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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

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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.

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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

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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

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

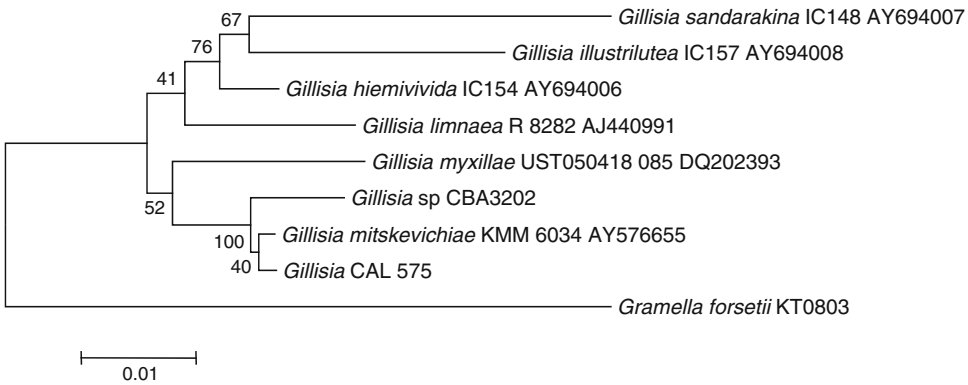

Fig. 1 Phylogenetic tree constructed using the 16S rRNA gene sequences representative of all the known *Gillisia* species

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

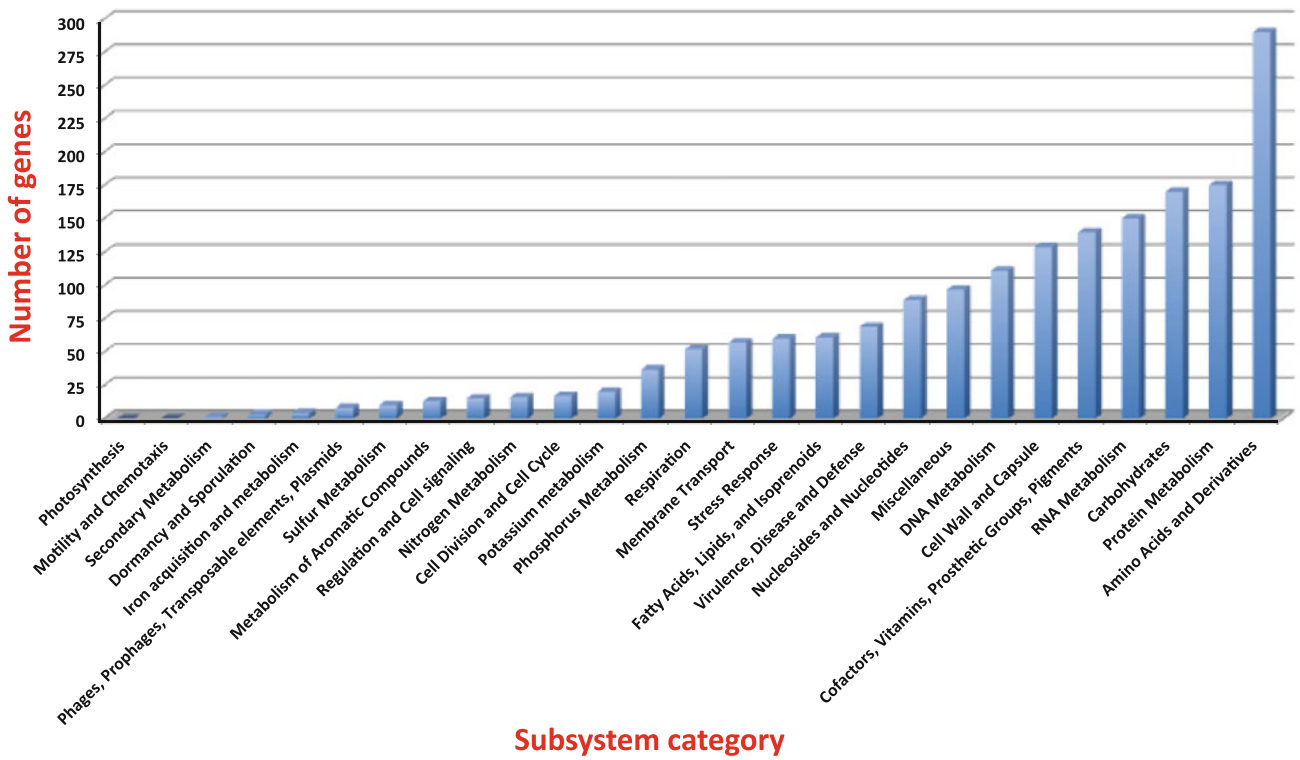

Fig. 2 Distribution of genes involved in the subsytems. The order is from the smallest to the larger number of genes

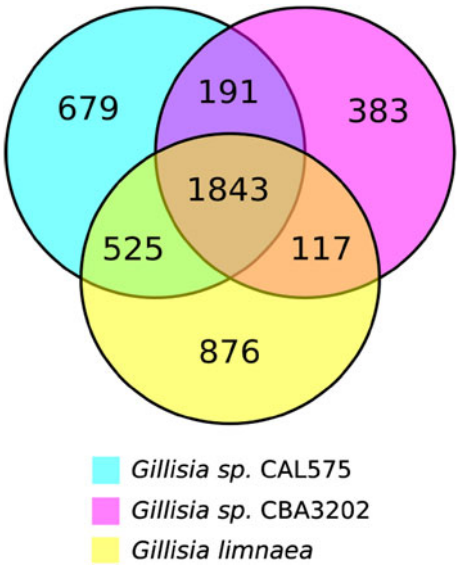

Fig. 3 Venn diagram representing the pangenome of the three *Gillisia* strains analyzed in this work

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

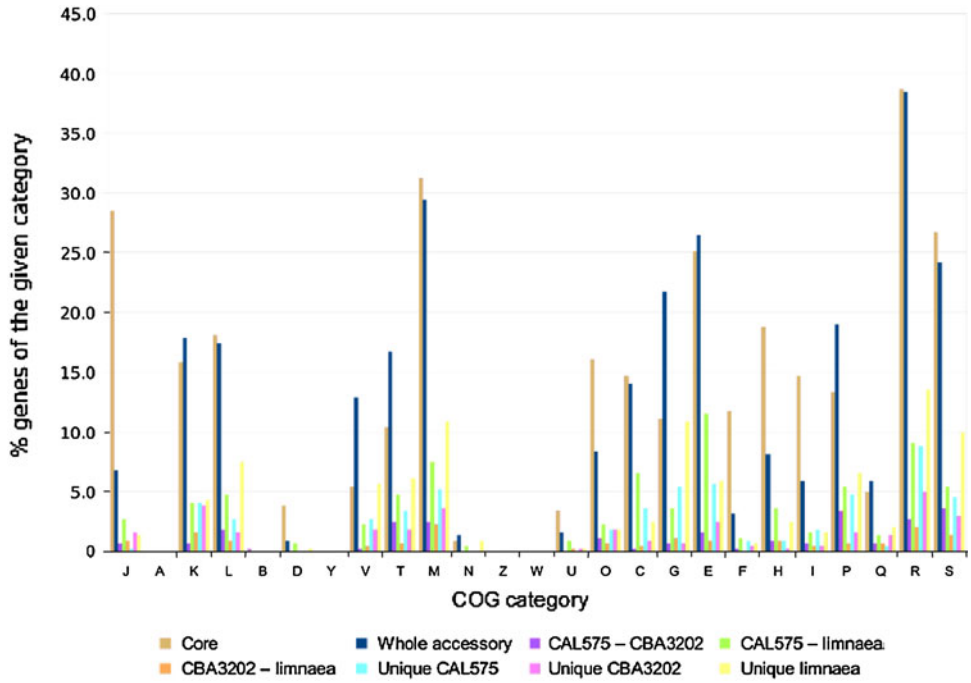

Fig. 4 Functional characterization, according to the COG database, of genes found in the pangenome of the three *Gillisia* strains taken into account. Genes belonging to the core and to the accessory genomes are split into two different *bars*. Genes embedded in the accessory genome are further split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

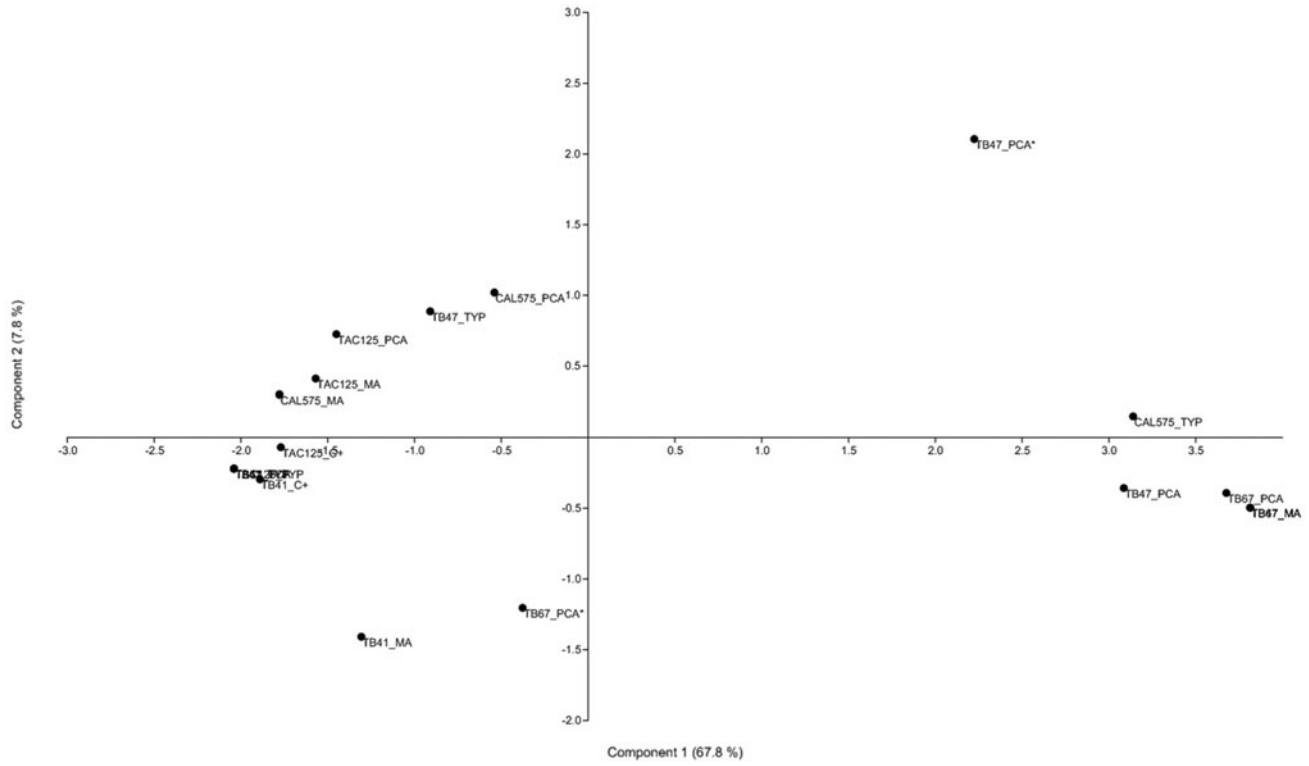

Fig. 5 Principal component analysis of inhibitory patterns of *Gillisia* sp. CAL575, *Pseudoalteromonas* TB41 and TAC125, and *Psychrobacter* TB47 and TB67 strains toward a panel of 38 Bcc strains. Percentages indicate the variance explained by the first two components

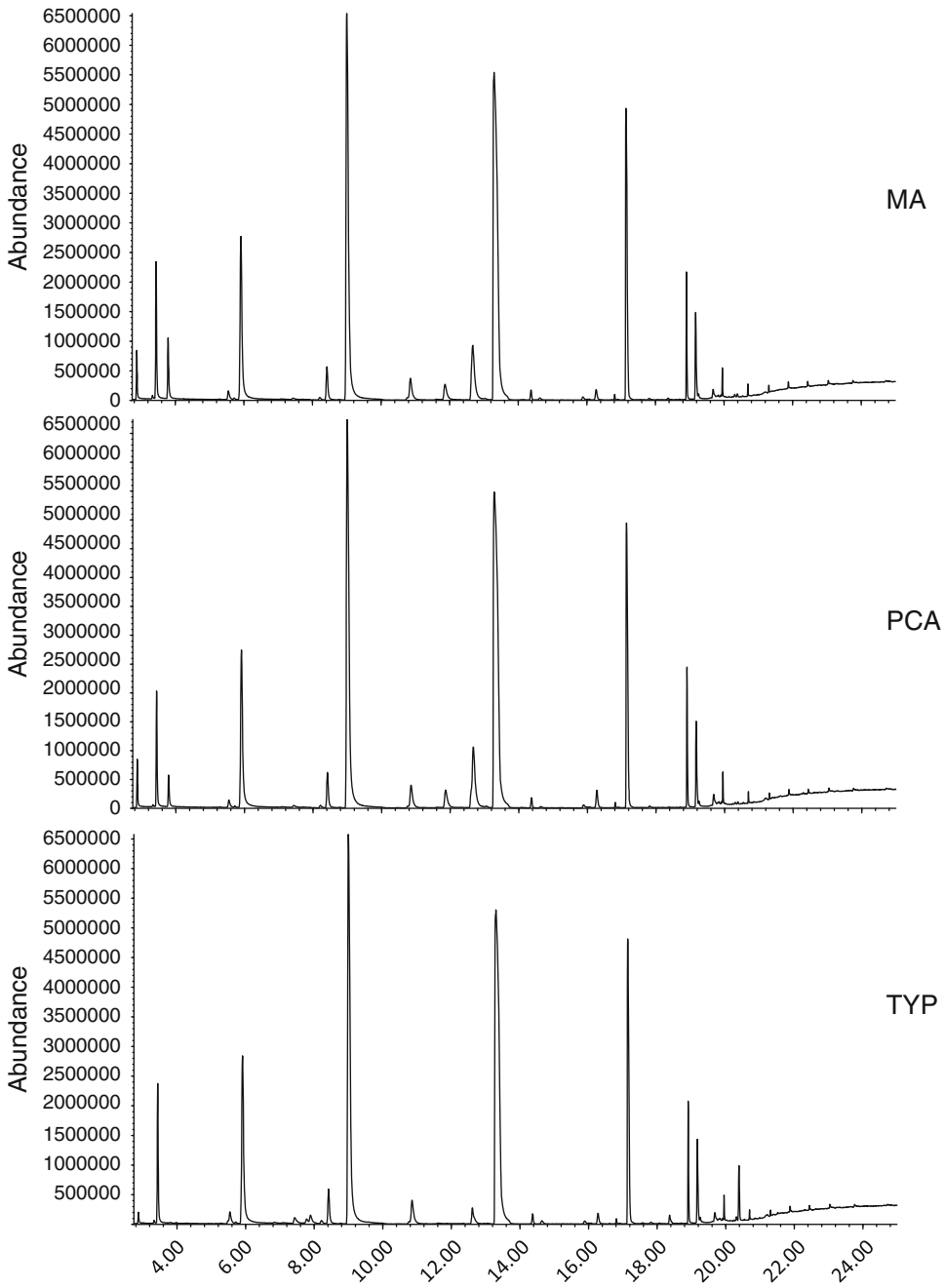

Fig. 6 mVOCs profiles obtained from *Gillisia* sp. CAL575 grown under aerobic conditions after 3 days of incubation in three different media

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

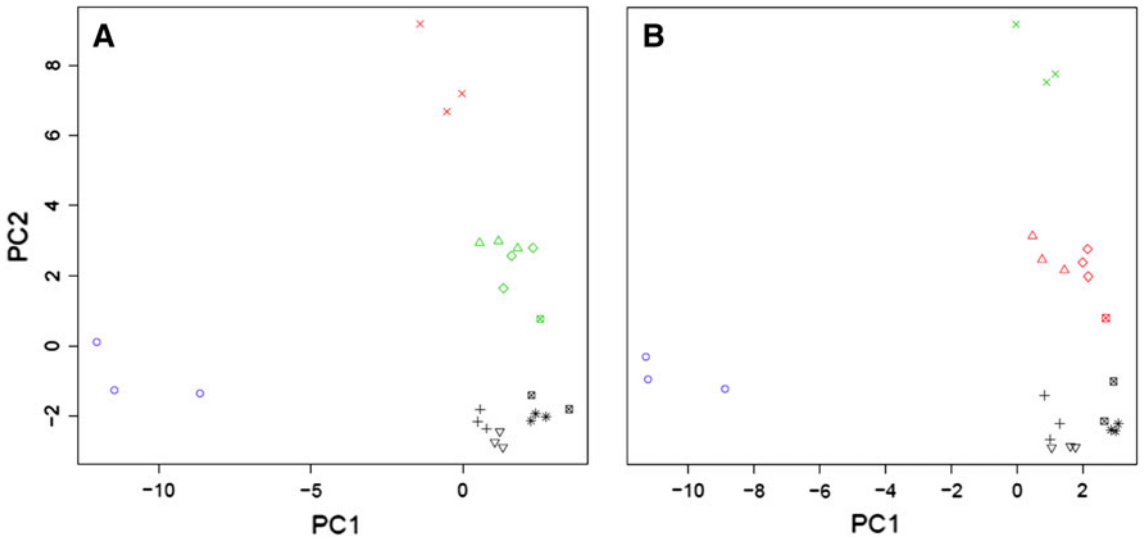

Fig. 7 PCA analysis of *Gillisia* sp. CAL575 grown in the three different media after one (**a**) and three (**b**) days of incubation at 21 °C. X *B. cenocepacia* LMG 16654 grown in PCA medium, ◇ *Gillisia* sp. CAL575 grown in PCA, ▽ PCA negative control, ⊠ *Gillisia* sp. CAL575 grown in TYP, * TYP 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.secondarymetabolites.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 18GX11/2 (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.

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