Bacterium–bacterium inhibitory interactions among psychrotrophic bacteria isolated from Antarctic seawater (Terra Nova Bay, Ross Sea)

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Abstract

One hundred and forty bacteria isolated from Antarctic seawater samples were examined for their ability to inhibit the growth of indigenous isolates and their sensitivity to antibacterial activity expressed by one another. On the basis of 16S rRNA gene sequencing and analysis, bacterial isolates were assigned to five phylogenetically different taxa, *Actinobacteria*, alpha and gamma subclasses of *Proteobacteria*, *Bacillaceae*, and *Bacteroidetes*. Twenty-one isolates (15%), predominantly *Actinobacteria*, exhibited antagonistic properties against marine bacteria of Antarctic origin. Members of *Bacteroidetes* and *Firmicutes* did not show any inhibitory activity. Differences were observed among inhibition patterns of single isolates, suggesting that their activity was more likely strain-specific rather than dependent on phylogenetic affiliation. A novel analysis based on network theory confirmed these results, showing that the structure of this population is probably robust to perturbations, but also that it depends strongly on the most active strains. The determination of plasmid incidence in the bacterial strains investigated revealed that there was no correlation between their presence and the antagonistic activity. The data presented here provide evidence for the antagonistic interactions within bacterial strains inhabiting Antarctic seawater and suggest the potential exploitation of Antarctic bacteria as a novel source of antibiotics.

Introduction

Antagonistic interactions among bacteria represent an interesting evolutionary strategy, conferring a selective advantage in competition for food and space in the environment, and acting as an effective control of microbial populations inhabiting the same ecological niche (Hentschel *et al.*, 2001). Marine bacteria have been intensely screened for their inhibitory effect against terrestrial microorganisms (Isnansetyo & Kamei, 2003). Conversely, few reports have regarded the inter-specific interactions among bacteria of the same or related marine environments, but they certainly demonstrate that antagonistic effects, expressed by phylogenetically different bacterial groups, are a widespread trait in marine habitats (Lemos *et al.*, 1985; Nair & Simidu, 1987; Long & Azam, 2001; Brinkhoff *et al.*, 2004; Grossart *et al.*, 2004; Bhattarai *et al.*, 2006). Antarctic marine ecosystems are among the less-explored environments on Earth and offer to researchers a unique opportunity for studying microbial diversity and evolution (Nichols *et al.*, 1999; Vincent, 2000). In particular, microbiological investigations have been mainly focused on bacterial diversity in Antarctic sea-ice, sediments and seawater. To our knowledge, antagonistic interaction among Antarctic marine isolates has never been considered and only one study was performed on the inhibitory properties of soil Antarctic bacteria towards food-borne microorganisms (O'Brien *et al.*, 2004). Bacteria inhabiting Antarctica have to cope with adverse environmental conditions and require peculiar survival strategies to achieve a competitive advantage. In addition to cellular modifications, antagonistic features may contribute to the adaptation of Antarctic bacteria to permanently low temperatures by reducing the presence of competitive microorganisms. Moreover, isolation and characterization of bacteria able to inhibit efficiently microorganisms at low temperatures will provide insight into the possibility to use cold-adapted bacteria as a new source of industrially exploitable antibiotics.

In this context, the aim of the present study was to investigate the antagonistic potential of Antarctic marine bacteria against bacteria inhabiting the same environment. Moreover, all isolates were phylogenetically characterized by 16S rRNA gene sequencing to (1) determine whether both the inhibitory activity and sensibility to inhibition were peculiar of a certain phylogenetic group, (2) establish the inter-specific interactions and (3) assess whether a relationship occurred between the presence of plasmid and antagonistic activity.

Materials and methods

Sampling area

Terra Nova Bay occupies the western coast of the Ross Sea, being delimited north by Cape Washington and south by the Drygalski Ice Tongue. The sea bottom reaches the greatest depth of the Ross Sea, with a pit of about 1100 m elongated along shore and bounded by 500 m isobaths (Buffoni *et al.*, 2002). At the sampling time, Terra Nova Bay was characterized by an evident water column stratification, resulting from prior melting of the pack ice. The mixed layer waters, composed of melted ice and Antarctic surface waters, ranged from 3 to 48 m in depth and were different from the deeper layers in terms of particulate organic matter composition (Fabiano *et al.*, 1996). Suspended particulate matter was mostly composed of autochthonous material, but also included some terrestrial components (Fabiano *et al.*, 1995). Seawater temperature was always above 0 °C ranging between 0.85 and 2.76 °C (Maugeri *et al.*, 1996).

Bacterial strains

The 140 psychrotrophic isolates used in this study were retrieved from seawater samples collected along the water column at two fixed stations (Mergellina, MER: 74°41′33″ S–164°07′15″E, about 250 m from the coast; Santa Maria Novella, SMN: 74°43′S–164°16′E, in the middle of the Terra Nova Bay, about 10.5 km from MER) in the Terra Nova Bay (Ross Sea, Antarctica) (Bruni *et al.*, 1995; Maugeri *et al.*, 1996). Samples were collected using Niskin bottles previously washed with a solution of HCl 10N. Serial dilutions were prepared (1 : 10 and 1 : 100, using filter-sterilized seawater) and 100 μL of each dilution was plated on two replicate plates of Marine Agar 2216 (MA, Difco). Inoculated plates were incubated in the dark for 21 days at 4 °C. Colonies were selected at random from the cultures on MA and isolates were streaked at least three times before being considered pure. All the isolates belong to the Italian Collection of Antarctic Bacteria (CIBAN) of the National Antarctic Museum (MNA) 'Felice Ippolito' kept at the University of Messina. They are maintained on MA slopes at 4 °C and routinely streaked on agar plates from tubes every 6 months to control purity and viability. Antarctic strains are also preserved by freezing cell suspensions at − 80 °C in Marine Broth (MB, Difco) to which 20% (v/v) glycerol is added.

PCR amplification, sequencing and analysis of 16S rRNA gene

PCR amplification, sequencing and phylogenetic analysis of 16S rRNA gene from bacterial isolates were carried out as previously described by Michaud *et al.* (2004). The first half (about 700 nucleotides) of each amplification product was sequenced by using the primer 27f. Each sequence was then used as a query in a BLASTN search (Altschul *et al.*, 1997) and further aligned using the program CLUSTAL W (Thompson *et al.*, 1994) to the most similar orthologous sequences retrieved from database. The 16S rRNA gene sequences were submitted to GenBank and assigned to the following accession numbers: DQ646848–DQ646868, DQ652544–DQ652563, DQ667067–DQ667136 and DQ831958–DQ831975.

Screening for antagonistic interactions among isolates

Experiments were performed on a solid medium containing (w/v): 0.2% Bacto-peptone, 0.2% casein hydrolysate, 0.2% yeast extract, 0.1% glucose, 0.02% KH_2PO_4, 0.005% $MgSO_4 \times 7H_2O$, 0.1% $CaCl_2$, 0.01% KBr and 1.5% Bacto-agar (Ivanova *et al.*, 1998). The medium was prepared in a mixture of 75% (v/v) natural seawater and 25% (v/v) distilled water (pH 7.8). Antibacterial activity was detected by using a 140×140 array of tests (19 600 tests) and the cross-streak method. Hereinafter, bacteria tested for inhibitory activity will be defined as 'tester strains', whereas those used as a target will be called 'target strains'. Tester strains were streaked across one-third of an agar plate and incubated at 15 °C (due to the psychrotrophic nature of the isolates). After good growth was obtained (generally in 7–10 days, depending on growth of the test strains), target strains were streaked perpendicular to the initial streak and plates were further incubated at 15 °C. The antagonistic effect was indicated by the failure of the target strain to grow in the confluence area. Inhibition had to be observed at least twice to be considered positive. If the first two assays showed ambiguous results, an additional assay was performed to re-assess inhibitory activity.

Analysis of plasmid content

Plasmid incidence in the bacterial population analyzed was determined as previously reported (Michaud *et al.*, 2004). Plasmid molecules were extracted from 3-mL bacterial cultures grown in MB using the commercial kit Plasmid

Miniprep (Qiagen) according to the manufacturer's instructions.

Antimicrobial susceptibility testing

Resistant Antarctic isolates were screened for susceptibility towards eight different commercial antibiotics: ampicillin (25 μg), penicillin G (10 μg), polymixin B (30 μg), nalidixic acid (30 μg), tobramycin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), and vibriostatic agent O/129 (10 μg) (Bauer *et al*., 1966). Antibiotic-impregnated disks (Oxoid) were laid on MA plates that had been previously surface inoculated with the test strains. Any sign of growth inhibition was scored as sensitivity to that antimicrobial compound. Resistance to an antimicrobial drug was indicated if a strain did not show any inhibition zone. This meant that resistance was strictly defined and results are reported as susceptible (+) or resistant (−).

Network analysis of the data

Data were used to derive an adjacency matrix for a network analysis of the inhibition patterns. The network derived is composed by nodes, representing each strain in the original dataset, and directed links: a node *A* can both send and receive a link; in the first case, it inhibits another strain, otherwise, it is inhibited. Networks were visualized using the free software VISONE (www.visone.info) and all the analyses were performed using self-written JAVA classes available from two of the authors (contact matteo.brilli@dbag.unifi.it and r_fani@dbag.unifi.it). We performed analyses of in- and outdegree distributions (see text for details) (Barabasi & Oltvai, 2004) to characterize the structure of the network and make inferences on the population structure.

Results

Sequencing and analysis of 16S rRNA gene of Antarctic isolates

The phylogenetic affiliation of our isolates was obtained by sequencing and analysis of 16S rRNA gene sequence. The 140 isolates were placed within five different taxa: (1) most isolates (56%) fell in the *Actinobacteria*; (2) 10% and (3) 21% of them were affiliated with alpha and gamma subclasses of *Proteobacteria*, respectively; (4) 12% belonged to the *Bacillaceae* of *Firmicutes* and, finally, (5) 1% of the bacteria to *Bacteroidetes*. Among *Actinobacteria*, six isolates shared the highest degree of sequence identity with undescribed glacial ice bacteria.

All the isolates of the *Gammaproteobacteria* and the *Actinobacteria* belonged to six and five different families, respectively. Isolates from the *Firmicutes* clustered into two families, whereas members of both *Alphaproteobacteria* and CFB group of *Bacteroidetes* belonged to a single family (*Rhodobacteraceae* and *Crenotrichaceae*, respectively).

The highest diversity of isolates was found within the *Actinobacteria* (with *Rhodococcus* spp. and *Arthrobacter* spp. as the dominant representatives) and the *Gammaproteobacteria* (with *Pseudoalteromonas* spp. as the most abundant). *Paracoccus* spp. and *Planococcus* spp. were the most frequent isolates within *Alphaproteobacteria* and *Firmicutes*, respectively. Most genera were represented by only one or two isolates.

The phylogenetic affiliation of all isolates tested is reported in Table 1.

Antagonistic interactions among isolates

Based on data obtained from the preliminary screening, isolates were operationally grouped into three different interactivity clusters (active, sensitive or resistant). A number of active isolates was also among sensitive or resistant ones. The screening made it possible to select 21 active Antarctic isolates (final detection rate of 15%), generally isolated from depths of 5–25 m. Inhibition patterns vary greatly for different isolates (see below), even though they were affiliated to the same phylogenetic group. As previously reported by Grossart *et al*. (2004), closely related isolates sharing a degree of 16S rRNA gene sequence similarity $> 99\%$ showed large differences in inhibitory activities. Thus, in our experiments all isolates were treated as distinct entities, even though they were often closely related according to their 16S rRNA gene sequences.

Data analysis revealed that no great difference between the numbers of active isolates was observed when SMN and MER samples were compared, as they yielded 12 (out of 81) and nine (out of 59) producers, respectively. Members of three different phylogenetic groups showed inhibitory activities: 16 *Actinobacteria*, three *Gammaproteobacteria* and two *Alphaproteobacteria* (Table 2). No member of both *Bacteroidetes* and *Firmicutes* showed a detectable antagonistic activity.

Overall, the mean number of the inhibited isolates was 19.9, but the number of sensitive target isolates was generally highest for members of the *Actinobacteria*. For example, five strains belonging to the genus *Arthrobacter* (isolates D27, D70, E49, F15 and F40) and one identified as *Corynebacterium* (isolate H22) inhibited 30 or more of all other isolates. In particular, a broad spectrum of antibacterial activity was observed for the strain F40, able to inhibit the growth of 82 isolates used as a target.

Inhibition occurred between strains belonging to both different and the same bacterial species. Within the same taxon, different isolates showed different inhibitory activity. Isolates inhibited the growth of both closely related and taxonomically distant bacteria (Table 3). Members of

Table 1. 16S rRNA gene sequence affiliation to the closest phylogenetic neighbors, inhibition activity and susceptibility of Antarctic isolates

Strain	Origin (St, depth)*	AN†	Next relative by GenBank alignment (AN, organism)	Hom‡ (%)	Phylum or class§	Family	Number of inhibited strains	Inhibited by (number of strains)
B3	SMN, 5 m	DQ646851	DQ341415, *Arthrobacter* sp. Antarctic IS03	98	ACT	*Micrococcaceae*	0	4
B20	SMN, 15 m	DQ646858	DQ172990, *Arthrobacter* sp. TSBY-20	99		*Micrococcaceae*	0	1
C7	SMN, 0 m	DQ646862	AF041789, *Arthrobacter* sp. S23H2	99		*Micrococcaceae*	0	3
C13	SMN, 0 m	DQ646863	AF134184, *Arthrobacter agilis* strain LV7	99		*Micrococcaceae*	0	2
D1	SMN, 0 m	DQ646866	DQ341415, *Arthrobacter* sp. Antarctic IS03	98		*Micrococcaceae*	0	4
D15	SMN, 10 m	DQ652544	DQ366002, *Arthrobacter* sp. 20/4	99		*Micrococcaceae*	0	9
D27	SMN, 10 m	AY451328	AJ639830, *Arthrobacter parietes* LMG 22281T	99		*Micrococcaceae*	30	4
D61	MER, 5 m	DQ652553	AJ639830, *Arthrobacter parietes* LMG 22281T	99		*Micrococcaceae*	25	4
D68	MER, 10 m	DQ652556	AF041789, *Arthrobacter* sp. S23H2	99		*Micrococcaceae*	0	7
D70	MER, 10 m	DQ652558	AJ639830, *Arthrobacter parietes* LMG 22281T	99		*Micrococcaceae*	31	6
D72	MER, 10 m	DQ667114	AJ639830, *Arthrobacter parietes* LMG 22281T	98		*Micrococcaceae*	23	5
E17	MER, 15 m	DQ667069	AY526657, *Arthrobacter* sp. Mutz-E03	99		*Micrococcaceae*	0	8
E28	MER, 25 m	AY316680	AF041789, *Arthrobacter* sp. S23H2	98		*Micrococcaceae*	0	8
E30	MER, 25 m	DQ667072	DQ341415, *Arthrobacter* sp. Antarctic IS03	99		*Micrococcaceae*	0	10
E36	SMN, 0 m	DQ667115	AJ639830, *Arthrobacter parietes* LMG 22281T	99		*Micrococcaceae*	25	0
E37	SMN, 0 m	DQ667073	AF134184, *Arthrobacter agilis* strain LV7	99		*Micrococcaceae*	0	7
E49	SMN, 100 m	DQ667079	AJ639830, *Arthrobacter parietes* LMG 22281T	99		*Micrococcaceae*	32	3
E54	SMN, 100 m	DQ667081	AF134184, *Arthrobacter agilis* strain LV7	99		*Micrococcaceae*	0	9
F15	SMN, 20 m	DQ667086	DQ366002, *Arthrobacter* sp. 20/4	99		*Micrococcaceae*	50	7
F27	SMN, 25 m	DQ667089	AY571801, *Arthrobacter* sp. clone 433F	99		*Micrococcaceae*	0	6
F37	MER, 25 m	DQ667093	DQ513408, *Arthrobacter* sp. B-5	98		*Micrococcaceae*	0	9
F40	MER, 25 m	DQ667096	DQ366002, *Arthrobacter* sp. 20/4	99		*Micrococcaceae*	83	5
G13	SMN, 50 m	DQ667111	AF041789, *Arthrobacter* sp. S23H2	99		*Micrococcaceae*	0	7
G15	SMN, 50 m	DQ667117	AJ313024, *Micrococcus sp*. MN 8.1d.1c	99		*Micrococcaceae*	0	1
G18	SMN, 100 m	DQ667118	AF409000, *Arthrobacter* sp. Ellin158	98		*Micrococcaceae*	0	7
G42	MER, 0 m	DQ667125	AF041789, *Arthrobacter* sp. S23H2	99		*Micrococcaceae*	0	9
G61	MER, 15 m	DQ667126	DQ366002, *Arthrobacter* sp. 20/4	97		*Micrococcaceae*	12	7
G75	MER, 25 m	AY451331	AY383043, *Arthrobacter* sp. DY12-2	99		*Micrococcaceae*	7	8
H21	MER, 25 m	DQ667135	AF041789, *Arthrobacter* sp. S23H2	99		*Micrococcaceae*	0	6
I33	SMN, 0 m	DQ831965	AF041789, *Arthrobacter* sp. S23H2	99		*Micrococcaceae*	0	3
I34	SMN, 0 m	DQ831966	AF134184, *Arthrobacter agilis* strain LV7	99		*Micrococcaceae*	0	9
H22	MER, 25 m	DQ667136	AJ244681, *Corynebacterium aquaticum* V4BO26	98		*Corynebacteriaceae*	42	5
L16	SMN, 25 m	DQ831972	AJ244681, *Corynebacterium aquaticum* V4BO26	98		*Corynebacteriaceae*	0	5
B8	SMN, 5 m	AY444156	Y08540, *Janibacter thuringensis*	97		*Intrasporangiaceae*	11	5
F21	SMN, 25 m	DQ667087	Y08540, *Janibacter thuringensis*	99		*Intrasporangiaceae*	0	1
F34	MER, 10 m	DQ667092	Y08540, *Janibacter thuringensis*	99		*Intrasporangiaceae*	0	2
F39	MER, 25 m	DQ667095	Y08540, *Janibacter thuringensis*	99		*Intrasporangiaceae*	0	1
G4	SMN, 10 m	DQ667104	Y08540, *Janibacter thuringensis*	98		*Intrasporangiaceae*	0	0
G5	SMN, 25 m	DQ667105	Y08540, *Janibacter thuringensis*	99		*Intrasporangiaceae*	0	0
I44	SMN, 25 m	DQ831968	Y08540, *Janibacter thuringensis*	97		*Intrasporangiaceae*	5	2

Table 1. Continued.

Strain	Origin (St, depth)*	AN†	Next relative by GenBank alignment (AN, organism)	Hom‡ (%)	Phylum or class§	Family	Number of inhibited strains	Inhibited by (number of strains)
I36	SMN, 0 m	DQ831967	DQ195813, *Nocardioidaceae* bacterium SM2	98	ACT	*Nocardioidaceae*	0	3
B7	SMN, 5 m	DQ646853	AY771765, *Rhodococcus fascians* clone SE59	99		*Nocardiaceae*	11	5
B11	SMN, 5 m	DQ646855	AY730713, *Rhodococcus fascians*	99		*Nocardiaceae*	0	1
B15	SMN, 5 m	DQ646857	AY730713, *Rhodococcus fascians*	99		*Nocardiaceae*	0	1
B21	SMN, 15 m	DQ646859	AB010908, *Rhodococcus* sp. SGB1168-118	99		*Nocardiaceae*	1	1
D4	SMN, 0 m	DQ646867	AY771765, *Rhodococcus fascians* clone SE59	98		*Nocardiaceae*	0	2
D13	SMN, 0 m	DQ646868	AY730713, *Rhodococcus fascians*	99		*Nocardiaceae*	0	2
D51	MER, 0 m	DQ652551	AY771765, *Rhodococcus fascians* clone SE59	99		*Nocardiaceae*	0	4
D58	MER, 5 m	DQ652552	AF181690, *Rhodococcus* sp. 5/14	99		*Nocardiaceae*	0	2
E38	SMN, 0 m	DQ667074	AY785731, *Rhodoccus erythropolis* isolate OUCZ20	99		*Nocardiaceae*	0	1
E46	SMN, 25 m	DQ667076	AF181690, *Rhodococcus* sp. 5/14 16S	98		*Nocardiaceae*	0	2
E48	SMN, 50 m	DQ667078	AY730713, *Rhodococcus fascians*	99		*Nocardiaceae*	0	2
E56	SMN, 200 m	DQ831960	AY771765, *Rhodococcus fascians* clone SE59	99		*Nocardiaceae*	0	2
E57	SMN, 200 m	DQ667082	AY730713, *Rhodococcus fascians*	99		*Nocardiaceae*	0	1
E60	SMN, 200 m	AY316681	AY512642, *Rhodococcus* sp. *A1XB1-5*	99		*Nocardiaceae*	0	2
F30	MER, 0 m	DQ667090	AY730713, *Rhodococcus fascians*	99		*Nocardiaceae*	0	1
F32	MER, 10 m	DQ667091	AY730713, *Rhodococcus fascians*	99		*Nocardiaceae*	0	1
G3	SMN, 10 m	DQ667103	AY771765, *Rhodococcus fascians* clone SE59	98		*Nocardiaceae*	1	7
G9	SMN, 50 m	DQ667109	AY730713, *Rhodococcus fascians*	99		*Nocardiaceae*	0	2
G31	SMN, 200 m	DQ667123	AY730713, *Rhodococcus fascians*	99		*Nocardiaceae*	0	1
G33	MER, 0 m	DQ667124	AB010908, *Rhodococcus* sp. SGB1168-118	98		*Nocardiaceae*	0	1
G76	MER, 25 m	DQ667128	AB180236, *Rhodococcus fascians*	99		*Nocardiaceae*	0	2
G77	MER, 25 m	DQ667129	AY771765, *Rhodococcus fascians* clone SE59	99		*Nocardiaceae*	0	2
H14	MER, 10 m	DQ667133	AJ002093, *Rhodococcus* strain 40	98		*Nocardiaceae*	0	3
H24	MER, 25 m	DQ831961	AY730713, *Rhodococcus fascians*	98		*Nocardiaceae*	0	2
I14	MER, 10 m	DQ831962	AY730713, *Rhodococcus fascians*	99		*Nocardiaceae*	0	2
I21	MER, 10 m	DQ831964	AY730713, *Rhodococcus fascians*	98		*Nocardiaceae*	0	2
N44	MER, 25 m	DQ831975	AY730713, *Rhodococcus fascians*	99		*Nocardiaceae*	0	1
D69	MER, 10 m	DQ652557	AJ438585, *Leifsonia rubeus* strain CMS 76r	99		*Microbacteriaceae*	0	9
F13	SMN, 20 m	DQ667085	DQ172995, *Frigoribacterium* sp. TSBY-26	99		*Microbacteriaceae*	0	8
N37	MER, 25 m	DQ831974	AY571814, *Microbacterium* sp. 44/18	99		*Microbacteriaceae*	0	2
B24	SMN, 15 m	DQ646861	AF479355, Glacial ice bacterium G50-TB8	99		*Microbacteriaceae*	0	7
D16	SMN, 10 m	DQ652545	AF479355, Glacial ice bacterium G50	99		*Microbacteriaceae*	0	1
D21	SMN, 10 m	DQ652546	AY571813, *Frigoribacterium* sp. 34/19	97		*Microbacteriaceae*	0	0
D49	MER, 0 m	DQ652550	AF479355, Glacial ice bacterium G50	99		*Microbacteriaceae*	0	9
E27	MER, 25 m	DQ667071	AF479355, Glacial ice bacterium G50	99		*Microbacteriaceae*	0	10
G1	SMN, 0 m	DQ667101	AF479329, Glacial ice bacterium G500K-10	98		*Microbacteriaceae*	0	3
G21	SMN, 100 m	DQ667120	AF479355, Glacial ice bacterium G50-TB8	99		*Microbacteriaceae*	0	6
B22	SMN, 15 m	DQ646860	AY745834, *Paracoccus* sp. NPO-JL-65	99	ALF	*Rhodobacteraceae*	1	1
D39	SMN, 200 m	AY451333	DQ270725, *Paracoccus* sp. B-101	99		*Rhodobacteraceae*	0	0

Table 1. Continued.

Strain	Origin (St, depth)*	AN†	Next relative by GenBank alignment (AN, organism)	Hom‡ (%)	Phylum or class§	Family	Number of inhibited strains	Inhibited by (number of strains)
D40	SMN, 200 m	AY309073	AY771745, *Loktanella salsilacus* clone SE10	99	ALF	*Rhodobacteraceae*	0	2
D41	SMN, 200 m	DQ652549	AY771745, *Loktanella salsilacus* clone SE10	99		*Rhodobacteraceae*	0	1
D38	SMN, 100 m	DQ652548	AY745834, *Paracoccus* sp. NPO-JL-65	99		*Rhodobacteraceae*	0	1
D67	MER, 5 m	DQ652555	AY494629, *Paracoccus* sp. clone ALPHA2D	99		*Rhodobacteraceae*	0	1
D71	MER, 0 m	DQ652559	DQ270725, *Paracoccus* sp. B-101	99		*Rhodobacteraceae*	0	0
F12	SMN, 20 m	DQ667084	AY745834, *Paracoccus* sp. NPO-JL-65	99		*Rhodobacteraceae*	9	2
F38	MER, 25 m	DQ667094	AY745834, *Paracoccus* sp. NPO-JL-65	99		*Rhodobacteraceae*	0	0
G8	SMN, 25 m	DQ667108	DQ270725, *Paracoccus* sp. B-1018	99		*Rhodobacteraceae*	0	2
G14	SMN, 50 m	DQ667112	AY745834, *Paracoccus* sp. NPO-JL-65	98		*Rhodobacteraceae*	0	4
G29	SMN, 200 m	DQ667121	DQ270725, *Paracoccus* sp. B-1018	98		*Rhodobacteraceae*	0	1
G30	SMN, 200 m	DQ667122	DQ298023, *Paracoccus marcusii* strain T1947D	99		*Rhodobacteraceae*	0	2
G65	MER, 15 m	DQ667127	AY771745, *Loktanella salsilacus* clone SE10	100		*Rhodobacteraceae*	0	1
A14	MER, 10 m	DQ646849	AJ238597, *Marinomonas protea*	100	GAM	*Oceanospirillaceae*	4	0
E12	MER, 5 m	DQ667067	AY771708, *Marinomonas primoryensis*	99		*Oceanospirillaceae*	10	0
B1	SMN, 5 m	DQ646850	AY576005, *Pseudoalteromonas* sp. D32	99		*Pseudoalteromonadaceae*	0	0
D48	MER, 0 m	DQ831959	DQ520886, *Pseudoalteromonas* sp. BSi20325	97		*Pseudoalteromonadaceae*	0	0
F26	SMN, 25 m	DQ667088	AY664306, *Pseudoalteromonas* clone JL-BS-K14	98		*Pseudoalteromonadaceae*	0	0
F43	MER, 10 m	DQ667097	AY576005, *Pseudoalteromonas* sp. D32	99		*Pseudoalteromonadaceae*	0	0
F46	MER, 10 m	AY451334	AM110975, *Pseudoalteromonas* sp. 3020	97		*Pseudoalteromonadaceae*	0	0
F47	MER, 10 m	DQ667098	AY576005, *Pseudoalteromonas* sp. D32	99		*Pseudoalteromonadaceae*	0	0
F48	MER, 10 m	DQ667099	AY576005, *Pseudoalteromonas* sp. D32	99		*Pseudoalteromonadaceae*	0	0
F53	SMN, 200 m	DQ667100	AY536560, *Pseudoalteromonas* sp. TP-C	98		*Pseudoalteromonadaceae*	0	0
G19	SMN, 100 m	DQ667119	AY576005, *Pseudoalteromonas* sp. D32	99		*Pseudoalteromonadaceae*	0	0
G24	SMN, 100 m	AY451336	AM110950, *Pseudoalteromonas* sp. 1002	99		*Pseudoalteromonadaceae*	5	0
H17	MER, 10 m	DQ667134	AY576005, *Pseudoalteromonas* sp. D32	99		*Pseudoalteromonadaceae*	0	0
M4	MER, 0m	DQ831973	AY576005, *Pseudoalteromonas* sp. D32	99		*Pseudoalteromonadaceae*	0	0
D94	MER, 15 m	DQ652563	U85853, *Glaciecola punicea* ACAM 611T	99		*Alteromonadaceae*	0	1
D81	MER, 15 m	DQ652561	AY771710, *Marinobacter lipolyticus*	98		*Alteromonadaceae*	0	3
E45	SMN, 25 m	DQ667075	U65012, *Pseudomonas stutzeri*	99		*Pseudomonadaceae*	0	1
I45	SMN, 100 m	DQ831969	U65012, *Pseudomonas stutzeri*	100		*Pseudomonadaceae*	0	0
B26	SMN, 15 m	DQ667113	CP000082, *Psychrobacter arcticus* 273-4	99		*Moraxellaceae*	0	6
C1	SMN, 0 m	AY316679	AY771717, *Psychrobacter fozii*	98		*Moraxellaceae*	0	2
C11	SMN, 0 m	DQ831958	AY316679, *Psychrobacter* sp. isolate C1	98		*Moraxellaceae*	0	5
C16	SMN, 10 m	DQ646864	AY660685, *Psychrobacter* sp. cryopeg_5	99		*Moraxellaceae*	0	1
E59	SMN, 200 m	DQ667083	AY639872, *Psychrobacter cibarius* strain JG-220	98		*Moraxellaceae*	0	2
H2	MER, 0 m	DQ667132	AJ431338, *Psychrobacter* sp. ikaite c11	99		*Moraxellaceae*	0	1
A7	MER, 0 m	DQ646848	AY77173, *Shewanella frigidimarina* isolate S5-8	99		*Shewanellaceae*	0	0
B6	SMN, 5 m	DQ646852	AY771713, *Shewanella frigidimarina isolate S5-8*	99		*Shewanellaceae*	0	0
D64	MER, 5 m	DQ652554	AY771736, *Shewanella frigidimarin* isolate S5-8	99		*Shewanellaceae*	0	0
D79	MER, 15 m	DQ652560	AY771736, *Shewanella frigidimarina* isolate S5-8	99		*Shewanellaceae*	0	1

Table 1. Continued.

Strain	Origin (St, depth)*	AN†	Next relative by GenBank alignment (AN, organism)	Hom‡ (%)	Phylum or class§	Family	Number of inhibited strains	Inhibited by (number of strains)
D82	MER, 15 m	DQ652562	AY771736, *Shewanella frigidimarina* isolate S5-8	100		*Shewanellaceae*	0	1
E9	MER, 5 m	DQ667116	AY771736, *Shewanella frigidimarina* isolate S5-8	99		*Shewanellaceae*	0	8
E14	MER, 5 m	DQ667068	AY576726, *Salinibacterium* sp. 18III/A01/077	99	BAC	*Crenotrichaceae*	0	6
C17	SMN, 10 m	DQ646865	AJ237708, *Bacillus marinus* strain DSM 1297	98	FIR	*Bacillaceae*	0	2
D37	SMN, 100 m	DQ652547	DQ019166, *Exiguobacterium aurantiacum*	99		*Bacillaceae*	0	2
G2	SMN, 0 m	DQ667102	AM111065, *Bacillus* sp. 7327	97		*Bacillaceae*	0	3
B10	SMN, 5 m	DQ646854	AY745836, *Planococcus* sp. NPO-JL-69	98		*Planococcaceae*	0	2
B13	SMN, 5 m	DQ646856	AY745836, *Planococcus* sp. NPO-JL-69	99		*Planococcaceae*	0	2
D3	SMN, 0 m	AY451329	AM111009, *Planococcus sp. 3059*	93		*Planococcaceae*	0	3
E18	MER, 15 m	DQ667070	AY745836, *Planococcus* sp. NPO-JL-69	99		*Planococcaceae*	0	4
E47	SMN, 50 m	DQ667077	AY745836, *Planococcus* sp. NPO-JL-69	99		*Planococcaceae*	0	8
E53	SMN, 100 m	DQ667080	AY745836, *Planococcus* sp. NPO-JL-69	99		*Planococcaceae*	0	7
G6	SMN, 25 m	DQ667106	AY745836, *Planococcus* sp. NPO-JL-69	98		*Planococcaceae*	0	3
G7	SMN, 25 m	DQ667107	AY745836, *Planococcus* sp. NPO-JL-69	98		*Planococcaceae*	0	2
I15	MER, 10 m	DQ831963	AY745836, *Planococcus* sp. NPO-JL-69	99		*Planococcaceae*	0	1
G78	MER, 25 m	DQ667130	AM111008, *Planomicrobium* sp. 3057	99		*Planococcaceae*	0	2
G87	MER, 25 m	DQ667131	AM111008, *Planomicrobium* sp. 3057	99		*Planococcaceae*	0	2
I46	SMN, 100 m	DQ831970	AM111008, *Planomicrobium* sp. 3057	98		*Planococcaceae*	0	1
G11	SMN, 50 m	DQ667110	DQ236259, Uncultured *Sporosarcina* sp. clone 68	99		*Planococcaceae*	0	5
I50	SMN, 200 m	DQ831971	AY745836, *Planococcus* sp. NPO-JL-69	99		*Planococcaceae*	0	0

*St, sampling station; SMN, Santa Maria Novella; MER, Mergellina.

†AN, accession number.

‡Hom, sequence homology.

§ALF, *Alphaproteobacteria*; GAM, *Gammaproteobacteria*; ACT, *Actinobacteria*; BAC, *Bacteroidetes*; FIR, *Firmicutes*.

Actinobacteria generally inhibited isolates clustered in all five phylogenetic groups detected. In particular, *Arthrobacter* and *Corynebacterium* isolates tended to be predominantly active against members belonging to the same class. Active *Alphaproteobacteria* were represented by two members of the genus *Paracoccus*, showing highly different inhibition patterns; neither expressed inhibitory activity against isolate of *Firmicutes*. Among *Gammaproteobacteria* the only *Pseudoalteromonas* isolate (G24) showed antibiotic activity exclusively against bacteria grouped in the *Firmicutes*, whereas members of the *Marinomonas* genus (isolates A14 and E12) were able to inhibit *Gammaproteobacteria* and *Actinobacteria*, too. The auto-inhibition phenomenon was never observed.

Antagonism assays demonstrated that each producer generally inhibited the growth of the other producers as well as of some nonproducer strains. Active isolates were generally sensitive to one to eight Antarctic strains; strains D70, F15, G61 and G75 (*Arthrobacter*) and G3 (*Rhodococcus*) were the most inhibited. Among active strains, A14 and E12 (both identified as member of the genus *Marinomonas*), E36 (*Arthrobacter*) and G24 (*Pseudoalteromonas*) were resistant. Isolates of the genera *Janibacter* (B7 and I44), *Rhodococcus* (G3 and B21), *Paracoccus* (B22) and *Pseudoalteromonas* (G24), as well as only one member of the genus *Arthrobacter* (G75), lacked activity towards all other antagonistic strains.

All the sensitive isolates (nearly 82%) were inhibited by 1–10 active strains, with the 51% (58 out of 114) susceptible to only one or two producers. Sensitive strains belonged to all phylogenetic clusters detected by sequencing the 16S rRNA gene, with the majority of them (65%) falling into the *Actinobacteria* phylum.

Only 26 isolates (18.6% of total isolates) were resistant to the inhibitory activity of other Antarctic bacteria. Most of them belonged to the *Gammaproteobacteria* and, in particular, to the genus *Pseudoalteromonas*. Four active strains, affiliated with the *Gammaproteobacteria* (isolates A14, E12 and G24) and *Actinobacteria* (isolate E36), were among resistant bacteria, too.

Table 2. Total number of isolates belonging to the different phylogenetic groups and the percentage showing inhibitory activity

Phylogenetic affiliation	Number of isolates	Active isolates (%)
Actinobacteria	78	20.5
Alphaproteobacteria	14	14.3
Gammaproteobacteria	30	10
Bacteroidetes	1	0
Firmicutes	17	0

Network analysis of data

The interrelationships existing between the 140 bacterial isolates are visualized in Figs 1 and 2. In the inhibitory networks, constructed as described in 'Materials and

Table 3. Antagonistic interactions among phylogenetic groups

			Affiliation*				
	Genus	Isolate	ALF	GAM	FIR	ACT	BAC
Actinobacteria	*Arthrobacter*	D27	1	3	–	25	1
	Arthrobacter	D61	1	2	–	22	–
	Arthrobacter	D70	1	1	1	27	1
	Arthrobacter	D72	–	–	–	23	–
	Arthrobacter	E36	–	2	–	23	–
	Arthrobacter	E49	–	3	–	29	–
	Arthrobacter	F15	–	1	6	42	1
	Arthrobacter	F40	5	4	8	64	1
	Arthrobacter	G61	–	–	1	11	–
	Arthrobacter	G75	1	2	3	1	–
	Corynebacterium	H22	–	4	13	24	1
	Janibacter	B8	4	1	3	3	–
	Janibacter	I44	1	2	1	1	–
	Rhodococcus	B7	3	2	4	2	–
	Rhodococcus	B21	–	–	–	1	–
	Rhodococcus	G3	–	–	1	–	–
Alphaproteobacteria	*Paracoccus*	B22	–	1	–	–	–
	Paracoccus	F12	1	–	–	7	1
Gammaproteobacteria	*Marinomonas*	A14	–	1	–	3	–
	Marinomonas	E12	–	4	2	4	–
	Pseudoalteromonas	G24	–	–	5	–	–

*ALF, *Alphaproteobacteria*; GAM, *Gammaproteobacteria*; ACT, *Actinobacteria*; FIR, *Firmicutes*; BAC, *Bacteroidetes*.

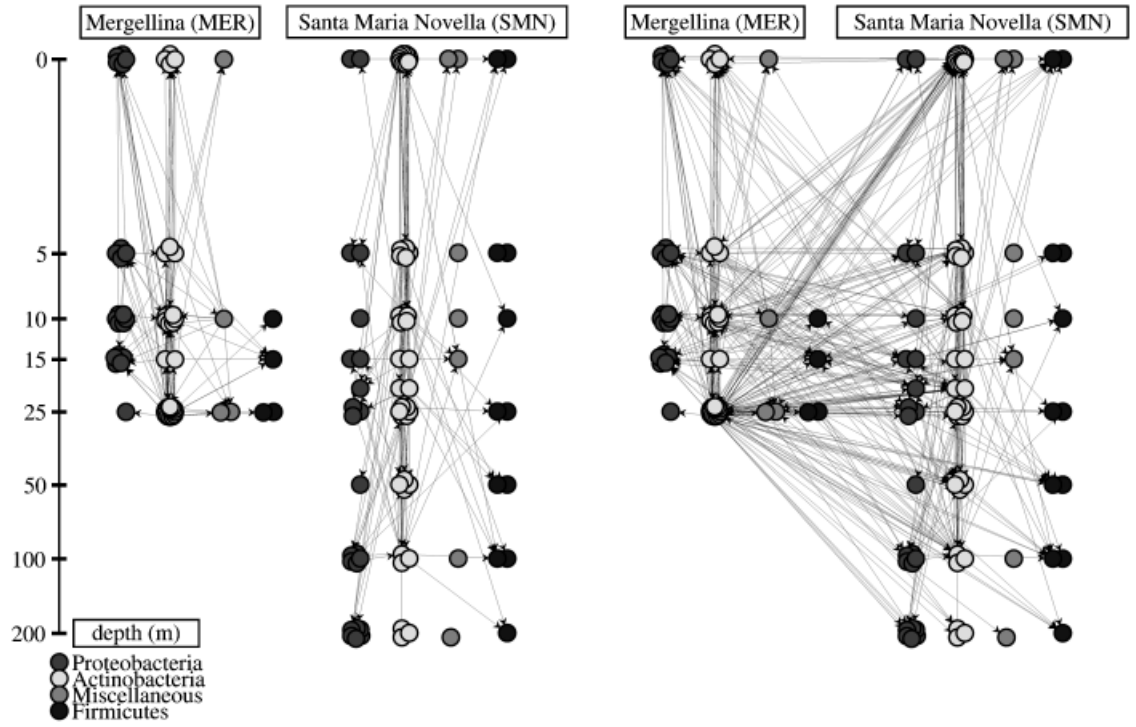

Fig. 1. Original output of 'ecoNetwork'. The three networks showed the interactions existing bewteen strains isolated from (i) Mergellina (MER) station (left panel), (ii) Santa Maria Novella (SMN) (middle panel), and (iii) between all of the 140 strains (right panel). The arrows are directed towards the inhibited strains.

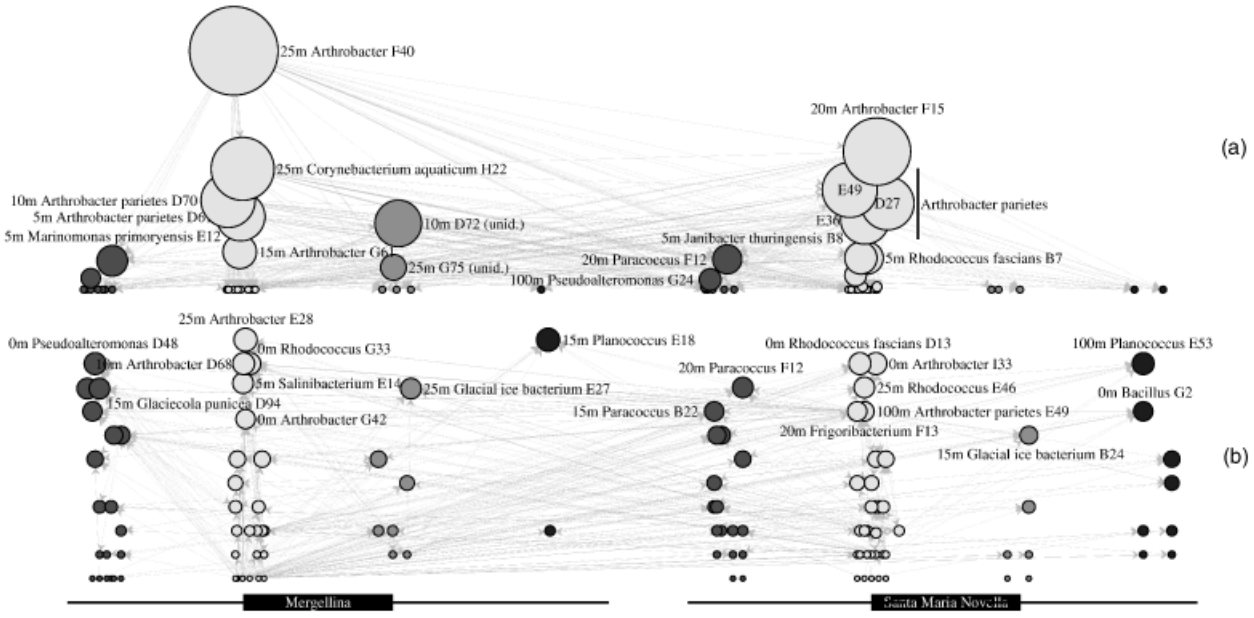

Fig. 2. Schematic representation of strains stratification. Each strain is represented by a circle (node) whose size (score) is proportional to the number of inhibited isolates (a), or inhibitor strains (b). The nodes are stratified from highest (up) to lowest size (down). The name of the top strains for each panel is indicated. In (a), the top strain is the best inhibitor, while the top strains in (b) are the most inhibited ones. (a) shows that isolates with the highest inhibiting activity belong to *Actinobacteria*, while no taxonomic group emerges as top inhibited, the number of inhibitions being more or less constant for different taxonomic groups.

methods', nodes represent bacterial isolates, whereas the inhibitory action of a strain (A) against another one (B) is shown as an arrow pointing from node A to node B. The global inhibitory pattern is shown in Fig. 1, which gives a general view of the inhibitory relationships existing between the 140 isolates. A stratification of nodes based on the number of inhibited strains (*outdegree* analysis) or the number of inhibitor strains (*indegree* analysis) for each isolate is reported in Fig. 2a and b, respectively. As shown in Fig. 2a, isolates possessing the highest inhibitory activity belong to the *Actinobacteria*, while strains from other taxonomic groups have only mild inhibitory ability. No taxonomic group or strain emerges as the most inhibited one in the *indegree* panel (Fig. 2b), where the bacterial isolates are homogeneously distributed over the entire taxonomic dataset.

Analysis of plasmid content

Plasmid molecules of different size (ranging from 1.7 to about 10 kb) were found in 18 isolates; in just a few bacterial isolates, two different plasmids coexist within the same cell. Most of the isolates belonged to the γ-subdivision of *Proteobacteria*, represented by *Pseudoalteromonas* (isolates F43, F46, F48, G19, H17 and M4) and *Psychrobacter* (isolates B26, C1, C11, C16 and H2). The other seven plasmids were detected in bacteria belonging to the *Alphaproteobacteria* subclass: *Paracoccus* (isolates D38, D67, D71, G29 and G30) and *Loktanella* (isolates D40 and G65). It is worth noting that some *Pseudoalteromonas* strains harboured the same very little plasmid of about 1.7 kb. Another plasmid of larger size (about 3 kb) was found in *Pseudoalteromonas* isolate F46. Apparently, no plasmid sharing occurred between isolates belonging to different species, suggesting the transfer of these molecules between different species might be difficult.

Plasmids were found in seven and 11 isolates which were resistant or susceptible, respectively, to the antibacterial activity of other cold-adapted bacteria. Conversely, plasmids were not detected in antagonistic isolates.

Antimicrobial susceptibility testing

Results from the susceptibility tests to antibiotics of resistant Antarctic isolates are displayed in Table 4. Isolates were sensitive to at least three of the antibiotics tested. All strains were susceptible to chloramphenicol and ampicillin (except for G4), whereas only three of them (*Janibacter* spp. G4 and G5, and *Pseudomonas* sp. I45) were inhibited by the vibriostatic agent O/129. Susceptibility to the remaining

Table 4. Susceptibility to different antibiotics of resistant Antarctic isolates

		Antibiotic tested*†							
Phylum or class	Isolate	Amp	Tob	Tet	Pol B	Chl	Pen G	N.A.	O/129
Actinobacteria	*Frigoribacterium* sp. D21	+	–	–	–	+	+	–	–
	Janibacter sp. G4	–	–	–	–	+	w	–	+
	Janibacter sp. G5	w	–	–	–	+	+	–	+
Alphaproteobacteria	*Paracoccus* sp. D39	+	+	+	+	+	–	+	–
	Paracoccus sp. D71	+	+	+	+	+	+	+	–
	Paracoccus sp. F38	+	+	+	+	+	+	+	–
Gammaproteobacteria	*Pseudoalteromonas* sp. B1	+	+	–	+	+	–	+	–
	Pseudoalteromonas sp. D48	w	+	–	+	+	–	+	–
	Pseudoalteromonas sp. F26	w	+	–	+	+	+	+	–
	Pseudoalteromonas sp. F43	+	+	+	+	+	+	+	–
	Pseudoalteromonas sp. F46	+	+	–	+	+	–	+	–
	Pseudoalteromonas sp. F47	+	+	–	–	+	–	+	–
	Pseudoalteromonas sp. F48	+	+	+	+	+	+	+	–
	Pseudoalteromonas sp. F53	+	+	–	+	+	+	+	–
	Pseudoalteromonas sp. G19	+	+	+	+	+	–	+	–
	Pseudoalteromonas sp. H17	+	+	+	+	+	+	+	–
	Pseudoalteromonas sp. M4	w	+	–	+	+	–	+	–
	Pseudomonas sp. I45	+	–	–	–	+	+	–	+
	Shewanella sp. A7	+	+	+	+	+	+	+	–
	Shewanella sp. B6	+	+	+	+	+	+	+	–
	Shewanella sp. D64	+	+	+	+	+	+	+	–
Firmicutes	*Planococcus* sp. I50	+	+	+	+	+	+	+	–

Grey boxes indicate strains harbouring plasmid molecules.

Amp, ampicillin; Tob, tobramycin; Tet, tetracycline; Pol B, polymixin B; Chl, chloramphenicol; Pen G, penicillin G; N.A., nalidixic acid; O/129, vibriostatic O/129; w, weak activity.

antibiotics was rather variable: tobramycin and nalidixic acid > polymixin B > penicillin G > tetracycline. *Actinobacteria* were among more resistant isolates, being susceptible to three or four antibiotics, whereas both *Alphaproteobacteria* and the sole representative of *Firmicutes* were among more sensitive ones. Within *Gammaproteobacteria*, members of the genus *Pseudoalteromonas* showed different inhibition patterns, whereas all *Shewanella* isolates were sensitive to the same antibiotics.

Discussion

Identification of Antarctic isolates

Altogether, 16S rRNA gene sequencing revealed that the 68% of total isolates were Gram-positive bacteria (i.e. *Actinobacteria* and *Firmicutes*). Even though this finding might be considered unusual for marine water column, similar data have been previously reported also by Grossart *et al.* (2004) for bacteria isolates from organic aggregates of the Wadden Sea. Most Gram-positive analyzed in this work were affiliated to *Actinobacteria*. Although evidence exists supporting their physiological adaptation and growth in seawater, it has been frequently assumed that *Actinomycetes* isolates from marine samples are merely of terrestrial origin and their inclusion within autochthonous marine microbiota has not been widely accepted (Mincer *et al.*, 2002). The high percentage of *Actinobacteria* detected in this study might be due to the environmental features of the marine area investigated: Terra Nova Bay is a semi-enclosed area and, therefore, it is strongly influenced by continental inputs, mainly deriving from frequent katabatic wind events or from the extension of both Campbell glacier tongue and pack-ice; furthermore, in the sampling period a continental input, even though limited, was detected (Fabiano *et al.*, 1995).

Additionally, as previously observed by Grossart *et al.* (2004), our Gram-positive isolates generally grew at high salt concentrations (up to 11% NaCl; data not shown), suggesting their strong adaptation to marine environment and the capability to compete efficiently with strictly marine bacteria.

As a consequence of the high percentage of *Actinobacteria* and *Firmicutes*, Gram-negative bacteria constituted only a small fraction of our isolates, with *Gammaproteobacteria* predominating.

Antagonistic interaction among Antarctic isolates

Each of the 140 bacterial isolates analyzed in this work was examined for its ability to inhibit the growth of indigenous isolates and its sensitivity to antibacterial activity expressed by one another. The finding that just 15% of isolates showed an inhibitory activity suggests that it was not a common feature of our isolates. Similar results (nearly 17%) for epiphytic marine bacteria were reported by Lemos *et al.* (1985), whereas other studies found lower (Nair & Simidu, 1987, 5–8%) or much higher percentages of bacterial antagonisms (Long & Azam, 2001; Grossart *et al.*, 2004; more than 50%). However, it has to be underlined that bacteria used in this study were probably free-living as they were isolated from unfiltered seawater that contained no visible particles (although microparticles could be present), whereas previous studies mainly report on particle-attached or epibiotic bacteria. As observed by Long & Azam (2001) and Nair & Simidu (1987), attached bacteria were more likely prone to produce inhibitory compounds than their free-living counterparts, suggesting that bacterial antagonism is more common on particles than in the surrounding waters. Strains analyzed in this study were isolated from seawater at different depths, and it could be assumed that they were not likely to share exactly the same environment. Nevertheless, marine bacteria probably spend most of their time freely swimming in the water while searching for particles to colonize. Thus, in the free-living stage the production of antimicrobial compounds may involve an unnecessary energy waste, whereas antagonistic interactions may become a key factor in regulating bacterial populations when high bacterial densities occur in an ecological niche (Long & Azam, 2001; Gram *et al.*, 2002).

Isolation procedure never reflects the real marine bacterial community due to biases linked to the media utilized or to the intrinsic microbial cultivability. Nevertheless, some ecological interpretations about the *in situ* effects of the bacterial inhibitory activity occurring in Antarctic seawater can be extrapolated from data reported in this work. Inhibition activity was predominantly expressed by yellow- or orange-pigmented isolates (14 out of 21: all *Actinobacteria*). This finding strengthened the assumption that antibiotic production is often linked to the presence of pigments (Lemos *et al.*, 1985; Sobolevskaya *et al.*, 2005). Holmström *et al.* (1998) observed the production and release in the culture medium by *Pseudoalteromonas tunicata* of a dark pigment at the same time as antibiotic components. In that case, the pigment lacked inhibitory action, but it was probably involved in the same pathway (or in one of its branches) leading to the production of active components.

Hentschel *et al.* (2001), assessing sponge isolates for antimicrobial interactions against each other, observed that Gram-positive strains were generally active against Gram-positive bacteria and Gram-negative strains were generally active against Gram-negative bacteria, with the exception of an *Alphaproteobacterium* isolate which was able to inhibit both types of target strains. As observed also by Grossart *et al.* (2004), this general pattern was not so noticeable for our Antarctic isolates.

Differences recorded among the inhibition patterns of the active strains, together with the sensitivity of nearly 80% of all isolates to one or more producers, suggested that antagonistic activity was probably due to several environmental factors, such as the alteration of pH and nutrient availability (because live bacteria were used in the bioassays) in the culture medium or the production of multiple inhibitory compounds by a single species. Moreover, the possibility that the production of secondary metabolites was induced or enhanced by the presence of other bacteria or by the growth on a solid surface cannot be excluded *a priori*.

Antagonism in relation to affiliation

The main purpose of the present investigation was to establish the antagonistic interactions among Antarctic isolates, representing five classes of cultivable marine bacteria. Unlike *Actinobacteria* and both *Alpha-* and *Gammaproteobacteria*, members of *Bacteroidetes* and *Firmicutes* were not able to inhibit the growth of other isolates. Our results are quite different from those previously reported by other authors. Long & Azam (2001) reported *Gammaproteobacteria* as dominant producers, followed by *Alphaproteobacteria* and, at a lesser extent, by *Bacteroidetes*. In the same study, *Actinobacteria* were surprisingly absent among active isolates. Grossart *et al.* (2004), studying isolates belonging to the same five phylogenetic groups we recognized, found the highest antagonistic activity in *Actinobacteria* and *Alphaproteobacteria*. Comparing antagonism among bacteria from additional marine environments will probably afford a deeper understanding of this ecological process.

Altogether, no correlation seemed to exist between the inhibitory effect or sensitivity of the isolates screened and their phylogenetic affiliations. All groups included isolates which did not show antagonistic activity at all. Differences were observed between inhibition patterns of single isolates, suggesting that antagonistic activity was more likely strain-specific rather than dependent on phylogenetic affiliation.

As reported in the section above, active isolates were mainly affiliated with *Actinobacteria*. *Arthrobacter* spp. are generally isolated from soil, but they were also recovered from marine samples (Bowman *et al.*, 1997a; Junge *et al.*, 1998; Hentschel *et al.*, 2001). Although *Actinobacteria* are well known microorganisms producing antimicrobial agents, inhibitory properties of *Arthrobacter* members have been seldom reported (Kamigiri *et al.*, 1996; Hentschel *et al.*, 2001; O'Brien *et al.*, 2004). Conversely, in this study nine *Arthrobacter* isolates were among the most active producers detected. Moreover, their inhibitory effect against closely related bacteria, as well as the only *Corynebacterium* isolate, suggests the potential production of bacteriocin-like compounds. This finding is in disagreement with observations made by Grossart *et al.* (2004) as their closely related isolates never inhibited each other. *Rhodococcus* and *Janibacter* isolates were much less active than other *Actinobacteria*: they inhibited few target bacteria (1–11) in a mean number comparable with that recorded for both *Alpha-* and *Gammaproteobacteria*.

The antimicrobial activity of these two latter bacterial classes has been frequently reported. Among *Alphaproteobacteria*, members of the *Roseobacter* clade seem to be promising antibiotic producers and their ecological significance has been investigated (Hentschel *et al.*, 2001; Long & Azam, 2001; Gram *et al.*, 2002; Brinkhoff *et al.*, 2004; Grossart *et al.*, 2004; Bruhn *et al.*, 2005). In our study, two *Paracoccus* isolates were active and characterized by strongly different inhibition spectra. Among *Gammaproteobacteria*, the dominant cultivable marine bacteria, members of the genus *Pseudoalteromonas* are frequently found in seawater and in association with living surfaces, such as algae and sponges (Holmström *et al.*, 1998; Hentschel *et al.*, 2001). Although several *Pseudoalteromonas* species are known to produce bioactive metabolites with antimicrobial and antialgal properties (Ivanova *et al.*, 1998; Egan *et al.*, 2001; Isnansetyo & Kamei, 2003; Sobolevskaya *et al.*, 2005), antagonistic activity was observed only for the isolate G24. Conversely, the majority of *Pseudoalteromonas* affiliates were among resistant isolates.

As well as *Pseudoalteromonas* species, *Marinomonas* members have been isolated from different Antarctic environments (Bowman *et al.*, 1997b; Bozal *et al.*, 2003; Prabagaran *et al.*, 2005; Shivaji *et al.*, 2005; Gupta *et al.*, 2006). Because their antibacterial activity has, to our knowledge, not been reported previously, these bacteria could be a novel source of antimicrobial compounds.

Analysis of plasmid content and antibiotic susceptibility

Plasmids often contain genes encoding for antibiotic production, as well as for antibiotic resistance, conferring a selective advantage to the bacterium bearing them (Martin & Liras, 1989). A plasmid extraction method was used to determine plasmid incidence in the bacterial populations investigated. The percentage of plasmid-harbouring isolates varied between interactivity clusters. In fact, plasmid molecules were not detected in bacteria expressing antagonistic effects, whereas they were extracted from seven resistant strains. Thus, in the present case, plasmid presence seemed to be mainly linked to antibiotic resistance rather than to antibiotic production. On the other hand, both results from susceptibility tests carried out using commercial antibiotics and plasmid detection in sensitive isolates suggest that plasmids were probably not involved in the antagonistic activity observed.

Concluding remarks

The aim of this work was to study the antagonistic interactions among psychrotrophic bacteria isolated from Antarctic seawater. On the basis of 16S rRNA gene sequencing and analysis, isolates were assigned to five phylogenetically different taxa, *Actinobacteria* predominating. This finding does not reflect the typical bacterial community in marine environments and might be a specific feature of the two sites during the sampling period.

The results establish that (1) antagonistic activity was not a common feature of all the phylogenetic groups detected through the study, being *Firmicutes* and *Bacillaceae* members unable to inhibit the growth of other isolates, and, further, strain-specific rather than dependent on bacterial affiliation; (2) inter-specific interactions can occur among Antarctic bacteria; and (3) plasmid molecules were not involved in the inhibition process.

Data presented here enlarge our knowledge of bacterium–bacterium interactions, extending it from marine temperate regions to the Southern Ocean. It could be argued that the antimicrobial activity observed may constitute a particular advantage in reducing inter-species competition in a severe environment such as Antarctica. In addition to their ecological significance, results from this study highlight the potential exploitation of the Antarctic marine bacteria as a source of new compounds with antibacterial properties. Further studies are in progress to elucidate the nature of the antagonistic activities observed that could derive from changes in nutrient availability or alteration of the pH in the environment, as well as from the production of inhibitor compounds involved in bacterial communication.

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References

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

Barabasi AL & Oltvai ZN (2004) Network biology: understanding the cell's functional organization. *Nat Rev Genet* **5**: 101–113.

Bauer AN, Kirby WMM, Sherry JC & Turck M (1966) Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol* **45**: 493–496.

Bhattarai HD, Lee YK, Cho KH, Lee HK & Shin HW (2006) The study of antagonistic interactions among pelagic bacteria: a promising way to coin environmental friendly antifouling compounds. *Hydrobiologia* **568**: 417–423.

Bowman JP, McCammon SA, Brown MV, Nichols DS & McMeekin TA (1997a) Diversity and association of psychrophilic bacteria in Antarctic sea ice. *Appl Environ Microbiol* **63**: 3068–3078.

Bowman JP, Nichols DS & McMeekin TA (1997b) *Psychrobater glacincola* sp. nov., a halotolerant, psychrophilic bacterium isolated from Antarctic sea ice. *Syst Appl Microbiol* **20**: 209–215.

Bozal N, Montes MJ, Tutela E & Guinea J (2003) Characterization of several *Psychrobacter* strains isolated from Antarctic environments and description of *Psychrobacter luti* sp. nov. and *Psychrobacter fozii* sp. nov. *Int J Syst Evol Microbiol* **53**: 1093–1100.

Brinkhoff T, Bach G, Heidorn T, Liang L, Schlingloff A & Simon M (2004) Antibiotic production by a *Roseobacter* clade-affiliated species from the German Wadden Sea and its antagonistic effect on indigenous isolates. *Appl Environ Microbiol* **70**: 2560–2565.

Bruhn JB, Nielsen KF, Hjelm M, Hansen M, Bresciani J, Schulz S & Gram L (2005) Ecology, inhibitory activity and morphogenesis of a marine antagonistic bacterium belonging to the *Roseobacter* clade. *Appl Environ Microbiol* **71**: 7263–7270.

Bruni V, La Ferla R, Acosta Pomar MCL & Salomone L (1995) Structural differences of the microbial community in two sites of the Terra Nova Bay (Ross Sea, Antarctica): a statisical analysis. *Microbiologica* **18**: 409–422.

Buffoni G, Cappelletti A & Picco P (2002) An investigation of thermohaline circulation in Terra Nova Bay polynya. *Antarct Sci* **14**: 83–92.

Egan S, James S, Holmström C & Kjelleberg S (2001) Inhibition of algal spore germination by the marine bacterium *Pseudoalteromonas tunicata*. *FEMS Microbiol Ecol* **35**: 67–73.

Fabiano M, Danovaro R, Crisafi E, La Ferla R, Povero P & Acosta Pomar L (1995) Particulate organic matter composition and bacterial distribution in Terra Nova Bay (Antarctica) during summer 1989–1990. *Pol Biol* **15**: 393–400.

Fabiano M, Povero P & Danovaro R (1996) Particulate organic matteri composition in Terra Nova Bay (Ross Sea, Antarctica) during summer 1990. *Antarct Sci* **8**: 7–13.

Gram L, Grossart HP, Schlingloff A & Kiørboe T (2002) Possible quorum sensing in marine snow bacteria: production of acylated homoserine lactones by *Roseobacter* strains isolated from marine snow. *Appl Environ Microbiol* **68**: 4111–4116.

Grossart HP, Schlingloff A, Bernhard M, Simon M & Brinkhoff T (2004) Antagonistic activity of bacteria isolated from organic aggregates of the German Wadden Sea. *FEMS Microbiol Ecol* **47**: 387–396.

Gupta P, Chaturvedi P, Pradhan S, Delille D & Shivaji S (2006) *Marinomonas polaris* sp. nov., a psychrotolerant strain isolated from coastal sea water off the subantarctic Kerguelen islands. *Int J Syst Evol Microbiol* **56**: 361–364.

Hentschel U, Schmid M, Wagner M, Fieseler L, Gernert C & Hacker J (2001) Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean sponges *Aplysina aerophoba* and *Aplysina cavernicola*. *FEMS Microbiol Ecol* **35**: 305–312.

Holmström C, James S, Neilan BA, White DC & Kjelleberg S (1998) *Pseudoalteromonas tunicata* sp. nov., a bacterium that produces antifouling agents. *Int J System Bacteriol* **48**: 1205–1212.

Isnansetyo A & Kamei Y (2003) MC21-A, a bactericidal antibiotic produced by a new marine bacterium, *Pseudoalteromonas phenolica* sp. nov. O-BC30^{T}, against methicillin-resistant *Staphylococcus aureus*. *Antim Ag Chem* **47**: 480–488.

Ivanova EP, Nicolau DV, Yumoto N & Taguchi T (1998) Impact of conditions of cultivation and adsorption on antimicrobial activity of marine bacteria. *Mar Biol* **130**: 545–551.

Junge K, Gosink JJ, Hoppe HG & Staley JT (1998) *Arthrobacter*, *Brachybacterium* and *Planococcus* isolates identified from Antarctic sea ice brine. Description of *Planococcus mcmeekinii*, sp. nov. *Syst Appl Microbiol* **31**: 306–314.

Kamigiri K, Tokunaga T, Shibazaki M, Setiavan B, Rantiatmodjo RM, Morioka M & Suzuki KI (1996) YM-3009, a novel quinolone antibiotic produced by *Arthrobacter* sp. *J Antibiot* **49**: 823–825.

Lemos ML, Toranzo AE & Barja JB (1985) Antibiotic activity of epiphytic bacteria isolated from intertidal seaweeds. *Microb Ecol* **11**: 149–163.

Long RA & Azam F (2001) Antagonistic interactions among marine pelagic bacteria. *Appl Environ Microbiol* **67**: 4975–4983.

Martin JF & Liras P (1989) Organization and expression of genes involved in the biosynthesis of antibiotics and other secondary metabolites. *Ann Rev Microbiol* **43**: 173–206.

Maugeri TL, Gugliandolo C & Bruni V (1996) Heterotrophic bacteria in the Ross Sea (Terra Nova Bay, Antarctica). *Microbiologica* **19**: 67–76.

Michaud L, Di Cello F, Brilli M, Fani R, Lo Giudice A & Bruni V (2004) Biodiversity of cultivable psychrotrophic marine bacteria isolated from Terra Nova Bay (Ross Sea, Antarctica). *FEMS Microbiol Lett* **230**: 63–71.

Mincer TJ, Jensen PR, Kauffman CA & Fenical W (2002) Widespread and persistent populations of a major new marine actinomycetes taxon in ocean sediments. *Appl Environ Microbiol* **68**: 5005–5011.

Nair S & Simidu U (1987) Distribution and significance of heterotrophic marine bacteria with antibacterial activity. *Appl Environ Microbiol* **53**: 2957–2962.

Nichols D, Bowman J, Sanderson K, Mancuso Nichols C, Lewis T, McMeekin T & Nichols PD (1999) Development with Antarctic microorganisms: culture collections, bioactivity screening, taxonomy, PUFA production and cold-adapted enzymes. *Curr Op Biotechnol* **10**: 240–246.

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

Prabagaran SR, Suresh K, Manorama R, Delille D & Shivaji S (2005) *Marinomonas ushuaiensis* sp. nov., isolated from coastal seawater in Ushuaia, Argentina, sub-Antarctica. *Int J Syst Evol Microbiol* **55**: 309–313.

Shivaji S, Reddy GSN, Suresh K, Gupta P, Chintalapati S, Schumann P, Stackebrandt E & Matsumoto GI (2005) *Psychrobacter vallis* sp. nov. and *Psychrobacter aquaticus* sp. nov., from Antarctica. *Int J Syst Evol Microbiol* **55**: 757–762.

Sobolevskaya M, Smetanina OF, Speitling M, Shevchenko LS, Dmitrenok PS, Laatsch H, Kuznetsova TA, Ivanova EP & Elyakov GB (2005) Controlling production of brominated cyclic depsipeptides by *Pseudoalteromonas maricaloris* KMM 636^{T}. *Lett Appl Microbiol* **40**: 243–248.

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

Vincent WF (2000) Evolutionary origins of Antarctic microbiota: invasion, selection and endemism. *Antarct Sci* **12**: 374–385.