*IIIB*)	CF	−	−	+
FCF 20	*B. cenocepacia* (*IIIB*)	CF	−	−	+
FCF 23	*B. cenocepacia* (*IIIB*)	CF	−	−	+
FCF 27	*B. cenocepacia* (*IIIB*)	CF	−	−	+
FCF 29	*B. cenocepacia* (*IIIB*)	CF	−	−	+
FCF 30	*B. cenocepacia* (*IIIB*)	CF	−	−	+
LMG 16654	*B. cenocepacia* (*IIIB*)	CF	−	−	+
C5424	*B. cenocepacia* (*IIIB*)	CF	+/−	−	+
CEP511	*B. cenocepacia* (*IIIB*)	CF	−	−	−
MVPC 1/16	*B. cenocepacia* (*IIIB*)	Environmental	−	−	+
MVPC 1/73	*B. cenocepacia* (*IIIB*)	Environmental	+	−	+
LMG 19230	*B. cenocepacia* (*IIIC*)	Environmental	+	+	+
LMG 19240	*B. cenocepacia* (*IIIC*)	Environmental	+	−	+
FCF 38	*B. cenocepacia* (*IIID*)	CF	−	−	−
LMG 21462	*B. cenocepacia* (*IIID*)	CF	−	−	−
FCF 41	*B. stabilis* (*IV*)	CF	+	−	+
TVV 75	*B. vietnamiensis* (*V*)	Environmental	−	−	+
LMG 18941	*B. dolosa* (*VI*)	CF	−	−	+
LMG 18942	*B. dolosa* (*VI*)	CF	+	−	+
LMG 18943	*B. dolosa* (*VI*)	CF	−	−	+
MCI 7	*B. ambifaria* (*VII*)	Environmental	−	−	+
LMG 19467	*B. ambifaria* (*VII*)	CF	−	−	+
LMG 19182	*B. ambifaria* (*VII*)	Environmental	−	−	+
LMG 16670	*B. anthina* (*VIII*)	Environmental	−	−	−
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*	Animal Infection	+/− −	−	+
LMG 24067	*B. seminalis*	CF	+	−	+
LMG 24068	*B. metallica*	CF	+	+	+
LMG 24066	*B. arboris*	Environmental	+/− −	−	+
LMG 24263	*B. ubonensis*	Nosocomial Infection	+/−	−	+

+, growth; +/−, reduced growth; +/− −, very reduced growth; −, no growth

is known that VOCs profiles produced by microorganisms might depend on cultivation conditions, environment and inputs and thus relate to population dynamics (Sunesson et al. 1997), we tested the influence of the growth medium on the ability of this Antarctic bacterium to inhibit the growth of a panel of targets consisting of 38 Bcc strains representative of the 17 described Bcc species either of clinical or environmental origin. With this aim, cross-streak experiments were carried out using Petri dishes with a central septum, which allows the physical separation of media on which tester and target strains were grown. *Gillisia* sp. CAL575 was grown on three different media (i.e. PCA, MA and TYP), while the Bcc (target) strains were always grown on PCA medium. Data obtained for the three culture media are reported in Table 2 and showed that *Gillisia* sp. CAL575 exhibited a very different pattern of Bcc inhibition depending on the growth medium; in particular, the growth of almost all the Bcc strains was inhibited when CAL575 was grown on MA, and very few Bcc strains were inhibited when CAL575 was grown in TYP. Interestingly, when the Antarctic strain displayed a reduced ability to inhibit the growth of Bcc bacteria, most, if not all, strains with an environmental origin were "resistant" to the presence of tester strains. Finally, we compared the data from the cross-streaking analysis using the three different media (PCA, MA, TYP) with those obtained using tester strains *Pseudoalteromonas*

haloplanktis TAC125 (Medigue et al. 2005), isolated from Antarctic seawater column, *Pseudoalteromonas* sp. TB41 and *Psychrobacter* sp. TB47 and TB67 isolated from *L. nobilis* and *A. joubini* Antarctic sponges, respectively. *Gillisia* sp. CAL575 and *Psychrobacter* TB47 and TB67 possess different inhibition patterns depending on the growth media. However, the two strains belonging to the genus *Psychrobacter* were able to inhibit most of the target strains when grown on TYP media (Papaleo et al. 2013), differently from *Gillisia* sp. CAL575 that in TYP was able to inhibit a few Bcc strains and a higher number of Bcc strains when grown in MA. A principal component analysis (PCA) (Fig. 5) of the inhibition pattern showed by these five strains was performed and it revealed that *Gillisia* sp. CAL575, grown in TYP, had an inhibition pattern similar to that exhibited by the *Psychrobacter* strains grown in PCA and MA media, whereas CAL575 grown in MA was placed very close to *Pseudoalteromonas* (TAC125 and TB41) strains and *Psychrobacter* strains (TB47 and TB67) grown in TYP.

Solid phase micro extraction GC–MS analysis

In order to identify the mVOCs produced by the Antarctic bacteria, the SPME technique was used. Tests were performed in aerobic conditions (resembling the cross-streaking conditions) as recently described in "Materials and methods" (Romoli et al. 2013).

We checked whether the *Gillisia* sp. CAL575 strain synthesized different mVOCs when grown on different media. The mVOCs profile of each sample was checked every 24 h for 6 days in order to determine the dynamics of mVOCs production. The volatile profiles obtained from *Gillisia* sp. CAL575 after 3 days of incubation are shown in Fig. 6 and clearly revealed that the mVOCs produced by this strain, in the three different media, gave very similar patterns. Moreover, the analysis of the dynamics of mVOCs production revealed that the mVOCs profile did not change over the time, with an increase in the mVOCs relative quantity (data not shown). The obtained mVOCs profiles of the samples, excluding the components that are present both in negative control and blank samples, were characterized by 30 different compounds (Table 3). Some of these compounds were also detected in previous experiments performed under microaerophilic conditions (Romoli et al. 2011), even though the number of compounds produced under aerobic conditions was much lower than that present in low oxygen concentration conditions. Some of these are sulphur-containing compounds. The relative percentage composition of the different compounds produced by *Gillisia* sp. CAL575 in different media is reported in Table 3, whose analysis revealed that the

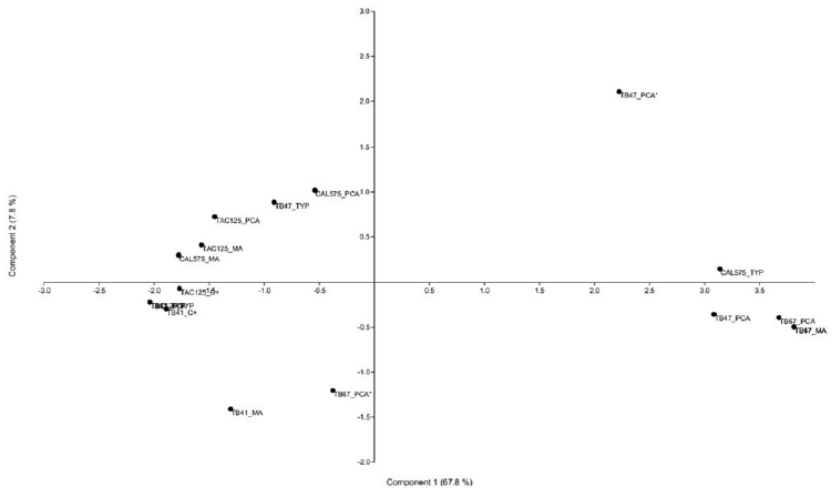


Fig. 5 Principal component analysis of inhibitory patterns of *Gillisia* sp. CAL575, *Pseudoalteromonas* TB41 and TAC125, and *Psychrobacter* TB47 and TB67 strains toward a panel of 38 Bcc strains. Percentages indicate the variance explained by the first two components

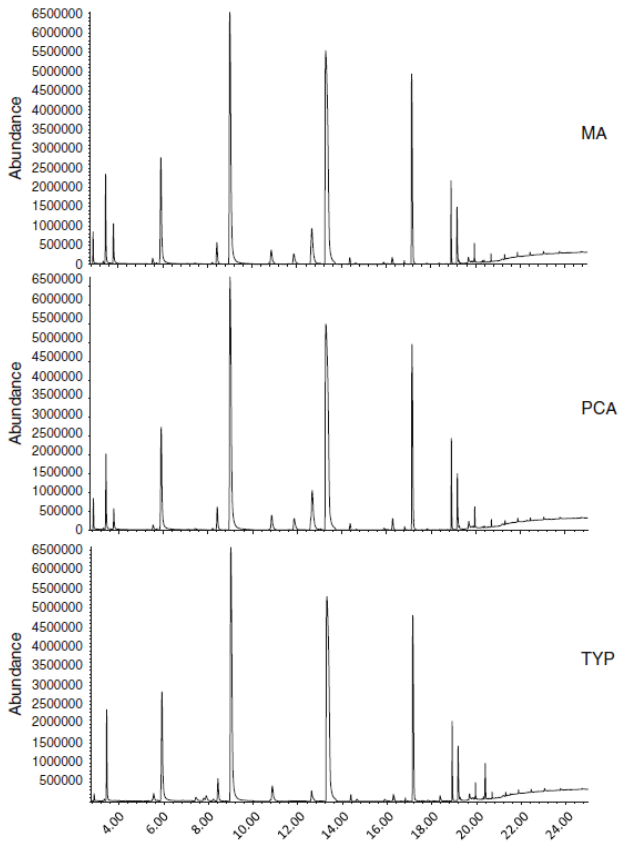


Fig. 6 mVOCs profiles obtained from *Gillisia* sp. CAL575 grown under aerobic conditions after 3 days of incubation in three different media

relative percentage of each of the thirty compounds varied in the different samples. Thus, in spite of the high similarity of mVOCs profiles exhibited by the different samples, the combination of different relative quantity of the thirty compounds might account for the different ability of *Gillisia* sp. CAL575 to antagonize the growth of Bcc strains when the tester strain was grown in different media. Accordingly, the PCA analysis reported in Fig. 7 gave results in agreement with cross-streaking experiments. Indeed, the three mVOCs profiles produced by the three *Gillisia* sp. CAL575 samples grown in TYP were placed very close to the negative controls. This might be due to the reduced growth of CAL575 in this medium, in agreement with cross-streaking data showing that strain CAL575 had a very low inhibitory activity when grown in this medium. The PCA analysis placed the *Gillisia* samples grown either in PCA medium or MA very close to each other, in agreement with cross-streaking experimental data (see Table 2).

Since the analysis of mVOCs under cross-streaking conditions was performed without a physical barrier between the target and tester strains, we could not a priori

Table 3 Percentage composition of different components in *Gillisia* CAL575 in three different media (MA, PCA e TYP) obtained by HS–SPME–GC–MS analysis

Target compound	Concentration (%)						
	TYP—*Gillisia* cal575	TYP—negative control	PCA—Burkholderia 16654	PCA—*Gillisia* cal575	PCA—negative control	MA—*Gillisia* cal575	MA—negative control
Carbon dioxide	9.31	23.27	14.38	6.61	46.73	22.14	48.51
2-Butene	0.15	0.49	0.04	0.09	0.62	0.10	1.62
2-Methyl-2-butene	0.01	0.02	0.00	0.00	0.03	0.00	0.02
2-Methyl-1, 3-butadiene	0.00	0.00	0.08	0.00	0.01	0.00	0.02
Methanethiol	0.89	0.17	14.82	2.64	0.26	8.40	0.35
Carbon disulfide	0.52	1.24	0.04	0.03	0.40	0.01	0.08
Dimethyl sulfide	0.00	0.01	0.08	0.00	0.00	0.02	0.01
Furan	0.01	0.05	0.00	0.01	0.06	0.01	0.08
Acetone	32.46	47.82	2.25	11.63	28.08	18.41	30.01
Dimethyl selenide	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Furan, 3-methyl-	0.01	0.01	0.00	0.01	0.02	0.00	0.03
Butanal	0.19	2.07	0.00	0.00	1.81	0.00	0.14
2-Butanone	4.26	10.66	0.29	0.33	8.06	0.37	5.16
2-Propanol	1.35	0.91	0.07	3.82	1.25	0.86	0.53
3-Methyl-2-Butanone	0.21	0.21	0.17	0.09	0.27	0.04	0.19
4-Methyl-2-Pentanone	0.03	0.07	0.18	0.03	0.12	0.01	0.12
Ethanethioic acid, *S*-methyl ester	2.37	0.01	0.07	19.21	0.01	14.03	0.02
Dimethyl disulfide	34.96	1.82	65.97	27.28	0.40	31.66	0.39
2-Methyl-1-propanol	0.96	0.45	0.05	4.48	1.05	0.31	0.69
1-Butanol, 3-Methyl	0.02	0.00	0.00	1.36	0.01	0.14	0.01
S-Methyl propanethioate	0.01	0.00	0.02	0.02	0.00	0.10	0.00
1-Butanol	4.17	6.01	0.03	1.11	2.83	1.27	3.85
1-Butanol, 3-methyl-	6.60	0.69	0.04	20.64	1.40	1.63	1.13
S-methyl-3-methylbutanethioate	0.22	0.88	0.04	0.06	1.95	0.02	2.16
3-Methyl-3-buten-1-ol	0.00	0.01	0.01	0.01	0.01	0.02	0.01
Dimethyl trisulfide	0.46	0.06	0.84	0.13	0.09	0.15	0.10
Oxime-, methoxy-phenyl-	0.48	1.81	0.13	0.34	3.44	0.26	4.21
Acetophenone	0.29	1.15	0.03	0.05	0.98	0.01	0.45
Disulfide methyl(methylthio)methyl	0.02	0.01	0.35	0.01	0.05	0.02	0.04
Phenylethyl alcohol	0.03	0.08	0.00	0.03	0.05	0.01	0.06

exclude the possibility that other (non-volatile compounds) might be responsible for the inhibition of the growth of the target strain. For this reason, we performed preliminary experiments spotting different aliquots of supernatant of CAL575 liquid cultures onto PCA plates containing Bcc bacteria. However, we did not find any inhibition of Bcc growth (data not reported), a finding, which supports the idea that mVOCs might represent the main (if not the unique) responsible for the Bcc growth inhibition.

Conclusions

In this work we have characterized, from different perspectives, the *Gillisia* sp. CAL575 strain, which was isolated from the Antarctic sponge *H. verrucosa*. Cross-streaking experiments revealed that it was able to inhibit the growth of several bacteria belonging to the Bcc, which are responsible for the lung infection of immune-compromised patients affected by Cystic Fibrosis, and that the

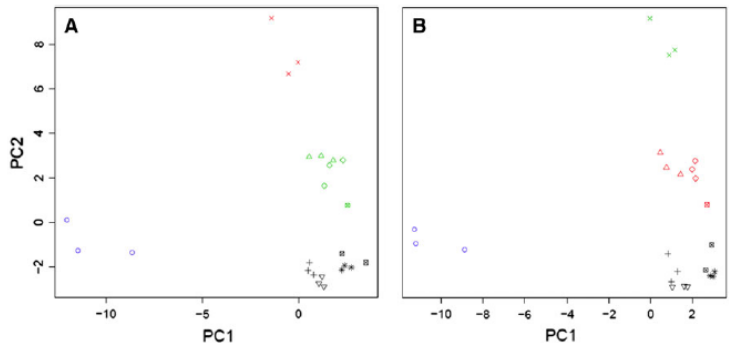


Fig. 7 PCA analysis of *Gillisia* sp. CAL575 grown in the three different media after one (a) and three (b) days of incubation at 21 °C. X *B. cenocepacia* LMG 16654 grown in PCA medium, ◇ *Gillisia* sp. CAL575 grown in PCA, ▽ PCA negative control, ☒ *Gillisia* sp. CAL575 grown in TYP, * TYP negative control, △ Gillisia sp. CAL575 grown in MA, + MA negative control

inhibition efficiency was strongly dependent on the medium used for growing *Gillisia* sp. CAL575. This is in agreement with other recent data obtained on *Psychrobacter* and *Pseudoalteromonas* Antarctic strains (Papaleo et al. 2013). However, the maximum inhibitory effect by the latter bacteria was exhibited on TYP, whereas *Gillisia* sp. CAL575 showed the maximum inhibitory effect on MA, suggesting that the composition of the growth medium might strongly and differently affect the production of antimicrobial compounds by strains belonging to different Antarctic genera. Data obtained also revealed that these antimicrobial compounds might be very likely VOCs. This was confirmed by the GC-SPME experiments, which allowed identifying some of the VOCs produced, that they are constitutively produced by *Gillisia* sp. CAL575, and whose relative concentration varied if this bacterium was grown onto different media. Even though the synthesis of non-volatile antimicrobial compounds cannot be a priori excluded, preliminary data from spot-tests experiments do not apparently support this hypothesis. Moreover, according to previous experimental data (Papaleo et al. 2012) we did not find in this gene set any gene involved in secondary metabolites biosynthesis (*pks* or *nrps*), except for the presence of a *pks* type III gene and a terpene biosynthetic cluster. Thus, the whole body of data obtained demonstrated that *Gillisia* sp. CAL575 might represent a very important source of bioactive molecules of volatile nature. Even though the biological significance of such production is still unclear, these compounds are synthesized under aerobic conditions by *Gillisia* sp. CAL575 through unknown metabolic pathways. The mechanism of action of these mVOCs is still unclear. However, in spite of the strong similarity of the VOCs profile obtained in growing the *Gillisia* sp. CAL575 strain in different media, in our opinion, the relatively different concentrations of VOCs might influence their inhibitory power. Thus, it is possible that a combination and the relative concentration of different volatile molecules are responsible for the growth inhibition of Bcc strains. This finding strongly resembles the antibacterial activity of essential oils extracted from medicinal plants, which are a heterogeneous mixture of a variety of different VOCs.

Regarding the molecular target(s) of these VOCs, both the nature and the number are still unknown. However, the observation that during the cross-streaking experiments we did not find any Bcc mutant resistant to mVOCs produced by Antarctic (data not shown) suggests the possibility that these VOCs might act on different molecular targets.

The availability of the genome sequence of *Gillisia* sp. CAL575 provided us the possibility to have also an insight on the strategy adopted by this strain for cold adaptation. Thus, we checked for the presence of genes related to the life in cold environment (Medigue et al. 2005) in the draft genomes of Antarctic *Gillisia* sp. CAL575 and *G. limnaea* DSM 15749 (Riedel et al. 2012), and *Gillisia* sp. CBA3202 (Nam et al. 2012) (Table 2). Data obtained showed that the three genomes most likely differentiate their gene set according to the environment they live in: indeed, there are little differences between the two Antarctic strains *Gillisia* sp. CAL575 and *Gillisia limnaea*, while there is a major gap between these two and *Gillisia* sp. CBA3202. Furthermore, the analysis of the presence of ROS response

genes and energy production genes (Table 3) revealed the existence of some differences among the three genomes; particularly, *Gillisia* sp. CAL575 is more similar to the Antarctic *G. limnaea* DSM 15749, especially for what concerns the presence of genes involved in ROS response. Thus, in this analysis, *Gillisia* sp. CAL575 was shown to possess many particular features in coping with opportunistic pathogens and genetic determinants to adapt at lowest temperature.

These features, together with the finding that *Gillisia* sp. CAL575 produces bioactive molecules of volatile nature, make this bacterium a promising tool for biotechnology uses.

Materials and methods

Strain isolation

During the characterization of bacteria isolated from the sponge *Haliclonissa verrucosa* (Burton 1932) collected from Terra Nova Bay (Ross Sea, Antarctica) in January 2005, the strain *Gillisia* sp. CAL575 (AN HQ702270; Papaleo et al. 2012) was isolated on marine agar 2216 (MA; Difco) after 1 month incubation at 4 °C. The strain belongs to the Italian Collection of Antarctic Bacteria (CIBAN) of the National Antarctic Museum (MNA) "Felice Ippolito" at the University of Messina.

Phenotypic characterization

Phenotypic analysis was performed using previously described methods (Lo Giudice et al. 2012). Gram reaction, oxidase, catalase, motility and endospore presence were determined as reported by Smibert and Krieg (1994). Colony morphology and pigmentation were recorded from growth on MA at 4 °C. Flagellar arrangement was determined using the Bacto Flagella Stain (Difco). The growth of the strain CAL575 at different temperatures was tested in marine broth (MB, Difco) incubated at 4, 10, 15, 20, 25, 30 and 37 °C for up to 4 weeks. The pH range for growth was determined in MB with pH values of separate batches of medium adjusted to 4, 5, 6, 7, 8 and 9 by the addition of HCl and NaOH (0.01, 0.1 and 1 M solutions). Salt tolerance tests were performed on Nutrient Agar (NA) with NaCl concentrations ranging from 0 to 15 % (w/v). The strain CAL575 was tested for the ability to grow on Trypticase Soy Agar (TSA; Oxoid) and TCBS agar (Difco). Chitin hydrolysis was assayed by adding colloidal chitin to MA plates (0.1 %, w/v). Starch hydrolysis was screened as described by Smibert and Krieg (1994). Susceptibility to antibiotics was assayed using the antibiotic-impregnated disks (Oxoid), which were laid on MA plates previously surface-inoculated with the test strains. The following antibiotics were tested: ampicillin (25 µg), penicillin G (10 µg), polymyxin B (30 µg), nalidixic acid (30 µg), tobramycin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), and vibriostatic agent O/129 (10 µg). Any sign of growth inhibition was scored as sensitivity to that antimicrobial compound. Resistance to an antimicrobial drug was indicated if did not show any inhibition zone. Additional biochemical and enzymatic tests were performed using API tests (BioMerieux), including API 20E and API 20NE galleries, according to the manufacturer's instructions. For tests carried out on solid and liquid media cultures were incubated at 15 °C for 21 days. All analyses were performed at least twice to confirm results.

Phylogenetic affiliation

Two hundred and seventy one 16S rRNA coding nucleotide sequences belonging to *Flavobacteriae* strains were retrieved from the Ribosomal Data Project DataBase (RDP) (http://rdp.cme.msu.edu/) (Cole et al. 2009). After a preliminary phylogenetic analysis (see below) we selected 88 16S rRNA gene sequences from the *Flavobacteria* representing each accepted species included in the RDP. Moreover, the 16S rRNA gene complete sequences from *G. forsetii* KT08023 were retrieved from the complete deposited genome sequences that showed high similarity with the *Gillisia* sp. CAL575 genome and were used as out-group; since in the Ribosomal database only partial 16S rRNA genes were deposited for *Gillisia* species, those two complete sequences were utilized as template to obtain the complete 16S rRNA gene from the contigs. The ClustalW program (Thompson et al. 1994) was used to align the 16S rRNA gene sequences with the most similar ones retrieved from the database. The phylogenetic tree was inferred using the Neighbor-Joining method (Saitou and Nei 1987) using the Kimura 2-parameter model (Kimura 1980). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches (Felsenstein 1985). The genetic distances were computed using the maximum composite likelihood method (Tamura et al. 2004) and are in the units number of base substitutions per site. Phylogenetic analyses were conducted in MEGA 5 (Tamura et al. 2011).

DNA isolation and sequencing

Gillisia sp. CAL575 was grown at 21 °C on PCA medium and the DNA was extracted with the CTAB methods as previously described (Papaleo et al. 2012). Whole genome sequencing was performed by the Institute of Applied Genomics and IGA Technology Services srl (University of

Udine, Italy) using an Illumina (Solexa) Genome Analyzer II platform (Zhou et al. 2010).

Genome assembly and annotation

SolexaQA software package was used to evaluate the quality of read pairs obtained and to remove poor quality bases and reads (Cox et al. 2010). Reads were then assembled in contigs using Ray software (Boisvert et al. 2010) with a k-mer length of 31. Genome annotation was performed using the Rapid Annotation by Subsystem Technology (RAST) pipeline (http://rast.nmpdr.org/rast.cgi). The on-line version of the server was used (Aziz et al. 2008). Additional functional annotation was performed querying other functional databases, including KAAS (Moriya et al. 2007), Blast2GO (Conesa et al. 2005)and COG (Tatusov et al. 2003).

Search for genes involved in cold adaptation, salt adaptation, osmotic shock protection, and energy production and in ROS response

The analysis was performed using *E. coli* sequences available in the KEGG database as seeds for the genes involved in energy production and in ROS response. For the genes involved in cold adaptation the seed sequences were retrieved from the annotated genome of *P. halo-planktis* TAC125. All the genes were searched using BLAST analyses with default parameter, and with an e-value of 0.05 (Altschul et al. 1997).

Secondary metabolite search

We used the antiSMASH (http://antismash.secondary metabolites.org/), a software allowing the rapid genome-wide identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genomes (Medema et al. 2011).

Cross-streaking

Antibacterial activity was detected using the cross-streak method (Lo Giudice et al. 2007b; Papaleo et al. 2012). As previously described by Papaleo et al. (2013), Petri dishes with a septum separating two hemi-cycles were used, in order to permit the growth of the tester and target strains on different media without any physical contact. Antarctic (tester) strains were streaked across one-half of an agar plate containing either PCA, TYP or MA and incubated at 21 °C. After 4 days, Bcc (target) strains were perpendicularly streaked to the initial streak and plates were further incubated at 21 °C, since it allowed the growth of both tester and target strains. The 38 Bcc target strains belonging to the 17 known species and of either environmental or clinical origin utilized in this work are listed in Table 4.

Solid phase micro extraction GC–MS analysis

The volatile compounds profile was obtained by Solid Phase Micro Extraction (SPME) GC–MS technique according to the method previously described (Romoli et al. 2011) and in aerobic conditions (resembling the cross-streaking conditions) as reported by Romoli et al. (2013).

After acquisition of chromatographic raw data, a tentative identification of volatile compounds was made by matching EI deconvoluted mass spectra, obtained using NIST-AMDIS (v.2.68) software (Stein 1999), against NIST 08 and Wiley 07 spectral libraries. The NIST-MS-Search program was used for mass spectra comparisons. The use of AMDIS allows true deconvolution and cleaning of the mass spectra; a complete analysis of noise for component perception; correction for baseline drift and the extraction of closely coeluting peaks, i.e. within a single scan of each other.

A dynamic programming approach, using the R package "Flagme—fragment-level analysis of GCMS-based metabolomics data" (Robinson 2013; Robinson et al. 2007), was used to analyze the chromatographic raw data after the deconvolution step.

Standard methods of chemometrics (Massart et al. 1997; Varmuza and Filzmoser 2009; Schleyr et al. 1998), such as principal component analysis (PCA) and successive cluster analysis (CA), were applied to evaluate the relationships among variables. In this paper the scree plot approach, i.e. a plot of the eigenvalues vs. the number of principal components was used, by selecting the number of PCs before a visible "big gap" or "elbow" as a first approach. The choice of the clustering algorithm was performed according to a cluster validity measure based on within- and between-sum-of-squares for the k-means, fuzzy c-means, and k-medoid clustering methods. All the statistical analyses were performed using the abundance of the reconstructed total ion current (TIC) after the spectral deconvolution, the alignment, and the clean up.

All statistical analyses were performed by means of R (R Development Core Team 2012) version 3.0.1 software, with Ubuntu 12.04 (precise), kernel Linux 3.2.0-48-generic, a 2.20 GHz CoreDuo CPU and 4GiB memory.

Samples preparation for determination of microbial volatile organic compounds (mVOCs) under aerobic conditions

The mVOCs analysis of Antarctic strains was tested both alone and in presence of *B. cenocepacia* LMG 16654 strain

(i.e. under cross-streak conditions) according to Romoli et al. (2013). Also for the *B. cenocepacia* LMG 16654 strain, the mVOC profile was analyzed, according to Romoli et al. (2011). Briefly, nine ml of PCA medium was added to each pre-sterilized 20 HS vial with aluminum crimp caps (Gerstel, Mülheim an der Ruhr, Germany). For the analysis in the absence of *B. cenocepacia* LMG16654 cells, Antarctic strains were streaked on the surface of PCA medium and the vial was crimped after one day, whereas to maintain aerobic conditions, 2 needles 18GX1 1/2 (40 length, 1,4 ∅, BD Microlance™) were inserted in the caps to allow air to go through and cut just at the top vial septum. All vials were incubated at room temperature (20 ± 1 °C) and the mVOCs production was evaluated every 24 h for 6 days consecutively. The same experiment was also carried out for *B. cenocepacia* LMG16654 strain.

The evaluation of mVOCs production in the presence of *B. cenocepacia* strain LMG16654, i.e. under cross-streak conditions, was carried out as above, except that Antarctic strain was streaked across one-third of the surface of PCA medium of each 20 HS vial, which was crimped with 2 needles inserted in the caps. All vials were incubated at room temperature (20 ± 1 °C) and the mVOCs production was checked every 24 h for 4 days consecutively. To the fourth day, the vials were quickly opened and *B. cenocepacia* strain LMG16654 was streaked perpendicular to the initial streak and vials were further crimped in the previous conditions and incubated at room temperature (20 ± 1 °C) and the mVOCs production checked after 24 h.

Acknowledgments This work was supported by grants from the Italian Cystic Fibrosis Research foundation (Grant FFC#12/2011), the Ente Cassa di Risparmio di Firenze (Grant 1103#2008), and the MNA (Museo Nazionale dell'Antartide). Valerio Orlandini and Marco Fondi are financially supported by an Italian Cystic Fibrosis Research Foundation fellowship and a FEMS Advanced Fellowship (FAF2012), respectively. We also thank the EU KBBE Project Pharmasea 2012–2016, Grant Agreement No: 312184. Lastly, we are very grateful to two anonymous referees for their suggestions in improving the manuscript.

References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O (2008) The RAST Server: rapid annotations using subsystems technology. BMC Genomics 9:75

Baker JT, Borris RP, Carte B, Cordell GA, Soejarto DD, Cragg GM, Gupta MP, Iwu MM, Madulid DR, Tyler VE (1995) Natural product drug discovery and development: new perspectives on international collaboration. J Nat Prod 58:1325–1357

Boisvert S, Laviolette F, Corbeil J (2010) Ray: simultaneous assembly of reads from a mix of high-throughput sequencing technologies. J Comput Biol 17:1519–1533

Bowman JP, McCammon SA, Rea SM, McMeekin TA (2000) The microbial composition of three limnologically disparate hypersaline Antarctic lakes. FEMS Microbiol Lett 183:81–88

Burton M (1932) Sponges. Discov Rep 6:237–392

Casanueva A, Tuffin M, Cary C, Cowan DA (2010) Molecular adaptations to psychrophily: the impact of 'omic' technologies. Trends Microbiol 18:374–381

Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res 37:D141–145

Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, Robles M (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21:3674–3676

Cox MP, Peterson DA, Biggs PJ (2010) SolexaQA: at-a-glance quality assessment of Illumina second-generation sequencing data. BMC Bioinforma 11:485

Felitsk DJ, Cannon JG, Capp MW, Hong J, Van Wynsberghe AW, Anderson CF, Record MT (2004) The exclusion of glycine betaine from anionic biopolymer surface: why glycine betaine is an effective osmoprotectant but also a compatible solute. Biochemistry 43:14732–14743

Felsenstein J (1985) Confidence limit on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Ivanova EP, Alexeeva YV, Flavier S, Wright JP, Zhukova NV, Gorshkova NM, Mikhailov VV, Nicolau DV, Christen R (2004) Formosa algae gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae*. Int J Syst Evol Microbiol 54:705–711

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Kirchman DL (2002) The ecology of Cytophaga-Flavobacteria in aquatic environments. FEMS Microbiol Ecol 39:91–100

Lee SJ, Gralla JD (2004) Osmo-regulation of bacterial transcription via poised RNA polymerase. Mol Cell 14:153–162

Lee OO, Lau SC, Tsoi MM, Li X, Plakhotnikova I, Dobretsov S, Wu MC, Wong PK, Qian PY (2006) *Gillisia myxillae* sp. nov., a novel member of the family Flavobacteriaceae, isolated from the marine sponge *Myxilla incrustans*. Int J Syst Evol Microbiol 56:1795–1799

Lo Giudice A, Brilli M, Bruni V, De Domenico M, Fani R, Michaud L (2007a) Bacterium-bacterium inhibitory interactions among psychrotrophic bacteria isolated from Antarctic seawater (Terra Nova Bay, Ross Sea). FEMS Microbiol Ecol 60:383–396

Lo Giudice A, Bruni V, Michaud L (2007b) Characterization of Antarctic psychrotrophic bacteria with antibacterial activities against terrestrial microorganisms. J Basic Microbiol 47:496–505

Lo Giudice A, Mangano S, Bruni V, De Domenico M, Michaud L (2012) Marine bacterioplankton diversity and community composition in an Antarctic coastal environment. Microb Ecol 63:210–223

Massart D, Vandeginste B, Buydens L, De Jong S, Smeyers-Verbeke J (1997) Handbook of chemometrics and qualimetrics: part A. Elsevier, Amsterdam

Medema MH, Blin K, Cimermancic P, de Jager V, Zakrzewski P, Fischbach MA, Weber T, Takano E, Breitling R (2011) antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. Nucleic Acids Res 39:W339–W346

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

Moriya Y, Itoh M, Okuda S, Yoshizawa AC, Kanehisa M (2007) KAAS: an automatic genome annotation and pathway reconstruction server. Nucleic Acids Res 35:W182–185

Nam YD, Lee HW, Lee M, Yim KJ, Kim KN, Roh SW, Kim D (2012) Draft genome sequence of *Gillisia* sp. strain CBA3202, a novel member of the genus *Gillisia*, which belongs to the family *Flavobacteriaceae*. J Bacteriol 194:3739

Nedashkovskaya OI, Kim SB, Lee KH, Mikhailov VV, Bae KS (2005) *Gillisia mitskevichiae* sp. nov., a novel bacterium of the family *Flavobacteriaceae*, isolated from sea water. Int J Syst Evol Microbiol 55:321–323

Newman DJ, Cragg GM (2004) Advanced preclinical and clinical trials of natural products and related compounds from marine sources. Curr Med Chem 11:1693–1713

O'Brien A, Sharp R, Russell NJ, Roller S (2004) Antarctic bacteria inhibit growth of food-borne microorganisms at low temperatures. FEMS Microbiol Ecol 48:157–167

Orlandini V, Maida I, Fondi M, Perrin E, Papaleo MC, Bosi E, de Pascale D, Tutino ML, Michaud L, Lo Giudice A, Fani R (2013) Genomic analysis of three sponge-associated Arthrobacter Antarctic strains, inhibiting the growth of *Burkholderia cepacia* complex bacteria by synthesizing volatile organic compounds. Microbiol Res (in press)

Papaleo MC, Fondi M, Maida I, Perrin E, Lo Giudice A, Michaud L, Mangano S, Bartolucci G, Romoli R, Fani R (2012) Sponge-associated microbial Antarctic communities exhibiting antimicrobial activity against *Burkholderia cepacia* complex bacteria. Biotechnol Adv 30:272–293

Papaleo MC, Romoli R, Bartolucci G, Maida I, Perrin R, Fondi M, Orlandini V, Mengoni A, Emiliani G, Tutino ML, Parrilli E, de Pascale D, Michaud L, Lo Giudice A, Fani R (2013) Bioactive volatile organic compounds from Antarctic (sponges) bacteria. New Biotechnol. pii: S1871-6784(13)00044-7

Pherobase (2011). http://www.pherobase.com/database/kovats/kovats-index.php

Ponce E, Garcia M, Munoz ME (2005) Participation of the Entner–Doudoroff pathway in Escherichia coli strains with an inactive phosphotransferase system (PTS-Glc+) in gluconate and glucose batch cultures. Can J Microbiol 51:975–982

R Development Core Team (2012) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. ISBN 3-900051-07-0

Riedel T, Held B, Nolan M, Lucas S, Lapidus A, Tice H, Glavina T, Rio D, Cheng JF, Han C, Tapia R, Goodwin LA, Pitluck S, Liolios K, Mavromatis K, Pagani I, Ivanova N, Mikhailova N, Pati A, Chen A, Palaniappan K, Land M, Rohde M, Tindall BJ, Detter JC, Göker M, Bristow JM, Eisen JA, Markowitz V, Hugenholtz P, Kyrpides NC, Klenk HP, Woyke T (2012) Genome sequence of the Antarctic rhodopsins-containing flavobacterium *Gillisia limnaea* type strain (R- 8282T). Stand Genomic Sci 7:107–119

Robinson M (2013) flagme: analysis of metabolomics GC/MS Data. R package version 1.16.2

Robinson M, De Souza D, Keen W, Saunders E, McConville M, Speed T, Likic V (2007) A dynamic programming approach for the alignment of signal peaks in multiple gas chromatography–mass spectrometry experiments. BMC Bioinforma 8(1):419

Rodrigues DF, Tiedje JM (2008) Coping with our cold planet. Appl Environ Microbiol 74:1677–1686

Romoli R, Papaleo MC, de Pascale D, Tutino ML, Michaud L, LoGiudice A, Fani R, Bartolucci G (2011) Characterization of the volatile profile of Antarctic bacteria by using solid-phase microextraction-gas chromatography–mass spectrometry. J Mass Spectrom 46:1051–1059

Romoli R, Papaleo MC, de Pascale D, Tutino ML, Michaud L, LoGiudice A, Fani R, Bartolucci G (2013) GC–MS volatolomic approach to study the antimicrobial activity of the antarctic bacterium *Pseudoalteromonas* sp. TB41. Metabolomics. doi:10.1007/s11306-013-0549-2

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Schleyr P, Allinger N, Clark T, Gasteiger J, Kollman P, Schaefer III, HSF (eds) (1998) Encyclopedia of computational chemistry. Wiley, Chichester

Smibert RM, Krieg NR (1994) Phenotypic characterization. In: Methods for general and molecular microbiology, methods for general and molecular microbiology. American Society for Microbiology, pp 611–654

Staunton J, Weissman KJ (2001) Polyketide biosynthesis: a millennium review. Nat Prod Rep 18:380–416

Stein S (1999) An integrated method for spectrum extraction and compound identification from gc/ms data. J Am Soc Mass Spectrom 10:770–781

Sunesson AL, Nilsson CA, Carlson R (1997) Influence of temperature, oxygen and carbon dioxide levels on the production of volatile metabolites from streptomyces albidoflavus cultivated on gypsum board and tryptone glucose extract agar. Ann Occup Hyg 41:393–413

Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci USA 101:11030–11035

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Tatusov RL, Fedorova ND, Jackson JD, Jacobs AR, Kiryutin B, Koonin EV, Krylov DM, Mazumder R, Mekhedov SL, Nikolskaya AN, Rao BS, Smirnov S, Sverdlov AV, Vasudevan S, Wolf YI, Yin JJ, Natale DA (2003) The COG database: an updated version includes eukaryotes. BMC Bioinforma 4:41

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Varmuza K, Filzmoser P (2009) Introduction to multivariate statistical analysis in chemometrics. Taylor & Francis Group CRC Press, UK

Zhou X, Ren L, Meng Q, Li Y, Yu Y, Yu J (2010) The next-generation sequencing technology and application. Protein Cell 1:520–536

G Model
MICRES-25627; No. of Pages 9

ARTICLE IN PRESS

Microbiological Research xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Genomic analysis of three sponge-associated *Arthrobacter* Antarctic strains, inhibiting the growth of *Burkholderia cepacia* complex bacteria by synthesizing volatile organic compounds

Valerio Orlandini[a,b,1], Isabel Maida[a,1], Marco Fondi[a], Elena Perrin[a], Maria Cristiana Papaleo[a], Emanuele Bosi[a], Donatella de Pascale[b], Maria Luisa Tutino[c,d], Luigi Michaud[e], Angelina Lo Giudice[e], Renato Fani[a,*]

[a] Laboratory of Microbial and Molecular Evolution, Department of Biology, University of Florence, Via Madonna del Piano 6, 50019 Sesto Fiorentino, Firenze, Italy
[b] Institute of Protein Biochemistry, National Research Council, Via P. Castellino, 111, I-80134 Naples, Italy
[c] Department of Chemical Sciences, University of Naples Federico II, Naples, Italy
[d] School of Biotechnological Sciences, University of Naples Federico II, Complesso Universitario M. S. Angelo, Via Cintia, I-80126 Naples, Italy
[e] Department of Biological and Environmental Sciences (DiSBA-CIBAN), University of Messina, Viale Ferdinando Stagno d'Alcontrès 31, I-98166 Messina, Italy

ARTICLE INFO

Article history:
Received 22 July 2013
Received in revised form
27 September 2013
Accepted 30 September 2013
Available online xxx

Keywords:
Arthrobacter
Pangenome
Burkholderia
VOCs
Cystic fibrosis

ABSTRACT

In this work we analyzed the ability of three *Arthrobacter* strains (namely TB23, TB26 and CAL618), which were isolated from the Antarctic sponges *Haliclonissa verrucosa* and *Lyssodendrix nobilis*, to specifically inhibit the growth of a panel of 40 *Burkholderia cepacia* complex strains, representing a major cause of infections in patients that are affected by Cystic Fibrosis. The inhibitory activity was due to the synthesis of antimicrobial compounds, very likely volatile organic compounds (VOCs), and was partially dependent on the growth media that were used for Antarctic strains growth. The phylogenetic analysis revealed that two of them (i.e. CAL 618 and TB23) were very close and very likely belonged to the same *Arthrobacter* species, whereas the strain TB26 was placed in a distant branch. The genome of the strains TB26 and CAL618 was also sequenced and compared with that of the strain TB23. The analysis revealed that TB23 and CAL618 shared more genomic properties (GC content, genome size, number of genes) than with TB26. Since the three strains exhibited very similar inhibition pattern *vs* Bcc strains, it is quite possible that genes involved in the biosynthesis of antimicrobial compounds very likely belong to the core genome.

1. Introduction

Species that are members of the *Arthrobacter* genus are Gram positive, obligate aerobes, and chemoorganotrophs, using a variety of substrates in their oxidative metabolism including nicotine, nucleic acids, and other less usual organic molecules (Mongodin et al., 2006). *Arthrobacter* species are commonly found amongst soil bacteria, but they have been isolated also in other places such as deep subsurface, Arctic ice, chemically contaminated sites, and radioactive environments, where they have been reported to exert interesting ecological functions. For instance, some *Arthrobacter* species are naturally adapted to mineralize pesticides and herbicides, making these strains quite active in the bioremediation of polluted groundwater (Michaud et al., 2004). This metabolic flexibility relies on the notable adaptability of their genomes, which allow them to efficiently cope with stressful conditions and environments. The plasticity of *Arthrobacter* genomes is witnessed by the diversity of the almost twenty whole sequences deposited in public databanks, since December 2012. This particular feature is shared with *Actinobacteria*, to which phylum *Arthrobacter* belongs.

Arthrobacter strains have been also isolated from extreme environments such as the Antarctic Sea. This is particularly intriguing since marine microorganisms represent a still largely untapped reservoir of biologically active products (Debbab et al., 2010). Marine habitats are sometimes characterized by stressful parameters, such as low average temperature, reduced or absent sun irradiation and high-pressure conditions. Organisms thriving in these environments adopt a plethora of variegate and original strategies to cope with the physiological issues imposed by apparently hostile habitat parameters (Casanueva et al., 2010) and they are capable to produce an almost unknown repertoire of bioactive molecules (Debbab et al., 2010). This chemical diversity, and

* Corresponding author. Tel.: +39 0554574742.
E-mail address: renato.fani@unifi.it (R. Fani).
[1] Equal contributors.

0944-5013/$ – see front matter © 2013 Published by Elsevier GmbH.
http://dx.doi.org/10.1016/j.micres.2013.09.018

Please cite this article in press as: Orlandini V, et al. Genomic analysis of three sponge-associated *Arthrobacter* Antarctic strains, inhibiting the growth of *Burkholderia cepacia* complex bacteria by synthesizing volatile organic compounds. Microbiol Res (2013), http://dx.doi.org/10.1016/j.micres.2013.09.018

the associated biological properties, make these organisms and their metabolome a promising "land of discovery" for biotechnologists willing to find novel industrial for biomedical applications. We have recently reported that Antarctic marine bacteria, some of which isolated from tissues of different sponges, are able to synthesize a chemically heterogeneous group of biomolecules, i.e. volatile organic compounds (VOCs) (Papaleo et al., 2012; Fondi et al., 2012), whose role in the ecology and/or physiology of these bacteria in still unclear. However, it has been demonstrated that these VOCs are responsible for the specific inhibition of the growth of *Burkolderia cepacia* complex (Bcc) strains (Papaleo et al., 2012). The Bcc consists of 18 closely related species occupying very different ecological niches, and many of them are opportunistic human pathogens and represent a serious concern for Cystic Fibrosis (CF) patients and immuno-compromised individuals (Drevinek and Mahenthiralingam, 2010). The two most clinically relevant species are *Burkolderia cenocepacia* and *Burkolderia multivorans*, accounting for >85% of all Bcc infections, such as the "cepacia syndrome", characterized by high fever, severe progressive respiratory failure, leucocytosis and elevated erythrocyte sedimentation rate. Bcc strains are naturally resistant to many antibiotics such as cephalosporin, β-lactams, polymyxins and aminoglycosides; therefore, Bcc infections are very problematic to eradicate (Drevinek and Mahenthiralingam, 2010; Bazzini et al., 2011). The Multi Drug Resistance (MDR) exhibited by Bcc bacteria is mainly attributed to: (i) the ability of its members to reduce outer membrane permeability, (ii) the production of modifying β-lactamase enzymes, (iii) a variety of virulent factors with most notable lipases, proteases and other secreted products including system-associated effectors; (iv) the presence of multidrug efflux pumps with emphasis in members of the Resistance-Nodulation cell Division (RNDs) (Perrin et al., 2010, 2013). In addition to this, Bcc bacteria may acquire resistance to one (or more) antibiotic(s) through the alteration of antibiotic targets, i.e. mutations in the gene(s) coding for the target molecule(s) or in genes controlling their expression.

Therefore, our recent studies regarding the finding that the VOCs synthesized by Antarctic bacteria are able to completely inhibit the growth of several Bcc strains seems to be very promising (Papaleo et al., 2012, 2013; Fondi et al., 2012). It has also been shown that the ability of Antarctic bacteria to inhibit the growth of Bcc strains strongly depends on the growth medium used (Papaleo et al., 2013; Maida et al., 2013). Besides, different Antarctic bacteria belonging to dissimilar genera/species exhibited diverse inhibitory activity *vs* Bcc strains. One of the most active Antarctic bacteria analyzed so far is the Gram-positive *Arthrobacter* sp. strain TB23, whose draft genome has been recently reported (Fondi et al., 2012) and that was isolated from the sponge *Lissodendoryx nobilis* tissue (Mangano et al., 2009). This strain very likely produces an array of molecules endowed with a potent antimicrobial activity *vs* either other Antarctic (Mangano et al., 2009) or Bcc strains (Papaleo et al., 2012), making it a model organism to unravel metabolic pathways responsible for the production of bioactive primary and/or secondary metabolites. The *Arthrobacter* sp. TB23 draft genome sequence harbours three interesting gene clusters, including a type III polyketide synthase (PKS), a non-ribosomal peptide synthetase gene, and terpene biosynthetic genes, respectively (Fondi et al., 2012).

In the present paper, two other *Arthrobacter* Antarctic strains (namely TB26 and CAL618) were characterized at both phenotypic and genomic level, in order to (i) check whether different strains exhibit different/similar inhibitory activity *vs* Bcc strains and (ii) check if this activity was dependent on the growth media and (iii) if might be attributed to the synthesis of mVOCs, (iv) establish if additional (non volatile molecules) might be involved in the inhibition of Bcc growth, (v) identify genes/metabolic pathways involved in the biosynthesis of antimicrobial compounds and, lastly, (vi) try to shed some light on the mechanism(s) of inhibition of Bcc strains growth.

2. Materials and methods

2.1. Bacterial strains, growth conditions, DNA isolation and sequencing

2.1.1. Bacterial strains and growth conditions

During the characterization of bacteria isolated from the sponges *Haliclonissa verrucosa* and *L. nobilis* collected from Terra Nova Bay (Ross Sea, Antarctica) in January 2005, the three *Arthrobacter* sp. strains TB23, TB26 (from *L. nobilis*) and CAL618 (from *H. verrucosa*) (Papaleo et al., 2012) were isolated on marine agar 2216 (MA; Difco) after 1-month incubation at 4 °C. The three strains belong to the Italian Collection of Antarctic Bacteria (CIBAN) of the National Antarctic Museum (MNA) "Felice Ippolito" at the University of Messina. Antarctic strains were grown in PCA, TYP or MA at 21 °C as described in Papaleo et al. (2013). Bcc bacteria (listed in Table 1) were grown on PCA at either 21 °C or 37 °C (Papaleo et al., 2013).

2.1.2. DNA isolation and sequencing

Total DNA from the strains TB26 and CAL618 was extracted using the CTAB method as described previously (Papaleo et al., 2012). Whole genome sequencing was performed by the Institute of Applied Genomics and IGA Technology Services S.r.l. (University of Udine, Italy), using an Illumina (Solexa) Genome Analyzer II. The CAL618 and TB26 whole Genome Shotgun project have been deposited at DDBJ/EMBL/GenBank under the accession ASZW00000000 and AUPJ00000000, respectively. The versions described in this paper are version ASZW01000000 and AUPJ01000000, respectively.

2.2. Cross-streaking experiments

Antibacterial activity was determined by using the cross-streak method (Lo Giudice et al., 2007; Papaleo et al., 2012). Hereinafter, Antarctic bacterial isolates to be tested for inhibitory activity will be termed "tester" strains, whereas Bcc strains used, as a target, will be called "target" strains. Cross-streaking experiments were carried out as previously described (Papaleo et al., 2013) by using Petri dishes either without or with a central septum separating two hemi-cycles. In the first case (experiments performed using Petri dishes without a central septum) tester strains were streaked across one-half of an agar plate with PCA medium and incubated at 21 °C. In the second case (experiments performed using Petri dishes with a central septum) tester strains were streaked across one-half of an agar plate with PCA, TYP or MA and incubated at 21 °C. In both cases, after 4 days of incubation, target strains were streaked on PCA medium perpendicular to the initial streak and plates were further incubated at 21 °C. The antagonistic effect was indicated by the failure of the target strains to grow. The target strains utilized in this work were the 40 Bcc strains listed in Table 1.

2.3. Bioinformatic analyses

Total DNA extracted from *Arthrobacter* spp. TB26 and CAL628 was sequenced using Illumina HiSeq2000. The obtained paired-end reads were trimmed with the utility DynamicTrim from the package SolexaQA version 2.1 (Cox et al., 2010). The assemblies were performed with ABySS 1.3.4 (Simpson et al., 2009), trying different *k*-mer values automatically using a custom made software written in C++ and Ruby. This software evaluates at which *k*-mer value (inside a range specified by the user and based on the mean

Please cite this article in press as: Orlandini V, et al. Genomic analysis of three sponge-associated *Arthrobacter* Antarctic strains, inhibiting the growth of *Burkholderia cepacia* complex bacteria by synthesizing volatile organic compounds. Microbiol Res (2013), http://dx.doi.org/10.1016/j.micres.2013.09.018

Table 1
Growth of 40 *Burkholderia cepacia* complex strains in the presence/absence of Antarctic *Arthrobacter* sp. CAL618, TB23 or TB26 grown on three different media (PCA, MA, TYP).

Target strain			*Arthrobacter* tester strain																	
Strain	Species	Origin	CAL618					TB23						TB26						
			C-	PCA*	PCA	TYP	MA	C-	PCA*	PCA	TYP	MA	C-	PCA*	PCA	TYP	MA
FCF1	*B. cepacia(I)*	CF	+	–	–	–	–	+	–	–	–	–	+	–	–	–	–
FCF3			+	–	–	–	–	+	–	–	–	–	+	–	–	–	–
LMG17588	*B. multivorans(II)*	ENV	+	–	–	–	–	+	–	–	–	–	+	–	–	–	–
FCF16	*B. cenocepacia(IIIA)*	CF	+	–	–	–	–	+	–	–	–	–	+	–	–	–	–
J2315			+	–	–	–	–	+	–	–	–	–	+	–	–	–	–
FCF18			+	–	–	–	–	+	–	–	–	–	+	–	–	–	–
FCF20			+	+	+	+	+	+	–	–	–	–	+	–	–	–	–
FCF23			+	–	–	–	–	+	–	–	–	–	+	–	–	–	–
FCF24			+	–	–	–	–	+	+/–	–	–	–	+	+/–	–	–	–
FCF27			+	–	–	–	–	+	–	–	–	–	+	–	–	–	–
FCF29		CF	+	–	–	–	–	+	–	–	–	–	+	–	–	–	–
FCF30	*B. cenocepacia(IIIB)*		+	–	–	–	–	+	–	–	–	–	+	–	–	–	–
LMG16654			+	–	–	–	–	+	–	–	–	–	+	–	–	–	–
C5424			+	–	–	–	–	+	–	–	–	–	+	–	–	–	–
CEP511			+	–	–	–	–	+	–	–	–	–	+	–	–	–	–
MVPC1/16		ENV	+	–	–	–	–	+	–	–	–	–	+	+/–	–	–	–
MVPC1/73			+	–	–	–	+/–	+	+/–	–	–	–	+	+/–	+/–	–	–
LMG19230	*B. cenocepacia(IIIC)*	ENV	+	–	–	–	+/–	+	–	–	–	+/–	+	–	+/–	–	–
LMG19240			+	–	–	–	+	+	–	–	–	–	+	–	–	–	–
FCF38	*B. cenocepacia(IIID)*	CF	+	–	–	–	+/–	+	–	–	–	–	+	–	–	–	–
LMG21462			+	–	–	–	+/–	+	–	–	–	–	–	–	–	–	–
FCF41	*B. stabilis(IV)*	CF	+	–	–	–	+	+	–	–	–	–	+	–	–	–	–
FCF42	*B. vietnamiensis(V)*	CF	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–
TVV75		ENV	+	–	–	–	–	+	–	–	–	–	+	–	–	–	–
LMG18941			+	–	–	–	–	+	–	–	–	–	+	–	–	–	–
LMG18942	*B. dolosa(VI)*	CF	+	–	–	–	+/–	+	–	–	–	+	+	–	–	–	+/–
LMG18943			+	–	–	–	–	+	–	–	–	–	+	–	–	–	–
MCI7		ENV	+	–	–	–	–	+	–	–	–	–	+	–	–	–	–
LMG19467	*B. ambifaria*	CF	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–
LMG19182		ENV	+	–	–	–	–	+	–	–	–	–	+/–	–	–	–	–
LMG16670	*B. anthina(VIII)*	ENV	+	–	–	+/–	–	+	–	–	–	–	+	–	–	–	–
FCF43	*B. pyrrocinia(IX)*	CF	+	+/–	–	–	–	+	–	–	–	–	+	–	–	–	–
LSED4	*B. lata*	CF	+	–	–	+/–	–	+	–	–	–	–	+	–	–	–	–
LMG24064	*B. latens*	CF	+	–	–	–	–	+	–	–	–	–	+	–	+	–	–
LMG24065	*B. diffusa*	CF	+	–	–	–	–	+	–	+/–	–	–	+	–	–	–	+/–
LMG23361	*B. contaminans*	AI	+	–	–	–	–	+	–	+/–	–	–	+	–	–	–	+/–
LMG24067	*B. seminals*	CF	+	–	–	–	+/–	+	–	+/–	–	–	+	–	–	+/–	+/–
LMG24068	*B. metallica*	CF	+	–	+	–	+	+	–	+/–	–	–	+	–	+/–	+/–	+/–
LMG24066	*B. arboris*	ENV	+	–	+/–	–	+/–	+	–	–	–	–	+	–	–	–	+/–
LMG24263	*B. ubonensis*	NI	+	–	–	–	–	+	–	–	–	–	+	–	–	–	–

Abbreviations: CF, strains isolated from cystic fibrosis patients; AI, strains isolated from animal infection; NI, strains isolated from nosocomial infection; ENV, environmental strain.
Symbols: +, growth; +/–, reduced growth; +/ —, very reduced growth; –, no growth; PCA*, Petri dishes without a central septum; C-, Petri dishes containing only the target strains.

Please cite this article in press as: Orlandini V, et al. Genomic analysis of three sponge-associated *Arthrobacter* Antarctic strains, inhibiting the growth of *Burkholderia cepacia* complex bacteria by synthesizing volatile organic compounds. Microbiol Res (2013), http://dx.doi.org/10.1016/j.micres.2013.09.018

length of the trimmed reads) the assembly gives the best total contigs/total assembly length ratio (that is, the highest mean contig length value). Abnormal assemblies, with assembly lengths that are too different from the mean value obtained from the various assemblies, are automatically discarded. Protein predictions and the tRNA identification were carried out using RAST annotation system (Aziz et al., 2008).

The determination of the pangenome of the three strains was made using a custom made Perl script, which uses InParanoid (O'Brien et al., 2005) and MultiParanoid (Alexeyenko et al., 2006) and automatically performed comparisons among each couple of genomes of the dataset under analysis. The proteins of the various sets of the pangenome were characterized according to the COG (*Cluster of Orthologous Groups*) database, which classifies the protein sequences according to their predicted function. The detection of secondary metabolite producing cluster was made with antiSMASH 2.0 (Blin et al., 2013).

The phylogenetic tree was constructed with MEGA 5 (Tamura et al., 2011), using the 16S rDNA sequences of the three strains we sequenced and of the other *Arthrobacter* strains available in the Ribosomal Database Project (Cole et al., 2009). The statistical method used was Maximum Likelihood, with 500 bootstrap replications.

The comparison of some genomic *loci* in the three *Arthrobacter* strains was performed using Mauve 2.3.1 (Darling et al., 2004).

3. Results and discussion

3.1. Cross-streaking

Previous findings demonstrated that *Arthrobacter* spp. TB23, TB26 and CAL618 were able to specifically inhibit the growth of some Bcc strains (Papaleo et al., 2012) and that this inhibition was very likely due to (at least partly) the production of VOCs. However, these experiments were carried out growing both tester and the target strains on the same growth medium (PCA), whose composition allowed the growth of both of them. In this work, we tested the influence of the growth medium composition on the ability of the three Antarctic *Arthrobacter* strains to inhibit the growth of a panel consisting of 40 Bcc strains representative of the 17 described Bcc species either of clinical or environmental origin. Experiments were performed using Petri dishes either with or without a central septum. Tester strains were grown on three different media (i.e. PCA, MA and TYP), while the Bcc (target) strains were grown on PCA medium. Furthermore, an additional cross-streaking experiment was performed using PCA plates without septum, to evaluate whether the absence of a physical barrier to the flow of molecules from Antarctic tester strains might enhance the inhibition of Bcc growth. Data obtained are reported in Table 1 and revealed that the pattern of inhibition of *Arthrobacter* spp. TB23, TB26 and CAL618 were very similar one to each other. In particular, the growth of almost all the Bcc strains was inhibited when the three *Arthrobacter* strains were grown in PCA and TYP, and there was a slight decrease of the inhibitory efficiency when they were grown in MA. Finally, the comparative analysis of data obtained from cross-streaking experiments performed on PCA medium using Petri dishes with and without septum revealed that the absence of the septum allowed the three *Arthrobacter* strains to increase their inhibitory efficiency (Fig. 1), especially against *Burkolderia diffusa*, *Burkolderia contaminans*, *Burkolderia seminalis*, and *Burkolderia metallica*, thus suggesting that also non-volatile organic compounds might interfere with the growth of Bcc strains.

Concerning the molecular target(s) of the antimicrobial compounds in the Bcc cells, both the nature and the number are still unknown. However, during the cross-streaking experiments no Bcc mutant resistant to the compounds (either mVOCs or non volatile ones) synthesized by the three strains was detected, strongly suggesting the possibility that these compounds might interfere with the functionality of different molecular targets within the Bcc cells.

3.2. Genome sequencing and phylogenetic analysis

The genome of *Arthrobacter* strains TB26 and CAL618 was determined using Illumina HiSeq2000 and compared with each other

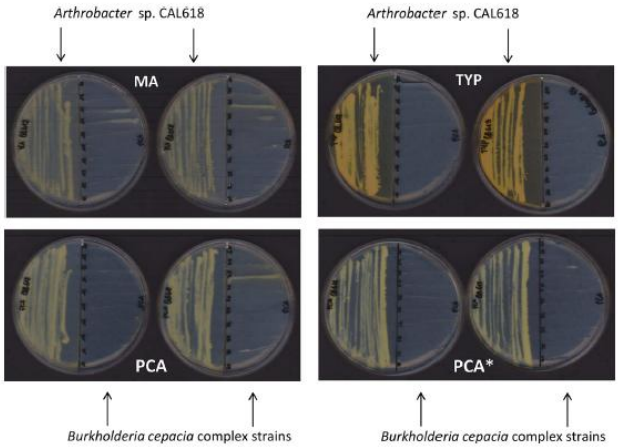


Fig. 1. Cross streaking experiments performed using the *Arthrobacter* sp. strain CAL618 grown in Petri dishes with a central septum and containing MA, TYP medium (above) or PCA in a Petri dish with or without (PCA*) septum (below).

Please cite this article in press as: Orlandini V, et al. Genomic analysis of three sponge-associated *Arthrobacter* Antarctic strains, inhibiting the growth of *Burkholderia cepacia* complex bacteria by synthesizing volatile organic compounds. Microbiol Res (2013), http://dx.doi.org/10.1016/j.micres.2013.09.018

Table 2
Overview of the main features of three *Arthrobacter* sp. genomes analyzed in this work.

Strain	Origin	Assembly size (bp)	Number of				GC content (%)
			Contigs	ORFs	rRNA	tRNA	
TB23	*Lissodendoryx nobilis*	3,542,528	104	3298	6	46	63.32
TB26		4,383,124	574	4004	6	46	66.33
CAL618	*Haliclonissa verrucosa*	3,657,222	289	3430	6	47	63.75

and that of *Arthrobacter* sp. TB23 strain, which has been recently published (Fondi et al., 2012). The two genomes were sequenced obtaining 15,922,741 and 7,216,198 reads for TB26 and CAL618, respectively (reads length 101 bp). The sequence depth has been estimated to be at least 40×. The reads were first trimmed with SolexaQA (Phred cutoff = 20, obtaining reads 55 bp long, on average), and then assembled using ABySS software, version 1.3.4 (*k*-mer value = 50). The *Arthrobacter* sp. TB26 assembled genome is 4,383,124 bp long, distributed into 574 contigs (>500 bp, an average length of 7636 bp), whereas a smaller assembled genome was obtained for *Arthrobacter* sp. CAL618, consisting of 3,657,222 bp long sequence, distributed into 289 contigs (>500 bp, average length: 12,655 bp) (Table 2). The overall GC contents, 66.33% (TB26) and 63.75% (CAL618), and the size of the three *Arthrobacter* genomes are in line with the majority of genomes of *Actinobacteria* species members. Genome annotation, using the RAST annotation system, highlighted the presence of a quite homogeneous content of tRNA encoding genes (46 in TB26, 47 in CAL618) and 6 rRNA operons in both strains. As it might be expected from the different assembled genome size, a different number of open reading frames (ORFs) were predicted for TB26 and CAL618 genomes (4004 and 3430, respectively) (Table 2). Overall, data reported in Table 2 revealed that the *Arthrobacter* strain CAL618 share more genomic features with strain TB23 than with TB26.

To confirm this finding, we compared two genomic regions embedding the *gyrB*, and the *dapC* genes, encoding the β subunit of DNA gyrase, and the L,L-diaminopimelate aminotrasnferase, respectively. Data concerning the *gyrB*, and the *dapC* regions are shown in Fig. 2A and B, whose analysis revealed that CAL618 and TB23 share a degree of sequence identity much higher than that shared with TB26. In addition to this, we have also analyzed the gene clusters involved in the biosynthesis of secondary metabolites (see below). Data reported in Fig. 2C clearly supported the high degree of similarity of this region shared by strains CAL618 and TB23.

For both genomes, approximately 70% of ORFs were assigned to (at least) one COG category (Fig. 3).

In order to establish the evolutionary relationships between the three *Arthrobacter* strains, a phylogenetic tree using the 16S rDNA sequences from the three strains and the most similar orthologous sequences retrieved from RDP database was constructed. The analysis of the phylogenetic tree (shown in Fig. 4) revealed that *Arthrobacter* sp. CAL618 and TB23 share a 100% sequence identity between them and with the orthologous sequence from *Arthrobacter flavus* JCM11496, suggesting that these three strains might belong to the same species. On the contrary, strain TB26 (sharing a 96% degree of 16S rRNA gene sequence identity with strains CAL618 and TB23) was placed very close to *Arthrobacter*

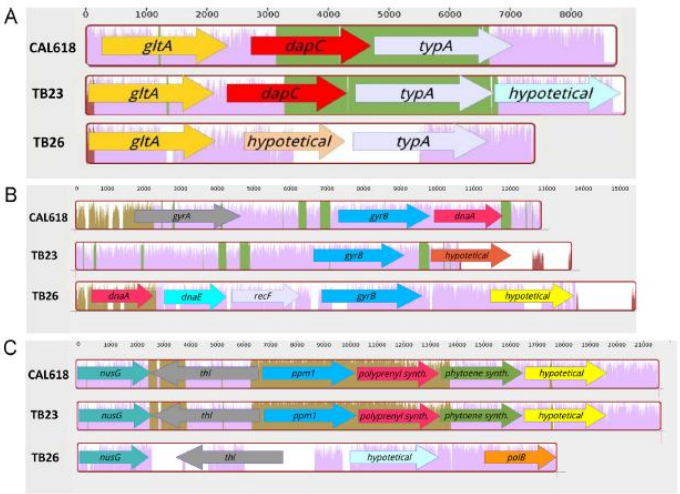


Fig. 2. Genomic organization of the regions surrounding *dapC* (A), *gyrB* (B) and *thl* (C) in the *Arthrobacter* sp. strains CAL618, TB23 and TB26. The same colour inside the sequences of different strains represents a highly similar region.

Please cite this article in press as: Orlandini V, et al. Genomic analysis of three sponge-associated *Arthrobacter* Antarctic strains, inhibiting the growth of *Burkholderia cepacia* complex bacteria by synthesizing volatile organic compounds. Microbiol Res (2013), http://dx.doi.org/10.1016/j.micres.2013.09.018

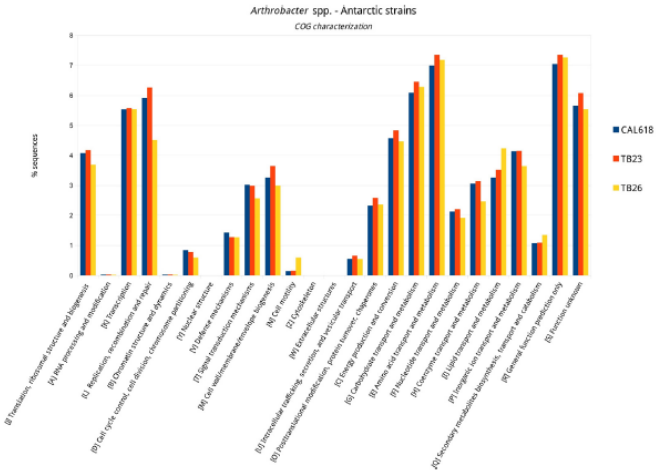


Fig. 3. Characterization of the proteins of the three *Arthrobacter* spp. analyzed in this work according to the COG (Cluster of Orthologous Groups) database.

sulfonivorans, which in turn, is placed on a branch distant from that occupied by the other two strains.

3.3. The pangenome of Antarctic Arthrobacter strains

Once we obtained the protein predictions, we used them to perform the identification of the pangenome, i.e. the sets of proteins shared by all the strains (*core* genome), by two strains (*accessory* genomes) and unique to only one strain (*unique* genomes – one for each strain).

Data obtained are summarized in Fig. 5 and revealed that (i) the three *Arthrobacter* strains share 1753 genes, (ii) strains CAL618 and TB23 share much more genes (813) than with TB26 (102 and 121, respectively), a finding that might be expected on the basis of their higher phylogenetic vicinity, (iii) accordingly, TB26 possesses a large set of unique genes (1920 sequences). The characterization according to COG database of the genes belonging to the *core* genome is reported in the Supplemental Table 1.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micres.2013.09.018.

The COG characterization (Fig. 6) confirms the diversity of TB26 compared to the other two strains: there are several categories (especially J–Translation, ribosomal structure and biogenesis; N–Cell motility and Q–Secondary metabolite biosynthesis, transport and catabolism) for which the unique TB26 genome greatly differs from the other two unique genomes, while being, in most of the cases, similar to the percent of genes shared by the two other strains only. The COG characterization of the three strains done separately (i.e. each set corresponds to the entire protein set of a strain, not considering whether they are shared by the other strains or not) (Fig. 3) revealed that, with just a few minor exceptions, the percent distribution of the proteins of each strain among the various COG categories is almost the same. This finding is apparently in conflict with the different genome size of TB26 strain in respect to TB23 and CAL618 strains. However, a comparison of the genome size of the three *Arthrobacter* sp. strains analyzed in this work with those of their close relatives reported in the phylogenetic tree (Fig. 4), revealed that the genome size of TB26 strain is in line with that of its relatives whose genome has been sequenced, i.e. *Arthrobacter phenanthrenivorans* Sphe3 and *Arthrobacter chlorophenolicus* A-6, whereas no information is available about the whole genome sequence of TB23 and CAL618

Table 3
Overview of the main features of three *Arthrobacter* sp. genomes analyzed in this work.

Taxonomy	Strain	Assembly size (Mbp)	Plasmids		Number of ORFs	GC content (%)
			Name	Size (Mbp)		
Arthrobacter sp.	TB26	4.38	–	–	4004	66.33
A. chlorophenolicus	A-6	4.98	pACHL01 pACHL02	0.426 0.158	4590	66.00
A. phenanthrenivorans	Sphe 3	4.54	pASPHE01 pASPHE02	0.190 0.094	4131	65.40
Arthrobacter sp.	CAL618	3.66	–	–	3430	63.75
Arthrobacter sp.	TB23	3.54	–	–	3298	63.32
A. flavus	JC11496	Unknown	–	–	Unknown	64.00

Please cite this article in press as: Orlandini V, et al. Genomic analysis of three sponge-associated *Arthrobacter* Antarctic strains, inhibiting the growth of *Burkholderia cepacia* complex bacteria by synthesizing volatile organic compounds. Microbiol Res (2013), http://dx.doi.org/10.1016/j.micres.2013.09.018

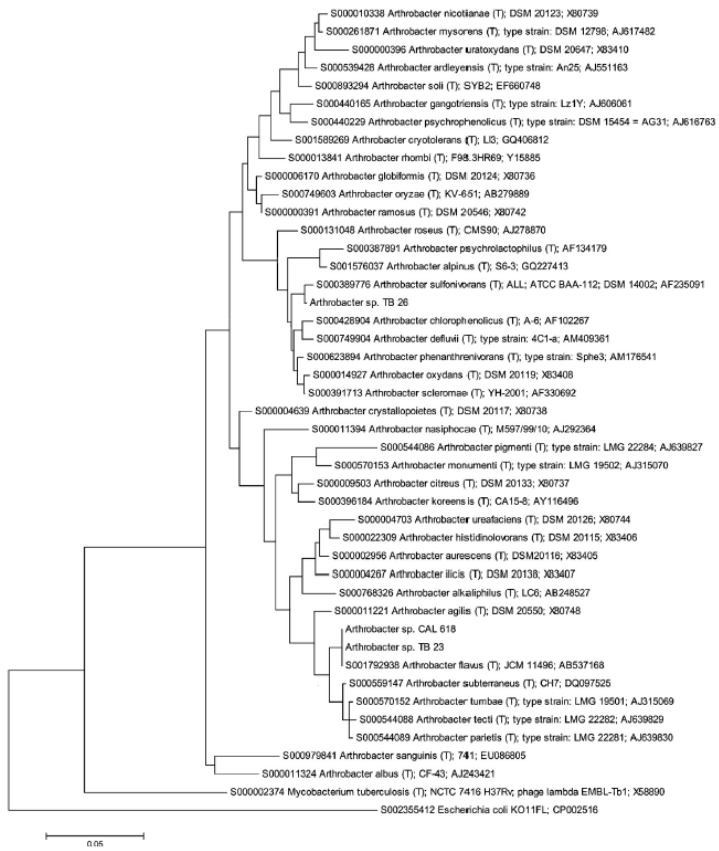


Fig. 4. Phylogenetic tree of the *Arthrobacter* species constructed using the 16S rRNA gene sequences.

close relatives (Table 3). However, it is worth noting that both *A. phenanthrenivorans* Sphe3 and *A. chlorophenolicus* A-6 harbour two (very) large plasmids (Table 3); as shown in Table 4, a BLAST scanning (using the following parameters: BLAST+ 2.2.27, with 0.05 as *e*-value threshold and 40.0% for the identity threshold) of the TB26, TB23 and CAL618 genomes using as query the nucleotide sequence of the four plasmids, retrieved a much higher number of bp from TB26 genome than from TB23 and CAL618 genomes. Therefore, the

Table 4
Basepairs matching between plasmids from *A. chlorophenolicus* and *A. phenanthrenivorans* and the *Arthrobacter* sp. Genomes of strains CAL618, TB23 and TB26.

Query sequence				Number of bp retrieved in the genome of:		
Species	Strain	Plasmids		*Arthrobacter* sp. CAL618	*Arthrobacter* sp. TB23	*Arthrobacter* sp. TB26
		Name	Size (Mbp)			
A. chlorophenolicus	A-6	pACHL01	0.426	3644	4327	10,928
		pACHL02	0.158	4296	483	31,373
A. phenanthrenivorans	Sphe 3	pASPHE01	0.190	13,515	16,879	95,218
		pASPHE02	0.094	0	0	18,111

Please cite this article in press as: Orlandini V, et al. Genomic analysis of three sponge-associated *Arthrobacter* Antarctic strains, inhibiting the growth of *Burkholderia cepacia* complex bacteria by synthesizing volatile organic compounds. Microbiol Res (2013), http://dx.doi.org/10.1016/j.micres.2013.09.018

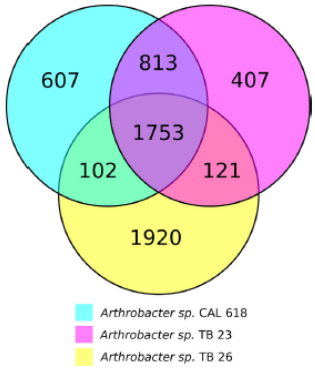


Fig. 5. Venn diagram showing the structure of the pangenome of the three *Arthrobacter* strains analyzed in this work.

presence of (large) plasmids in the TB26 cells might, at least partially, account for the different genome size between on the other one.

A further analysis of the proteome of these strains consisted in looking for secondary metabolite productions with antiSMASH (Blin et al., 2013). *Arthrobacter* spp. CAL618 and TB23 were recognized to have three clusters related to secondary metabolites: one for type 3 polyketide synthases (PKS3), one for terpene (Fig. 2C) and the last one with an unknown function. The TB26 strain has only two clusters, one for the polyketide synthases and the other one whose function was not predicted by the software; this confirms the overall distinctness of the genomic context of this strain in respect to the other two examined in this study.

4. Conclusions

In this work we have analyzed three strains belonging to the genus *Arthrobacter* living in association with Antarctic sponges. We tested their ability to inhibit the growth of *B. cepacia* complex cells, and the results were very encouraging. Indeed data obtained from the cross-streaking experiments showed that *Arthrobacter* spp. TB23, TB26, and CAL618 (the first two isolated from the sponge *L. nobilis* and the latter from *H. verrucosa*) were able to inhibit the growth of (almost) all the Bcc strains, probably through the production of a combination of diffusible and volatile molecules. Moreover, we observed also an effect of the growth medium on the ability of *Arthrobacter* strains to inhibit the growth of Bcc bacteria, suggesting that the metabolic status of the *Arthrobacter* cells might influence the production antimicrobial compounds by the bacterial cells. This is in agreement with previous data obtained with *Pseudoalteromonas* and *Psychrobacter* strains (Papaleo et al., 2013), even though the antimicrobial activity of those strains was much more dependent on the growth medium than that exhibited by the three *Arthrobacter* strains.

Then, we sequenced the genomes of TB26 and CAL618, which were analyzed and compared between them and with that of strain TB23. We found that the strains TB23 and CAL618 are much more similar than TB26, the former sharing more proteins between them. This probably does not have reflections on their metabolism, since

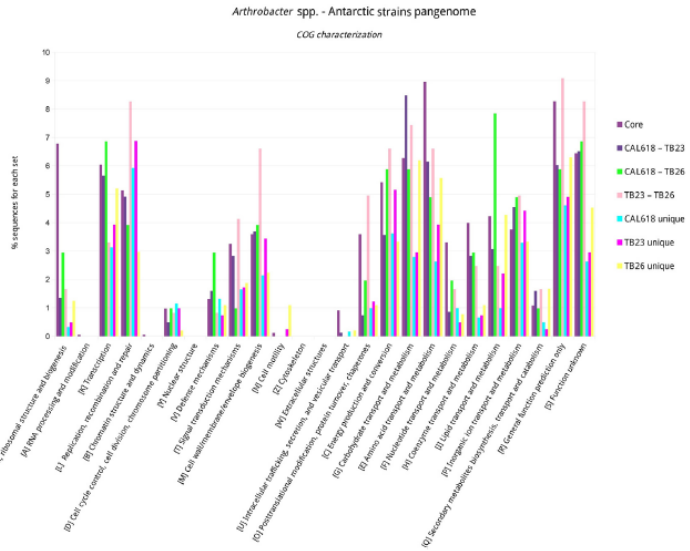


Fig. 6. *In silico* characterization of the proteins belonging to each subset of the pangenome of the three *Arthrobacter* spp. strains according to the COG database.

Please cite this article in press as: Orlandini V, et al. Genomic analysis of three sponge-associated *Arthrobacter* Antarctic strains, inhibiting the growth of *Burkholderia cepacia* complex bacteria by synthesizing volatile organic compounds. Microbiol Res (2013), http://dx.doi.org/10.1016/j.micres.2013.09.018

the distribution of the proteins of the three strains in the categories of the COG database was very similar. This was probably due to the strong environmental pressure driving different strains to adopt the same strategies to survive in an extreme environment like the Antarctic Ocean.

A phylogenetic tree made by using the 16S rRNA gene sequences of all the *Arthrobacter* confirmed the differences between CAL618 and TB23 in respect to TB26.

TB26 revealed to be more similar to *A. sulfonivorans* than the other two strains we isolated, which, on the contrary, seemed to be strictly related to each other.

These results, which represent a preliminary explorative step, brought new knowledge about this bacterial genus. With a solid basic knowledge made of experimental tests and genomic analysis (genome sequences and annotation, protein predictions comparative genomics metrics), a whole plethora of possible further insights are now possible. The most promising field of research will probably be the production of molecules with antibiotic properties by these strains, which could find an important place in the real world applications.

Acknowledgments

This work was supported by grants from the Italian Cystic Fibrosis Research Foundation (Grant FFC#12/2011), the Ente Cassa di Risparmio di Firenze (Grant 1103#2008), and the MNA (Museo Nazionale dell'Antartide). Valerio Orlandini and Marco Fondi are financially supported by an Italian Cystic Fibrosis Research Foundation Fellowship and a FEMS Advanced Fellowship (FAF2012), respectively. We also thank the EU KBBE Project PharmaSea 2012–2016, Grant Agreement No. 312184. Lastly, we are very grateful to anonymous referees for their suggestions in improving the manuscript.

References

Alexeyenko A, Tamas I, Liu G, Sonnhammer EL. Automatic clustering of orthologs and inparalogs shared by multiple proteomes. Bioinformatics 2006;22(14):e9–15.

Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, et al. The RAST Server: rapid annotations using subsystems technology. BMC Genomics 2008;9:75.

Bazzini S, Udine C, Riccardi G. Molecular approaches to pathogenesis study of *Burkholderia cenocepacia*, an important cystic fibrosis opportunistic bacterium. Appl Microbiol Biotechnol 2011;92(5):887–95.

Blin K, Medema MH, Kazempour D, Fischbach MA, Breitling R, Takano E, et al. antiSMASH 2.0 – a versatile platform for genome mining of secondary metabolite producers. Nucleic Acids Res 2013;41(W1):W204–12.

Casanueva A, Tuffin M, Cary C, Cowan DA. Molecular adaptations to psychrophily: the impact of 'omic' technologies. Trends Microbiol 2010;18(8):374–81.

Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, et al. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res 2009;37(Database issue):D141–5.

Cox MP, Peterson DA, Biggs PJ. SolexaQA:. At-a-glance quality assessment of Illumina second-generation sequencing data. BMC Bioinform 2010;11:485.

Darling AC, Mau B, Blattner FR, Perna NT. Mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome Res 2004;14(7):1394–403.

Debbab A, Aly AH, Lin WH, Proksch P. Bioactive compounds from marine bacteria and fungi. Microb Biotechnol 2010;3(5):544–63.

Drevinek P. , Mahenthiralingam E. *Burkholderia cenocepacia* in cystic fibrosis: epidemiology and molecular mechanisms of virulence. Clin Microbiol Infect 2010;16(7):821–30.

Fondi M, Orlandini V, Maida I, Perrin E, Papaleo MC, Emiliani G, et al. Draft genome sequence of the volatile organic compound-producing Antarctic bacterium *Arthrobacter* sp. strain TB23, able to inhibit cystic fibrosis pathogens belonging to the *Burkholderia cepacia* complex. J Bacteriol 2012;194(22):6334–5.

Lo Giudice A, Bruni V, Michaud L. Characterization of Antarctic psychrotrophic bacteria with antibacterial activities against terrestrial microorganisms. J Basic Microbiol 2007;47(6):496–505.

Maida I, Fondi M, Papaleo MC, Perrin E, Orlandini V, Emiliani G, et al. Phenotypic and genomic characterization of the Antarctic bacterium *Gillisia* sp. CAL575, a producer of antimicrobial compounds. Extremophiles 2013, http://dx.doi.org/10.1007/s00792-013-0590-0.

Mangano S, Michaud L, Caruso C, Brilli M, Bruni V, Fani R, et al. Antagonistic interactions between psychrotrophic cultivable bacteria isolated from Antarctic sponges: a preliminary analysis. Res Microbiol 2009;160(1):27–37.

Michaud L, Lo Giudice A, Saitta M, De Domenico M, Bruni V. The biodegradation efficiency on diesel oil by two psychrotrophic Antarctic marine bacteria during a two-month-long experiment. Mar Pollut Bull 2004;49(5/6):405–9.

Mongodin EF, Shapir N, Daugherty SC, DeBoy RT, Emerson JB, Shvartzbeyn A, et al. Secrets of soil survival revealed by the genome sequence of *Arthrobacter aurescens* TC1. PLoS Genet 2006;2(12):e214.

O'Brien KP, Remm M, Sonnhammer EL. Inparanoid: a comprehensive database of eukaryotic orthologs. Nucleic Acids Res 2005;33(Database issue):D476–80.

Papaleo MC, Fondi M, Maida I, Perrin E, Lo Giudice A, Michaud L, et al. Sponge-associated microbial Antarctic communities exhibiting antimicrobial activity against *Burkholderia cepacia* complex bacteria. Biotechnol Adv 2012;30(1):272–93.

Papaleo MC, Romoli R, Bartolucci G, Maida I, Perrin E, Fondi M, et al. Bioactive volatile organic compounds from Antarctic (sponges) bacteria. Nat Biotechnol 2013;30:824–38.

Perrin E, Fondi M, Papaleo MC, Maida I, Buroni S, Pasca MR, et al. Exploring the HME and HAE1 efflux systems in the genus *Burkholderia*. BMC Evol Biol 2010;10:164.

Perrin E, Fondi M, Papaleo MC, Maida I, Emiliani G, Buroni S, et al. A census of RND superfamily proteins in the *Burkholderia* genus. Future Microbiol 2013;8:923–37.

Simpson JT, Wong K, Jackman SD, Schein JE, Jones SJ, Birol I. ABySS: a parallel assembler for short read sequence data. Genome Res 2009;19(6):1117–23.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28(10):2731–9.

Please cite this article in press as: Orlandini V, et al. Genomic analysis of three sponge-associated *Arthrobacter* Antarctic strains, inhibiting the growth of *Burkholderia cepacia* complex bacteria by synthesizing volatile organic compounds. Microbiol Res (2013), http://dx.doi.org/10.1016/j.micres.2013.09.018

Draft genomes of three Antarctic *Psychrobacter* strains producing antimicrobial compounds against *Burkholderia cepacia* complex, opportunistic human pathogens.

Marco Fondi[1,2§], Valerio Orlandini[1,3], Elena Perrin[1], Isabel Maida[1], Emanuele Bosi[1], Maria Cristiana Papaleo[1], Luigi Michaud[4], Angelina Lo Giudice[4], Donatella de Pascale[3], Maria Luisa Tutino[5], Pietro Liò[2] and Renato Fani[1].

[1]*Laboratory of Microbial and Molecular Evolution, Department of Biology, University of Florence, Via Madonna del Piano 6, 50019 Sesto Fiorentino (Firenze)*

[2]*Computer Laboratory, Cambridge University, William Gates Building 15, JJ Thomson Avenue, Cambridge, United Kingdom*

[3]*Institute of Protein Biochemistry, National Research Council, Via P. Castellino, 111 I-80134, Naples, Italy*

[4]*Department of Biological and Environmental Sciences (DiSBA-CIBAN), University of Messina, Viale Ferdinando Stagno d'Alcontrès 31, I-98166 Messina, Italy*

[5]*Department of Chemical Sciences, University of Naples Federico II, Complesso Universitario M. S. Angelo, Via Cintia, I-80126, Naples, Italy*

[§]*Corresponding author*

Dr. Marco Fondi

Laboratory of Microbial and Molecular Evolution, Department of Biology, University of Florence, Via Madonna del Piano 6, 50019 Sesto Fiorentino (Firenze)

Tel +390554574736

Email: marco.fondi@unifi.it

Keywords: Comparative genomics – Antarctic bacteria – Genomics –*Burkholderia* infections

Abstract

Herein we present the draft genomes of three *Psychrobacter* strains isolated from Antarctic sponges and able to inhibit the growth of bacteria belonging to the *Burkholderia cepacia* complex, responsible for infections of the respiratory system in patients affected by Cystic Fibrosis. The comparative analysis of the annotated genomes of these *Psychrobacter* strains highlighted their differences in terms of overall genomic content (e.g. shared gene sets) and allowed the identification of gene clusters hypothetically involved in the biosynthesis of antimicrobial compounds.

Main text

Bacteria inhabiting extreme and isolated environments represent potential sources of novel bioactive molecules. In particular, Antarctic bacteria have been shown to be capable of synthesizing compounds with antimicrobial activity [1, 2], particularly active against bacteria belonging to the *Burkholderia cepacia* complex (Bcc).

In this work, we report the genome sequences of three strains belonging to the *Psychrobacter* genus isolated from different Antarctic sponges. Two of them (*Psychrobacter* sp. TB2 and TB15) were isolated from samples of the Antarctic sponge *Lissodendoryx nobilis*, whereas the remaining one (*Psychrobacter* sp. AC24) was isolated from *Haliclonissa verrucosa*. Strains TB2, TB15 and AC24 belong to the Italian Collection of Antarctic Bacteria of the National Antarctic Museum (CIBAN-MNA, Italy).

Their inhibitory activity against representatives of the Bcc was assessed as described in Papaleo et al. (2012) [1]. Results of these tests revealed the capability of *Psychrobacter* sp. AC24 to efficiently inhibit the growth of almost all the Bcc strains tested in this work, regardless of the growth medium. Conversely, TB2 and TB15 displayed a reduced inhibitory ability compared to AC24 and, in some cases, the effect on the growth of Bcc strains was influenced by the corresponding growth medium (Supporting Information, Table S1).

Genome sequencing (using Illumina HiSeq2000) was performed in order to provide a genomic and taxonomic background able to guide future research on these strains. Obtained reads were trimmed with SolexaQA DynamicTrim [3]. The resulting reads (28,229,244 for AC24, 26,667,670 for TB15 and 17,211,784 for TB2) were assembled using ABySS 1.3.6 [4]. The optimal parameters for the assemblies were determined after carrying out several trials, automatically performed with an *ad hoc* developed software (available at http://www.dbefcb.unifi.it/CMpro-v-p-8.html). Among obtained assemblies, we chose those for which the highest average contigs length was obtained. After filtering out the contigs with a length < 500 bp, we obtained an assembly size of 3,574,524 bp, 3,066,842 bp and 3,033,234 bp for AC24, TB15 and TB2, respectively, distributed into 88, 43 and 47 contigs. Further details for genome assemblies are shown in Table 1.

Contigs were submitted to RAST annotation server [5], allowing the identification of 3,076, 2,627 and 2,587 ORFs for AC24, TB15 and TB2, respectively. A total of 2,300 (75%) ORFs of AC24, 2,064 (79%) of TB15 and 2,040 (79%) of TB2 were assigned to at least one of the Clusters of Orthologous Groups (COG) [6].

Particular attention was devoted to the search of genes involved in the biosynthesis of secondary metabolites, known to often possess antimicrobial activity. A search for secondary metabolites related genes was thus carried out with antiSMASH [7], revealing a variable number of clusters putatively involved in

such biosynthesis; 12, 8 and 7 clusters were retrieved for AC24, TB15 and TB2 strains, respectively (Supporting Information, File S1). From a structural viewpoint, all these gene clusters showed GC% content values in the range of the ones possessed by the corresponding genome (i.e. from 39% to 43%). Unfortunately, on the basis of performed sequence-similarity searches, no hints could be derived concerning the product(s) synthesized by those clusters. This, in turn, suggests that the metabolic strategies exploited by the three *Psychrobacter* strains to inhibit the growth of *Burkholderia* representatives fall outside the range of already characterized biochemical systems and that more experimental effort will be necessary to fully elucidate them.

The evolutionary distance among these strains was assessed through a phylogenetic analysis based on their 16S rDNA sequences. The obtained phylogenetic tree (grouping TB2 and TB15 strains away from AC24) is available in Supporting Information, File S2. However, to gain a deeper knowledge of the genomic background of the isolated *Psychrobacter* strains, comparative genomics analyses were performed. A custom made Perl script (available at http://www.dbefcb.unifi.it/CMpro-v-p-8.html) that iteratively uses InParanoid [8] and MultiParanoid [9] to make multiple comparisons between pairs of proteins sets, was run to identify which protein sequences are shared among all the strains (core genome), by only two of them (accessory genome) or are genome specific (unique genomes). Results of this analysis are reported in Figure 1. This analysis is in overall agreement with the relative phylogenetic position of the *Psychrobacter* representatives analysed in this work. Moreover, it allows reducing the search space of genes related to their antimicrobial activity. Indeed, the different inhibitory activity of the three strains (higher in AC24 with respect to TB2 and TB15, see Table 1) suggests the presence of specific metabolic circuits in *Psychrobacter sp.* AC24 strain which, in turn, are likely to be encoded by its unique genome. A BLAST search confirmed this hypothesis since clusters from TB2 and TB15 all belong

to core and accessory genomes whereas four of those from AC24 are encoded by its unique genome.

In conclusion, the analysis of the annotated genomes of *Psychrobacter* strains AC24, TB2 and TB15 (Genbank accessions AYXM01000000, AYUI01000000 and AYXN01000000, respectively) revealed the presence of several (still uncharacterized) gene clusters involved in secondary metabolites production that may be the object of further investigation and major differences in terms of shared gene sets. These data represent a solid platform for further characterization/exploitation of the metabolic features linked to bioactive compound biosynthesis.

Acknowledgements

Marco Fondi is financially supported by a FEMS advanced fellowship (FAF 2012). This work was supported by grants from the Italian Cystic Fibrosis Research foundation (Grant FFC#12/2011), the Ente Cassa di Risparmio di Firenze (Grant 1103#2008), and the MNA (Museo Nazionale dell'Antartide). We also thank the EU KBBE Project PharmaSea 2012-2016, Grant Agreement no: 312184. Valerio Orlandini is financially supported by the EU KBBE Project PharmaSea 2012-2016, Grant Agreement no: 312184.

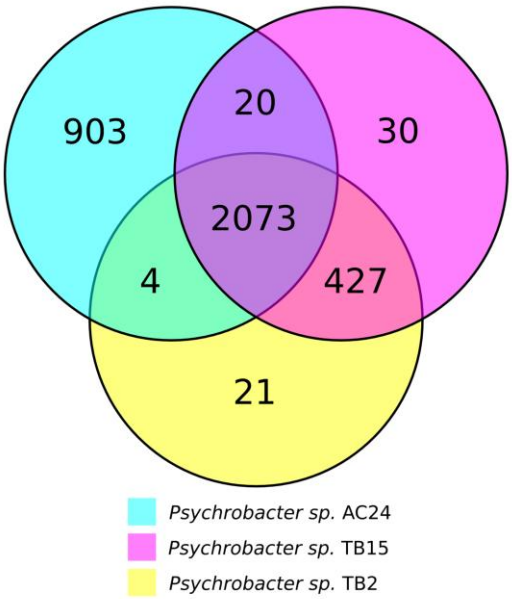
Tables

Table 1. General features of *Psychrobacter* draft genomes

Strain	Origin	Assembly size (bp)	N. contigs	GC content %	Predicted ORFs	tRNA	rRNA
AC24	*H. verrucosa*	3,574,524	88	43.01	3,076	52	12
TB15	*L. nobilis*	3,066,842	43	43.89	2,627	44	6
TB2		3,033,234	47	43.76	2,587	44	6

Figures

Figure 1. Pangenome structure of the three *Psychrobacter* strains



References

[1] M.C. Papaleo, M. Fondi, I. Maida, E. Perrin, A. Lo Giudice, L. Michaud, S. Mangano, G. Bartolucci, R. Romoli, R. Fani, Sponge-associated microbial Antarctic communities exhibiting antimicrobial activity against Burkholderia cepacia complex bacteria, Biotechnol Adv, 30 (2012) 272-293.
[2] M.C. Papaleo, R. Romoli, G. Bartolucci, I. Maida, E. Perrin, M. Fondi, V. Orlandini, A. Mengoni, G. Emiliani, M.L. Tutino, E. Parrilli, D. de Pascale, L. Michaud, A. Lo Giudice, R. Fani, Bioactive volatile organic compounds from Antarctic (sponges) bacteria, N Biotechnol, 30 (2013) 824-838.
[3] M.P. Cox, D.A. Peterson, P.J. Biggs, SolexaQA: At-a-glance quality assessment of Illumina second-generation sequencing data, BMC Bioinformatics, 11 (2010) 485.
[4] J.T. Simpson, K. Wong, S.D. Jackman, J.E. Schein, S.J. Jones, I. Birol, ABySS: a parallel assembler for short read sequence data, Genome Res, 19 (2009) 1117-1123.
[5] R.K. Aziz, D. Bartels, A.A. Best, M. DeJongh, T. Disz, R.A. Edwards, K. Formsma, S. Gerdes, E.M. Glass, M. Kubal, F. Meyer, G.J. Olsen, R. Olson, A.L. Osterman, R.A. Overbeek, L.K. McNeil, D. Paarmann, T. Paczian, B. Parrello, G.D. Pusch, C. Reich, R. Stevens, O. Vassieva, V. Vonstein, A. Wilke, O. Zagnitko, The RAST Server: rapid annotations using subsystems technology, BMC Genomics, 9 (2008) 75.
[6] R.L. Tatusov, M.Y. Galperin, D.A. Natale, E.V. Koonin, The COG database: a tool for genome-scale analysis of protein functions and evolution, Nucleic Acids Res, 28 (2000) 33-36.
[7] K. Blin, M.H. Medema, D. Kazempour, M.A. Fischbach, R. Breitling, E. Takano, T. Weber, antiSMASH 2.0--a versatile platform for genome mining of secondary metabolite producers, Nucleic Acids Res, 41 (2013) W204-W212.
[8] K.P. O'Brien, M. Remm, E.L. Sonnhammer, Inparanoid: a comprehensive database of eukaryotic orthologs, Nucleic Acids Res, 33 (2005) D476-480.
[9] A. Alexeyenko, I. Tamas, G. Liu, E.L. Sonnhammer, Automatic clustering of orthologs and inparalogs shared by multiple proteomes, Bioinformatics, 22 (2006) e9-15.

Bibliography

Baldi, F., N. Ivosevic, A. Minacci, M. Pepi, R. Fani, V. V. Svetlicic & V. V. utic, (1999) Adhesion of *Acinetobacter venetianus* to diesel fuel droplets studied with In situ electrochemical and molecular probes. *Appl Environ Microbiol* **65**: 2041-2048.

Baldi, F., M. Pepi, A. Capone, C. della Giovampaola, C. Milanesi, R. Fani & R. Focarelli, (2003) Envelope glycosylation determined by lectins in microscopy sections of *Acinetobacter venetianus* induced by diesel fuel. *Res Microbiol* **154**: 417-424.

Baptist, J. N., R. K. Gholson & M. J. Coon, (1963) Hydrocarbon oxidation by a bacterial enzyme system. I. Products of octane oxidation. *Biochim Biophys Acta* **69**: 40-47.

Bax, R., N. Mullan & J. Verhoef, (2000) The millennium bugs--the need for and development of new antibacterials. *Int J Antimicrob Agents* **16**: 51-59.

Dams-Kozlowska, H., M. P. Mercaldi, B. J. Panilaitis & D. L. Kaplan, (2008) Modifications and applications of the *Acinetobacter venetianus* RAG-1 exopolysaccharide, the emulsan complex and its components. *Appl Microbiol Biotechnol* **81**: 201-210.

Di Cello, F., M. Pepi, F. Baldi & R. Fani, (1997) Molecular characterization of an n-alkane-degrading bacterial community and identification of a new species, *Acinetobacter venetianus*. *Res Microbiol* **148**: 237-249.

Feng, L., W. Wang, J. Cheng, Y. Ren, G. Zhao, C. Gao, Y. Tang, X. Liu, W. Han, X. Peng, R. Liu & L. Wang, (2007) Genome and proteome of long-chain alkane degrading *Geobacillus thermodenitrificans* NG80-2 isolated from a deep-subsurface oil reservoir. *Proc Natl Acad Sci U S A* **104**: 5602-5607.

Fondi, M., V. Orlandini, G. Emiliani, M. C. Papaleo, I. Maida, E. Perrin, M. Vaneechoutte, L. Dijkshoorn & R. Fani, (2012a) Draft genome sequence of the hydrocarbon-degrading and emulsan-producing strain *Acinetobacter venetianus* RAG-1T. *J Bacteriol* **194**: 4771-4772.

Fondi, M., V. Orlandini, I. Maida, E. Perrin, M. C. Papaleo, G. Emiliani, D. de Pascale, E. Parrilli, M. L. Tutino, L. Michaud, A. Lo Giudice & R. Fani, (2012b) Draft genome sequence of the volatile organic compound-producing Antarctic bacterium *Arthrobacter* sp. strain TB23, able to inhibit cystic fibrosis pathogens belonging to the *Burkholderia cepacia* complex. *J Bacteriol* **194**: 6334-6335.

Fondi, M., E. Rizzi, G. Emiliani, V. Orlandini, L. Berna, M. C. Papaleo, E. Perrin, I. Maida, G. Corti, G. De Bellis, F. Baldi, L. Dijkshoorn, M. Vaneechoutte & R. Fani, (2013) The genome sequence of the hydrocarbon-degrading *Acinetobacter venetianus* VE-C3. *Res Microbiol* **164**: 439-449.

Ishige, T., A. Tani, Y. Sakai & N. Kato, (2000) Long-chain aldehyde dehydrogenase that participates in n-alkane utilization and wax ester synthesis in *Acinetobacter* sp. strain M-1. *Appl Environ Microbiol* **66**: 3481-3486.

Leahy, J. G., Z. M. Khalid, E. J. Quintero, J. M. Jones-Meehan, J. F. Heidelberg, P. J. Batchelor & R. R. Colwell, (2003) The concentrations of hexadecane and inorganic nutrients modulate the production of extracellular membrane-bound vesicles, soluble protein, and bioemulsifier by *Acinetobacter venetianus* RAG-1 and *Acinetobacter* sp. strain HO1-N. *Can J Microbiol* **49**: 569-575.

Li, J. W. & J. C. Vederas, (2009) Drug discovery and natural products: end of an era or an endless frontier? *Science* **325**: 161-165.

Lo Giudice, A., M. Brilli, V. Bruni, M. De Domenico, R. Fani & L. Michaud, (2007a) Bacterium-bacterium inhibitory interactions among psychrotrophic bacteria isolated from Antarctic seawater (Terra Nova Bay, Ross Sea). *FEMS Microbiol Ecol* **60**: 383-396.

Lo Giudice, A., V. Bruni & L. Michaud, (2007b) Characterization of Antarctic psychrotrophic bacteria with antibacterial activities against terrestrial microorganisms. *J Basic Microbiol* **47**: 496-505.

Maida, I., M. Fondi, M. C. Papaleo, E. Perrin, V. Orlandini, G. Emiliani, D. de Pascale, E. Parrilli, M. L. Tutino, L. Michaud, A. Lo Giudice, R. Romoli, G. Bartolucci & R. Fani, (2013) Phenotypic and genomic characterization of the Antarctic bacterium *Gillisia* sp. CAL575, a producer of antimicrobial compounds. *Extremophiles*.

Maier, T., H. H. Forster, O. Asperger & U. Hahn, (2001) Molecular characterization of the 56-kDa CYP153 from *Acinetobacter* sp. EB104. *Biochem Biophys Res Commun* **286**: 652-658.

Mangano, S., L. Michaud, C. Caruso, M. Brilli, V. Bruni, R. Fani & A. Lo Giudice, (2009) Antagonistic interactions between psychrotrophic cultivable bacteria isolated from Antarctic sponges: a preliminary analysis. *Res Microbiol* **160**: 27-37.

Mara, K., F. Decorosi, C. Viti, L. Giovannetti, M. C. Papaleo, I. Maida, E. Perrin, M. Fondi, M. Vaneechoutte, A. Nemec, M. van den Barselaar, L. Dijkshoorn & R. Fani, (2012) Molecular and phenotypic characterization of *Acinetobacter* strains able to degrade diesel fuel. *Res Microbiol* **163**: 161-172.

Nakar, D. & D. L. Gutnick, (2001) Analysis of the wee gene cluster responsible for the biosynthesis of the polymeric bioemulsifier from the oil-degrading strain *Acinetobacter lwoffii* RAG-1. *Microbiology* **147**: 1937-1946.

O'Brien, A., R. Sharp, N. J. Russell & S. Roller, (2004) Antarctic bacteria inhibit growth of food-borne microorganisms at low temperatures. *FEMS Microbiol Ecol* **48**: 157-167.

Orlandini, V., I. Maida, M. Fondi, E. Perrin, M. C. Papaleo, E. Bosi, D. de Pascale, M. L. Tutino, L. Michaud, A. Lo Giudice & R. Fani, (2013) Genomic analysis of

three sponge-associated *Arthrobacter* Antarctic strains, inhibiting the growth of *Burkholderia cepacia* complex bacteria by synthesizing volatile organic compounds. *Microbiol Res*.

Papaleo, M. C., M. Fondi, I. Maida, E. Perrin, A. Lo Giudice, L. Michaud, S. Mangano, G. Bartolucci, R. Romoli & R. Fani, (2012) Sponge-associated microbial Antarctic communities exhibiting antimicrobial activity against *Burkholderia cepacia* complex bacteria. *Biotechnol Adv* **30**: 272-293.

Papaleo, M. C., R. Romoli, G. Bartolucci, I. Maida, E. Perrin, M. Fondi, V. Orlandini, A. Mengoni, G. Emiliani, M. L. Tutino, E. Parrilli, D. de Pascale, L. Michaud, A. Lo Giudice & R. Fani, (2013) Bioactive volatile organic compounds from Antarctic (sponges) bacteria. *N Biotechnol* **30**: 824-838.

Pathom-Aree, W., J. E. Stach, A. C. Ward, K. Horikoshi, A. T. Bull & M. Goodfellow, (2006) Diversity of actinomycetes isolated from Challenger Deep sediment (10,898 m) from the Mariana Trench. *Extremophiles* **10**: 181-189.

Rojo, F., (2009) Degradation of alkanes by bacteria. *Environ Microbiol* **11**: 2477-2490.

Romoli, R., M. C. Papaleo, D. de Pascale, M. L. Tutino, L. Michaud, A. LoGiudice, R. Fani & G. Bartolucci, (2011) Characterization of the volatile profile of Antarctic bacteria by using solid-phase microextraction-gas chromatography-mass spectrometry. *J Mass Spectrom* **46**: 1051-1059.

Throne-Holst, M., S. Markussen, A. Winnberg, T. E. Ellingsen, H. K. Kotlar & S. B. Zotchev, (2006) Utilization of n-alkanes by a newly isolated strain of *Acinetobacter venetianus*: the role of two AlkB-type alkane hydroxylases. *Appl Microbiol Biotechnol* **72**: 353-360.

Throne-Holst, M., A. Wentzel, T. E. Ellingsen, H. K. Kotlar & S. B. Zotchev, (2007) Identification of novel genes involved in long-chain n-alkane degradation by *Acinetobacter* sp. strain DSM 17874. *Appl Environ Microbiol* **73**: 3327-3332.

van Beilen, J. B. & E. G. Funhoff, (2007) Alkane hydroxylases involved in microbial alkane degradation. *Appl Microbiol Biotechnol* **74**: 13-21.

van Beilen, J. B., S. Panke, S. Lucchini, A. G. Franchini, M. Rothlisberger & B. Witholt, (2001) Analysis of *Pseudomonas putida* alkane-degradation gene clusters and flanking insertion sequences: evolution and regulation of the alk genes. *Microbiology* **147**: 1621-1630.

Wentzel, A., T. E. Ellingsen, H. K. Kotlar, S. B. Zotchev & M. Throne-Holst, (2007) Bacterial metabolism of long-chain n-alkanes. *Appl Microbiol Biotechnol* **76**: 1209-1221.

Ringraziamenti

Alla fine di questo percorso vorrei ringraziare tutte le persone che mi sono state vicine e mi hanno aiutato durante questi tre anni.

Il primo ringraziamento va al Prof.Fani, senza il quale questo lavoro non sarebbe stato possibile. Grazie per tutta la fiducia che ha sempre riposto in me.

Un grazie enorme va a Marco per essere sempre disponibile per qualsiasi cosa, e ad Isa per tutto il sostegno che ci diamo a vicenda. Grazie anche a Cristiana, con la quale ho condiviso gran parte di questo percorso, e ad Alessio per i suoi consigli sempre utilissimi.

Grazie a tutti coloro che hanno contribuito ai lavori presenti in questa tesi. In particolare grazie a Francesca e Silvia entrambe delle ottime insegnanti.

Grazie a tutte le tesiste che con il loro lavoro hanno contribuito ai risultati presenti in questa tesi, in particolare a Camilla , ossessiva e precisa quasi più di me, ma anche a Beatrice, Federica, Alessandra, Melania, Marta e Vanessa.

Infine, ultimo ma non meno importante, grazie per tutto a babbo, mamma e Francesco...