Appendix 2

In addition to the projects I've worked on during the PhD, other projects have been carried out by our research group, on which I worked to a lesser extent.

These works are part of two main lines of research. The first concerns the characterization of *Acinetobacter* strains able to degrade diesel fuel, while the second is the search of new antimicrobials from bacteria isolated from Antarctica.

Acinetobacter venetianus: a diesel-fuel degrading bacterium

The characterization of the *Acinetobacter* strains represents a critical step for their possible use in the field of bioremediation, the technique that involves the use of (micro)organisms to remove or neutralize pollutants from a contaminated site.

Crude oil is a complex mixture, in which alkanes are the major components, that can produce serious environmental problems when spills occur and over the last decade, extensive research has focused on its degradation by pure culture or mixed bacterial consortia isolated from oil-contaminated soils (Rojo, 2009).

Degradation of *n*-alkanes has been extensively studied mainly in *Pseudomonas putida* GPo1, but in recent years various microbial species involved in alkane degradation and/or capable of thriving on these highly reduced organic compounds have been revealed and studied including bacteria of the genus *Acinetobacter* (Baptist *et al.*, 1963, van Beilen *et al.*, 2001, van Beilen & Funhoff, 2007, Di Cello *et al.*, 1997, Ishige *et al.*, 2000, Throne-Holst *et al.*, 2007).

The process of bacterial *n*-alkane degradation consists of two main steps. The first is the interaction of bacterial cells with diesel fuel drops, for which different bacteria have developed distinct strategies. For instance, for *Acinetobacter venetianus* VE-C3, the interaction between the diesel fuel droplets and the cell envelope is a complex process, which involves some changes in cell envelope (Baldi *et al.*, 1999, Baldi *et al.*, 2003), while a different strategy is adopted by *A. venetianus* RAG-1^{T}, whose cells produce a strong biosurfactant, the lipopolysaccharide emulsan that interfaces between cell membranes and oil . A 27

kbp cluster of genes responsible for the biosynthesis of this amphipathic, polysaccharide bioemulsifier was isolated and characterized (Dams-Kozlowska *et al.*, 2008, Nakar & Gutnick, 2001). Other *Acinetobacter* strains, like strain HO1-N, solubilize hydrocarbons in vesicles composed of proteins, phospholipids and lipopolysaccharides (Leahy *et al.*, 2003).

The second step is the enzymatic degradation of hydrocarbons. In most described cases, the *n*-alkane is oxidized to the corresponding primary alcohol by substrate-specific terminal monooxygenases/hydroxylases. For long-chain *n*-alkane oxidation, two unrelated classes of enzymes have been proposed: (1) cytochrome P450-related enzymes in both yeasts and bacteria and (2) bacterial alkane hydroxylases (pAHs) (Wentzel *et al.*, 2007). The latter class of integral membrane non-heme diiron alkane monooxygenases of the AlkB-type allows a wide range of microorganisms to grow on *n*-alkanes with carbon chain lengths from C5 to C16 (van Beilen & Funhoff, 2007). AlkB-type enzymes form a complex with two electron transfer proteins, a dinuclear iron rubredoxin, and a mononuclear iron rubredoxin reductase channeling electrons from NADH to the active site of the alkane hydroxylase (van Beilen & Funhoff, 2007). After the initial oxidation of the *n*-alkane, the corresponding alcohol is oxidized step by step by alcohol dehydrogenase and aldehyde hydrogenase to the corresponding aldehyde and carboxylic acid, respectively. The carboxylic acid then serves as a substrate for acyl-CoA-synthase, and the resulting acyl-CoA enters the b-oxidation pathway (van Beilen & Funhoff, 2007). Several bacterial strains able to degrade C5eC10 alkanes contain alkane hydroxylases that belong to a distinct family of soluble cytochrome P450 monooxygenases (Wentzel et al., 2007) as, for example, *Acinetobacter* sp. EB104 (Maier *et al.*, 2001). Alternative alkane hydroxylases have been found in those microorganisms capable of degrading alkanes longer than C20. Usually, these enzymes are not evolutionary related to known AlkB- and P450-like sequences and include AlmA (a flavin binding monooxygenase able to oxidize C20eC32 alkanes)

from *Acinetobacter* strain DSM 17874 (Throne-Holst *et al.*, 2006) and LadA from *Geobacillus thermodenitrificans* (Feng *et al.*, 2007), able to generate primary alcohols from C15eC36 alkanes.

The analysis performed in our laboratory started from the characterization of 17 *Acinetobacter* strains (13 species, including five *A. venetianus* strains) able to use diesel fuel oil as sole carbon and energy source (Mara *et al.*, 2012).

Most of these strains were able to grow in the presence of either diesel fuel or alkanes with variable chain length as the sole carbon and energy source, although to a very different extent. The drop-collapse test revealed that only three out of the 17 strains [RAG-1T and LUH 7437, and strain ATCC 17905 (genomic species 13BJ)] were able to produce a biosurfactant, suggesting that different molecular strategies to adhere to diesel fuel drops are adopted, while the MATH test showed that most strains had hydrophobic cell surface properties both when grown in LB and minimal medium containing high NaCl concentrations, with few exceptions, for example *A. venetianus* VE-C3 that was hydrophobic only in minimal medium.

The *alkM* gene encoding alkane hydroxylase was detected in the chromosome of 15 strains by PCR amplification and sequencing or Southern blot analysis, which also suggested that this gene was localized on the *Acinetobacter* chromosome rather than on plasmids.

The five *A. venetianus* strains showed different capacities and molecular mechanisms for interacting with diesel fuel droplets and degrading *n*-alkanes of different length, and their diversity was confirmed by the Phenotype Microarray (Biolog), which enabled testing them for their ability to utilize a variety of carbon and nitrogen sources. These strains used most C- and N-sources in (a very) similar way, are unable to use carbohydrates and differentially used purines as N-source.

VE-C3 was the most diverse strain, having a lower capacity to metabolize some organic acids than the other strains.

In conclusion, this work has shed light on the strategy adopted by *Acinetobacter* strains toward diesel fuel degradation. The five strains belonging to *A. venetianus* species showed better efficiency at degrading diesel fuel than the other species analyzed in this study, suggesting that the use of such microorganisms during bioremediation procedures might provide valuable advances in this important biological/ biotechnological field.

A further characterization of the *A. venetianus* strains was carried out at the genomic level through the genome sequencing and analysis of two of these strains: *A. venetianus* RAG1^{T}and VEC-3 (Fondi *et al.*, 2012a, Fondi *et al.*, 2013).

A. venetianus RAG-1^T (ATCC 31012) was first isolated from seawater near a beach in Tel Baruch, Israel. Its genome was sequenced using Illumina HiSeq2000.

Among the set of the genes that are commonly required for the metabolism of *n*-alkanes, *A. venetianus* RAG-1^T possesses *alkB*, *alkH*, *alkJ*, and *alkK*, which were found on different contigs, suggesting that they are scattered throughout the *A. venetianus* RAG-1^T chromosome. Additionally, the four genes encoding rubredoxin (*rubA*), rubredoxin reductase (*rubB*), AlmA, and LadA were found. No close homolog was found for AlkL, -S, -T, or -N. Lastly, despite the fact that *A. venetianus* RAG-1^T is able to grow in the presence of long-chain alkanes, it is missing the soluble cytochrome P450 monooxygenase that is probably involved in long-chain alkane degradation. Consistent with the presence of *A. venetianus* RAG-1^T in contaminated environments, its genome harbors several systems involved in resistance to or tolerance of toxic compounds, including cobalt, cobalt-zinc-cadmium, arsenic, and chromium, as well as 15 genes encoding multidrug resistance efflux pumps.

A. venetianus VE-C3 was isolated in 1993 from the superficial waters of the former industrialized Marghera Port in the Venice lagoon. Its genome was sequenced using Roche/454 and Illumina. Post sequencing analyses revealed that this strain is relatively distantly related to the other *Acinetobacter* strains completely sequenced

so far as shown by phylogenetic analysis and pangenome analysis (1285 genes shared with all the other *Acinetobacter* genomes sequenced so far).

Regarding the adhesion to *n*-alkanes the *wee* gene cluster, that is involved in the biosynthesis of emulsan in *A. venetianus* RAG-1^T, VE-C3 share with RAG-1^T two large portions, the first and the final part, while the central part of the cluster partially differs in the two strains. In particular some genes responsible for polymerisation of the apoemulsan were not found in the genome of *A. venetianus* VE-C3, explaining the different strategies adopted by these strains for the interaction with *n*-alkanes.

Among the genes probably involved in the metabolism of long-chain *n*-alkanes *A. venetianus* VE-C3 possesses a smaller set of *alk*-like sequences compared *to P. putida* GPo1 and these genes are scattered throughout the genome. In particular *A. venetianus* VE-C3 encodes two paralogous copies of *alkB*, *alkH* and *alkJ*, and a single copy of *alkK*. No ortholog of AlkG, AlkT, AlkN and AlkS sequences were retrieved. AlkG and AlkT (coding for rubredoxin and rubredoxin reductase, respectively) could be replaced by the *rubA-rubB* operon. Interestingly, a sequence embedding both a rubredoxin and a rubredoxin reductase domain was identified, suggesting its possible role in the alkane degradation process. Also ALkS and *alkN* (a regulator and a genes involved in chemotaxis transduction respectively) lacks in *A. venetianus* VEC-3.

A. venetianus VE-C3 encodes a single cytochrome P450 in an operon-like structure with genes encoding a ferredoxin, an FAD-dependent oxyreductase and a gene encoding an AraC transcriptional regulator. Orthologs of LadA and AlmA encoding genes were also found when probing the genome of VE-C3

A wide range of determinants involved in resistance to toxic metals (e.g. arsenic, cadmium, cobalt and zinc) were found. Genes belonging to these processes were found both on the chromosome and in plasmids.

Finally, the presence of a number of DNA mobilization-related genes (i.e. transposases, integrases, resolvases) strongly suggests an important role played by horizontal gene transfer in shaping the genome of *A. venetianus* VE-C3 and in its adaptation to its special ecological niche.

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Research in Microbiology 163 (2012) 161–172

www.elsevier.com/locate/resmic

Molecular and phenotypic characterization of *Acinetobacter* strains able to degrade diesel fuel

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Received 19 September 2011; accepted 23 November 2011
Available online 16 December 2011

Abstract

Characterization of bacterial communities in oil-contaminated soils and evaluation of their degradation capacities may serve as a guide for improving remediation of such environments. Using physiological and molecular methods, the aim of this work was to characterize 17 *Acinetobacter* strains (13 species) able to use diesel fuel oil as sole carbon and energy source. The strains were first tested for their ability to grow on different alkanes on minimal medium containing high NaCl concentrations. The envelope hydrophobicity of each strain was assessed by microbial adhesion to the hydrocarbon test (MATH) when grown in LB medium or minimal medium containing succinate or diesel fuel. Most strains were hydrophobic both in LB and minimal medium, except for strain *Acinetobacter venetianus* VE-C3 that was hydrophobic only in minimal medium. Furthermore, two *A. venetianus* strains, RAG-1^T and LUH 7437, and strain ATCC 17905 (genomic species 13BJ) displayed biosurfactant activity. The *alkM* gene encoding alkane hydroxylase was detected in the chromosome of the 15 strains by PCR amplification, sequencing and Southern blot analysis. Phenotype microarray analysis performed on the five *A. venetianus* strains revealed that they differentially used purines as N-source and confirmed that they are unable to use carbohydrates.

Keywords: *Acinetobacter venetianus*; Phenotype microarray; *alkM* gene

1. Introduction

Crude oil is a complex mixture of hydrocarbons and other organic compounds that can produce serious environmental problems when spills occur. Bioremediation is an efficient, economic and versatile alternative to physicochemical treatment of oil contaminants. Over the last decade, extensive research has focused on oil bioremediation, and crude oil degradation has been carried out with pure culture or mixed bacterial consortia isolated from oil-contaminated soils (Rojo, 2009).

Characterization of bacterial populations living in oil-contaminated soils and evaluation of their degradation capacities may serve as a guide for improving remediation of such environments (van Hamme et al., 2003; Zhengzhi et al., 2010). Degradation of n-alkanes has been extensively studied in *Pseudomonas putida* GPo1 (formerly *Pseudomonas oleovorans*; Baptist et al., 1963; van Beilen et al., 2001). Further to

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doi:10.1016/j.resmic.2011.12.002

that, over the last decades, various microbial species involved in alkane degradation and/or capable of thriving on these highly reduced organic compounds have been revealed and studied (van Beilen and Funhoff, 2007), including bacteria of the genus *Acinetobacter* (Di Cello et al., 1997; Ishige et al., 2000; Throne-Holst et al., 2007) that might represent interesting model systems for studying this process. Overall, the process of bacterial *n*-alkane degradation is complex and consists of two main steps: i) the interaction of bacterial cells with diesel fuel drops; and ii) the oxidation process. Concerning the first step, different bacteria able to grow on hydrocarbons as sole carbon and energy source have developed distinct strategies for interacting with diesel fuel (Baldi et al., 1999; Rosenberg et al., 1979; Zuckerberg et al., 1979). For instance, different strains of *Acinetobacter venetianus* have adopted varying strategies to adhere to diesel fuel drops. In strain VE-C3, the interaction between the diesel fuel droplets and the cell envelope is a complex process during which *n*-alkanes induce glycosylation of membrane proteins involved in oil uptake (Baldi et al., 2003) and in biofilm formation due to cell-to-cell contact and synthesis of a composite material constituted by exopolysaccharides (EPSs) and *n*-alkanes (Baldi et al., 1999). The cell-to-cell aggregation is parallel to an increase in cell envelope hydrophobicity and is then followed by internalization of diesel fuel droplets (Baldi et al., 1999). A completely different strategy is adopted by in-depth-studied *A. venetianus* strain RAG-1^T, whose cells produce a strong biosurfactant, the lipopolysaccharide emulsan that interfaces between cell membranes and oil (Dams-Kozlowska et al., 2008a,b; Gutnick et al., 1991; Nakar and Gutnick, 2001). Other *Acinetobacter* strains, like strain HO1-N, solubilize hydrocarbons in vesicles composed of proteins, phospholipids and lipopolysaccharides (Leahy et al., 2003). The second step, enzymatic degradation of hydrocarbons, is usually catalyzed by the alkane monooxygenase complex formed by three different subunits: i) alkane hydroxylase (encoded by *alkM*); ii) rubredoxin; and iii) rubredoxin reductase. This complex has been characterized in detail in *P. putida* GPo1, where the *alk* genes are operonically organized into the octane utilization (OCT) plasmid (van Beilen et al., 2001). A different type of organization was found in some *Acinetobacter* strains, such as *Acinetobacter baylyi* ADP-1 (Ratajczak et al., 1998; Tani et al., 2001; van Hamme et al., 2003) and *A. venetianus* VE-C3, where genes responsible for degradation of *n*-alkanes are located in the bacterial chromosome. However, analysis of Alk$^-$ mutants suggested that genes involved in hydrocarbon uptake were also present on the two plasmids pAV1 (10,820 bp) and pAV2 (15,135 bp) (Decorosi et al., 2006; Di Cello et al., 1997; Mengoni et al., 2007). Recently, Vaneechoutte et al. (2009) described the novel species *A. venetianus* and reported preliminary analysis of the ability of strains belonging to this species to grow in the presence of diesel fuel. In the same paper, type and reference strains of a number of species were used to assess whether alkane degradation was an exclusive feature of *A. venetianus,* and data obtained showed that growth on long C-chain alkanes is not exclusive to *A. venetianus*.

The aim of this work was to gain better insight into mechanisms used to degrade alkanes by bacteria of the genus *Acinetobacter* through characterization of the set of strains previously analyzed by Vaneechoutte et al. (2009) using a combination of physiological and molecular methods. Characterization included growth in minimal medium with different alkanes as sole carbon and energy source, microbial adhesion to hydrocarbons (MATH test), biosurfactant activity, analysis of plasmid content and detection of the *alkM* gene coding for alkane hydroxylase. Lastly, *A. venetianus* strains were investigated for their utilization of different carbon and nitrogen sources by phenotype microarray analysis.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The *Acinetobacter* strains used are listed in Table 1 and comprised five well-defined *A. venetianus* strains of various origins and a set of type and reference strains of other *Acinetobacter* species previously tested for growth on C-sources of different length (Vaneechoutte et al., 2009). The strains were grown either in Luria-Bertani (LB) or Minimal Medium Venetia (MMV) (1.0 g l^{-1} of $MgSO_4.7H_2O$, 0.7 g l^{-1} of KCl, 2.0 g l^{-1} of KH_2PO_4, 3.0 g l^{-1} of Na_2HPO_4, 1.0 g l^{-1} of NH_4NO_3, and 24.0 g l^{-1} of NaCl in deionized water) (Mills et al., 1978) containing 0.4% diesel fuel or 0.4% succinate as sole carbon and energy source. Diesel fuel (Esso Italiana) was previously filtered through a 0.2 µm-pore-size filter (Sartorius) for sterilization and particle removal. Bacterial cultures were incubated overnight at 30 °C.

2.2. Drop-collapse assay to assess biosurfactant activity

The drop-collapse test was used for screening biosurfactant production by *Acinetobacter* liquid cultures as described by Tugrul and Cansunar (2005). All experiments were repeated four times.

2.3. Investigation of adherence to hydrocarbon (MATH test)

In order to check changes in the envelope of *Acinetobacter* cells grown under different conditions (i.e., in LB or MMV medium supplemented either with 0.4% succinate or 0.4% diesel fuel), microbial adhesion to the hydrocarbon test (MATH) was performed according to Hori et al. (2008).

2.4. Amplification and sequencing of alkM gene

The *alk*M gene was detected by PCR as described by Smits et al. (1999) using primers Ts2s and Deg1re and 2 µl of cell lysate prepared by lysing of 2–3 colonies grown overnight in LB (Papaleo et al., in press). Amplification products were analyzed by agarose gel (0.8% w/v) electrophoresis in TAE buffer (0.04 M Tris–Acetate, 0.01 M EDTA) containing 0.5 µg/ml (w/v) ethidium bromide.

Table 1
List of *Acinetobacter* strains used in this work and their *alkM* gene sequence accession numbers.

Strain[a]	Species	Origin	*alkM* accession number
LUH 3904^T (RAG-1^T)	*A. venetianus*	Seawater, Tel Baruch, Israel	JN384212
LUH 4379 (VE-C3)	*A. venetianus*	Venice lagoon, Adriatic Sea, Italy	
LUH 5627 (S1-2)	*A. venetianus*	Aquaculture pond, Denmark	JN384213
LUH 7437 (CUHK 7025)	*A. venetianus*	Vegetable market, Hong Kong	
LUH 8758 (T4, MBIC 1332)	*A. venetianus*	Japanese Sea	JN384214
RUH 2215^T (ATCC 17906^T	*A. haemolyticus*	Sputum	JN384215
RUH 2228^T(ATCC 17908^T)	*A. junii*	Urine	
RUH 2867 (ATCC 17979^T)	*A.* genomic species 6	Throat	
LUH 1717 (ATCC 17905)	*A.* genomic species 13BJ/14TU	Conjunctiva	
LUH 1726 (382[b])	*A.* genomic species 14BJ	Conjunctiva	
LUH 1729 (79[b])	*A.* genomic species 15BJ	Skin	
LUH 1731 (ATCC 17988)	*A.* genomic species 16	Urine	JN384216
LUH 1735 (641[b])	*A.* genomic species 17	Wound	
LUH 9346^T (CCM 7200, 7N16^T)	*A. tjembergiae*	Activated sludge	
RUH 2219^T (NCTC 5866)	*A. lwoffii*	Unknown	
RUH 2865^T (IAM 13186^T)	*A. radioresistens*	Cotton	
RUH 3023^T (CCUG 19096^T)	*A. baumannii*	Urine	

[a] All isolates were obtained from the strain collection of the Department of Infectious Diseases, Leiden University Medical Center, Leiden, Netherlands.
[b] Designations used by Bouvet PJM, Jeanjean S. Res Microbiol 1989;140:291–9.

For sequencing, amplicons were purified from agarose gel using the MinElute gel extraction purification kit (Qiagen) according to the manufacturer's instructions. The nucleotide sequence of a 550 bp *alk*M gene region was determined on both strands using an Applied Biosystems BigDye terminator cycle sequencing kit, version 3.1, according to the manufacturer's instructions.

2.5. Analysis of plasmid content

For each strain plasmid DNA was obtained from 3 ml bacterial cultures grown overnight using the alkaline lysis method (Sambrook et al., 1989). The presence of plasmid molecules was analyzed by agarose gel (0.8% w/v) electrophoresis as described in Section 2.4. Three replicates were performed and the same results in the three independent experiments were obtained (not shown).

2.6. Southern hybridization

Total and plasmid DNA of the seventeen bacterial strains were separated by electrophoresis on a 0.8% w/v agarose gel. DNA was transferred onto a nylon membrane (Hybond N, Amersham) and Southern blotting was performed as described (Sambrook et al., 1989). The probe for Southern hybridization was an *A. venetianus* LUH 8758 *alk*M gene fragment obtained via PCR amplification. The probe was labeled and hybridization signals detected with the "Digoxigenin Labeling and Detection Kit" (Roche, Switzerland) using the colorimetric method following the instructions of the supplier.

2.7. Analysis of sequence data

BLAST probing of DNA databases was performed with BLASTn and BLASTp options of the BLAST program (Altschul et al., 1997) using default parameters. Nucleotide sequences were retrieved from the GenBank database. The ClustalW program (Thompson et al., 1994) was used to align AlkM amino acid sequences obtained with the most similar ones retrieved from the databases. Each alignment was checked manually, corrected and then analyzed using the neighbor-joining method (Saitou and Nei, 1987) and the model of Kimura 2-parameter distances (Kimura, 1980). Phylogenetic trees were constructed with the aligned sequences using Molecular Evolutionary Genetics Analysis 5 software (Tamura et al., 2011). The robustness of the inferred trees was evaluated by 1000 bootstrap resamplings.

2.8. Phenotype microarray (PM) tests

The five *A. venetianus* strains listed in Table 1 were tested on PM 96-well plates (PM01–02 carbon sources and PM03 nitrogen sources for a total of 285 compounds). The complete list of compounds assayed by PM01, PM02 and PM03 can be obtained at http://www.biolog.com/pdf/PM1-PM10.pdf. PM uses tetrazolium violet reduction as a reporter of active metabolism (Bochner et al., 2001). The reduction of the dye causes the formation of a purple color that is recorded by a CCD camera at defined time intervals, providing quantitative and kinetic information about the response of the cells in the PM plates (Bochner et al., 2001). Strains were grown overnight at 30 °C on LB and then cells were picked up with a sterile cotton swab and suspended in 15 ml inoculation fluid (IF-0, Biolog). Cell density was adjusted to 81% transmittance (T) on a Biolog turbidimeter. PM plates were inoculated (100 µl per well) using cell suspensions complemented with 1% (v/v) Dye Mix E (Biolog). The bacterial suspensions used for inoculation of PM03 were supplemented with 20 mM sodium succinate and 2 µM ferric citrate as carbon source. PM plates were incubated at 30 °C in an Omnilog Reader (Biolog) and monitored automatically every 15 min for color changes in the wells. Readings were

recorded for 48 h, and data were analyzed with Omnilog-PM software (release OM_PM_109M) (Biolog), which generated a time-course curve for tetrazolium color formation. Data from Omnilog-PM software were filtered, using average height as a parameter and processed with Bionumerics software (Applied Math, Kortrijk, Belgium). An average height threshold of 100 arbitrary omnilog units (AOUs) was chosen to identify the carbon and nitrogen sources used by the strains (background curves in the control wells showed an average height of around 70 AOU). Pearson's coefficient and the UPGMA clustering method were used for cluster analysis. A co-phenetic correlation coefficient was computed to evaluate the quality of the cluster analysis. Two replicates (all trays) for each strain were performed.

3. Results

3.1. Growth of Acinetobacter strains on minimal medium containing either diesel fuel or different carbon sources

It has been previously shown (Vaneechoutte et al., 2009) that most of the 17 *Acinetobacter* strains were able to grow in the presence of either diesel fuel or alkanes with variable chain length as the sole carbon and energy source, although to a very different extent. The unpublished raw data from that study were reprocessed for the present work and depicted for each strain in Fig. 1, showing that: i) all *Acinetobacter* strains grew better on long-chain *n*-alkanes (C20) rather than on C14 and C10 molecules, with the exception of *Acinetobacter junii* RUH 2228^T and *Acinetobacter haemolyticus* RUH 2215^T; ii) several *Acinetobacter* strains did not utilize *n*-alkanes with C10 length; iii) different strains belonging to *A. venetianus* species exhibited a differing ability to grow in the presence of *n*-alkanes, in the following decreasing order: RAG-1^T (LUH 3904) > LUH 8758/LUH 7437 > VE-C3 (LUH 4379) > LUH 5627; iv) conversely, strains belonging to different species showed similar behavior, e.g. LUH 1717 *Acinetobacter* genomic species (gen. sp.) 13BJ/14TU and gen. sp. 17 LUH 1735; v) some strains (gen. sp. 6 RUH 2867, gen. sp. 14BJ LUH 1729, *Acinetobacter radioresistens* RUH 2865^T and *Acinetobacter baumannii* RUH 3023^T) exhibited very low capacity to grow on *n*-alkanes, utilized as the sole carbon and energy source.

3.2. Drop-collapse test

In order to check whether the 17 strains produced biosurfactants or not, all were tested with the drop-collapse assay. Tests were carried out on cells grown in three different media: LB and MMV containing either succinate or diesel fuel as the sole carbon and energy source. Data obtained (Table 2) confirmed that *A. venetianus* RAG-1^T produced a biosurfactant in MMV medium containing either diesel fuel or succinate, but not when grown in LB medium. Strains *A. venetianus* LUH 7437 and gen. sp. 13BJ/14TU LUH 1717 showed emulsifying activity only in medium

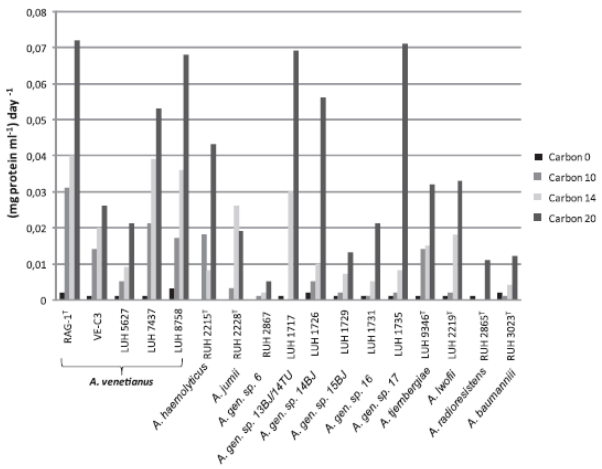

Fig. 1. Growth of 17 *Acinetobacter* strains after 72 h in mineral medium containing high sodium chloride concentrations and *n*-alkanes of variable chain lengths, expressed as (mg protein ml^{-1}) day^{-1}. Carbon 0: inoculated mineral medium (no carbon source), carbon 10, carbon 14, carbon 20: alkanes with different chain lengths. All measurements were done in quadruplicate except for the control ($n = 11$). Mean values are presented (modified from Vaneechoutte et al., 2009; supplementary material, Fig. S1).

Table 2
Overview of growth, emulsifying activity, surface hydrophobicity (MATH test), *alk*M plasmid presence and Southern blot analyses of the strains of study.

Strain	Species	Growth medium		Growth[a]	EA[b]	MATH test[c]	Plasmid presence[d]	alkM[e]	
								PCR	Southern blot
LUH 3904^T (RAG-1^T)	*A. venetianus*	MMV	+diesel fuel	+	+	+	−	+	+
			+succinate	+	+	+			
		LB		+	−	+			
LUH 4379 (VE-C3)	*A. venetianus*	MMV	+diesel fuel	+	−	+	+	+	+
			+succinate	+	−	+			
		LB		+	−	−			
LUH 5627	*A. venetianus*	MMV	+diesel fuel	+	−	+	+	+	+
			+succinate	+	−	+			
		LB		+	−	+			
LUH 7437	*A. venetianus*	MMV	+diesel fuel	+	+	+	+	+	+
			+succinate	+	−	+			
		LB		+	−	+			
LUH 8758	*A. venetianus*	MMV	+diesel fuel	+	−	+	+	+	+
			+succinate	+	−	+			
		LB		+	−	+			
RUH 2215^T	*A. haemolyticus*	MMV	+diesel fuel	+	−	+	+	+	+
			+succinate	+	−	+			
		LB		+	−	+			
RUH 2228^T	*A. junii*	MMV	+diesel fuel	+	−	+	−	−	+
			+succinate	+	−	−			
		LB		+	−	+			
RUH 2867	*A.* genomic species 6	MMV	+diesel fuel	−	nd	nd	−	−	+
			+succinate	+	−	−			
		LB		+	−	−			
LUH 1717	*A.* genomic species 13BJ/14TU	MMV	+diesel fuel	+	+	+	+	−	−
			+succinate	+	−	+			
		LB		+	−	+			
LUH 1726	*A.* genomic species 14BJ	MMV	+diesel fuel	+	−	−	−	+	+
			+succinate	+	−	+			
		LB		+	−	+			
LUH 1729	*A.* genomic species 15BJ	MMV	+diesel fuel	+	−	+	−	+	+
			+succinate	+	−	+			
		LB		+	−	−			
LUH 1731	*A.* genomic species 16	MMV	+diesel fuel	+	−	+	+	+	+
			+succinate	+	−	+			
		LB		+	−	+			
LUH 1735	*A.* genomic species 17	MMV	+diesel fuel	+	−	+	−	−	−
			+succinate	+	−	+			
		LB		+	−	+			
LUH 9346^T	*A. tjembergiae*	MMV	+diesel fuel	+	−	+	+	−	+
			+succinate	+	−	+			
		LB		+	−	+			
LUH 2219^T	*A. lwoffii*	MMV	+diesel fuel	+	−	+	+	−	+
			+succinate	−	nd	nd			
		LB		+	−	+			
RUH 2865^T	*A. radioresistens*	MMV	+diesel fuel	−	nd	nd	−	+	−
			+succinate	−	nd	nd			
		LB		+	−	−			

(*continued on next page*)

Table 2 (*continued*)

Strain	Species	Growth medium		Growth[a]	EA[b]	MATH test[c]	Plasmid presence[d]	alkM[e]	
								PCR	Southern blot
RUH 3023^T	*A. baumannii*	MMV	+diesel fuel	−	nd	nd	+	+	+
			+succinate	+	−	+			
		LB		+	−	−			

[a] +: Growth; −: no growth.
[b] EA: emulsifying activity investigated by the drop-collapse assay tested on the upper interface of the supernatant; +: Presence of EA; −: absence of EA.
[c] +: Hydrophobic cells; −: hydrophilic cells.
[d] +: Presence of plasmids; −: absence of plasmids.
[e] +: Presence of *alkM*; −: absence of *alkM* nd: not determined.

containing diesel fuel. All other strains were negative for this test. As shown in Table 2, strains RUH 2867, RUH 2865^T and RUH 3023^T did not grow in MMV containing diesel fuel as the sole carbon and energy source, in agreement with their weak ability to grow on *n*-alkanes of different length (Fig. 1).

3.3. Microbial adhesion to hydrocarbon (MATH) test

To check whether the 17 strains differed in their ability to adhere to hydrophobic surfaces, a MATH test was carried out on each strain grown either in LB or MMV medium containing 0.4% diesel fuel or 0.4% succinate. Data obtained are reported in Fig. 2 and show that four out of the five *A. venetianus* strains were highly hydrophobic both in LB and in MMV medium, with *A. venetianus* LUH 4379 (VE-C3) being the exception, as it was hydrophilic in LB, in agreement with previous data (Baldi et al., 1999). Most strains of the other species were also hydrophobic both in LB and MMV medium (Additional file 1 and Table 2).

3.4. Amplification and sequencing of the alkM gene

The presence of the *alk*M gene encoding alkane hydroxylase, in the *Acinetobacter* genome was assessed through PCR. An amplicon of the expected size (approximately

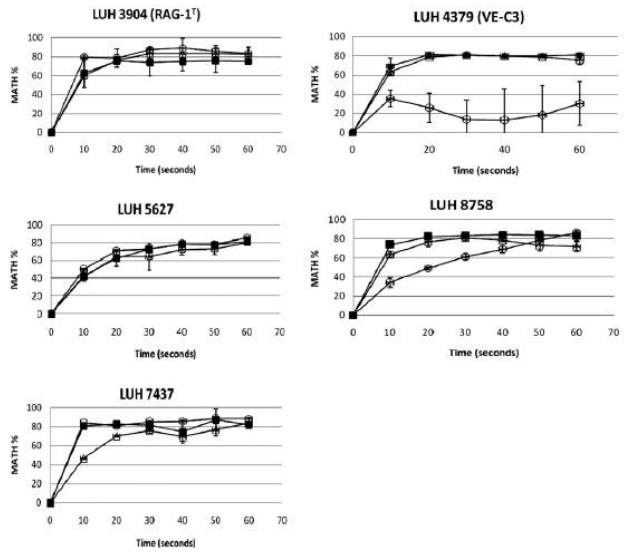

Fig. 2. MATH tests performed on *A. venetianus* strains under different growth conditions. Curves: -○- LB; -■- MMV + diesel fuel; -◇- MMV + succinate. MATH% was calculated as MATH% = $[(OD_{600\ \text{before treatment}} - OD_{600\ \text{after tratment}})/(OD_{600\ \text{before treatment}})] \times 100$. The *x* axis refers to the vortexing time.

550 bp) was obtained from the DNA of 11 out of 17 strains (Table 2). *A. junii* RUH 2228^T, gen. sp. 6 RUH 2867, gen. sp. 13BJ/14TU LUH 1717, gen. sp. 17 LUH 1735, *Acinetobacter tjernbergiae* LUH 9346^T and *Acinetobacter lwoffii* LUH 2219^T did not give an amplicon of the expected size (Table 2). In order to investigate whether the amplicons obtained actually corresponded to a fragment of a gene coding for an alkane hydroxylase, the nucleotide sequences of the amplicons were determined and compared to those available in databases (Altschul et al., 1997). Data obtained revealed that the nucleotide sequences (and the putative amino acid sequences they coded for), matched sequences corresponding to a fragment of alkane hydroxylase at the lowest E-value, suggesting that the amplification products actually represent a segment of the *alkM* gene. We also performed phylogenetic analysis using a region of the amino acid sequence of the AlkM protein from *A. baylyi* ADP-1, ranging from position 255 to position 347. For this purpose, the most similar sequences retrieved from BLASTp analysis were aligned using the ClustalW (Thompson et al., 1994) program to the AlkM sequences obtained in this work and the alignment was then used to construct the phylogenetic tree reported in Fig. 3. All *Acinetobacter* AlkM sequences clustered together and were not intermixed with AlkM sequences from other bacterial genera.

3.5. Analysis of plasmid content

Since it has been previously reported that the two VE-C3 plasmids pAV1 and pAV2 might harbor genes involved in diesel fuel uptake (Mengoni et al., 2007), we checked the presence of plasmids in the 17 strains of the study. Data obtained (Fig. 4) revealed that 10 out of the 17 strains harbored plasmids (Table 2), with four of these strains (LUH 4379, LUH 5627, LUH 7437, LUH 8758) belonging to *A. venetianus*. The plasmid size ranged between about 3 and 20 kb. Some strains carried multiple plasmids of different size. The absence of (visible) plasmids of higher size did not *per se* imply the absence of such molecules, because the plasmid extraction procedure used in this work did not permit isolation of plasmids larger than 40 kb.

3.6. Southern blotting experiments

Absence of *alk*M amplification products in six non-*A. venetianus* strains (Table 2) might be due either to absence of the gene or to divergence of the primer anchor sites. In order to discriminate between these two alternatives and to assess the localization of the *alk*M gene in *Acinetobacter* strains, a Southern blotting experiment was carried out using the *A. venetianus* LUH 8758 *alk*M amplification product as a probe and the total and plasmid DNA of the 17 strains as targets. The hybridization experiment (not shown) retrieved a signal from the genomic DNA of 13 strains (Table 2), whereas no signal was obtained from any plasmid molecule. These findings suggest that *alk*M gene is localized on the chromosome and not in plasmids.

3.7. Characterization of A. venetianus strains by phenotype microarrays

The five strains of *A. venetianus* (Table 1) were tested for utilization of a variety of carbon (PM01-02) and nitrogen sources (PM03) using the BIOLOG Phenotype MicroArray. Kinetic comparison of two independent experiments for each strain showed that reproducibility was very high and that there were no significant differences between the two kinetics curves (data not shown). As shown in Table 3, organic acids and amino acids were the two classes of compounds mainly used by the *A. venetianus* strains as carbon sources. All strains showed high activity on L-glutamic acid, L-proline, L-asparagine, L-alanine, L-histidine, pyruvic acid, L-malic acid, succinic acid, fumaric acid and acetic acid (Fig. 5). Furthermore, all strains used Tween 20, Tween 40 and Tween 80, molecules containing fatty acid moieties with long aliphatic chains (laurate, palmitate and oleate respectively). Carbohydrates were not used except for dextrin, a polymeric carbohydrate produced by hydrolysis of starch and glycogen, which was slightly utilized by all five strains.

The five strains used inorganic nitrogen compounds (ammonia, nitrate, nitrite) and amino acids (mainly L-glutamic acid, L-glutamine, L-tyrosine, L-proline, L-alanine, L-arginine, L-asparagine, L-histidine), but not dipeptides, as nitrogen sources. Strains utilized some purines but not pyrimidines.

Cluster analysis based on carbon source utilization showed that the five strains had a very similar metabolic profile (the similarity of the metabolic profiles ranged from 93.7% to 98%) and did not reveal a clear pattern with well-defined groups (Additional file 2). VE-C3 was the most diverse strain, having a lower capacity to metabolize some organic acid than the other strains, such as L-malic acid, butyric acid, α-ketobutyric acid, α-ketovaleric acid, β-hydroxybutiric acid, caproic acid and sorbic acid. LUH 8758 was the only strain able to use sebacic acid, while LUH 7437 was unable to use γ-amino-N-butyric acid.

Profiles of nitrogen utilization were similar for all strains (similarity 92–98%); however, two distinct groups could be distinguished, the former containing LUH 7437 and VE-C3 and the latter containing LUH 5627, RAG-1 and LUH 8758. The most discriminative compounds between the two groups were purines: all strains used guanine and adenine, but guanosine, xanthine, uric acid and allantoin (purine derivates) were used only by the latter group (Additional file 2).

4. Discussion

The aim of this work was to analyze a panel of 17 *Acinetobacter* strains belonging to different species (Vaneechoutte et al., 2009), able to preferentially degrade long-chain *n*-alkanes, although to very different extents (at both the inter- and intraspecies level).

The drop-collapse test revealed that only three (RAG-1^T, LUH 7437, and LUH 1717) out of the 17 strains were able to produce a biosurfactant, suggesting that different molecular

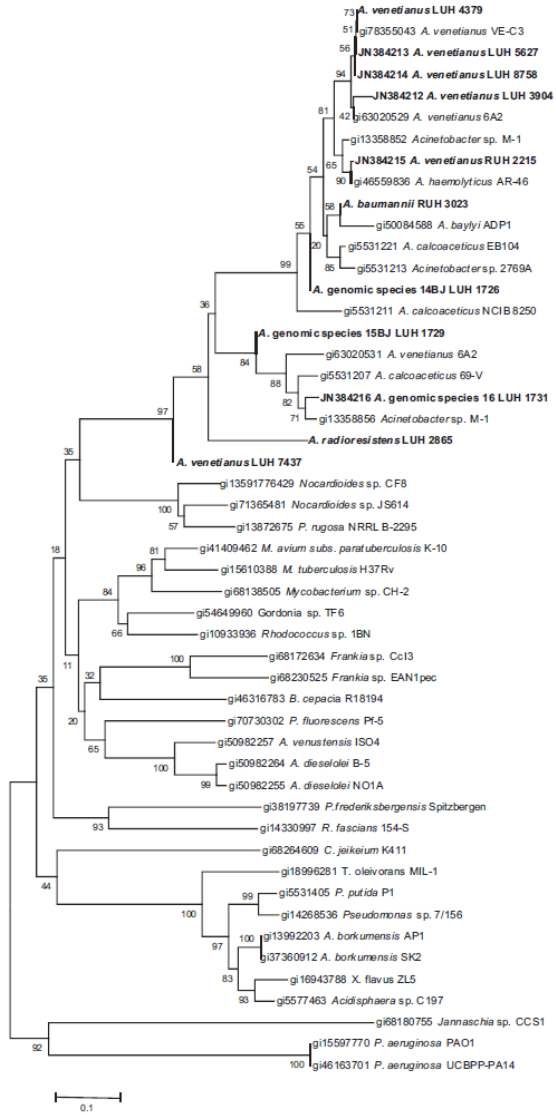

Fig. 3. Phylogenetic tree constructed using amino acid sequences that correspond to an amino acid sequence from *A. baylyi* ADP-1 (position 255 to position 347) encoding for alkane hydroxylase. Numbers at each node represent bootstrap values.

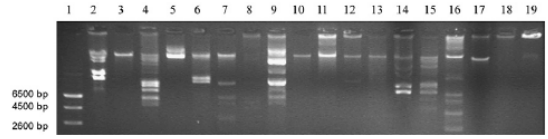

Fig. 4. Agarose gel electrophoresis of plasmid DNA extracted from *Acinetobacter* strains. Lanes: 1) plasmid marker; 2) RAG-1^T; 3) VE-C3; 4) LUH 5627; 5) LUH 8758; 6) LUH 7437; 7) LUH 2215^T; 8) LUH 2228^T; 9) LUH 1717; 10) LUH 1726; 11) LUH 1729; 12) LUH 1731; 13) LUH 1735; 14) RUH 3023^T; 15) LUH 9346^T; 16) LUH 2219^T; 17) RUH 2865^T; 18) RUH 2867.

strategies to adhere to diesel fuel drops are adopted by the different strains and species, all belonging to the genus *Acinetobacter*. Although most strains had hydrophobic cell surface properties, as displayed by the MATH test both when grown in LB and MMV, there were exceptions. These included VE-C3 that was hydrophilic only in LB, and strain gen. sp. 14BJ LUH 1726, which appeared to be hydrophobic when grown in MMV containing diesel fuel as the sole carbon and energy source.

Although several *Acinetobacter* strains harbor single or multiple plasmids of different size, Southern experiments suggested that *alk*M gene was localized on the *Acinetobacter* chromosome rather than on plasmids, in agreement with previous studies (Decorosi et al., 2006; Tani et al., 2001; van Hamme et al., 2003). In spite of this, we cannot *a priori* exclude the possibility that the *alk*M gene might be localized in a very large plasmid that cannot be disclosed by the methodology used in this work. Moreover, the topology of the AlkM phylogenetic tree strongly suggested that no recent (and possibly plasmid-mediated) horizontal transfer involving *alk*M occurred between *Acinetobacter* strains and other microorganisms belonging to the other genera. However, the finding that *A. venetianus* sequences are scattered throughout the *Acinetobacter* cluster suggested the possibility of horizontal transfer of this gene among strains belonging to different *Acinetobacter* species.

The pattern of growth in MMV compared to *alkM* analysis (PCR amplification and Southern hybridization) is rather intriguing and might be explained as follows: i) the ability to grow in the presence of diesel fuel as sole carbon and energy source was confirmed by both PCR and Southern analysis of *alkM* for nine strains (the five *A. venetianus* strains and RUH 2235^T, LUH 1726, LUH 1729, LUH 1731); ii) three *Acinetobacter* strains, that is, *A.* gen. sp. 6 RUH 2867, *A. radioresistens* RUH 2865^T and *A. baumannii* RUH 3023^T, exhibited weak ability to grow in the presence of *n*-alkanes of different lengths (Fig. 1) and in MMV supplemented with diesel fuel as sole carbon and energy source (Table 2). In spite of this, the three strains gave a positive signal in PCR, Southern or both experiments, suggesting the presence of the gene in their genome which, however, might be inactive in these microorganisms. It is noteworthy that *A. baumannii* strain RUH 3023^T, unable to degrade diesel fuel, is a human clinical isolate; it is possible that it might have evolved from living in the soil to

Table 3
Classes of compounds mainly used by the *A. venetianus* strains as Carbon and Nitrogen sources

C-sources	Tested	Used				
		LUH3904	LUH4379	LUH5627	LUH7437	LUH8758
Amino acids	30	10	10	11	12	9
Carboxylic acids	59	23	20	24	25	25
Carbohydrates	64	0	0	0	0	0
Polymers	11	0	1	1	1	1
Alcohols	5	0	0	0	0	0
Amines	5	0	0	0	0	1
Amides	3	0	0	0	0	0
Fatty acids	3	3	3	3	3	3
Totals	**180**	**36**	**34**	**39**	**42**	**39**
N-sources	Tested	Used				
		LUH3904	LUH4379	LUH5627	LUH7437	LUH8758
Inorganic compounds	3	3	3	3	3	3
Amino acids	33	24	21	22	24	25
Di-peptides	12	0	0	0	0	0
Purines	8	6	2	6	2	7
Pyrimidines	7	0	0	0	0	0
Amino sugar	6	0	0	0	0	0
Others	25	5	4	4	4	5
Totals	**94**	**38**	**30**	**35**	**33**	**40**

the human body, and during this evolutionary pathway lost its ability to break down *n*-alkanes. This scenario is in full agreement with very recent data (Maida et al., manuscript in preparation) showing that several clinical strains belonging to the *Burkholderia cepacia* complex have lost the ability to degrade diesel fuel, although they still harbor an *alkM* sequence. However, we cannot a priori exclude the possibility that these strains might be able to degrade diesel fuel under physiological conditions different from those used in this work, i.e., growth in MMV, a medium containing a high NaCl concentration. Since the source of some strains is not seawater, it is possible that the high NaCl concentration might interfere with diesel fuel degradation. Indeed, it has been very recently reported that the ability of *Acinetobacter* sp. strain DR1 to protect against diesel oil was influenced by NaCl concentration of the growth medium (Kang and Park, 2010). iii) The remaining five strains grew well in diesel fuel and on *n*-alkanes with different chain length. Three of them (RUH 2228^T, LUH 9346^T, and LUH 2219^T) gave a strong hybridization signal, suggesting the presence of *alkM* in their genome that, however, was not amplified via PCR, very likely because of the divergence of the primer and the annealing site sequences. The last two strains, LUH 1717 and LUH 1735, gave neither an amplification product nor a hybridization signal, which might be due to the divergence of the primers and probe used and the target sequences.

The five *A. venetianus* strains (isolated from different geographical areas) showed different capacities and molecular mechanisms for interacting with diesel fuel droplets and degrading *n*-alkanes of different length. Interestingly, Southern blot hybridization performed using the entire gene cluster responsible for synthesis of emulsan in RAG-1^T gave a strong hybridization signal with the DNA of some of the five *A. venetianus* strains analyzed in this work (Fondi et al., manuscript in preparation), suggesting that (at least) some of the genes coding for the enzymes involved in the metabolic pathway leading to emulsan might be present in the genome of these strains, which raises the intriguing question of the lack of emulsan in these strains. The diversity of the five *A. venetianus* strains was confirmed by the metabolic profiles obtained using the Phenotype Microarray (Biolog), which enabled testing them for their ability to utilize a variety of carbon and nitrogen sources. Organic acids and amino acids were the two classes of compounds mainly used by *A. venetianus* strains as carbon sources, whereas carbohydrates were not used. The failure of some *Acinetobacter* strains to use carbohydrates has already been reported (Knight et al., 1995) and is due both to the inability to transport these molecules into the cytoplasm and to the existence of different metabolic routes to synthesize sugars, as previously described (Cook and Fewson, 1973). The five *A. venetianus* strains in our study used inorganic nitrogen compounds and amino acids, but not dipeptides, as nitrogen

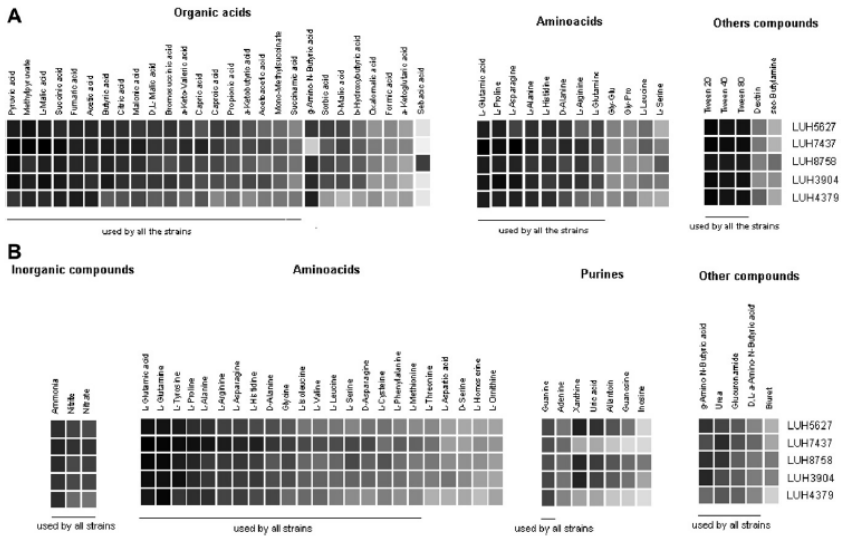

Fig. 5. Carbon (A) and nitrogen (B) sources utilized by the five *A. venetianus* strains. To represent the average height of the kinetic curve relative to each compound, a gray scale was used ranging from black (280 AOUs) to white (0 AOUs). A threshold of 100 AOUs was used to discriminate between used (>100 AOUs) and unused (<100 AOUs) carbon sources. Carbon sources PM 01-02. Nitrogen sources PM03.

sources. Strains did not grow on pyrimidines, which might be due to the inability to take up these compounds (Ovrebo and Kleppe, 1973). While the five strains used most C- and N-sources in (a very) similar way, the utilization of purines as N-source clearly splits the five strains into two groups, the former containing LUH 7437 and VE-C3 and the latter containing LUH 5627, RAG-1^T and LUH 8758. Indeed, all these strains used guanine and adenine, but guanosine, xanthine, uric acid and allantoin (purine derivates) were used only by the latter group. VE-C3 was the most diverse strain, having a lower capacity to metabolize some organic acids than the other strains. The reason for this difference is unclear.

In conclusion, our work has shed light on the strategy adopted by *Acinetobacter* strains toward diesel fuel degradation. The five strains belonging to *A. venetianus* species showed better efficiency at degrading diesel fuel than the other species analyzed in this study, suggesting that the use of such microorganisms during bioremediation procedures might provide valuable advances in this important biological/biotechnological field.

Acknowledgments

We thank the providers of the strains used in this study: D. Gutnick, E. Houang, L. Guardabassi, F. Baldi, S. Yamamoto, I. Tjernberg and Ph. Bouvet.

Marco Fondi is financially supported by a post-doctoral fellowship from "Fondazione Adriano Buzzati-Traverso".

Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.resmic.2011.12.002.

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GENOME ANNOUNCEMENT

Draft Genome Sequence of the Hydrocarbon-Degrading and Emulsan-Producing Strain *Acinetobacter venetianus* RAG-1^T

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We report the draft genome sequence of *Acinetobacter venetianus* strain RAG-1^T, which is able to degrade hydrocarbons and to synthesize a powerful biosurfactant (emulsan) that can be employed for oil removal and as an adjuvant for vaccine delivery. The genome sequence of *A. venetianus* RAG-1^T might be useful for bioremediation and/or clinical purposes.

Acinetobacter venetianus strain RAG-1^T (ATCC 31012) was first isolated from seawater near a beach in Tel Baruch, Israel (11, 12). It was affiliated with the genus *Arthrobacter* (12), species *A. lwoffii* (1) or *A. calcoaceticus* (5). More recently, it has been demonstrated that RAG-1^T belongs to the species *Acinetobacter venetianus* (7, 19, 20).

The importance of this strain mainly resides in its bioremediation potential, since it is capable of degrading *n*-alkanes and, also, because it produces a potent amphipathic polysaccharide bioemulsifier (emulsan) (12–14) that is involved in the capture and transport of *n*-alkanes into the cell (10, 21) and whose structure might be responsible for macrophage stimulation (9).

The genome sequence of *A. venetianus* RAG-1^T might provide useful insights into its metabolism with regard to the search for biodegradable surfactants and crude oil viscosity modifiers, as well as vaccine adjuvants and drug delivery vehicles (3, 8, 9).

The *A. venetianus* RAG-1^T genome was sequenced using Illumina HiSeq2000, and the 3,019,963 reads (109-bp long) were assembled using Abyss software version 1.2.6 (15). The assembled genome has a length of 3,464,338 bp, consists of 87 contigs (>500 bp; average length, 39,819 bp) and has an overall GC content of 39.38%, similar to that of the other *Acinetobacter* genomes sequenced so far. Genome annotation was performed with the RAST annotation system (2), allowing the identification of 3,196 open reading frames (ORFs), 73 tRNAs, and 8 rRNA operons. Of the identified ORFs, 2,403 (75.18%) could be assigned to at least one Cluster of Orthologous Groups (COG) (16).

The presence in the *A. venetianus* RAG-1^T genome of genes encoding homologs to the Alk (AlkB, -F, -G, -H, -L, -J, -K, -S, -T, and -N) from *Pseudomonas putida* GPo1 (18), the soluble cytochrome P450 monooxygenases from *Acinetobacter* sp. EB104 (6), AlmA from *Acinetobacter* sp. DSM 17874 (17), and the LadA protein from *Geobacillus thermodenitrificans* (4) was checked.

Among the set of the genes that are commonly required for the metabolism of *n*-alkanes, *A. venetianus* RAG-1^T possesses *alkB*, *alkH*, *alkJ*, and *alkK*, which were found on different contigs, suggesting that they are scattered throughout the *A. venetianus* RAG-1^T chromosome, unlike in *P. putida*, where all the *alk* genes are clustered in the OCT plasmid (18). Additionally, the four genes encoding rubredoxin (*rubA*), rubredoxin reductase (*rubB*), AlmA, and LadA were found. No close homolog was found for AlkL, -S, -T, or -N. Lastly, despite the fact that *A. venetianus* RAG-1^T is able to grow in the presence of long-chain alkanes, it is missing the soluble cytochrome P450 monooxygenase that is probably involved in long-chain alkane degradation (6).

Consistent with the presence of *A. venetianus* RAG-1^T in contaminated environments, its genome harbors several systems involved in resistance to or tolerance of toxic compounds, including cobalt, cobalt-zinc-cadmium, arsenic, and chromium, as well as 15 genes encoding multidrug resistance efflux pumps.

Nucleotide sequence accession numbers. This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession number AKIQ00000000. The version described in this paper is the first version, AKIQ01000000.

ACKNOWLEDGMENT

A FEMS Advanced Fellowship (FAF2012) financially supports Marco Fondi.

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Received 9 June 2012 Accepted 19 June 2012
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Research in Microbiology 164 (2013) 439–449

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The genome sequence of the hydrocarbon-degrading *Acinetobacter venetianus* VE-C3

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Received 27 July 2012; accepted 8 March 2013
Available online 23 March 2013

Abstract

Here we report the genome sequence of *Acinetobacter venetianus* VE-C3, a strain isolated from the Venice Lagoon and known to be able to degrade *n*-alkanes. Post sequencing analyses revealed that this strain is relatively distantly related to the other *Acinetobacter* strains completely sequenced so far as shown by phylogenetic analysis and pangenome analysis (1285 genes shared with all the other *Acinetobacter* genomes sequenced so far). *A. venetianus* VE-C3 possesses a wide range of determinants whose molecular functions are probably related to the survival in a strongly impacted ecological niche. Among them, genes probably involved in the metabolism of long-chain *n*-alkanes and in the resistance to toxic metals (e.g. arsenic, cadmium, cobalt and zinc) were found. Genes belonging to these processes were found both on the chromosome and on plasmids. Also, our analysis documented one of the possible genetic bases underlying the strategy adopted by *A. venetianus* VE-C3 for the adhesion to oil fuel droplets, which could account for the differences existing in this process with other *A. venetianus* strains. Finally, the presence of a number of DNA mobilization-related genes (i.e. transposases, integrases, resolvases) strongly suggests an important role played by horizontal gene transfer in shaping the genome of *A. venetianus* VE-C3 and in its adaptation to its special ecological niche.

Keywords: *Acinetobacter*; Alkane metabolism; Microbial genomics; Bioremediation

1. Introduction

The marine environment is subjected to the contamination by organic pollutants from a variety of sources, with crude oil being one of the most important substances (Head and Swannell, 1999). Alkanes are the major components of crude oils and are commonly found in oil-contaminated environments (Feng et al., 2007). Aerobic *n*-alkane degradation is a widespread phenomenon in nature, and several microbial species/strains and enzymes involved in *n*-alkane degradation

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http://dx.doi.org/10.1016/j.resmic.2013.03.003

have been identified, isolated and studied in detail (Throne-Holst et al., 2006).

In most described cases, the *n*-alkane is oxidized to the corresponding primary alcohol by substrate-specific terminal monooxygenases/hydroxylases. Two unrelated classes of enzymes for long-chain *n*-alkane oxidation have been proposed: (1) cytochrome P450-related enzymes in both yeasts and bacteria, e.g., bacterial CYP153 enzymes, and (2) bacterial alkane hydroxylases (pAHs) (Wentzel et al., 2007). The latter class of integral membrane non-heme diiron alkane monooxygenases of the AlkB-type allows a wide range of microorganisms to grow on *n*-alkanes with carbon chain lengths from C5 to C16 (van Beilen and Funhoff, 2007). AlkB-type enzymes function in complex with two electron transfer proteins, a dinuclear iron rubredoxin, and a mononuclear iron rubredoxin reductase channeling electrons from NADH to the active site of the alkane hydroxylase (van Beilen and Funhoff, 2007). After the initial oxidation of the *n*-alkane, the corresponding alcohol is oxidized step by step by alcohol dehydrogenase and aldehyde hydrogenase to the corresponding aldehyde and carboxylic acid, respectively. The carboxylic acid then serves as a substrate for acyl-CoA-synthase, and the resulting acyl-CoA enters the β-oxidation pathway (van Beilen and Funhoff, 2007).

Degradation of *n*-alkanes through this kind of catabolic pathway has been extensively studied in *Pseudomonas putida* GPo1 [formerly *Pseudomonas oleovorans* (Baptist et al., 1963; van Beilen et al., 2001)]. Several bacterial strains able to degrade C5–C10 alkanes contain alkane hydroxylases that belong to a distinct family of soluble cytochrome P450 monooxygenases (Wentzel et al., 2007) as, for example, *Acinetobacter* sp. EB104 (Maier et al., 2001) and representatives from mycobacteria, rhodococci and proteobacteria (Sekine et al., 2006; van Beilen et al., 2005, 2006). Alternative alkane hydroxylases have been found in those microorganisms capable of degrading alkanes longer than C_{20}. Usually, these enzymes are not evolutionary related to known AlkB- and P450-like sequences and include AlmA (a flavin binding monooxygenase able to oxidize C_{20}–C_{32} alkanes) from *Acinetobacter* strain DSM 17874 (Throne-Holst et al., 2006) and LadA from *Geobacillus thermodenitrificans* (Feng et al., 2007), able to generate primary alcohols from C_{15}–C_{36} alkanes.

Finally, the cell contact with hydrophobic substrates is crucial because the initial step of alkane degradation is usually carried out by oxidation reactions catalyzed by cell-surface associated oxygenases (Foster, 1962; Wentzel et al., 2007). The solubility of low-molecular-weight alkanes is sufficient to mediate the uptake of the alkane from water, whereas uptake of medium- and long-chain-length *n*-alkanes occurs by either adhesion to hydrocarbon droplets or by a surfactant-facilitated process (Rojo, 2009).

Many alkane-degrading bacteria secrete diverse surfactants that facilitate emulsification of hydrocarbons (Hommel, 1990; Ron and Rosenberg, 2002). In particular, among surfactant producers, *Acinetobacter venetianus* RAG-1^T (Reisfeld et al., 1972; Vaneechoutte et al., 1999) has been shown to produce an extracellular anionic lipoheteropolysaccharide, known as emulsan, to aid in the capture and transport of the carbon sources to the cell (Mercaldi et al., 2008; Pines et al., 1983; Zuckerberg et al., 1979). A 27 kbp cluster of genes responsible for the biosynthesis of this amphipathic, polysaccharide bioemulsifier from the oil-degrading *A. venetianus* RAG-1^T was isolated and characterized (Nakar and Gutnick, 2001). The draft genome of this strain was recently obtained (Fondi et al., 2012) and is likely to provide further insight into the genetic basis of its alkane degradation and emulsan production in this strain. Genomes of other oil-degrading bacteria have been obtained in recent years, including those of *Acinetobacter* sp. DR1 (Kang et al., 2011), *Alcanivorax borkumensis* (Schneiker et al., 2006) and *Marinobacter aquaeolei* VT8 (Kostka et al., 2011).

Some light has been shed on the different strategies for diesel fuel degradation adopted by different *Acinetobacter* strains, suggesting a good efficiency in this process by *A. venetianus* (Mara et al., 2012). Thus, use of strains of this species in bioremediation might provide valuable advances in this important biological/biotechnological field. One *A. venetianus* strain, VE-C3, was isolated in 1993 (Baldi et al., 1997) from the superficial waters of the former industrialized Marghera Port in the Venice lagoon. This area has been polluted due to oil refineries for decades up to late nineties although, today, it is a dismissed and remediated area. VE-C3 strain has been shown to grow on C10 and C14 (Mara et al., 2012). The overall genetic and functional understanding of its biologically and biotechnologically relevant phenotype, including its adhesion to hydrocarbon molecules, its subsequent metabolism and its adaption to its peculiar ecological niche is far from complete. To address these points whole genome sequencing of the marine, hydrocarbon-degrading bacterium *A. venetianus* VE-C3 was performed by the use of a comprehensive approach that combined the next-generations sequencing (NGS) platforms Roche/454 and Illumina with the classical Sanger sequencing of PCR products. Genome analysis yielded interesting insights into the biology of this strain, allowing the identification of putative niche-adaptation and bioremediation-related specific gene sets.

2. Material and methods

2.1. Sample preparation and genome sequencing

Genomic DNA extraction was carried out as previously described (Giovannetti et al., 1990). A first single stranded Roche/454 library was then prepared starting from 5 μg of *A. venetianus* VE-C3 DNA and used to perform the shotgun sequencing, following the procedure as reported in the Roche/454 standard protocol. A second library was prepared in order to obtain paired ends reads. For this purpose, another aliquot of 5 μg of genomic DNA was fragmented to obtain fragments of an average size of 3 kb using the HydroShear apparatus (Digilab Inc., Holliston, MA, USA). These fragments were converted into a paired ends single stranded library following the Roche/454 procedure as reported in the 3 kb paired ends library preparation method manual. Both libraries were

quantitated by the Ribo Green assay (Invitrogen Inc, Carlsbad, CA, USA), amplified by emulsion PCR as reported in the Roche/454 procedure and sequenced using the Titanium version of the Genome Sequencer FLX System. A total of about 1.2×10^5single shotgun reads and about 1.5×10^5 paired ends reads were obtained. Illumina sequencing of *A. venetianus* VE-C3 was carried out by IGA (Istituto di Genomica Applicata, Udine, Italy) with Illumina HiSeq2000.

2.2. Genome assembly

The Roche/454 paired ends reads were assembled with Roche assembler (Newbler). From this assembly, 111 contigs embedded in 14 different scaffolds were generated. Two scaffolds corresponded to already sequenced *A. venetianus* VE-C3 plasmids, namely pAV1 and pAV2 (Mengoni et al., 2007), and where therefore not considered in the further stages of genome assembly.

Illumina GA1 reads were first trimmed to eliminate low quality base callings. Trimming was performed adopting the dynamic trimming algorithm embedded in the SolexaQA suite (Cox et al., 2010), selecting a Phred score threshold value of 30. Further on, reads were assembled using the Abyss assembler v. 1.2.7 (Simpson et al., 2009) with a *k*-mer size of 51. This resulted in a preliminary assembly of 438 contigs.

In order to integrate the two different assemblies, we combined the contigs generated by Illumina and Roche/454 technologies in a hybrid assembly using Phrap assembler (de la Bastide and McCombie, 2007). This resulted in an improved assembly embedding 12 scaffold and 27 contigs. Part of the remaining gaps, within and among the different scaffolds were closed adopting a computational approach based on the mapping of original Roche/454 reads at the extremities of scaffolds (Fondi et al., submitted for publication).

The closure of the gaps among the different scaffolds was then validated with PCR amplification and Sanger sequencing of the overlapping regions. PCR amplification coupled with Sanger sequencing was also performed in those cases in which the adopted computational gap closure strategy (described above) failed to reconstruct the correct order of the scaffolds.

2.3. Genome annotation

Genome annotation was performed using the Rapid Annotation by Subsystem Technology (RAST) pipeline (Aziz et al., 2008). Additional functional annotation was performed querying other functional databases, including KAAS (Moriya et al., 2007), Interpro (*via* Interproscan (Quevillon et al., 2005)) and COG (Clusters of Orthologous Groups) (Tatusov et al., 2003). Atypical chromosomal regions were identified with AlienHunter tool (Vernikos and Parkhill, 2006) using default parameters.

2.4. Genomes retrieval and orthologs identification

Acinetobacter genomes were downloaded from NCBI database as on November 2011 and included *Acinetobacter baumannii* ATCC_17978 (NC_009085), *Acinetobacter calcoaceticus* PHEA-2 (CP002177), *Acinetobacter* sp. DR1 (NC_014259), *Acinetobacter baylyi* ADP1 (NC_014259) and the newly sequenced *A. venetianus* RAG-1^T (AKIQ01000000). When comparing the different *Acinetobacter* genomes (including the newly sequenced *A. venetianus* VE-C3), the groups of orthologous genes were identified with Inparanoid and Multiparanoid softwares (Alexeyenko et al., 2006; Remm et al., 2001). Amino acid sequences of the proteins used to build *Acinetobacter* reference phylogeny were retrieved adopting the Bidirectional Best Hit (BBH) criterion and using the *A. baylyi* ADP1 sequences (retrieved from the Ribosomal Database Project (RDP), http://rdp.cme.msu.edu/) as queries.

2.5. Sequence alignment and phylogenetic tree construction

Multiple sequence alignments were performed using Muscle (Edgar, 2004) and misaligned regions were visually inspected and removed where necessary.

Maximum Likelihood (ML) analysis was carried out using Phyml (Guindon et al., 2005, 2009), with a WAG model of amino acid substitution, including a gamma function with 6 categories to take into account differences in evolutionary rates at sites. Statistical support at nodes was obtained by non-parametric bootstrapping on 1000 re-sampled datasets.

2.6. Permutation tests

To assess whether the accessory genome was enriched in a particular functional category, the proportions of the COGs (Tatusov et al., 2003) in the core and accessory genome were compared. Statistical significance to the enrichment analysis was gained through permutation tests on the original gene sets, i.e. one million random samplings were performed and the COG proportions of each sample were compared to a sample from the whole genome. *P*-values below 0.05 were considered to be significant.

2.7. Genome accession numbers

This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession ALIG00000000. The version described in this paper is the first version, ALIG01000000.

3. Results and discussion

3.1. Genome overview

After Roche/454 and Illumina sequencing of the *A. venetianus* VE-C3 genome, a total of 3,564,836 bp bases were assembled; 10,820 bp and 15,135 bp corresponded to the already known pAV1 and pAV2 plasmids (Mengoni et al., 2007) respectively, whereas 186.446 corresponded to the newly identified pAV3 (large) plasmid (Fig. 1). The remaining

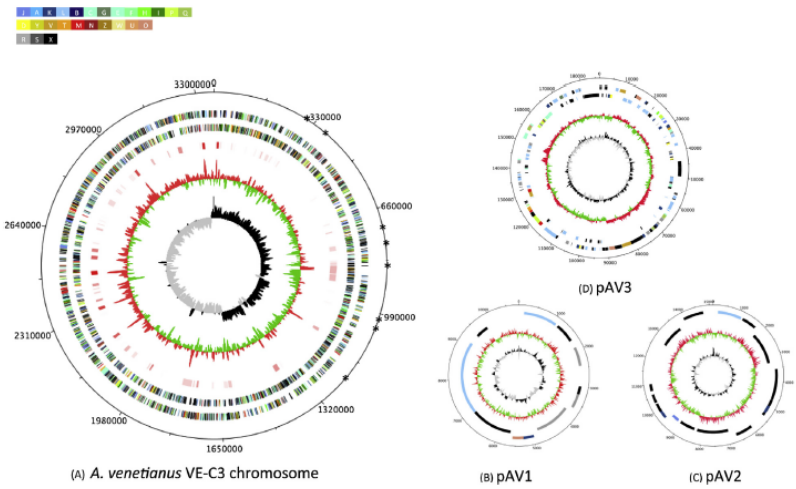

Fig. 1. Circular representations of *A. venetianus* VE-C3 chromosome and plasmids displaying relevant genome features. From the outer to the inner concentric circle: circle 1, genomic position in kb; circles 2 and 3, predicted protein coding sequences (CDS) on the forward (outer wheel) and the reverse (inner wheel) strands coloured according to the assigned COG classes; circle 4 represents atypical chromosomal regions identified by AlienHunter (see Material and methods); circle 5, G + C content showing deviations from the average (39%); circle 6, GC skew. The bar below the plot represents the COG colours for the functional groups (C, energy production and conversion; D, cell cycle control, mitosis and meiosis; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; J, translation; K, transcription; L, replication, recombination and repair; M, cell wall/membrane biogenesis; N, cell motility; O, post-translational modification, protein turnover, chaperones; P, inorganic ion transport and metabolism; Q, secondary metabolites biosynthesis, transport and catabolism; R, general function prediction only; S, function unknown; T, signal transduction mechanisms; U, intracellular trafficking and secretion; V, defence mechanisms; X, unknown function). Asterisks in (A) represent the regions of the scaffold still containing gaps.

3,352,435 bp were assembled in a single scaffold (embedding 8 contigs) and represented the *A. venetianus* VE-C3 chromosome. A total of 3472 coding sequences (CDS) were identified and a putative function was assigned to about 2675 of them (77%) (see Supplementary Material 1 for the complete list of encoded functions). Additionally, *A. venetianus* VE-C3 encodes 6 rDNA operons (16S-23S-5S) and 74 tRNAs. The likely origin of replication (*oriC*) on the chromosome was inferred with OriFinder tool (Gao and Zhang, 2008) and confirmed by GC skew analysis (Fig. 1); six DnaA boxes were found in the inferred *oriC* region. The general features of the *A. venetianus* VE-C3 are reported in Table 1.

A summary of the functional capabilities of *A. venetianus* VE-C3 as a result of a BLAST search of its ORFs only against the COG database (Tatusov et al., 2003) is reported in Supplementary Material 1. Remarkably, with the exception of sequences without a clear assigned function (embedded in functional COG categories X, R and S and representing 26.30%, 7.94% and 7.34% of the total gene content, respectively), those embedded in functional category L (replication, recombination and repair) are the most abundant. This general COG category embeds, among the others, those sequences involved in the recombination (e.g. COG1381), transposition (e.g. COG3676, COG3547) and integration (e.g. COG0582) of DNA fragments, thus revealing that the exogenous acquisition (or loss) of genes might have played a role in shaping the genome of this strain, possibly with the intervention of mobile genetic elements. Interestingly, the proportion of genes

Table 1
General features of the *A. venetianus* VE-C3 genome.

DNA molecule	Chromosome	pAV1	pAV2	pAV3
Size (nucleotides)	3,352,435	10,820	15,135	186,446
GC-content (%) of chromosome	39.11	34.56	36.41	39.56
Protein coding genes	3255	12	16	189
Hypothetical proteins	732	3	11	51
Functions assigned	2523	9	5	138
Average protein length (amino acids)	292.77	210.92	217.41	260.80
Maximum protein length (amino acids)	1797	738	446	1863
rRNA operons (16S-23S-5S)	6	0	0	0
tRNAs	74	0	0	0

involved in this process in *A. venetianus* VE-C3 is larger than that observed in genomes of other bacteria, oil degrading as well as non-degrading. Indeed, the number of genes belonging to the COG category of transposition/mobilization/integration (COG category "L") was calculated also in the genomes of 3 arbitrarily chosen oil-degrading bacteria (i.e.: *A. borkumensis* SK2, *Acinetobacter oleivorans* DR1, and *M. aquaeolei* VT8) and 3 non-degrading bacteria (*Acinetobacter baumanii* ATCC_17978, *Bacillus subtilis* subsp. *subtilis* str. 168 and *Escherichia coli* K12). Results showed that *A. venetianus* VE-C3 possesses, on average, more genes belonging to the L COG category than both oil-degrading bacteria (7.10% against 4.46%, 6.5% and 2.71% in *A. borkumensis* SK2, *M. aquaeolei* VT8 and *Acinetobacter* sp. DR1, respectively) and non-degrading bacteria (7.10% against 3.62%, 3.1% and 5.2% in *A. baumanii* ATCC_17978, *Bacillus subtilis* subsp. *subtilis* str. 168 and *E. coli* K12, respectively). Comparison between plasmids and chromosome-encoded functions (Supplementary Material 2) shows, as expected, an overall predominance of transposition/integration/recombination related genes in plasmids rather than in the chromosome.

The massive presence of recombination related genes in *A. venetianus* VE-C3 is also confirmed by the comparative analysis with the genomes of other representatives of this genus (see below) and from previous work that showed a high level of horizontal gene transfer (HGT) and recombination events (also occurring within the same cell) in the *Acinetobacter* genus (Fondi et al., 2010). Interestingly, *A. venetianus* RAG-1^T possesses about half of the amount of the recombination related genes in respect to *A. venetianus* VE-C3 [117 (3.4%) and 254 (7.3%), respectively] and to other *A. venetianus* strains (Fondi et al., manuscript in preparation), suggesting that the abundance of this particular class of genes might be a peculiarity of VE-C3 strain.

For a deeper inspection of the *A. venetianus* VE-C3 chromosome we adopted the computational strategy implemented in the AlienHunter software, which exploits compositional biases using variable order motif distributions and captures the local composition of a sequence compared with fixed-order methods (Vernikos and Parkhill, 2006). This allowed the identification, although with different confidence values, of 66 atypical regions (Fig. 1), probably the outcome of chromosomal recombination and/or HGT events. This observation is supported by the fact that, as shown in Fig. 2, most of the genes found in these regions encode proteins that either have no homologs in the COG database or code for proteins likely involved in the recombination/transposition/integration of DNA fragments (58.7% of the proteins found in atypical regions). Indeed, it is known that the pool of genes responsible in the horizontal flow of genetic information, usually encodes proteins whose function is unknown yet (Bosi et al., 2011; Brilli et al., 2008; Tamminen et al., 2012). Nevertheless, genes belonging to other functional categories not strictly associated to the process of HGT itself [e.g. transcription factors, inorganic ion transport and metabolism (e.g. arsenic resistance), and defence mechanisms (e.g. ABC-type multidrug transport system)] were found within these regions, supporting the idea that HGT might have been a key player in the adaptation of this microorganism to the heavily polluted ecosystem of Venice Lagoon.

3.2. Genetic basis of alkane degradation in A. venetianus *VE-C3*

3.2.1. Adhesion to oil fuel

It has been suggested that *A. venetianus* VE-C3 is capable of two types of adhesion, i.e. (i) cell-to-cell interactions, preceding the cell adhesion to the *n*-alkane molecules, and (ii)

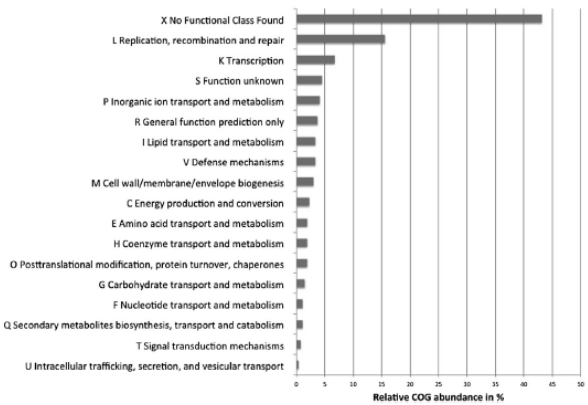

Fig. 2. Clusters of orthologous groups (COG) functional categories distribution of the genes that were found inside putative atypical regions.

n-alkane nano-micelles embedding into the capsular polysaccharide (Baldi et al., 2003), a mechanism for controlling the carbon source uptake, avoiding direct contact of *n*-alkanes with the membrane structures, which would be disruptive.

The genome sequence of *A. venetianus* VE-C3 offers the possibility to investigate the genetic basis of these two different *n*-alkane adhesion strategies, for which the genes and/or the pathways are still unknown.

Accordingly, the *wee* gene cluster of *A. venetianus* VE-C3 was identified (the complete annotation of the *wee*-like cluster is available in Supplementary Material 3) and compared to the one previously described in *A. venetianus* RAG-1^T. Results of this analysis (shown in Fig. 3) revealed that strains VE-C3 and RAG-1^T share two large portions (14 out of 22 genes are orthologous between RAG-1^T and VE-C3) of their *wee* gene cluster, i.e. its first part (including genes *mip*, *wzc*, *wzb*, *wza*, *weeB*, but lacking *weeA*) and the final one (from *weeH* to *pgm*) (Fig. 3). However, the central part of the cluster partially differs in the two strains: in the central region of its cluster, RAG-1^T harbors genes *weeA/C*, *wzx*, *wzy* and *weeD/E/F/G* while VE-C3 harbors a set of genes that are not orthologous but probably code for proteins which perform similar reactions, at least on the basis of *in silico* annotation, such as the *A. venetianus* VE-C3 *mviM*, encoding an oxidoreductase with similarity to RAG-1^T WeeA, or the three VE-C3 *rfaG* genes (not sharing significant sequence identity among each other), encoding a glucosyl transferase, in correspondence with the *weeG* gene of RAG-1^T. Anyway, it is important to stress that some genes of the RAG-1^T *wee* cluster, such as *wzx* and *wzy*, responsible for polymerisation of the apoemulsan (Nakar and Gutnick, 2001) were not found in the genome of *A. venetianus* VE-C3, explaining the different strategies adopted by these strains for the interaction with *n*-alkanes.

Interestingly, the central portions of both *A. venetianus* RAG-1^T and VE-C3 *wee* clusters are also different in terms of GC-content from the rest of the cluster in which they are embedded and also from the average GC-content of the corresponding genome (red dashed line in Fig. 3), indicating that past recombination and/or transposition event(s) might have shaped the *wee* clusters in both microorganisms.

3.2.2. Metabolism of n-alkanes

To search for the genetic determinants likely responsible for the catabolism of hydrocarbons, a set of sequences known to be involved in this pathway was assembled through bibliographical data mining. This seeds dataset included the complete set of Alk sequences (AlkB, G, H, L, J, K, S, T and N) from *P. putida* GPo1 (van Beilen et al., 2001), soluble cytochrome P450 monooxygenases from *Acinetobacter* sp. EB104 (Maier et al., 2001), AlmA from *Acinetobacter* sp. DSM 17874 (Throne-Holst et al., 2007) and LadA from *G. thermodenitrificans* (Feng et al., 2007).

The *A. venetianus* VE-C3 genome encodes a smaller set of *alk*-like sequences compared to *P. putida* GPo1. Moreover, unlike *P. putida* GPo1, in which all the genes are embedded in a single operon, these genes are scattered throughout the genome of VE-C3. *A. venetianus* VE-C3 encodes two paralogous copies of *alkB*, *alkH* (sharing 38% identity among themselves at the amino acid level) and *alkJ* (sharing 37% identity at the amino acid level), whereas AlkK was retrieved in a single copy. Conversely, no ortholog was retrieved from *A. venetianus* VE-C3 when probing its genome with AlkG, AlkT, AlkN and AlkS sequences. AlkG and AlkT (coding for rubredoxin and rubredoxin reductase, respectively) could be replaced by the *rubA-rubB* operon, as already suggested for *A. borkumensis* (Schelstraete et al., 2010) (Supplementary Material 4). Indeed rubredoxin reductase genes map in many alkane-degrading bacteria separately from the alkane hydroxylase genes (Abraham et al., 1998; Schneiker et al., 2006; Smits et al., 2002). Interestingly, a sequence embedding both a rubredoxin and a rubredoxin reductase domain was identified in the genome of *A. venetianus* VE-C3, suggesting its possible role in the alkane degradation process. Furthermore, the absence of ALkS, known to regulate the expression of the *alkBFGHJKL* operon in *P. putida* GPo1 (Eggink et al., 1987; Kok et al., 1989; Panke et al., 1999), raises the intriguing issue of how *alk*-like genes are regulated in *A. venetianus* VE-C3. Finally, *A. venetianus* VE-C3 lacks AlkN, a gene involved in chemotaxis transduction in *P. putida* GPo1 (van Beilen et al., 2001).

A. venetianus VE-C3 encodes a single cytochrome P450 in an operon-like structure with genes encoding a ferredoxin, an

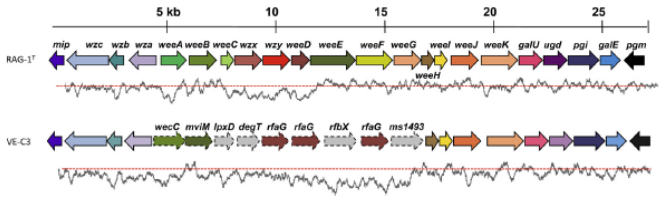

Fig. 3. Schematic representation of the *wee* cluster involved in emulsan production in *A. venetianus* RAG-1^T and VE-C3 strains. Solid line arrows represent orthologous genes between *A. venetianus* VE-C3 and RAG-1^T; dashed line arrows represent genes that are not othologous between the two strains, a similar colour indicates functional analogy. Grey lines below the gene clusters represent GC-content (calculated using a sliding window approach with steps of 100 nucleotides) in respect to the average of the corresponding genome (red dashed line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

FAD-dependent oxyreductase and a gene encoding an AraC transcriptional regulator (Supplementary Material 4). Interestingly, these genes are encoded by the largest plasmid harbored by *A. venetianus* VE-C3 (pAV3) and are flanked by others encoding a transposase and a resolvase. This, in addition to the observation that the organization of this cluster resembles that found, for example, in *A. borkumensis* SK2 (Schneiker et al., 2006), points to the possible acquisition of these genes by *A. venetianus* VE-C3 through one (or more) HGT event(s). Orthologs of LadA and AlmA encoding genes were also found when probing the genome of VE-C3 (Supplementary Material 4).

The presence/absence pattern of the genes coding for emulsan production (*A. venetianus* RAG-1 wee cluster) and for alkane degradation (*alk* genes from *P. putida* GPo1 (van Beilen et al., 2001), was also compared against the genomes of a set of 3 well-characterised oil-degrading bacteria: *A. borkumensis* SK2 (Schneiker et al., 2006), *Acinetobacter* sp. DR1 (Kang et al., 2011) and *M. aquaeolei* VT8 (Kostka et al., 2011) (Supplementary material 5). Concerning the *wee* cluster involved in fuel oil adhesion, the analysis showed a pattern for *Acinetobacter* sp. DR1 that was quite similar to the one observed in *A. venetianus* VE-C3 (lack of genes orthologous to their counterparts in *A. venetianus* RAG-1^T in the central part of the cluster); for *A. borkumensis* SK2 and *M. aquaeolei* VT8, only the genes from *weeJ* to *pgm* resulted to be present also in their genomes. Also in the case of *alk* genes set *A. venetianus* VE-C3 and *Acinetobacter* sp. DR1 showed a very similar pattern of presence/absence (except for *alkT*, absent in the first and present in latter). *A. borkumensis* SK2, showed a conserved *alkSB$_1$GJH* cluster, as already reported by Scheneiker et al. (2006), which, on the contrary, is absent in *M. aquaeolei* VT8.

3.3. Resistance to heavy metal

In the Venice Lagoon metal contamination by As, Cd, Co, Cr, Cu, Hg, Pb, Zn and others has been reported since decades and has been recently reviewed and elaborated based on the hazard quotients of sediments (Apitz et al., 2007). The *A. venetianus* VE-C3 genome encodes a high number of proteins potentially involved in the resistance to these heavy metals. Indeed, at least two gene clusters coding for CzcCBA complex systems [an RND family system involved in the efflux of such compounds, see (Silver and Phung le, 2005) for a review] were detected, the first also comprising a CzcD-like sequence, presumably involved in the efflux of Cu^{2+} and Zn^{2+} from the cell. Interestingly, two additional copies of CzcD-like coding genes were identified in the genome of *A. venetianus* VE-C3, both of them embedded in a bicistronic cluster together with a MerR-like transcriptional regulator. Besides, the first of these clusters is encoded by the pAV3 plasmid.

A. venetianus VE-C3 harbors three gene clusters potentially involved in arsenic resistance. The 5 genes embedded in each of the three clusters share the very same organization and encode for ArsH (NADPH-dependent FMN reductase), ACR3 (arsenite export protein), ArsC (arsenate reductase), ArsR (arsenic resistance operon repressor) and an additional copy of ArsC. Finally, an extra stand-alone copy of an ArsC coding gene was found in the genome of *A. venetianus* VE-C3. Interestingly, as in the case of cobalt–cadmium–zinc resistance related genes, one of the clusters is located on the major plasmid (pAV3).

Genes potentially involved in the resistance/tolerance to copper and chromium were also identified in the genome of *A. venetianus* VE-C3. In particular, two clusters carrying genes presumably involved in copper homeostasis were identified; one coding for CutE (copper homeostasis protein) and CorC (efflux protein) and another embedding multicopper oxidase and copper resistance protein encoding genes. Concerning chromium resistance, four chromate transport proteins (ChrA-like) coding genes were identified in the genome of *A. venetianus* VE-C3. Two of them are organized in cluster together with a LysR family transcriptional regulator. Another ChrA-like coding gene is embedded in a cluster together with a gene coding for ChrB (chromate resistance signal peptide protein). An additional stand-alone copy of a ChrA-like sequence was also identified. Finally, no genes encoding chromate reduction [from Cr(VI) to the less toxic and less insoluble Cr(III)] were identified in the genome of VE-C3 strain.

It must be stated clearly that all the genes mentioned above have been assigned to a particular functional category (i.e. resistance to a specific metal) only on the basis of their sequence similarity with other, better characterized, sequences. Thus, although in some cases the degree of sequence similarity is relatively high (i.e. above 50% at amino acid level) in cannot be excluded that their role might be slightly different (e.g. conferring resistance to a different compound).

3.4. Genomic comparison of A. venetianus VE-C3 to related species and the Acinetobacter pangenome

To establish the phylogenetic relationship existing between *A. venetianus* VE-C3 and the other representatives of the *Acinetobacter* genus sequenced so far, extensive phylogenetic analysis was conducted using a concatamer of a set of conserved proteins (FusA, IleS, LepA, LeuS, PyrG, RecA, RecG, RplB, RpoB). An alignment was built using these sequences, manually removing ambiguous positions. The result of this analysis (Fig. 4) reveals that *A. venetianus* is only distantly related to representatives of *A. baumannii*, *A. calcoaceticus* and *Acinetobacter* sp. strain DR1. Indeed, the clade embedding *A. venetianus* VE-C3, *A. venetianus* RAG-1^T and *A. baylyi* ADP1 is strongly supported as a sister group to the one embedding the aforementioned species, although the length of their branches suggests the presence of a massive evolutionary divergence between VE-C3 and ADP1 strains. This result was confirmed also when whole genome BLAST comparisons of the strains belonging to the different *Acinetobacter* species (and whose genome was completely sequenced) were carried out (*E*-value threshold: $1e^{-100}$). In this case, to avoid redundancy, only one representative from *A. baumannii* was maintained, namely strain ATCC_17978.

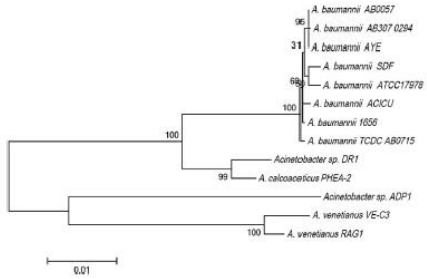

Fig. 4. Maximum likelihood phylogenetic tree showing the evolutionary relationships between *A. venetianus* VE-C3 and the other representatives of the *Acinetobacter* genus available in NCBI database.

BLAST comparisons were then transformed into a circular ideogram in which each genome is represented by an arc and the different genomes (arcs) are connected by vertices accounting for their shared sequence similarity (Fig. 5). It can be noted that *A. venetianus* VE-C3 and *A. baylyi* ADP1 are clearly less interconnected to the other *Acinetobacter* genomes analyzed, suggesting that these strains represent a distinct component of the *Acinetobacter* genus, at least among those strains whose genomes have been completely sequenced.

The genome sequence of *A. venetianus* VE-C3 genome allowed extending the analysis of the whole *Acinetobacter* genus pangenome compared to previous studies (Imperi et al., 2011; Peleg et al., 2012; Vallenet et al., 2008; Zhan et al., 2012). Accordingly, the *in silico* proteome of *A. venetianus* VE-C3 was compared with those of the other *Acinetobacter* representatives whose genome has been completely sequenced and that were previously used to build *Acinetobacter* reference phylogeny (Fig. 5). Also in this case only strain ATCC_17978 from *A. baumannii* was maintained to avoid possible biases due to overrepresentation of genomes belonging to the same species.

By comparing the 3472 CDSs found in the genome of *A. venetianus* VE-C3 with those of the other completely sequenced genomes, a set of orthologous groups was identified. A subset of 1940 CDSs was conserved across all the five genomes and, accordingly, was defined as the *core* genome of the strains belonging to the *Acinetobacter* genus completely sequenced so far.

The remaining 4200 orthologous groups were defined as members of the accessory genome for the five completely sequenced genomes and included both the unique genomes (that is the set of genes peculiar to each strain) and the sets of genes common to only few groups of strains (Fig. 6). In order to define possible differences in functions encoded by the core and/or the accessory genomes of the *Acinetobacter* genus, each protein was assigned to a COG category and the abundance of each COG category was plotted for both core and accessory genomes (Fig. 7). Statistically significant differences between core and accessory genome (computed as described in Material and methods) were found only for COG category L (DNA replication, recombination and repair), V (Defence mechanisms), and for proteins with no assigned COG (X): in these three categories, the accessory genome is enriched. In all the other COG categories (with the exception of functional category K, whose analysis resulted to be statistically unsound) the genes belonging to the core genome are more abundant than those belonging to the accessory genome.

It is interesting to notice that the genome of *A. venetianus* VE-C3 is the one possessing the highest number of unique genes (954, most of which do not have homologs in COG database (51.4% of the unique genes) or only have homologs with no assigned function (9.6%). Furthermore, genes belonging to COG categories L and V were those found to be

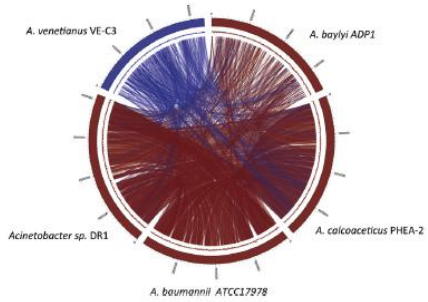

Fig. 5. Circular ideogram representing the comparison among *A. venetianus* VE-C3 genome and those of the other *Acinetobacter* representatives available in NCBI database. Each genome is represented as a bar on the outside of the ideogram together with its GC% content. Links connecting the different bars represent BLAST hits among the different genomes (threshold, *E*-value $1e^{-20}$).

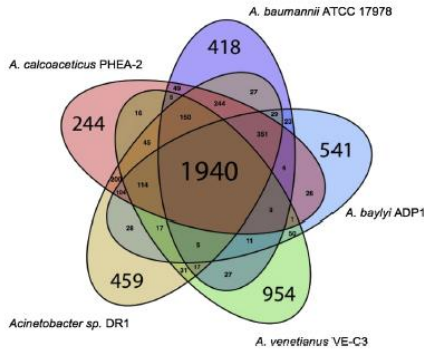

Fig. 6. The core, accessory and unique genomes of the *Acinetobacter* representatives.

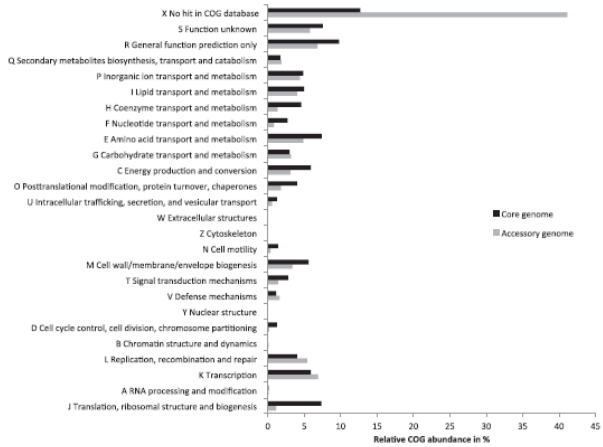

Fig. 7. Cluster of orthologous groups (COG) functional categories distribution of the genes of accessory and core genomes. Asterisks indicate statistically significant differences between accessory and core genomes.

significantly enriched (p-value < 0.05) in the unique genome of *A. venetianus* VE-C3.

Similar enrichment in genes with no assigned function has been previously reported in the accessory genome of other organisms (Bottacini et al., 2010; Galardini et al., 2011).

Given the small proportion of completely sequenced *Acinetobacter* genomes in respect to draft ones, the set of core genes found in this analysis involving only 5 strains, probably represents an overestimation of the actual *Acinetobacter* core genome. To overcome this, we also sampled a larger panel of *Acinetobacter* genomes, including all the complete and incomplete genomes available in NCBI database as on November 1st 2012 (37 genomes in total) and repeated the analysis for the identification of the core/accessory gene pool. In this case, as expected, the size of the core genome (1285 genes) is sensibly lower than in the analysis performed on the completely sequenced genomes only (1940 genes), probably representing a good approximation to the real universally shared gene pool of the *Acinetobacter* strains sequenced so far (Supplementary Material 6). Overall, we observed a general trend (Supplementary Material 6) towards the decrease of the core genome size parallel to the increase of the analyzed genomes, as recently observed by other authors (Imperi et al., 2011), when analyzing the pangenome size/dynamics of the *A. baumannii* species. Conversely, as expected, the size of the accessory genome is shown to increase as long as more strains are added to the analysis (Supplementary Material 6), indicating an open structure of the *Acinetobacter* pangenome as already shown for *A. baumannii* species (Imperi et al., 2011).

Besides providing interesting insight into bioremediation-related genes, data gained through our comparative genomics approach may also provide a basis for further analyses aimed at elucidating other important features of the overall *Acinetobacter* genus as, for example, pathogenicity (Peleg et al., 2012).

4. Conclusions

In this work we have reported the genome sequence of the strain *A. venetianus* VE-C3. In line with its environmentally strongly impacted ecological niche, the genome of this strain harbors a complete set of determinants whose functions are related to tolerance to various stresses. These include the genes probably involved in the metabolism of long-chain *n*-alkanes and in the resistance to toxic metals such as arsenic, cadmium, cobalt and zinc. We have also shown one of the possible genetic bases underlying the different strategies adopted by *A. venetianus* RAG-1^T and VE-C3 for the adhesion to oil fuel droplets. Furthermore, the presence of a number of DNA mobilization-related genes (i.e. transposases, integrases, resolvases) clearly points to a deep influence of HGT in shaping the genome of *A. venetianus* VE-C3 and in its adaptation to its special ecological niche.

Finally, the findings reported in this work provide a valuable background for future biotechnological applications as well as for deeper *in silico* analyses (e.g. metabolic network reconstruction and functional modelling) of *A. venetianus* VE-C3 metabolism.

Acknowledgments

Marco Fondi and Valerio Orlandini are financially supported by an FEMS advanced Fellowship (FAF2012) and an

Italian Cystic Fibrosis Research Foundation fellowship (grant 12#2011), respectively.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2013.03.003.

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New antimicrobial compounds from Antarctica

The second project regards the search of new antimicrobials compounds from microorganisms isolated from Antarctica.

The low number of new antibiotics discovered in the last years and the continuous spread of resistant bacteria has prompted research efforts towards the strengthening of existing antibiotics or the search for novel and efficient antibacterial molecules (Bax *et al.*, 2000). Traditionally terrestrial bacteria, fungi and higher plants, represented the main sources for drug discovery. Conversely, the antimicrobial potential of marine microorganisms has been investigated only in recent decades and most of them have been proven to be producers of natural products (Li & Vederas, 2009). Also bacterial communities from extreme environments have begun to capture the attention of scientists, because they could contain unusual and phylogenetically divergent microorganisms with unique adaptations to their habitats, that in some cases may be due to the synthesis of unusual natural products (Pathom-Aree *et al.*, 2006).

Bacteria from Antarctica represent a reservoir of unsampled biodiversity. Inhibitory activity against human pathogens has been reported for isolates from Antarctic soils (O'Brien *et al.*, 2004) and seawater (Lo Giudice *et al.*, 2007b). Moreover, the existence of inter-specific antagonistic interactions among bacteria from Antarctic seawater and sponges (i.e. *Lissodendoryx nobilis* and *Anoxycalyx joubini*) have been demonstrated (Lo Giudice *et al.*, 2007a, Mangano *et al.*, 2009). Therefore Antarctic sponge associated bacteria may represent a yet unexplored source of microorganisms with the ability to produce antibiotics targeting terrestrial organisms, integrating those recovered from temperate and tropical counterparts.

Our research project started with the analysis of three different microbial communities isolated from three different Antarctic sponges, *Haliclonissa verrucosa*, *Anoxycalyx joubini* and *Lissodendoryx nobilis* (Papaleo et al., 2012).First, the three cultivable communities were characterized from a molecular viewpoint,

and subsequently the inhibitory activity of these strains against some opportunistic pathogens was assayed.

The molecular characterization revealed that the three sponges harbored different microbial communities at genus, species and strain level, and that the genus/species/strain sharing is extremely low. These data are in agreement with previous studies (Lo Giudice et al., 2007a, Mangano et al., 2009) that demonstrated that the interaction between sponges and bacterial communities is specific and different sponges are inhabited by different microbial communities. This could be due to the production of antimicrobial compounds inhibiting the growth of other bacteria, thus the inhibitory activity of the Antarctic strains were tested against a panel of Bcc strains and also against few other human pathogens, revealing that most of the Antarctic strains were able to completely inhibit the growth of most Bcc strains, whereas the growth of the other pathogenic bacteria tested was not affected, suggesting that the inhibition is specific for Bcc bacteria. Through various type of experiments, it was also demonstrated that the antimicrobial compound(s) produced by Antarctic bacteria are thermo-stable and bacteriostatic.

The antimicrobial compounds synthesized by the most active Antarctic bacteria are very likely Volatile Organic Compounds (VOCs), a finding that was confirmed by the SPME–GC–MS technique, which revealed the production of a large set of VOCs by a representative set of Antarctic bacteria. The synthesis of these VOCs appeared to be related neither to the presence of *pks* genes nor the presence of plasmid molecules.

However, these first volatile profiles were obtained under anaerobic conditions, that is in normal HS sampling; because the Antarctic bacteria used are aerobic, these conditions might have probably caused some abiotic stresses modifying the composition of the volatile profile. For this reason a method that allows to detect the mVOCs produced by Antarctic bacteria under aerobic conditions and in cross-streaking conditions was developed (Romoli *et al.*, 2011). 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.

Then some of these Antarctic strains were further characterize from different viewpoints.

P. atlantica TB41, *Pseudoalteromonas. haloplanktis* TAC125, and *Psychrobacter* sp. TB47 and TB67 were characterized for their ability to inhibit Bcc in different culture media e for the kind of VOCs they produce. In addition the genome sequences of *P. atlantica* TB41, *Psychrobacter* sp.TB47 and TB67 were obtained (Papaleo *et al.*, 2013).

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 these 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.

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. 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. Recently, three other *Psychrobacter* strains, TB2, TB15 and AC24, were characterized in our laboratory (Fondi *et al.,* submitted fro publication). *Psychrobacter* sp. AC24 efficiently inhibit the growth of almost all the Bcc strains tested regardless of the growth media, 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. The genome sequences of these strains revealed a variable number of putative gene clusters involved in secondary metabolites in each genome: 12, 8 and 7 clusters were retrieved for AC24, TB15 and TB2 strains, respectively.

Gillisia sp. CAL575 strain was characterized from a phenotypic and genomic point of view (Maida *et al.*, 2013). Sequencing and analysis of its whole genome 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. Also in this case, the ability to inhibit the growth of Bcc strains was dependent on the medium used for growing *Gillisia* sp. CAL575, and this data was confirmed by the GC-SPME experiments, which allowed identifying some of the VOCs produced, whose relative concentration varied when the bacterium was grown onto different media.

Also three *Arthrobacter* strains, TB23, TB26 and CAL618 were further characterized (Fondi *et al.*, 2012b, Orlandini *et al.*, 2013): they also inhibit Bcc species differently depending on the type of culture media used and the genome sequencing of these strains revealed that *Arthrobacter* spp. CAL618 and TB23 have three clusters related to secondary metabolites, while the TB26 strain has only two clusters.

Although we are still far from obtaining the molecules able to inhibit the growth of Bcc species, the phenotypic and genomic characterization of the producer strains and the knowledge of the best conditions in which these molecules are produced, are essential for the continuation of the project.

Biotechnology Advances 30 (2012) 272–293

Contents lists available at ScienceDirect

Biotechnology Advances

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

Sponge-associated microbial Antarctic communities exhibiting antimicrobial activity against *Burkholderia cepacia* complex bacteria

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ARTICLE INFO

Available online 29 June 2011

Keywords:
Antibiotics
Cystic fibrosis
VOC

ABSTRACT

The aerobic heterotrophic bacterial communities isolated from three different Antarctic sponge species were analyzed for their ability to produce antimicrobial compounds active toward Cystic Fibrosis opportunistic pathogens belonging to the *Burkholderia cepacia* complex (Bcc).
The phylogenetic analysis performed on the 16S rRNA genes affiliated the 140 bacterial strains analyzed to 15 genera. Just three of them (*Psychrobacter*, *Pseudoalteromonas* and *Arthrobacter*) were shared by the three sponges. The further Random Amplified Polymorphic DNA analysis allowed to demonstrate that microbial communities are highly sponge-specific and a very low degree of genus/species/strain sharing was detected.
Data obtained revealed that most of these sponge-associated Antarctic bacteria and belonging to different genera were able to completely inhibit the growth of bacteria belonging to the Bcc. On the other hand, the same Antarctic strains did not have any effect on the growth of other pathogenic bacteria, strongly suggesting that the inhibition is specific for Bcc bacteria. Moreover, the antimicrobial compounds synthesized by the most active Antarctic bacteria are very likely Volatile Organic Compounds (VOCs), a finding that was confirmed by the SPME–GC–MS technique, which revealed the production of a large set of VOCs by a representative set of Antarctic bacteria.
The synthesis of these VOCs appeared to be related neither to the presence of *pks* genes nor the presence of plasmid molecules.
The whole body of data obtained in this work indicates that sponge-associated bacteria represent an untapped source for the identification of new antimicrobial compounds and are paving the way for the discovery of new drugs that can be efficiently and successfully used for the treatment of CF infections.

1. Introduction

The rapid development of antimicrobial compounds during the past century has vastly improved the treatment of infections and diseases (Davies, 2007). However, microbes possess extraordinary genetic capabilities and have benefited from man's overuse of antibiotics to develop multiple-resistance mechanisms for every antibiotic introduced into practice in clinical, agricultural or other application fields (Rohilla et al., 2010). Thus, the rise of bacterial resistance to existing antibiotics in hospitals, communities, and the environment, concomitant with their use, has become a public health problem (Davies and Davies, 2010). In addition, in recent decades there has been a dearth of new classes of antibiotics showing novel mechanisms of action. For such reasons, research efforts are now addressed to the strengthening of existing antibiotics or the search for novel and efficient antibacterial molecules (Bax et al., 2000). In the latter case, traditionally terrestrial bacteria (mainly actinomycetes), in addition to fungi and higher plants, represented the main sources for drug discovery. Conversely, the antimicrobial potential of marine microorganisms has been investigated only in recent decades and most of them have been proven to be producers of fascinating natural products (Li and Vederas, 2009).

In addition, unusual sources, such as extreme environments, have begun to capture the attention of scientists for the recovering of biotechnologically exploitable microbial candidates. In fact, these microbial communities are likely to contain unusual and phylogenetically divergent microorganisms with unique adaptations to their habitats. These, in turn, might be correlated at least in some cases with synthesis of unusual natural products, and would also tap into unexplored new

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doi:10.1016/j.biotechadv.2011.06.011

Table 1
List of Antarctic bacterial strains used in this work.

Tester Strain	Origin	AN	Next relative by GenBank alignment (AN, organism)	Seq. Id. (%)	Phylum or class	Family	RAPD Type	Plasmid
TB29	*L. nobilis*	JF273866	FJ205743, *Pseudoalteromonas sp. JS6*	100	GAM	*Pseudoalteromonadaceae*	*17*	
TB5		EU237121	EF382701, *Pseudoalteromonas sp. BSi20430*	99			*31*	
TB6		JF273870	EU330345, *Pseudoalteromonas sp.* BSs20061	99				
TB9		JF273871	DQ667099 *Pseudoalteromonas sp.* F48	99				
TB10								
TB12		JF273873	DQ667099 *Pseudoalteromonas sp.* F48	99				
TB14		JF273874	AB526340, *Pseudoalteromonas sp.* JAM-GA17	100				+
TB17		JF273875	EU330345, *Pseudoalteromonas sp.* BSs20061	99				
TB19		JF273876	HM584485, *Pseudoalteromonas sp.* Z18-3	98				+
TB22		JF273877	HM584485, *Pseudoalteromonas sp.* Z18-3	97				
TB24								
TB27		JF273878	EU330345, *Pseudoalteromonas sp.* BSs20061	99				
TB30		JF273879	EU330345, *Pseudoalteromonas sp.* BSs20061	99				
TB32		JF288186	DQ667099, *Pseudoalteromonas sp.* F48	98				
TB33		JF273867	GU584180, *Pseudoalteromonas sp. 204Z-28*	100				
TB34		JF273880	HM584485 *Pseudoalteromonas sp.* Z18-3	94				
TB25		HQ702265	HM584485, *Pseudoalteromonas sp. Z18-3*	100			*34*	
TB13		EU237124	AY657017, *Pseudoalteromonas sp. 41*	100			*36*	
TB41	*A. joubini*	HQ702264	FM992789, *Pseudoalteromonas sp. M71_D34*	100			*32*	
TB42		JF273855	HQ448932, *Pseudoalteromonas sp.*	100			*33*	
TB43		JF273854	HQ448944, *Pseudoalteromonas sp.*	100				+
TB51		JF273853	EU982330, *Pseudoalteromonas sp. UST020129-030*	100			*35*	
TB49		EU237134	EF635238, *Pseudoalteromonas sp. BSw20679*	99			*37*	
TB64		EU237138	EF409423, *Pseudoalteromonas sp. BSw10002*	99			*38*	
AC163	*H. verrucosa*	JF273924	HM593103, *Pseudoalteromonas sp. Z2*	100			*17*	
TB23	*L .nobilis*	EU237126	DQ831966, *Arthrobacter sp.I34*	99	ACT	*Micrococcaceae*	*46*	
TB16		JF273865	GQ454842, *Arthrobacter sp. VUG-A15*	100			*47*	
TB18		EU237125	DQ628958, *Arthrobacter sp.PSA A20(6)*	99			*48*	
TB26		EU237127	EF491954, *Arthrobacter sp.OS4*	99			*49*	
TB69	*A. joubini*	EU237140	EF540513, *Arthrobacter sp. 4_C16_51*	99			*50*	
CAL568	*H. verrucosa*	JF273881	GQ454842, *Arthrobacter sp. VUG-A15*	100	ACT	*Micrococcaceae*	*1*	
CAL569		JF273882	GQ454842, *Arthrobacter sp. VUG-A15*	100				
CAL571		JF273913	GQ454842, *Arthrobacter sp. VUG-A15*	100				
CAL587		JF273885	GQ454842, *Arthrobacter sp. VUG-A15*	100				
CAL594								
CAL563		HQ702268	GQ454842, *Arthrobacter sp. VUG-A15*	100			*n.d.*	
CAL567		HQ702269	GQ454842, *Arthrobacter sp. VUG-A15*	99			*2*	
CAL605		JF273910	GQ454842, *Arthrobacter sp. VUG-A15*	99				
CAL580		JF273908	GQ454842, *Arthrobacter sp. VUG-A15*	99			*3*	
CAL581		JF273909	GQ454842, *Arthrobacter sp. VUG-A15*	99				
CAL585		JF273911	GQ454842, *Arthrobacter sp. VUG-A15*	100				
CAL573		JF273914	FR682669, *Arthrobacter flavus*	99			*4*	
CAL576								
CAL578		JF273883	GQ454842, *Arthrobacter sp. VUG-A15*	100				
CAL583		JF273884	GQ454842, *Arthrobacter sp. VUG-A15*	100				
CAL590								
CAL591		JF273886	GQ454842, *Arthrobacter sp. VUG-A15*	100				
CAL593								
CAL572		JF273907	FR682669, *Arthrobacter flavus strain R-36538*	99			*5*	
CAL655		JF273906	FR691390, *Arthrobacter flavus strain R-43110*	100				
CAL607		JF273897	FR691390, *Arthrobacter flavus*	99			*6*	
CAL622		JF273920	FR691390, *Arthrobacter flavus*	100			*7*	
CAL612		JF273918	GQ454842, *Arthrobacter sp. VUG-A15*	99				
CAL618		JF273890	FR691390, *Arthrobacter flavus*	100				
CAL626								
CAL632								
CAL652								
CAL644							*8*	
CAL628		JF273912	GQ454842, *Arthrobacter sp. VUG-A15*	100				
CAL625		JF273934	FR691390, *Arthrobacter flavus*	100			*9*	
CAL602		JF273929	FR691390, *Arthrobacter flavus strain R-43110*	99			*10*	
CAL634								
CAL637	*H. verrucosa*	JF273894	FR691390, *Arthrobacter flavus*	99	ACT	*Micrococcaceae*	*11*	
CAL639		JF273905	FR691390, *Arthrobacter flavus*	100				
CAL640								
CAL641							*12*	
CAL645		HQ702271	FR682669, *Arthrobacter flavus*	100				
CAL647			FR691390, *Arthrobacter flavus*	100			*13*	
CAL649		JF273921	GQ454842, *Arthrobacter sp. VUG-A15*	99			*14*	
TB7	*L. nobilis*	EU237122	DQ646848, *Shewanella sp. A7*	99	GAM	*Shewanellaceae*	*39*	
TB3		JF273864	EU237128, *Shewanella sp. TB 31*	100				
TB21		JF273869	GU564403, *Shewanella sp. IR 26*	100			*40*	
TB28		HQ702262	AY771713, *Shewanella frigidimarina*	100			*41*	
TB8		JF273863	DQ533964, *Shewanella sp. ice-oil-255*	100				

(continued on next page)

Table 1 (*continued*)

Tester Strain	Origin	AN	Next relative by GenBank alignment (AN, organism)	Seq. Id. (%)	Phylum or class	Family	RAPD Type	Plasmid
TB31		EU237128	DQ530472, *Shewanella sp. gap-f-53*	99			*42*	
TB11		JF273862	FJ196028, *Shewanella sp. ZS4-23*	100			*43*	
TB37								
TB4		EU237120	AY771736, *Shewanella frigidimarina isolate S5-8*	100			*44*	
TB1		HQ702266	EU365540, *Shewanella sp. BSs20015*	99			*45*	
CAL635	*H. verrucosa*	HQ702273	EU365502, *Shewanella sp. BSs20115*	100			*21*	
CAL636		HQ702272	HM142581, *Shewanella livingstonensis strain NF1-17*	100				
CAL657		JF273902	HM142581, *Shewanella livingstonensis strain NF1-17*	100				
CAL603		JF273930	DQ533968, *Shewanella sp. ice-oil-417*	100			*22*	
CAL610							*23*	
CAL630		JF273892	HM142581, *Shewanella livingstonensis strain NF1-17*	100				
CAL606		JF273931	EU000237, *Shewanella donghaensis strain KOPRI_22224*	99			*n.d.*	
CAL631		JF273893	EU000237, *Shewanella donghaensis strain KOPRI_22224*	100			*n.d.*	
CAL614		JF273888	AY771713, *Shewanella frigidimarina*	100			*24*	
CAL615		F273919	EU000237, *Shewanella donghaensis strain KOPRI_22224*	99			*25*	
CAL617		JF273889	AB003190, *Shewanella sp. SC2A*	99			*26*	
CAL627		JF273899	HM142581, *Shewanella livingstonensis strain NF1-17*	99			*27*	
AC105		JF273927	EU000237, *Shewanella donghaensis strain KOPRI_22224*	99			*n.d.*	
CAL604		JF273887	EU000237, *Shewanella donghaensis strain KOPRI_22224*	100			*n.d.*	
TB15	*L. nobilis*	EU237123	DQ399762, *Psychrobacter sp. B-5161*	99	GAM	*Moraxellaceae*	*54*	
TB2		JF273871	DQ399762, *Psychrobacter sp. B-5161*	99				
TB20		JF273868	GQ358940, *Psychrobacter sp. BSw21516B*	100			*55*	
TB54	*A. joubini*	JF273859	AJ430827, *Psychrobacter fozii strain LMG 21280*	100			*53*	
TB55		EU237135	AJ430827, *Psychrobacter fozii strain LMG 21280*	99				
TB56		JF273860	AJ430827, *Psychrobacter fozii strain LMG 21280*	98				
TB57		JF273856	AJ430827, *Psychrobacter fozii strain LMG 21280*	99				
TB58		JF273857	GQ358940, *Psychrobacter sp. BSw21516B*	98				
TB61								
TB47		HQ702263	GU574735, *Psychrobacter sp. BSw21070*	100			*30*	
TB67		JF273861	AB094794, *Psychrobacter okhotskensis*	99				
TB66		JF273852	GU574735, *Psychrobacter sp. BSw21070*	100				
TB72		JF273858	EF202614, *Sulfitobacter donghicola strain DSW-25*	100				
TB40		EU237132	AB094794, *Psychrobacter Okhotskensis*	99				
CAL642	*H. verrucosa*	JF273900	AM419022, *Psychrobacter sp. Nj-79*	99			*29*	+
CAL643								+
AC 51		JF273926	EU237136, *Roseobacter sp. TB59*	99	ALF	*Rhodobacteraceae*	*30*	
AC 24		JF273923	FJ196029, *Psychrobacter sp. ZS2-14*	99	GAM	*Moraxellaceae*		+
AC43		JF273922	AY 167260, *Roseobacter sp ANT 9270*	96	ALF	*Rhodobacteraceae*		
CAL589		JF273916	DQ060402, *Marinobacter psychrophilus strain BSi20041*	99	GAM	*Alteromonadaceae*	*28*	
CAL619		JF273898	DQ060402, *Marinobacter psychrophilus* strain BSi20041	99				
CAL620								
CAL629		HQ702274	DQ060402, *Marinobacter psychrophilus* strain BSi20041	99				
CAL623		JF273891	DQ060402, *Marinobacter psychrophilus strain BSi20041*	99				
CAL633		JF273932	*AY167267*, *Marinobacter sp. ANT8277*	99				
CAL656		JF273895	DQ060402, *Marinobacter psychrophilus strain BSi20041*	99				
CAL575		HQ702270	NR025822, *Gillisia mitskevichiae* strain KMM 6034	99	Bacteroidetes	*Flavobacteriaceae*	*20*	
CAL577								
CAL579		JF273915	NR025822, *Gillisia* mitskevichiae strain KMM 6034	99				
CAL654		JF273901	NR025822, *Gillisia* mitskevichiae strain KMM 6034	99				
CAL648		JF273933	NR025822, *Gillisia* mitskevichiae strain KMM 6034	99			*n.d.*	
CAL596	*H. verrucosa*	JF273917	NR025822, *Gillisia mitskevichiae strain KMM 6034*	99		*Flavobacteriaceae*	*n.d.*	
TB59	*A. joubini*	EU237136	AY167262, Roseobacter *sp ANT9276a*	100	ALF	*Rhodobacteraceae*	*51*	
TB60		EU237137	AY167339, *Roseobacter sp. ARK9990*	96				
TB73		EU237142	AJ968651, *Roseobacter pelophilus strain SAM4T*	98			*52*	
TB44		JF273851	AJ968651, *Roseobacter pelophilus strain SAM4T*	99				
FAR19	*H. verrucosa*	JF273925	FN377730, *Colwellia sp. E4-4*	99	GAM	*Colwelliaceae*	*16*	
CAL574		JF273896	AY829232, *Colwellia sp. IE1-3*	99			*n.d.*	
CAL621							*18*	
CA 608		JF273903	AM945679, *Staphylococcus sp. J33*	100	FIRM	*Staphylococcaceae*		
CA 613		JF273904	FJ435350, *Rhodococcus sp. H2*	100	ACT	*Nocardiaceae*	*19*	
AC118		HQ702267	GU474988, *Oceanobacillus picturae*	100	FIRM	*Bacillales*	*15*	
AC164		JF273928	AY227267, *Sulfitobacter sp.* H25	99	ALF	*Rhodobacteraceae*	*n.d.*	+
TB71	*A. joubini*	EU237141	UI4583, *Octadecabacter antarcticus 307*	98			*56*	
TB79		EU237144	DQ781321, *Sphingopyxis sp. FR1093*	97		*Sphingomonadaceae*	*57*	
TB82		EU237146	DQ781320, *Sphingopyxis sp. FR1087*	97			*58*	
TB76		EU237143	AF320989, *Pseudomonas tolaasi strain NCPPB 2193*	99	GAM	*Pseudomonadaceae*	*59*	

ALF, α-proteobacteria; GAM, γ-proteobacteria; BAC, Bacteroidetes; ACT, Actinobacteria; FIRM, Firmicutes; +, presence of plasmid(s).

microbial sources of natural products including the gene for their synthesis (Pathom-Aree et al., 2006).

Among these, bacteria from Antarctica represent a reservoir of unsampled biodiversity. To date, the inhibitory activity against human pathogens has been reported exclusively for isolates from Antarctic soils (O'Brien et al., 2004) and seawater (Lo Giudice et al., 2007b). Moreover, the existence of inter-specific antagonistic interactions among bacteria from Antarctic seawater (Lo Giudice et al., 2007a) and

sponges (i.e. *Lissodendoryx nobilis* and *Anoxycalyx joubini*) (Mangano et al., 2009) have been demonstrated. In particular, Antarctic sponge-associated bacteria may represent a yet unexplored source of microorganisms with the ability to produce antibiotics targeting terrestrial organisms, integrating those recovered from temperate and tropical counterparts.

Antarctic microorganisms can produce, probably in response to environmental pressures (Baker et al., 1995), a wide range of potentially valuable natural compounds, most of them are soluble secondary metabolites, many of which can be volatile (Minerdi et al., 2009).

In this context, the aim of the present work was to check Antarctic sponge-associated bacteria for the production of new natural drugs that could be exploited in the control of infections in Cystic Fibrosis (CF) patients. Cystic Fibrosis (CF) is a hereditary disease that affects the normal function of body's epithelial cells, especially in the lungs and digestive system, causing progressive disability. Recurrent and chronic respiratory tract infections in CF patients result in progressive lung damage and represent the primary cause of morbidity and mortality. Infections are usually caused by Gram-negative organisms. Although the high detection frequency of *Pseudomonas aeruginosa* in CF patients, bacteria belonging to the *Burkholderia cepacia* complex (Bcc) have emerged as significant pathogens in CF patients mainly due to their resistance to most antibiotic treatments and the severity of respiratory infections observed in a subset of patients. Bcc is a complex taxonomic group and comprises seventeen closely related species, although *Burkholderia cenocepacia* and *Burkholderia multivorans* are the most common species recovered from CF patients (Coenye et al., 2001; Tablan et al., 1985). Some strains of the Bcc are resistant to several known antibiotics, including the front line drugs, trimethoprim/sulfamethoxazole, piperacillin, ceftazidime, ciprofloxacin, and pipericillin–tazobactam (Chen et al., 2001; Golini et al., 2006). Combination therapy with two or three agents is typically administered, but an optimal therapy has not been elucidated to date.

Since Antarctic sponges represent a potentially rich, untapped source of new antimicrobial agents, as previously described, in this study we screened a panel of bacterial strains isolated from three different sponge species for their ability to synthesize efficient antibacterial molecules against Bcc strains.

2. Materials and methods

2.1. Antarctic bacteria

2.1.1. Isolation of bacterial strains from Antarctic sponges

During the XX Italian Expedition to Antarctica (Austral summer 2004–2005), specimens of the sponges *Haliclonissa verrucosa*, *Anoxycalyx joubini* and *Lissodendoryx nobilis* were collected from five different sites along the Terra Nova Bay coast (Ross Sea). In details, two specimens of *H. verrucosa* were sampled from Adelie Cove (AC; coordinates 74° 45′ S–163° 59′ E), one from Faraglioni (FAR; coordinates: 74° 42′ S–164° 08′ E) and one from Caletta (CAL; coordinates: 74° 45′–164°05′). A single specimen of each sponge *A. joubini* and *L. nobilis* was collected from Tethys Bay (TB; coordinates: 74° 41′ S–164° 04′ E).

The preliminary treatment of samples was previously described (Mangano et al., 2009). Briefly, a central core of the sponge tissue was aseptically excised and manually homogenized. Tissue extracts were serially diluted by using filter-sterilized seawater. Aliquots (100 µl) of each dilution were plated in triplicate on Marine Agar 2216 (MA, Difco). Plates were incubated in the dark at 4 °C for one month. Bacterial colonies grown on MA were isolated at random and streaked at least three times before being considered pure. Cultures were routinely incubated in the dark at 4 °C, under aerobic conditions, on either MA or PCA medium (containing Tryptone 5 g/l, Yeast Extract 2.5 g/l, Glucose 1 g/l, NaCl 24 g/l and Agar Technical 16 g/l, OXOID).

The sponge-associated Antarctic bacteria analyzed in this work are listed in Table 1. All the isolates belong to the Italian Collection of Antarctic Bacteria (CIBAN) of the National Antarctic Museum (MNA) "Felice Ippolito" at the University of Messina.

2.1.2. Target microorganisms

Pathogenic bacteria used as targets in this work have been maintained at +37 °C either on Luria Bertani (LB) or PCA medium, and are listed in Table 2.

2.1.3. Preparation of cell lysates for DNA amplification

For preparation of cell lysates, Antarctic bacterial colonies grown overnight at 15 °C on MA plates were resuspended in 20 µl of sterile distilled water, heated to 95 °C for 10 min, and cooled on ice for 5 min.

2.1.4. RAPD analysis

Random amplification of DNA fragments was carried out in a total volume of 25 µl containing 1X Reaction Buffer, 300 µM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 200 µM, 0.5 U of Polytaq DNA polymerase (all reagents obtained from Polymed, Florence, Italy), 500 ng of primer 1253 (5′ GTTTCCGCCC 3′) (Mori et al., 1999) and 2 µl of lysate cell suspension prepared as described above.

The reaction mixtures were incubated in a MasterCycle Personal Thermal Cycler (Eppendorf) at 90 °C for 1 min, and 95 °C for 90 s. They were then subjected to 45 cycles, each consisting of incubation at 95 °C for 30 s, 36 °C for 1 min, and 75 °C for 2 min; finally, the reactions were incubated at 75 °C for 10 min and then at 60 °C for 10 min, 5 °C for 10 min. Reaction products were analyzed by agarose (2% *w*/*v*) gel electrophoresis in TAE buffer containing 0.5 µg/ml (*w*/*v*) of ethidium bromide.

2.1.5. PCR amplification of 16S rRNA and Polyketide Synthase genes from bacterial isolates

Two microliters of each cell lysate were used for the amplification *via* PCR of 16S rRNA and *pks* genes. Amplification of 16S rRNA genes was performed in a total volume of 50 µl containing 1X Reaction Buffer, 150 µM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 250 µM, and 2.0 U of Polytaq DNA polymerase (all reagents obtained from Polymed, Florence, Italy) and 0.6 µM of each primer [P0 5′ GAGAGTTTGATCCTGGCTCAG and P6 5′ CTACGGCTACCTTGTTACGA] (Grifoni et al., 1995). A primary denaturation treatment of 1.5 min at 95 °C was performed and amplification of 16S rRNA genes was carried out for 30 cycles consisting of 30 s at 95 °C, 30 s at 50 °C and 1 min at 72 °C, with a final extension of 10 min at 72 °C.

Polyketide Synthase (PKS coding gene) amplification was performed in a total volume of 50 µl containing 1X Reaction Buffer, 170 µM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 200 µM, and 1.25 U of Polytaq DNA polymerase (all reagents obtained from Polymed, Florence, Italy) and 0.1 µM of each primer [MDPQQRf (5′-RTRGAYCCNCAGCAICG-3′) and HGTGTr (5′-VGTNCCNGTGCCRTG-3′) (Kim et al., 2005)]. The following conditions were used: a primary denaturation at 95 °C for 5 min, followed by ten cycles of 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 1.5 min, with the annealing temperature reduced by 2 °C per cycle, followed by 30 cycles of 95 °C for 30 s, 45 °C for 30 s, and 72 °C for 1.5 min, with a final extension 72 °C for 7 min.

Each Thermal cycling was performed with a MasterCycle Personal Thermal Cycler (Eppendorf); 10 µl of each amplification mixture was analyzed by agarose gel (0.8% *w*/*v*) electrophoresis in TAE buffer containing 0.5 µg/ml (*w*/*v*) ethidium bromide.

2.1.6. Sequencing of 16S rRNA and pks genes

Amplicons corresponding to the 16S rRNA or *pks* genes (observed under UV, 312 nm) were excised from the gel and purified using the "QIAquick" gel extraction kit (QiAgen, Chatsworth, CA, USA) according to manufacturer's instructions. Direct sequencing was performed

Table 2
List of (opportunistic) pathogenic bacterial strains used in this work.

Species	Strain	Origin
Burkholderia cepacia	FCF1	Cystic Fibrosis patient
	FCF2	
	LMG1222	Environmental
Burkholderia multivorans	FCF5	Cystic Fibrosis patient
	FCF6	
	FCF7	
	FCF8	
	FCF9	
	FCF10	
	FCF11	
	LMG 13010	
	LMG18822	
	LMG17588	Environmental
Burkholderia cenocepacia IIIA	FCF12	Cystic Fibrosis patient
	FCF13	
	J2315	
	FCF14	
	FCF15	
	FCF16	
	FCF17	
Burkholderia cenocepacia IIIB	FCF18	
	FCF19	
	FCF20	
	FCF21	
	FCF22	
	FCF23	
	FCF24	
	FCF25	
	FCF27	
	FCF28	
	FCF29	
	FCF31	
	CEP511	
	LMG24506	
	MVPC1/16	Environmental
	MVPC1/73	
Burkholderia cenocepacia IIIC	LMG19230	
	LMG19240	
Burkholderia cenocepacia IIID	FCF32	Cystic Fibrosis patient
	FCF33	
	FCF34	
	FCF36	
	FCF37	
	FCF38	
	FCF39	
Burkholderia stabilis	LMG14294	Cystic Fibrosis patient
	FCF41	
Burkholderia dolosa	LMG18941	
	LMG 18942	
Burkholderia vietnamiensis	TVV75	Environmental
Burkholderiaa ambifaria	LMG 19467	Cystic Fibrosis patient
	MCI7	Environmental
Burkholderia anthina	LMG16670	
	LMG 20983	Cystic Fibrosis patient
Burkholderia pyrrocinia	FCF43	
	FCF44	
	LMG 21824	
	ATCC15958	Environmental
Burkholderia pyrrocinia	MVPC1/26	Environmental
	MVPC2/77	
Burkholderia lata	LMG6991	
	LMG 22485	
Burkholderia ubonensis	LMG 24263	Nosocomial infection
Burkholderia arboris	LMG 24066	Environmental
Burkholderia contaminans	LMG 23361	Animal infection
Burkholderia diffusa	LMG 24065	Cystic Fibrosis patient
Burkholderia latens	LMG 24064	
Burkholderia metallica	LMG 24068	
Burkholderia seminalis	LMG 24067	
Escherichia coli	ATCC 8739	–
Staphylococcus aureus	ATCC 6538	–
Bacillus subtilis	ATCC 6633	–
Enterobacter cloacae	ATCC 35030	–
Salmonella typhimurium	ATCC 14028	–
Aspergillus niger	ATCC 16404	–
Pseudomonas aeruginosa	ATCC9027	
	ATCC27853	
Stenotrophomonas maltophilia	ATCC51331	
Staphylococcus aureus MRSA	MRSA1	Cystic Fibrosis patient
	MRSA2	

on both DNA strands using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and the chemical dye terminator (Sanger et al., 1977). Each 16S rRNA gene sequence was submitted to GenBank and assigned the accession number shown in Table 1. The TB41 *pks* sequence was assigned the accession number JF268666.

2.1.7. Homologs retrieval and phylogenetic analysis

BLAST probing of DNA databases was performed with the BLASTn option of the BLAST program (Altschul et al., 1997), using default parameters. Nucleotide sequences were retrieved from the GenBank, EMBL, and RDP databases. The ClustalW program (Thompson et al., 1994) was used to align the 16S rRNA gene sequences obtained with the most similar ones retrieved from the databases. Each alignment was checked manually, corrected, and then analyzed. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) according to the model of Kimura 2-parameter distances (Kimura, 1980). The optimal tree with the sum of branch length = 1.62314544 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 1728 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

2.1.8. Cross-streaking

Antibacterial activity was detected by using the cross-streak method (Lo Giudice et al., 2007b). Hereinafter, bacteria tested for inhibitory activity will be defined as 'tester strains', whereas those used as a target will be referred to as 'target strains'. Tester strains were streaked across one-third of an agar plate with PCA medium and incubated at 20 °C. After growth (generally 4–6 days), target strains were streaked perpendicular to the initial streak and plates were further incubated at 20 °C. After a set of tests carried out at different temperatures (ranging from 4 °C to 37 °C) we choose a temperature incubation of 20 °C since it allowed the growth of both tester and target strains. Using incubation temperatures higher or lower than 20 °C resulted in the inability to grow of tester or target strains, respectively. The antagonistic effect was indicated by the failure of the target strains to grow in the confluence area.

2.1.9. Test to evaluate the presence of volatile organic compounds (VOCs)

The volatile nature of antimicrobial compounds synthesized by Antarctic bacteria was checked by a "double plate" method as follows: 1) the tester Antarctic strain was streaked homogeneously on a PCA plate, 2) a second PCA plate was then placed over the first one; both plates were without cover; in this way the VOCs (eventually) produced by the Antarctic strain grown on the bottom plate may flow through the air and embed the culture medium of the upper plate; 3) the "double plate" was then accurately surrounded by

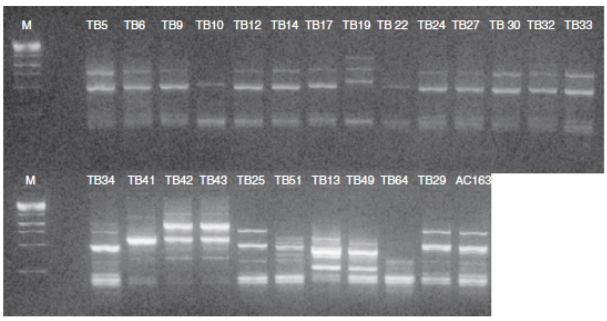

Fig. 1. Agarose gel electrophoresis of RAPD profiles obtained from the DNA of *Pseudoalteromonas* strains.

parafilm and incubated at 20 °C for four days; 4) then, the Bcc target strains were streaked on the upper plate and the plate was then re-positioned over the tester plate (containing the Antarctic bacterium); 5) the double plate was then incubated again at 20 °C for three days and the eventual growth of Bcc strains was checked.

2.1.10. Solid Phase Micro Extraction GC–MS analysis

The volatile compounds profile was obtained by Solid Phase Micro Extraction (SPME) GC–MS technique. An Agilent 7890 gas-chromatograph equipped with a 5975 °C MSD (Agilent, Palo Alto, CA, USA) with EI ionization was used for analysis. A three-phase DVB/Carboxen/PDMS 75 μm SPME fiber (Supelco, Bellafonte, PA, USA) was exposed in the head space of the vials at room temperature for 15 min to extract the volatile compounds. A Gerstel MPS2 XL autosampler (Gerstel, Mulheim an der Ruhr, Germany) was used to automation the procedure and ensuring consistent SPME extraction conditions.

Chromatographic conditions used were column J&W HP-Innovax (Agilent) 50 m × 0.20 mm ID, 0.4 μm film thickness; injection temperature 250 °C, splitless mode, oven program 40 °C for 3 min then 5 °C/min to 100 °C, then 25 °C/min to 260 °C for 3.6 min; the flow were adjusted to 1.6 ml/min. Mass spectra were acquired within the m/z interval 40–450 at a scan speed such to obtain 3.5 scans/s.

2.1.10.1. Data handling and statistics. After acquisition, volatile compounds were identified by matching EI deconvoluted mass spectra against NIST 05 and Wiley 07 spectral library and Kovats indices for each component were assigned. The NIST AMDIS 2.68 software was used for deconvolution of raw mass spectra data. An absolute quantization through calibration curves for each identified analyte was not done, because only the identification of volatile compounds was the primary purpose of this study. The peak area relative to each compound was determined on a specific target ion (base peak) and the identification was confirmed by the matching of the deconvoluted mass spectra using a minimum match factor of 80%.

Principal component analysis (PCA) and successive cluster discriminant analysis were applied to evaluate the relationships among variables with the aim of classifying the microorganisms by their volatile profile. All statistical analyses were performed by means of R version 2.11.1 software.

3. Results and discussion

3.1. Molecular analysis of microbial communities isolated from three different sponge species (H. verrucosa, A. joubini, L. nobilis)

The overall experimental strategy used in this work to characterize from a molecular viewpoint the bacterial communities isolated from the three sponges was based on the following steps:

i) The molecular analysis of microbial communities firstly relied on the RAPD fingerprinting of each Antarctic bacterial strain. The subsequent comparative analysis of RAPD profiles allowed the bacterial strains to be clustered in groups embedding bacterial isolates exhibiting the very same amplification profile (hereinafter RAPD type). Bacterial isolates with the same RAPD type were considered as the same strain.
ii) The phylogenetic affiliation of each bacterial strain was carried out by the analysis of the 16S rRNA genes amplified *via* PCR from at least one representative of each RAPD type.

3.1.1. RAPD fingerprinting

The RAPD fingerprinting (Welsh and McClelland, 1990; Williams et al., 1990) was performed on the 131 bacterial isolates from the three different sponges using the primer 1253 as described in Materials and methods. In order to ensure reproducibility, RAPD amplifications were carried out in triplicate for each isolate. The RAPD profiles obtained in the three replicates were identical; moreover, no amplicon was obtained in the negative controls (not shown). An example of the amplification profiles obtained is shown in Fig. 1. The entire set of RAPD profiles is available as Additional file 1. Each of the 131 RAPD profiles was then compared with each other in order to cluster bacterial isolates in the same RAPD type. In this way, 59 different RAPD types, which might include isolates corresponding to the same strain, were obtained suggesting a high degree of genetic variability. The comparative analysis of RAPD types revealed that: i) the 70 *H. verrucosa* bacterial isolates were splitted into 30 groups; ii) the 35 *L. nobilis* bacterial isolates were grouped into 16 clusters, and iii) the 26 *A. joubini* strains felt into 15 different groups (Table 3).

A very low degree of RAPD-type sharing between different sponges was detected, indeed, just one type was shared between *L. nobilis* and *H. verrucosa* and one between *A. joubini* and *H. verrucosa*. Thus, no strain was shared by the three sponges.

Table 3
Number of genera and RAPD types from bacterial communities isolated from three different sponges.

Sponge	No. of bacteria	No. of genera	Ratio bacteria/genera	No. of RAPD types	Ratio bacteria/RAPD types
H. verrucosa	70	12	5.8	30	2.3
L. nobilis	35	4	8.7	16	2.2
A. joubini	26	7	3.7	15	1.7

3.1.2. Phylogenetic affiliation

In order to affiliate each bacterial strain to a given taxon, the nucleotide sequence of the 16S rRNA genes from at least one representative *per* each RAPD type was determined. To this purpose the 16S rRNA genes were amplified *via* PCR from 103 strains as described in Materials and methods. An amplicon of the expected size was obtained from each strain (data not shown). Each amplicon was purified from agarose gel and the nucleotide sequence was then determined. Each of the 103 sequences obtained was used as seed to probe the nucleotide databases using the BLASTn option of the BLAST program (Altschul et al., 1997). The whole body of data obtained revealed that the 140 isolates were representative of 15 bacterial genera, 4 Gram positive (*Arthrobacter, Staphylococcus, Rhodococcus* and *Oceanobacillus*), 10 Gram negative (*Shewanella, Pseudoalteromonas, Psychrobacter, Marinobacter, Colwellia, Pseudomonas, Sulfitobacter, Roseobacter, Octadecabacter,* and *Sphingopyxis*) and 1 Bacteroidetes (*Gillisia*). The distribution of each genus within the three sponges is shown in Fig. 2 whose analysis revealed that *H. verrucosa* and *A. joubini* exhibited the highest degree of biodiversity at the genus level, since 12 and 7 different genera were detected, respectively, while the strains from *L. nobilis* belong only to 4 different genera. In addition to this, a low degree of genera sharing between the sponges was detected (Table 3). Indeed just three genera (*Arthrobacter, Pseudoalteromonas* and *Psychrobacter*), which are also the predominant ones are shared by the three sponges. Besides, each of these three genera is predominant in different sponges. The most similar sequences to each of the query sequence retrieved from the BLAST search were then aligned using the ClustalW (Thompson et al., 1994) program; each alignment was then used to construct the phylogenetic trees, four of which are shown in Fig. 3.

The analysis of the phylogenetic trees revealed that there was not a random distribution of 16S rRNA gene sequence through the trees, in that in all trees most of the sequences obtained in this study were grouped together in a few clusters. For instance, in the *Shewanella* tree, all the sequences were split into two clusters. A similar scenario was depicted by the *Arthrobacter* tree. In some cases an intermixing between sequences coming from bacteria isolated from different sponges occurred.

A deeper analysis of the phylogenetic trees regarding the distribution of RAPD types revealed that overall bacteria exhibiting the same or very similar RAPD types were clustered together, in agreement with the idea that they represent the same strains (or very closely related ones).

3.2. Cross-streaking

In order to check the ability of Antarctic sponge-associated bacteria to antagonize the growth of (opportunistic) human pathogenic bacteria, cross-streak experiments were carried out using each of the 132 isolates as tester vs (at least) 10 Bcc strains representative of the following eight species: *B. cepacia, B. multivorans, B. cenocepacia, Burkholderia stabilis, Burkholderia dolosa, Burkholderia ambifaria, Burkholderia anthina,* and *Burkholderia pyrrocinia.* In addition to this, the pathogenic strains listed in Table 2 were also used as targets. Data obtained using the *Pseudoalteromonas* strains as testers are shown in Table 4 (the entire set of data is shown in Additional files 2 and 4). The analysis of these data revealed that most, if not all, the tester strains were able to completely inhibit the growth of most Bcc strains, whereas the growth of the other pathogenic bacteria was not affected at all. Hence, data obtained highlighted the ability of the Antarctic bacteria to inhibit the growth of only Bcc strains tested, suggesting a specificity of action *vs* these microrganisms. In order to reinforce this hypothesis a selected set of Antarctic strains was tested against a much larger panel of Bcc strains consisting of additional 51 strains isolated from either the environment or CF patients in order to cover all the seventeen known species. Data obtained are shown in Additional file 4 whose analysis confirmed the hypothesis of the specificity of the inhibitory activity of Antarctic bacteria against Bcc strains.

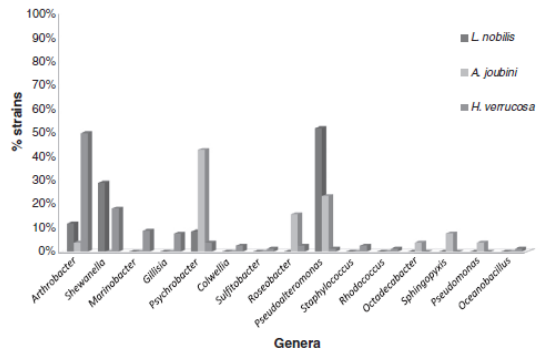

Fig. 2. Distribution of bacterial genera in the three Antarctic sponges.

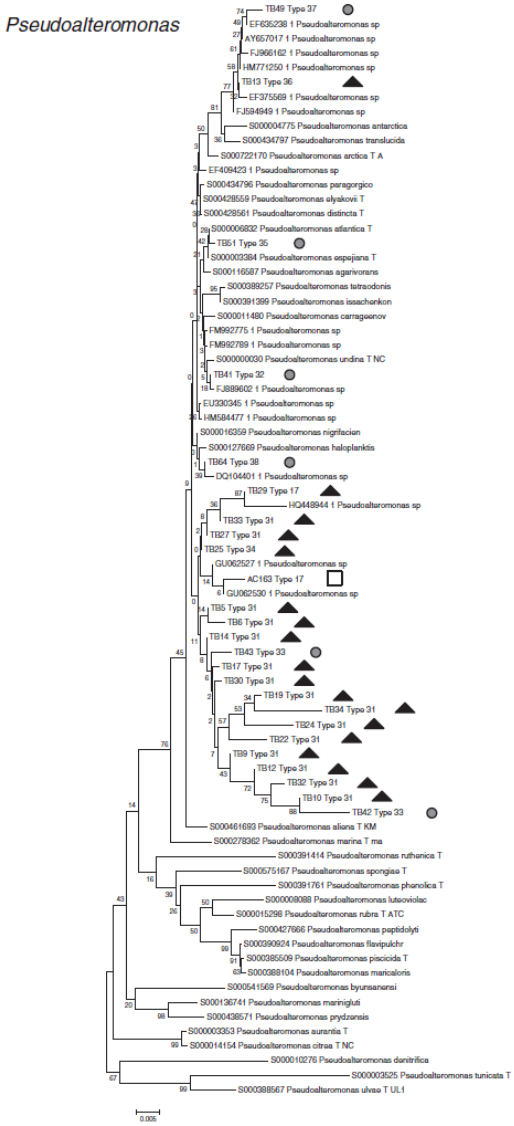

Fig. 3. 16S rRNA genes phylogenetic trees from *Pseudoalteromonas*, *Psychrobacter*, *Arthrobacter*, and *Shewanella* strains. Symbols: black triangles, gray circle, and squares represent strains isolated from *A. Joubini*, *L. nobilis*, and *H. verrucosa* sponges, respectively. Nd: not determined.

Fig. 3 (*continued*).

Fig. 3 (*continued*).

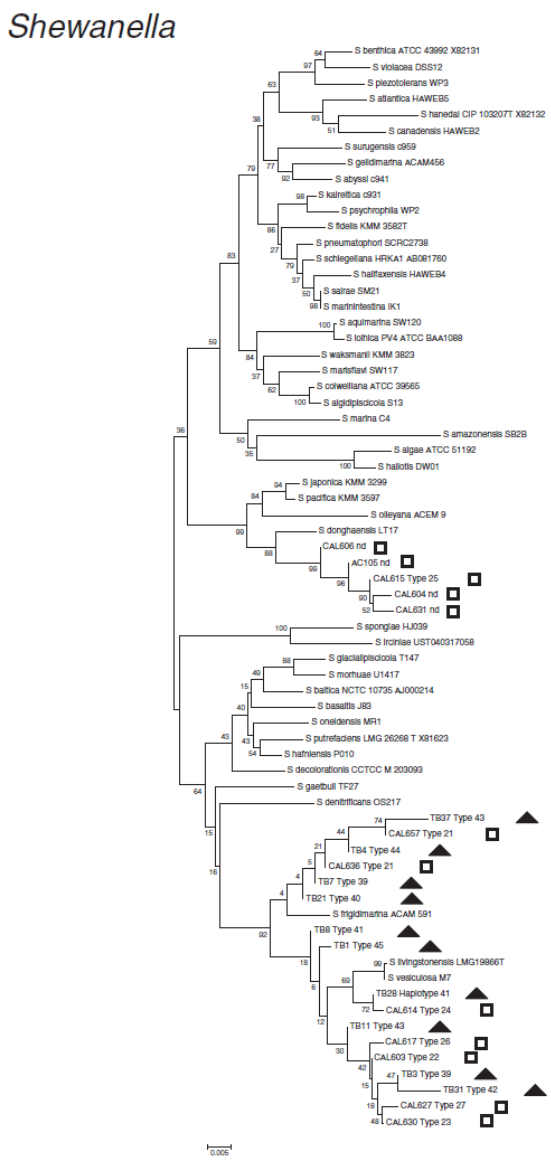

Fig. 3 (*continued*).

Table 4
Growth of (opportunistic) pathogens belonging to different species/genera in the presence of Antarctic *Pseudoalteromonas* strains.

Tester strain	Sponge	Genus	RAPD Type	Target strain									
				FCF2 *B. cepacia*	*LMG13010* *B. multivorans*	*J2315* *B. cenocepacia*	*FCF41* *B. stabilis*	*TVV75* *B. vietnamiensis*	*LMG18942* *B. dolosa*	*LMG19467* *B. ambifaria*	*LMG20983* *B. anthina*	*LMG21824* *B. pyrrocinia*	*LMG24263* *B. ubonensis*
TB29	*L. nobilis*	*Pseudoalteromonas*	17	−	−	−	−	−	−	−	−	−	
TB5			31	−	−	−	−	−	−	−	−	−	
TB6				−	−	−	−	−	−	−	−	−	
TB9				−	−	−	−	−	−	−	−	−	−
TB10				+/−	−	−	−	+/−	−	−	−	−	
TB12				−	−	−	−	−	−	−	−	−	
TB14				−	−	−	−	−	−	−	−	−	
TB17				+/−	−	−	−	−	−	−	−	−	
TB19				−	−	−	−	−	−	−	−	−	
TB22				−	−	−	−	−	−	−	−	−	
TB24				−	−	−	−	−	−	−	−	−	
TB27				−	−	−	−	−	−	−	−	−	
TB30				−	−	−	−	−	−	−	−	−	
TB32				+/−	−	−	−	−	−	−	−	−	
TB33				−	−	−	−	−	−	−	−	−	
TB34				−	−	−	−	−	−	−	−	−	
TB25			34	−	−	−	−	−	−	−	−	−	−
TB13			36	−	−	−	−	−	−	−	+/−	−	−
TB41	*A. joubini*		32	−	−	−	−	−	−	−	−	−	−
TB42			33	−	−	−	−	−	−	−	−	−	−
TB43				−	−	−	−	−	−	−	−	−	
TB51			35	−	−	−	−	−	−	−	−	−	−
TB49			37	−	−	−	−	−	−	−	−	−	−
TB64			38	−	−	−	−	−	−	−	−	−	−
AC163	*H. verrucosa*		17	−	−	−	−	−	−	−	+	−	−
C−				+	+	+	+	+	+	+	+	+	+

Tester strain	Sponge	Genus	RAPD Type	Target strain									
				LMG24066 *B. arboris*	*LMG23361* *B. contaminans*	*LMG24065* *B. diffusa*	*LMG22485* *B. lata*	*LMG24064* *B. latens*	*LMG24068* *B. metallica*	*LMG24067* *B. seminalis*	*ATCC 16404* *A. niger*	*ATCC 6633* *B. subtilis*	*ATCC 8739* *E. coli*
TB29	*L. nobilis*	*Pseudoalteromonas*	17										
TB5			31										
TB6													
TB9				−	−	−	−	−	−	−	−	+	+
TB10													
TB12													
TB14													
TB17													
TB19													
TB22													
TB24													
TB27													
TB30													
TB32													
TB33													
TB34													
TB25			34	−	−	−	−	−	−	−	−	+	+
TB13			36	−	−	−	−	−	−	−	−	+	+

(continued on next page)

Table 4 (continued)

Tester strain	Sponge	Genus	RAPD Type	Target strain										
				PCF2 *B. cepacia*	LMG13010 *B. multivorans*	J2315 *B. cenocepacia*	PCF41 *B. stabilis*	TVV75 *B. vietnamiensis*	LMG18942 *B. dolosa*	LMG19467 *B. ambifaria*	LMG20983 *B. anthina*	LMG21824 *B. pyrrocinia*	LMG24263 *B. ubonensis*
TB41	*A. joubini*		32	–	–	–	+/–	+/–	–	–	–	+	+
TB42			33	–	–	–	–	–	–	–	–	+	+
TB43													
TB51			35	–	–	–	–	–	–	–	–	+	+
TB49			37	–	–	–	–	–	–	–	–	+	+/–
TB64			38	–	–	–	–	–	–	–	–	+	+
AC163	*H. verrucosa*		17	–	–	–	–	–	–	–	–	+	+
C–				+	+	+	+	+	+	+	+	+	+

Tester	Sponge	Genus	RAPD Type	Target strain		
				ATCC 14028 *S. typhimurium*	ATCC 6538 *S. aureus*	ATCC 35030 *E. cloacae*
TB29	*L. nobilis*	*Pseudoalteromonas*	17			
TB5			31			
TB6						
TB9				+	+	+
TB10						
TB12						
TB14						
TB17						
TB19						
TB22						
TB24						
TB27						
TB30						
TB32						
TB33						
TB34						
TB25			34	+	+	+
TB13			36	+	+	+
TB41	*A. joubini*		32	+	+	+
TB42			33	+	+	+
TB43						
TB51			35	+	+	+
TB49			37	+	+	+
TB64			38	+	+	+
AC163	*H. verrucosa*		17	+	+	+
C–				+	+	+

+ Growth; +/– Reduced growth; – No growth; C–, negative control.

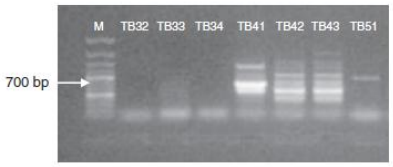

Fig. 4. Agarose gel electrophoresis of amplicons obtained from the DNA of a representative set of antarctic bacteria using sets of primers targeted toward the *pks* genes.

3.3. Thermostability of the antibiotic compound

To evaluate the thermo-stability of antimicrobial compounds cross streaking experiments were carried out in different conditions. After target organisms were streaked perpendicular to the initial streak of tester strains, plates were further incubated for 48 h at 37 °C. Identical results were obtained after incubation at 20 °C or 37 °C, suggesting that the antibacterial compounds eventually produced by Antarctic bacteria could be thermo-stable (data not shown).

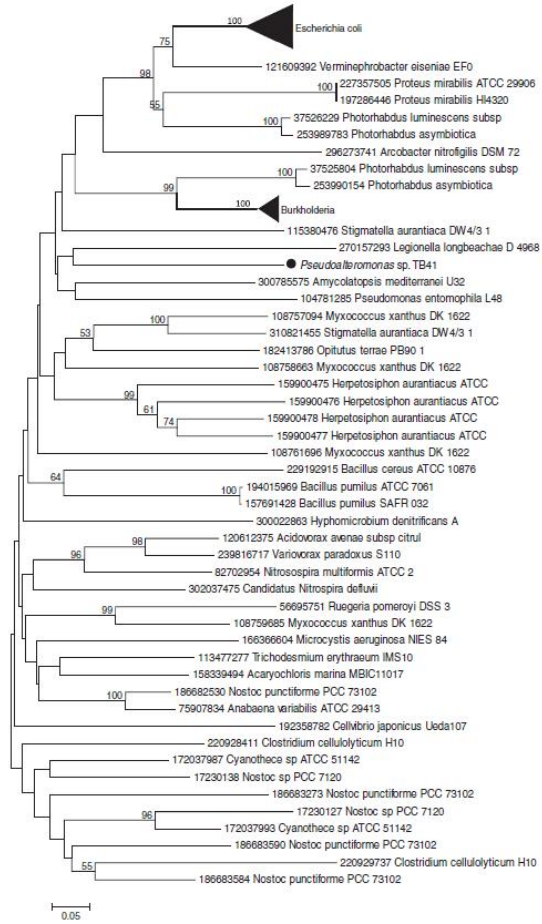

Fig. 5. Phylogenetic tree constructed using the aminoacid sequence of the protein encoded by *pks* genes.

Table 5
Results of cross-streak experiments: growth of pathogenic target strains in the presence of Antarctic tester bacteria.

Tester strain	Sponge	Genus	RAPD Type	Target strain										
				FG72 *B. cepacia*	LMG13010 *B. multivorans*	J2315 *B. cenocepacia*	FC41 *B. stabilis*	TVV75 *B. vietnamiensis*	LMG18942 *B. dolosa*	LMG19467 *B. ambifaria*	LMG20983 *B. anthina*	LMG21824 *B. pyrrocinia*	LMG24263 *B. ubonensis*
TB5	*L. nobilis*	*Pseudoalt*	31	–	–	–	–	–	–	–	–	–	+
TB25			34	+/–	–	–	+/–	+/–	+/–	+/–	+	–	+/–
TB13			36	++	–	–	+	+	+	+	+	–	+
TB29			17	–	–	–	+/–	–	+/–	+/–	+	+/–	+/–
TB41		*A. joubini*	32	+	+/–	–	+	+	+	+	+	–	+
TB42			33	+/–	–	–	–	+/–	+/–	+/–	+/–	–	+
TB51			35	–	–	+/–	+/–	–	+/–	+/–	+/–	–	+/–
TB49			37	+/–	–	–	+	+	+	+	+	–	+
TB64			38	++	–	–	+/–	+/–	+/–	+/–	+/–	–	+
AC163		*H. verrucosa*	17	+	–	–	+/–	+/–	+/–	+/–	+/–	–	+/–
TB23		*L. nobilis*	46	+	–	–	+	+	+/–	+/–	+	–	+
TB16		*Arthrobacter*	47	+	+/–	+/–	+	+/–	+/–	+/–	+	–	+
TB18			48	–	–	–	+/–	+/–	+/–	+	+	–	+/–
TB26			49	+	–	–	+/–	+/–	+/–	+/–	+	–	+
TB69		*A. joubini*	50	++	+/–	–	+	+	+	+	+	–	+
CAL569		*H. verrucosa*	1	+	+	–	+	+	+	+	+	–	+
CAL605			2	+	–	–	+	+	+	+	+	–	+
CAL580			3	+	–	–	+	+	+	+	+	–	+
CAL591			4	+	++	–	+	+	+	+	+	+/–	+
CAL572			5	+	+/–	–	+	+	+	+	+	–	+
CAL607			6	+	+	–	+	+	+	+	+	+/–	+
CAL612			7	++	++	+/–	+	+	+	+	+	–	+
CAL628			8	+	+/–	–	+	+	+	+	+	–	+
CAL625			9	–	–	–	+/–	+/–	+/–	+/–	+/–	–	–
CAL602			10	+	+/–	–	+/–	+	+/–	+/–	+	–	+
CAL639			11	+	+	+/–	+	+	+	+	+	–	+
CAL645			12	+	+	–	+	+	+/–	+/–	+	–	+/–
CAL 647			13	+	++	+/–	+	+	+	+	+	–	+
CAL649			14	+	++	+/–	+	+	+	+	+	–	+
C-				++	++	+/–	++	++	++	++	++	++	++
TB3		*L. nobilis*	*Shewanella*	39	+/–	–	–	–	+/–	–	–	–	–
TB21			40	+/–	–	–	+/–	+/–	+/–	+/–	+	–	+/–
TB28			41	+	–	–	+	+/–	+	+	+	+	+
TB31			42	+/–	–	–	+	+/–	+	+/–	+	–	+
TB11			43	+	–	–	+/–	+/–	+/–	+/–	+	–	+/–
TB4			44	+	–	–	+/–	+	+/–	+	+	–	+
TB1			45	+	+/–	+/–	+	+	+	+	+	+/–	+

CA1636	*H. vernacosa*		21	–	++	–	–	+/–	+/–	+/–	+/–	–	–	–	–			
CA1603			22	+	++	–	–	+/–	+	+	+/–	+/–	+/–	+/–	+/–			
CA1630			23	–	–	–	+/–	+/–	+	+	+/–	+/–	–	–	–			
CA1614			24	+	+	+/–	+/–	+/–	+/–	+	+/–	+/–	–	–	–			
CA1617			26	+	++	+/–	+	+	+	+	+	+	–	–	–			
CA1627			27	–	–	–	+/–	–	–	–	+/–	+	–	–	–			
TB15	*L. nobilis*	*Psychrobacter*	54	+	–	–	+/–	+/–	+/–	+/–	+/–	+	–	+/–	+/–			
TB20			55	++	–	–	+	+/–	+	+	+	+	+/–	+/–	+/–			
TB55	*A. joubini*		53	+	–	–	+	+	+	+	+	+	+/–	+/–	+/–			
TB40			30	+	+/–	.	+	+	+	+	+	+	+/–	+/–	+			
CA1642	*H. vernacosa*		29	+	+/–	–	+	+/–	+/–	+/–	+	+	–	–	+			
TB60	*A. joubini*	*Roseobacter*	51	+	++	+/–	+	+	+	+	+	+	–	–	+			
TB73			52	–	–	–	+/–	+/–	–	+	+	+	–	–	+/–			
TB71		*Octadecabacter*	56	+/–	–	–	–	+/–	+/–	+/–	–	–	–	–	+			
TB79		*Sphingopyxis*	57	–	–	–	–	.	+/–	–	–	–	–	–	+/–			
TB82			58	+	–	–	+	+	+	+	+	+	+	+	+			
TB76		*Pseudomonas*	59	++	–	–	+	+/–	+	+	+	+	+/–	+/–	+			
AC118	*H. vernacosa*	*Oceanobacillus*	15	+	++	–	+	+	+	+	+	+	+/–	+/–	+/–			
FAR19		*Cobetella*	16	+	++	+/–	+/–	+	+/–	+	+	+	–	–	+			
CA1608		*Staphylococcus*	18	++	+	+/–	+	+	+	+	+	+	+	+	+			
CA1613		*Rhodococcus*	19	+/–	–	–	+	+/–	+	+	+/–	++	–	–	+			
CA1579		*Gillisia*	20	+/–	++	–	+/–	+	–	+	+	+	–	–	+/–			
C–				++	++	+/–	++	++	++	++	++	++	++	++	++			
CA1633	*H. vernacosa*	*Martinobacter*	28	+	++	+/–	+/–	+/–	+/–	+/–	+	+	–	–	+			
AC51		*Roseobacter*	30	+	–	–	–	+/–	–	+/–	–	+/–	+/–	–	–			
C–				++	++	+/–	++	++	++	++	++	++	++	++	++			

Tester strain	Sponge	Genus	RAPD Type	Target strain												
				LMG24066 *B. arbens*	*LMG23361* *B. contaminans*	*LMG24065* *B. diffusa*	*LMG23485* *B. lata*	*LMG24064* *B. latens*	*LMG24068* *B. metallica*	*LMG24067* *B. seminalis*	*ATCC 16404* *A. niger*	*ATCC 6633* *B.subtilis*	*ATCC 8739* *E. coli*			
TB5	*L. nobilis*	*Pseudoalteromonas*	31	–	–	–	–	+/–	+/–	–	+/–	+/–				
TB25			34	+/–	+/–	+/–	+/–	+	+	–	–	+	+			
TB13			36	+/–	+	+	+/–	+	+	+/–	–	+	+			
TB29			17	+/–	+/–	+/–	+/–	+/–	+/–	+/–	–	+	+			
TB41	*A. joubini*		32	+/–	+/–	+	+	+	+	+/–	–	+	+			
TB42			33	+/–	+/–	–	+/–	+/–	+	+/–	+/–	+	+			
TB51			35	+/–	+/–	+/–	+/–	+/–	+/–	+/–	–	+	+			
TB49			37	+	+	+/–	+/–	+/–	+/–	+/–	–	–	+			
TB64			38	–	–	–	+/–	+/–	++	+/–	–	+	+			
AC163	*H. vernacosa*		17	–	–	–	+/–	.	+/–	–	–	+	+			
TB23	*L. nobilis*	*Arthrobacter*	46	+	+	+	+/–	+	+	+/–	–	+	+			
TB16			47	+	+	+/–	+/–	+/–	+	+	–	+	++			
TB18			48	+/–	+/–	+/–	+/–	+	+	–	–	++	++			
TB26			49	+/–	+/–	–	+/–	+	+	–	–	+	+			
TB69	*A. joubini*		50	+	+	+	+	+/–	+	–	–	+	+			
CA1569	*H. vernacosa*		1	+	+	+	+	+/–	+	+/–	–	+	++			
CA1605			2	+	+	+	+	+	+	+	–	+	++			
CA1580			3	+	+	+	+	+	+	–	–	+	++			
CA1591			4	+	+	+	+	+	+	+/–	–	+	+			
CA1572			5	+	+	+	+	+	+	+/–	–	++	++			
CA1607			6	+	+	+	+	+	+	+	–	+	+			
CA1612			7	–	+	+	+	+	+	+	–	+	++			
CA1628			8	+	+	+	+	+	+	+/–	–	++	++			
CA1625			9	–	–	–	+/–	+/–	+/–	–	–	+	+			
CA1602			10	+	–	+	+	+	+	+/–	–	++	++			

(continued on next page)

Table 5 (*continued*)

Tester strain	Sponge	Genus	RAPD Type	Target strain									
				FCF2 *B. cepacia*	*LMG13010* *B. multivorans*	*J2315* *B. cenocepacia*	*FCF41* *B. stabilis*	*TVV75* *B. vietnamiensis*	*LMG18942* *B. dolosa*	*LMG19467* *B. ambifaria*	*LMG20983* *B. anthina*	*LMG21824* *B. pyrrocinia*	*LMG24263* *B. ubonensis*
CAL639			11	+	+	+	+	+	+	+/−	−	++	++
CAL645			12	+/−	+/−	+	+	+	+/−	+/−	−	+	+
CAL 647			13	+	+	+	+	+	+	+/−	−	++	++
CAL649			14	+	+	+	+	+	+	−	−	++	++
C−				++	++	++	++	++	++	++	++	++	++
TB3	*L. nobilis*	*Shewanella*	39	−	+/−	−	−	+/−	−	+/−	−	+	+
TB21			40	+/−	+/−	−	−	+/−	+/−	−	−	+	+
TB28			41	+	+	+	+	+	+	+	−	+	+
TB31			42	+	+	+/−	+	+	+	+/−	−	+	+
TB11			43	+/−	+/−	+	+/−	+/−	+	+/−	−	+	+
TB4			44	−	−	−	+	−	+	−	−	+	+
TB1			45	+	+	+/−	+/−	+	+	+	−	+	+
CAL636	*H. verrucosa*		21	−	−	−	−	+/−	+/−	−	−	+	+
CAL603			22	+/−	+/−	+/−	+/−	+/−	+	+/−	−	+	+
CAL630			23	−	+/−	−	+/−	+/−	+	−	−	+	+
CAL614			24	−	−	−	−	+/−	+ −	−	−	+	+
CAL617			26	+	+	+/−	+/−	+	+	−	−	+	+
CAL627			27	−	−	−	−	+/−	+/−	−	−	+/−	+
TB15	*L. nobilis*	*Psychrobacter*	54	+	+	+	+/−	+/−	+	−	−	++	++
TB20			55	+	+	+	+	+	+	+/−	−	++	++
TB55	*A. joubini*		53	+	+	+/−	+/−	+	+	+/−	−	++	++
TB40			30	+	+	+/−	+	+	+	+	−	+	+
CAL642	*H. verrucosa*		29	+	+	+/−	+/−	+	+	+	−	++	++
TB60	*A. joubini*	*Roseobacter*	51	+	+	+	+	+	+	+/−	−	++	++
TB73			52	+/−	+	+	+/−	+/−	+	−	−	+	+
TB71		*Octadecabacter*	56	−	−	−	−	+/−	+	−	−	+	+
TB79		*Sphingopyxis*	57	−	−	−	−	+/−	−	−	−	+	+
TB82			58	+	+	+	+	+	+	+	+	+	+
TB76		*Pseudomonas*	59	+	+/−	−	+	+/−	+	+	−	++	++
AC118	*H. verrucosa*	*Oceanobacillus*	15	+	+	+	+	+	+	+	−	+	+
FAR19		*Colwellia*	16	+/−	+	−	−	+/−	+/−	−	−	+	+
CAL608		*Staphylococcus*	18	+	+	++	++	++	++	+	−	++	++
CAL613		*Rhodococcus*	19	+	+	+/−	+	+	+	+/−	+/−	++	++
CAL579		*Gillisia*	20	+	+	−	−	−	−	−	−	++	++
CAL633		*Marinobacter*	28	+	+	+/−	+	+	+	+/−	−	+	+
AC51		*Roseobacter*	30	−	−	−	−	+/−	+/−	+/−	+/−	−	+
CAL633	*H. verrucosa*	*Marinobacter*	28	+	+	+/−	+	+	+	+/−	−	+	+
AC51		*Roseobacter*	30	−	−	−	−	+/−	+/−	+/−	+/−	−	+
C−				++	++	++	++	++	++	++	++	++	++

Tester strain	Sponge	Genus	RAPD Type	Target strain		
				ATCC 14028 *S. typhimurium*	*ATCC 6538* *S. aureus*	*ATCC 35030* *E. cloacae*
TB5	*L. nobilis*	*Pseudoalteromonas*	31	+	+	+
TB25			34	+	+	+
TB13			36	+	+	+
TB29			17	+	+	+
TB41	*A. joubini*		32	+	+	+
TB42			33	+	+	+
TB51			35	+	+	+
TB49			37	+	+	+
TB64			38	+	+	+
AC163	*H. verrucosa*		17	+	+	+

TB23	*L. nobilis*	*Arthrobacter*	46	+	+	+
TB16			47	++	++	++
TB18			48	+	+	+
TB26			49	+	+	+
TB69	*A. joubini*		50	+	+	+
CAL569	*H. verrucosa*		1	++	++	++
CAL605			2	++	++	++
CAL580			3	++	++	++
CAL591			4	+	+	+
CAL572			5	++	++	++
CAL607			6	+	+	+
CAL612			7	+	+	+
CAL628			8	++	++	++
CAL625			9	+	+	+
CAL602			10	++	++	++
CAL639			11	++	++	++
CAL645			12	+	+	+
CAL 647			13	++	++	++
CAL649			14	++	++	++
TB3	*L. nobilis*	*Shewanella*	39	+	+	+
TB21			40	+	+	+
TB28			41	+	+	+
TB31			42	+	+	+
TB11			43	+	+	+
TB4			44	+	+	+
TB1			45	+	+	+
CAL636	*H. verrucosa*		21	+	+	+
CAL603			22	+	+	+
CAL630			23	+	+	+
CAL614			24	+	+	+
CAL617			26	+	+	+
CAL627			27	+	+	+
TB15	*L. nobilis*	*Psychrobacter*	54	++	++	++
TB20			55	++	++	++
TB55	*A. joubini*		53	++	++	++
TB40			30	+	+	+
CAL642	*H. verrucosa*		29	++	++	++
TB60	*A. joubini*	*Roseobacter*	51	++	++	++
TB73			52	+	+	+
TB71		*Octadecabacter*	56	+	+	+
TB79		*Sphingopyxis*	57	+	+	+
TB82			58	+	+	+
TB76		*Pseudomonas*	59	++	++	++
AC118	*H. verrucosa*	*Oceanobacillus*	15	+	+	+
FAR19		*Colwellia*	16	+	+	+
CAL608		*Staphylococcus*	18	++	++	++
CAL613		*Rhodococcus*	19	++	++	++
CAL579		*Gillisia*	20	++	++	++
CAL633	*H. verrucosa*	*Marinobacter*	28	+	+	+
AC51		*Roseobacter*	30	+	+	+
C−				++	++	++

++, optimal growth; +, growth; +/−, reduced growth; −, no growth; C−, negative control.

3.4. Amplification of pks genes from Antarctic bacteria

It is known that secondary metabolites that can act as antimicrobial molecules are sometimes synthesized by enzymes coded for by *pks* genes. In order to check whether bacteria isolated from the three sponges harbor the *pks* genes, a PCR amplification was carried out on the DNA of each of the 140 bacterial strains using a set of degenerate primers targeted towards *pks* genes. Data obtained revealed that an amplicon of the expected size (about 700 bp) was obtained only from the DNA of *Pseudoalteromonas* strain TB41. Amplicons of different sizes were obtained from the DNA of other strains. An example of the amplicons obtained is shown in Fig. 4.

Thus, the amplicons obtained from the *Pseudoalteromonas* TB41 and from the other three strains TB42, TB43, and TB51 (with a size similar to that expected) were purified from agarose gel and the nucleotide sequence determined. Each sequence was then used as a query in a BLAST search in order to retrieve the most similar sequences from the public databases. Data obtained revealed that only the sequence from strain TB41 produced a significant match ($3.E^{-57}$) with sequences corresponding to proteins encoded by *pks* genes (the other three sequences produced a significant match with genes not related to *pks* ones, data not shown). It was quite interesting that the TB41 sequence retrieved showed only a limited degree of sequence similarity with proteins coded for by genes belonging to *Pseudoalteromonas* strains whose genome has been completely sequenced, suggesting that such gene might have been acquired via HGT from other bacteria. The phylogenetic tree shown in Fig. 5 is in agreement with this idea.

3.5. Chemico-physical nature of antimicrobial compounds produced by Antarctic bacteria

It has been recently shown that some (micro)organisms are able to synthesize volatile organic compounds (VOCs) that inhibit the growth of other (antagonistic) microorganisms (Minerdi et al., 2009 and references therein). To check the possibility that also Antarctic bacteria might produce VOCs, "double-plate" experiments were carried out on 60 different strains representative of each RAPD type, and using as target strains ten *Burkholderia* strains and some of other pathogenic strains listed in Table 2. Data obtained are shown in Table 5 and revealed that:

a) Inhibition of bacterial growth occurred only for strains belonging to Bcc, confirming data on the specificity of action coming from cross-streak experiments reported in the previous paragraph.
b) Most of tester strains also inhibited the growth of *Aspergillus niger*.
c) Inhibition of Bcc growth was affected at a different extent by Antarctic bacteria belonging to different genera. Overall the most active strains belong to the genera *Pseudoalteromonas* and *Shewanella*.

The whole body of data obtained revealed that most of the bacteria tested were able to inhibit the growth of Bcc strains by producing one (or more) antimicrobial molecules that very likely are VOCs. To the best of our knowledge this is the first time that a production of VOCs by Antarctic marine bacteria has been reported.

3.6. Quantitative analysis of the inhibitory action of VOCs produced by Pseudoalteromonas sp. strain TB41

In order to quantify the inhibitory effect of Antarctic bacteria on *Burkholderia* growth the following experiment was carried out using as tester strain the *Pseudoalteromonas* sp. strain TB41 and as target the *B. cenocepacia* J3215 strain. We chose these two strains since TB41 was one of the most effective inhibitory Antarctic strains and J2315 is one of the most frequently Bcc clinical strains isolated from CF patients and thus might represent a good model for the study of antibiotic resistance/sensitivity.

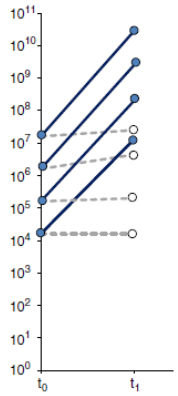

Fig. 6. Bacteriostatic nature of the antimicrobial compound revealed by double-plate experiments. In the y-axis the number of Colony Forming Unit (CFU) of strain J2315 is reported. Black and open circles represent the CFU of target strain J2315 grown in the absence or in the presence of tester strain TB41, respectively, at the beginning (t0) and the end (t1) of the experiment.

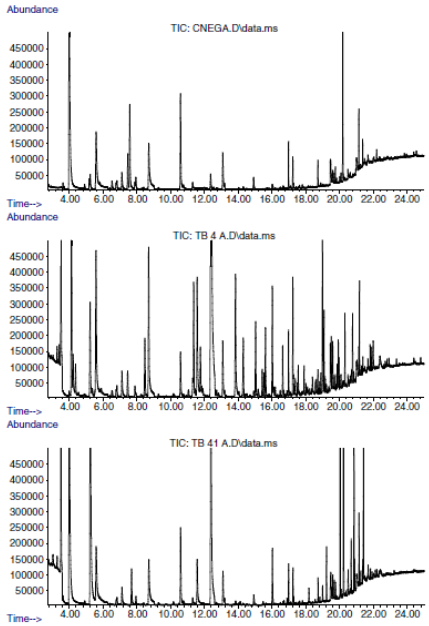

Fig. 7. Chromathograms obtained from *Shewanella* TB4 and *Pseudoalteromonas* TB41.

Table 6
Importance of components.

	PC1	PC2	PC3	PC4
Standard deviation	1.12e+06	4.64e+05	3.51e+05	1.75e+05
Proportion of variance	0.755	0.130	0.744	0.184
Cumulative proportion	0.755	0.885	0.960	0.978

Hence, the "double plate" method was used to evaluate the degree of growth inhibition of J2315 cells. To this purpose TB41 cells were streaked over the entire surface of a bottom PCA plate, a second PCA plate with no bacteria was placed over the bottom one and incubated for 4 days at 20 °C. Then, 10 fold different dilutions of a *B. cenocepacia* J2315 fresh liquid culture grown at 37 °C in LB medium up to an $OD_{550} = 0.5$, corresponding to about $1–2 \times 10^8$ cells were spread onto the up PCA plates embedded with the VOCs produced by *Pseudoalteromonas* sp. TB41. The double-plate was then filled again and incubated at 20 °C for 3 days. Cells from the upper plates were then recovered with LB medium, diluted and spread onto PCA plates containing Ampicillin (50 ug/ml) (an antibiotic that do not affect the growth of strain J2315), in order to avoid the growth of possible contaminants. Data obtained are shown in Fig. 6 and clearly revealed that the viable title of the *B. cenocepacia* J2315 did not changed over time when cells were plated onto PCA medium embedded with the VOCs produced by TB41, whereas the viable title of the control plates increased of about 1000 times.

3.7. Solid Phase Micro Extraction GC–MS analysis

In order to try to identify the VOCs produced by the Antarctic bacteria the SPME technique was used, which affords the possibility to extract the volatile compounds in head space with a minimal sample perturbation. Moreover, conversely to the classic head space techniques, the analytes are concentrated on the fiber allowing the detection of molecules present in trace amounts. Having no information about characteristics of the analytes, we decided to use a three-phase SPME fiber (DVB/Carboxen/PDMS) that ensures us wide affinity range. The analysis was performed in triplicate on the following five bacterial strains: *Pseudoalteromonas* sp. TB41 and AC163, *Shewanella* sp. TB4, *Psychrobacter* sp. TB47 and TB67, which were streaked into filled tubes containing PCA medium; the production of VOCs was then checked every day for a five-days period in order to determine the dynamics of VOCs production.

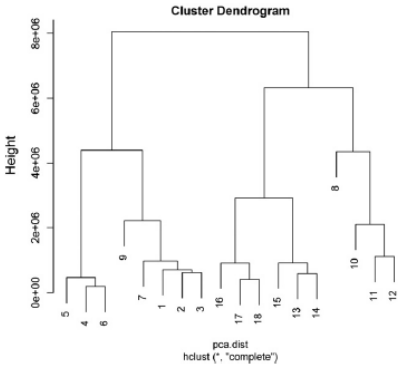

Fig. 9. Dendrogram of hierarchical cluster analysis. Numbers: 1–3, Strain AC163; 7–9, strain TB41; 10–12, strain TB-47; 13–15, strain TB4; 16–18, strain 67; 4–6, negative control (CNEG).

The obtained volatile profile of the samples analyzed was characterized by more than 130 different compounds. Some of these are not assigned by their mass spectra and they were processed by PCA analysis as unknowns (Fig. 7). The list of the entire set of VOCs synthesized by Antarctic bacteria is available as Additional file 3.

The PCA analysis generated ten principal components (PCs) but as much as 97.8% of the total variance was explained by the first four PCs (Table 6, Fig. 8).

The score plot of the samples, the result of a discriminant analysis is reported in Fig. 3 and shows the distance and the similarities between the groups. The hierarchical cluster analysis was made using the City Block Distance that is the sum of the absolute differences of the variables (Fig. 9). A hierarchy of object was constructed according to their similarity. The vertical axis represents the similarity between the clusters and the horizontal axis shows the object in a special ordering to avoid line crossing in the dendrogram. Horizontal lines

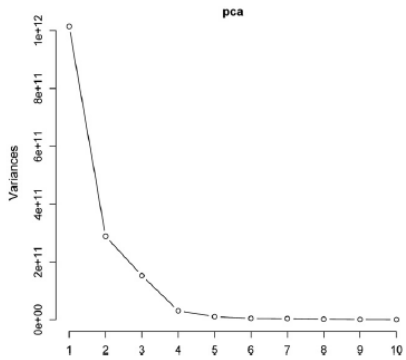

Fig. 8. Summary PCA. Explained variance.

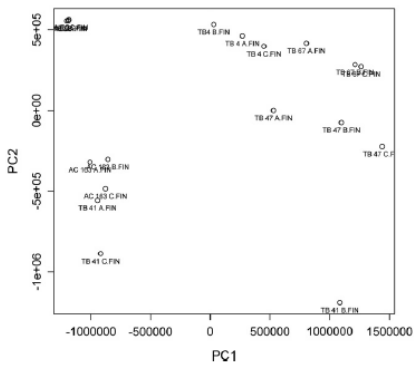

Fig. 10. Projection of the first two PCs.

indicate when the clusters are combined, and their vertical position show the cluster similarity.

Both the dendrogram and the projection of the first two PCs (Figs. 9 and 10) show a clear separation between microorganisms and the blank samples; furthermore the TB41-AC163 groups are separated from negative controls (CNEG), TB4, TB67 and TB47.

The dynamics of production of VOCs by the six bacterial strains revealed that in most cases most of the VOCs are synthesized at a very high extent just one day after bacteria inoculation, suggesting that the production of such molecule is constitutive and not induced by the presence of the target microorganism (data not shown). This finding is in agreement with cross-streak experiments carried out previously (data not shown).

4. Conclusions

The aim of this work was the characterization of cultivable microbial communities isolated from three different Antarctic sponge species in order to check the possibility that some of these strains were able to inhibit the growth of (at least) some opportunistic pathogens affecting Cystic Fibrosis (CF) patients.

The whole body of data obtained revealed that the three sponges harbored different microbial communities at genus, species and strain level, and that the genus/species/strain sharing is extremely low.

The degree of sharing apparently is as follows: genus>species> strain. Hence, these data highlight the idea that the sponge-associated bacterial communities might be sponge-specific and that the interaction between bacteria and sponge is also strain-specific. This specificity might rely also on the production of antimicrobial compounds able to inhibit antagonistic bacteria (Mangano et al., 2009). Thus, sponge associated bacteria might represent a novel source for the detection of new drugs that can antagonize the growth of human (opportunistic) pathogens.

A set of 132 bacterial strains were tested for their ability to inhibit the growth of a panel of more than 70 opportunistic pathogens. The whole body of data obtained clearly revealed that most of these sponge-associated Antarctic bacteria, belonging to different genera, were able to completely inhibit the growth of bacteria belonging to the *B. cepacia* complex, representing one of the most important pathogens in CF. On the other hand, the same Antarctic strains did not have any effect on the growth of other pathogenic bacteria, strongly suggesting that the inhibition is specific for Bcc bacteria. This finding is particularly relevant for the treatment of CF infection caused by Bcc bacteria, since the antimicrobial compound(s) is/are specifically targeted toward these pathogens. Overall, the most active Antarctic bacteria cross-streak experiments also revealed that the antimicrobial compounds are very likely VOCs, a finding that was further confirmed by the SPME–GC–MS technique, which revealed the production of a large set of VOCs by a representative set of Antarctic bacteria. Interestingly, strains belonging to the same or to different genus/species exhibiting a different activity on the panel of target strains, also exhibited a different set of VOCs, whereas strains with similar activity are clustered together in the Principal Component Analysis. The analysis of the activity of the VOCs produced by some Antarctic bacteria revealed that they are more effective in inhibiting the growth of Bcc bacteria than most of the commonly used antibiotics (ampicillin, tetracycline, rifampicine, chloramphenicol, ciprofloxacine, gentamicin, nalidixic acid) (data not shown). This finding was confirmed by the experiment carried out using the *Pseudoalteromonas* sp. strain TB41 and the *B. cenocepacia* J2315 as target strain, which showed that the VOCs are able to completely inhibit the growth of J2315 cells.

Moreover, the synthesis of these VOCs appeared to be related neither to the presence of *pks* genes (since just the *Pseudoalteromonas* TB41 genome apparently harbors such genes), nor the presence of plasmid molecules since just seven of sponge-associated Antarctic bacteria (that is TB14, TB19, TB43, CAL642, CAL643, AC24, and AC164) harbor plasmid molecules of different size, ranging between about 2.5 kb and 4.5 kb (data not shown).

Even though, on the basis of the available data, it is not still possible to clearly identify the VOCs responsible for the inhibition of Bcc strains, in our opinion data obtained in this work indicate that sponge-associated bacteria represent an untapped source for the identification of new antimicrobial compounds and are paving the way for the discovery of new drugs that can be efficiently and successfully used for the treatment of CF infections. The sequencing of the complete genome of *Pseudoalteromonas* strain TB41 is in progress as well as the isolation of Bcc mutants strains able to grow in the presence of VOCs, in order to identify the molecular targets of VOCs.

Supplementary materials related to this article can be found online at doi:10.1016/j.biotechadv.2011.06.011.

Acknowledgments

This work was supported by Italian Cystic Fibrosis Research foundation (Grant FFC#12/2011) and by Ente Cassa di Risparmio di Firenze. Marco Fondi is supported by a post-doctoral fellowship from "Fondazione Adriano Buzzati-Traverso".

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GENOME ANNOUNCEMENT

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

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***Arthrobacter* sp. strain TB23 was isolated from the Antarctic sponge *Lissodendoryx nobilis*. This bacterium is able to produce antimicrobial compounds and volatile organic compounds (VOCs) that inhibit the growth of other Antarctic bacteria and of cystic fibrosis opportunistic pathogens, respectively. Here we report the draft genome sequence of *Arthrobacter* sp. TB23.**

Volatile organic compounds (VOCs) are a class of heterogeneous molecules that are synthesized by various organisms, and the function, for most of them, has not been clarified. There is, however, increasing evidence that supports the idea that VOC production is a common strategy that is widespread among distantly related bacteria (3). Particularly interesting is the novel finding that several Antarctic bacteria affiliated with diverse genera (both Gram positive and Gram negative) and isolated from diverse ecological niches (sponges, seawater, and sediments) produce VOCs (7). The analysis of VOC profiles performed using gas chromatography–solid-phase microextraction technology also revealed that these VOCs belong to quite different chemical classes, including sulfur compounds (8). The biological significance of VOC production by Antarctic bacteria is still unknown, but it has been recently demonstrated that many sponge-associated Antarctic bacteria possess the ability to inhibit the growth of other Antarctic strains (4). Furthermore, these bacteria are also effective toward some human opportunistic pathogens. Indeed, some Antarctic bacteria are able to specifically inhibit the growth of *Burkholderia cepacia* complex (Bcc) strains (7). Bcc strains are among the most dangerous pathogens in immunocompromised patients, such as those affected by cystic fibrosis (CF) (6), and are known to be resistant to several antibiotics (1, 2). It is also noteworthy that the ability to inhibit the growth of Bcc bacteria is related to the production of VOCs (7, 8). One of the most interesting Antarctic bacteria is *Arthrobacter* sp. strain TB23, a strain isolated from a sponge affiliated with the species *Lissodendoryx nobilis*, which exhibited a very high inhibitory activity toward both other Antarctic and Bcc strains (4, 7). Therefore, the knowledge of the genome of this strain represents the first mandatory step toward the identification of the metabolic pathways responsible for new antimicrobial molecule production.

Herein we report the draft genome sequence of *Arthrobacter* sp. strain TB23. The TB23 genome was sequenced using Illumina HiSeq2000, and the 16,927,441 reads (101 bp long) were first trimmed with SolexaQA. The resulting reads, having an average length of 63 bp, were assembled using ABySS software version 1.3.4 ($k = 50$). The assembled genome was 3,542,528 bp long, distributed into 104 contigs (>1,000 b; average length, 34,062 bp), displaying an overall GC content of 63.32%, a rather high but expected value for a genome of a member of the *Actinobacteria*.

Genome annotation was performed using the RAST annotation system and allowed the identification of 3,298 open reading frames (ORFs), 46 tRNA, and 6 rRNA operons. Of the 3,298 ORFs, 2,418 (73%) were assigned to at least one of the Clusters of Orthologous Groups (9) families.

The presence of antibiotic and secondary metabolite biosynthesis genes was checked with antiSMASH (5), and that work revealed that the *Arthrobacter* sp. TB23 draft genome sequence harbors three interesting gene clusters, including a type III polyketide synthase (PKS), a nonribosomal peptide synthetase gene, and terpene biosynthetic genes, respectively. A deeper functional annotation of the predicted ORFs also revealed that the genome contains the full gene set responsible for the biosynthesis of the terpenoid backbone trough the nonmevalonate 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway.

Nucleotide sequence accession numbers. The results of this whole-genome shotgun project have been deposited at DDBJ/EMBL/GenBank under the accession number ALPM00000000. The version described in this paper is the first version, ALPM01000000.

ACKNOWLEDGMENTS

This work was supported by the Italian Cystic Fibrosis Research Foundation (FFC project 12 number 2011) and Ente Cassa di Risparmio (grant 2008.1103). Marco Fondi is financially supported by a FEMS Advanced Fellowship (FAF2012).

Received 8 August 2012 **Accepted** 10 September 2012
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doi:10.1128/JB.01432-12

Arthrobacter sp. TB23 belongs to the Italian Collection of Antarctic Bacteria of the National Antarctic Museum (CIBAN-MNA, Italy).

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