

## INCIDENCE OF PLASMID AND ANTIBIOTIC RESISTANCE IN PSYCHROTROPHIC BACTERIA ISOLATED FROM ANTARCTIC SPONGES

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**ABSTRACT.** A total of 297 bacterial strains were isolated from five Antarctic sponge species and tested by agarose gel electrophoresis for the presence of plasmid molecules. At least one kind of plasmid was carried by 69 isolates (about 23%). The disc diffusion susceptibility test was used to assay the resistance of plasmid-harboring bacteria towards 11 antibiotics. A multiple resistance was observed for the 72% of strains, among which the 33% were resistant to only two antibiotics. Bacteria showed a high degree of resistance towards O/129 (71%), tetracycline (42%) and nalidixic acid (25%), whereas any isolate showed resistance to gentamicin. The 16S rDNA sequencing revealed that plasmid-harboring strains were mainly affiliated to the *Gamma*proteobacteria (81%), whereas the other detected phylogenetic groups (i.e. *Firmicutes*, *Alphaproteobacteria*, *Actinobacteria* and CFB group of *Bacteroidetes*) were less abundant, each representing between 1% and 6% of the total isolates. The present study will contribute to the poor and fragmentary knowledge on plasmid incidence in natural microbial populations. In addition, monitoring antibiotic resistance in bacteria from remote areas, such as Antarctica, could also be a useful tool to evaluate the impact of anthropic pressure.

### 1. Introduction

The easy availability and the indiscriminate prescription practice of antibiotics have lead to their widespread use and therefore to the fast spreading of antibiotic resistance and virulence factors among bacteria [1]. As a consequence, the bacterial antibiotic resistance has become a global phenomenon and an emerging medical issue threatening the public health [2]. More and more pathogenic bacteria have shown resistance to one or a suite of antibiotics [3]. In some cases, the antibiotic resistance is due to the presence of plasmids which are among the most widely investigated characteristics in bacteria. The ability of plasmids to be horizontally transferred from one bacterium to another, even occasionally between phylogenetically distant bacteria, has greatly contributed to the widespread dissemination of antibiotic resistance genes in the environment [4]. Under the selection pressure derived from different antibiotics occurring in the environment, exposed bacterial population may evolve being capable of resisting to a wide spectrum of antibiotics through

multiple gene transfer and exchange process [5]. Existing knowledge on the incidence of plasmids in aquatic bacterial populations is limited to a small number of studies which have mainly regarded bacteria from marine water and sediment, therefore resulting in poor and fragmentary information [6, 7, 8, 9]. More limited data are available on plasmid-carrying bacteria from living organisms. In fact, only Kobory et al. [10] reported on nine strains which were obtained from specimens of Antarctic pycnogonids, amphipods and sponges. In particular, such a report is among those few which have dealt with the occurrence of plasmids in natural bacterial assemblages of polar regions [10, 11, 2]. The present study focuses on bacteria which have been isolated from five Antarctic sponge species (*Anoxycalyx joubini*, *Lissodendoryx nobilis*, *Myxodoryx hanitschi*, *Phorbas glaberrima* and *Haliclona pilosa*). The biological nature of the sponge-bacterium association is not well known. Bacteria may be either symbiotic, specific and permanently associated, but also not symbiotic or merely commensally present. In general, marine sponges provide a protected and nutrient-rich niche in which extensive interaction (including plasmid transfer) among the diverse microbial populations is fostered and probably unavoidable [12]. In this context, the present study was aimed at gaining further understanding of the frequency of plasmid presence in sponge-associated Antarctic bacteria and further establishing the possible relationship between such occurrence and the bacterial resistance to antibiotics.

## 2. Materials and methods

**Sample collection.** Specimens of the marine sponges *Anoxycalyx (Scolymastra) joubini* (Topsent, 1910), *Lissodendoryx (Ectyodoryx) nobilis* (Ridley and Dendy, 1886), *Myxodoryx hanitschi* (Kirkpatrick, 1907), *Phorbas glaberrima* (Topsent, 1916) and *Haliclona (Gellius) pilosa* (Kirkpatrick, 1907) were collected during the XX Italian Expedition to Antarctica (Austral Summer 2004-2005) in different sites at Terra Nova Bay (Ross Sea). Sponge specimens were immediately washed at least three times with filter-sterilized seawater to remove transient and loosely attached bacteria and/or debris. Specimens were then placed in individual sterile plastic bags containing filter sterilized seawater and transported directly to the laboratory at 4°C for microbiological processing (within 2 h after sampling). A fragment of each specimen was also preserved in 70% ethanol for taxonomic identification.

**Bacterial isolation.** Bacterial isolation from sponge tissues was carried out as previously described by Mangano et al. [13]. Briefly, a central core of the sponge tissue was cut using an EtOH sterilized corkborer or a sterile scalpel. The sponge tissue was then aseptically weighed and manually homogenized in 0.22  $\mu\text{m}$  filtered seawater in a sterile mortar. Tissue extracts were serially diluted using filter-sterilized seawater. Aliquots (100  $\mu\text{l}$ ) of each dilution were spread in triplicate on Marine Agar 2216 (MA, Difco). Plates were incubated in the dark at 4°C for 1 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. All the bacterial strains isolated from the sponges were included in the Italian Collection of Antarctic Bacteria (CIBAN) of the National Antarctic Museum (MNA) "Felice Ippolito" kept at the Department of Animal Biology and Marine Ecology of the University of Messina (Italy).

*PCR amplification of 16S rDNA.* PCR-amplification of 16S rDNA from bacterial isolates was carried out as described previously by Michaud et al. [11]. Briefly, a single colony of each strain was lysed by heating at 95°C for 10 min. Amplification of 16S rDNA was performed with an ABI 9600 thermocycler (PE, Applied Biosystems) using the forward primer P0 (5'-GAGAGTTTGATCCTGGCTCAG-3') and the reverse primer P6 (5'-CTACGGCTACCTTGTACGA-3'). The reaction mix was assembled at 0°C and contained 1-10 ng DNA, 10X buffer, 1.5 mM MgCl<sub>2</sub>, 150 ng of each forward and reverse primer, 250 μM dNTP, 0.5 units of PolyTaq polymerase (Polymed, Italy) and sterile distilled water to a final volume of 20 μl. The PCR program was as follows: 3 min at 95°C, followed by 30 cycles of 1 min at 94°C, 1 min at 50°C, 2 min at 72°C and a final extension step of 10 min at 72°C. The results of the amplification reactions were analyzed by agarose gel electrophoresis (1%, w/v) in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA), containing 1 μg/ml of ethidium bromide.

*Amplified rDNA Restriction Analysis (ARDRA).* 5 μl of each PCR mixture, containing approximately 1.5 μg of amplified 16S rDNA, were digested with 3 U of the restriction enzyme *AluI* (Fermentas) in a total volume of 20 μl at 37°C for 3 h. The enzyme was inactivated by heating at 65°C for 15 min and the reaction products were analyzed by agarose (2.5%, w/v) gel electrophoresis in TAE buffer containing 1 μg/ml of ethidium bromide [11]. On the basis of the restriction patterns obtained (and visually compared one to each other), Antarctic isolates were grouped into Operational Taxonomic Units (OTUs), assuming that one OTU was made up of strains belonging to the same species.

*Sequencing and analysis of 16S rDNA.* One to three representative strains (where possible), showing the identical ARDRA pattern, were randomly selected for sequencing by using the primer P0. All singletons (strains from OTUs made of a single member) were sequenced. When possible, representative strains were chosen among plasmid-harboring isolates (see below). Each sequence was then used as a query in a BLASTn search [14].

*Screening for plasmid-harboring Antarctic bacteria.* Plasmid molecules were extracted from 3 ml bacterial cultures grown in Marine Broth (MB, Difco) using the commercial kit QIAprep-spin Plasmid Miniprep (Qiagen) according to the supplier's instructions. The presence of plasmid molecules was checked by agarose (0.8%, w/v) gel electrophoresis in TAE buffer containing 1 μg/ml of ethidium bromide. Hereinafter, bacteria showing plasmid bands will be defined as "P<sup>+</sup>", while bacteria that didn't show any plasmid band will be defined as "P<sup>-</sup>".

*Antibiotic resistance test.* Resistance to antibiotics was determined by the method of Bauer et al. [15], using Sensi-Discs (Oxoid). The antibiotics tested were penicillin G (10 μg), polymyxin B (30 μg), chloramphenicol (30 μg), tobramycin (10 μg), tetracycline (30 μg), ampicillin (25 μg), nalidix acid (30 μg), vibriostatic compound O/129 (10 μg), gentamicin (10 μg), kanamycin (10 μg) and neomycin (30 μg). In order to establish the potential relation existing between the plasmid presence and the antibiotic resistance, this latter was tested for both P<sup>+</sup> strains and an identical number of P<sup>-</sup> strains, which were chosen to give a representative selection of pigmented and non pigmented strains.

**Table 1.** Relative number of isolates *per* sponge species and phylogenetic group.

Sponge species	Phylogenetic affiliation					
	ALF <sup>a</sup>	GAM <sup>b</sup>	BAC <sup>c</sup>	ACT <sup>d</sup>	FIR <sup>e</sup>	NA <sup>f</sup>
<i>A. joubini</i>	6	16	2	1	-	12
<i>L. nobilis</i>	2	85	-	4	-	1
<i>H. pilosa</i>	-	43	1	23	-	10
<i>M. hanitschi</i>	-	41	-	1	4	1
<i>P. glaberrima</i>	5	14	-	3	3	19
Total number	13	199	3	32	7	42

<sup>a</sup> *Alphaproteobacteria*    <sup>b</sup> *Gammaproteobacteria*

<sup>c</sup> CF group of *Bacteroidetes*    <sup>d</sup> *Actinobacteria*

<sup>e</sup> *Firmicutes*    <sup>f</sup> not assigned

### 3. Results

**Bacterial isolation and identification.** Overall, a total of 297 bacterial strains were isolated from the considered Antarctic sponges. In particular, 92 strains were obtained from *L. nobilis*, 76 from *H. pilosa*, 47 from *M. hanitschi*, 46 from *P. glaberrima*, and 36 from *A. joubini*. A total of 108 different *AluI* patterns (OTUs) were distinguished by ARDRA. Based on the results of the 16S rDNA sequencing of isolates representing each OTU, these were mainly affiliated to the *Gammaproteobacteria* (199 strains), followed by the *Actinobacteria* (32 strains), *Alphaproteobacteria* (13 strains), *Firmicutes* (7 strains), and *Bacteroidetes* (3 strains). A total of 42 strains were not assigned to a given phylogenetic group due to the PCR reaction failure with the used primers. The relative number of isolates *per* sponge species and phylogenetic group is reported in Table 1.

**Screening for plasmid-harboring Antarctic bacteria.** A total of 69 strains (out of 297; 23%) showed at least one plasmid band on agarose gel. Among these P<sup>+</sup> strains, 90% were Gram-negative and about 10% were pigmented (not shown). P<sup>+</sup> strains were obtained from all the considered sponge species, with the higher frequencies which were found in isolates from *H. pilosa* (29.0% of tested strains for such sponge), *M. hanitschi* (27.7%) and *L. nobilis* (27.2%), followed at a lesser extent by *A. joubini* (11.1%) and, finally, *P. glaberrima* (10.9%). The P<sup>+</sup> strains were mainly affiliated to the *Gammaproteobacteria* (81%) and at a lesser extent to the *Firmicutes* (6%), *Alphaproteobacteria* (4%) *Actinobacteria* (4%) and CFB group of *Bacteroidetes* (1%). Two isolates (3%) were classified as unidentified bacteria, since the first hits in BLASTn analysis were uncultured bacteria (Table 2). The *Alphaproteobacteria* were represented by the genera *Sphingomonas* and *Roseobacter*, while *Gammaproteobacteria* were affiliated to *Pseudoalteromonas*, *Shewanella*, *Psychrobacter* and *Alteromonas*. The *Firmicutes* were represented by three genera, *Oceanobacillus*, *Bacillus* and *Planococcus*. Finally, the *Actinobacteria* encompassed two genera of cultivable bacteria, namely *Arthrobacter* and *Citricoccus*. The sole CFB isolate was identified as a *Flavobacteriaceae* member.

**Antibiotic resistance test.** As stated in the materials and methods, the antibiotic resistance test was carried out on both the 69 P<sup>+</sup> strains and an identical number of P<sup>-</sup> strains. Results from the multiple drug resistance showed that the 72% of P<sup>+</sup> strains were resistant

**Table 2.** 16S rRNA gene sequence affiliation to their closest phylogenetic neighbours of P<sup>+</sup> strains.

Phylum or class	Next relative by GenBank alignment (AN <sup>a</sup> , organism)	Hom <sup>b</sup> (%)	Sponge				
			Aj <sup>c</sup>	Hp <sup>d</sup>	Ln <sup>e</sup>	Mh <sup>f</sup>	Pg <sup>g</sup>
ALF <sup>h</sup>	AJ968651, <i>Roseobacter pelophilus</i>	99	1				
	AY336556, <i>Sphingomonas</i> sp. pfB27	100			2		
GAM <sup>i</sup>	AY536572, <i>Alteromonas</i> sp. WED7C	98					1
	FJ161266, <i>Pseudoalteromonas haloplanktis</i> D4052	100		1			
	DQ642813, <i>Pseudoalteromonas</i> sp. 10	99	1				
	AY664366, <i>Pseudoalteromonas</i> sp. JL-BS-K82	97			11		
	DQ834998, <i>Pseudoalteromonas</i> sp. FOH-40	99			1	2	
	EF382701, <i>Pseudoalteromonas</i> sp. BSi20430	99			1		
	EU330352, <i>Pseudoalteromonas</i> sp. BSi20555	97				1	
	EU365532, <i>Pseudoalteromonas</i> sp. BSs20061	99			3		
	EU330345, <i>Pseudoalteromonas</i> sp. BSs20005	98			1		
	GQ452866, <i>Pseudoalteromonas</i> sp. BSw21530	97			1		
	AB526340, <i>Pseudoalteromonas</i> sp. JAM-GA17	100			1		
	EF613488, <i>Pseudoalteromonas</i> sp. NJ70	99		2			
	GQ149233, <i>Pseudoalteromonas</i> sp. ArcN81.11	100		6		7	
	FJ161250, <i>Pseudoalteromonas</i> sp. D4011	97		3			
	FJ594949, <i>Pseudoalteromonas</i> sp. Mn13	99		1			
	EU090718, <i>Psychrobacter cryohalolentis</i> KOPRI22219	100		1			
	FJ161365, <i>Psychrobacter nivimaris</i> D7084	99		4		2	
	FN377742, <i>Psychrobacter</i> sp. KB3-14	99		1			
	EU000237, <i>Shewanella donghaensis</i>	99			1		
	DQ533968, <i>Shewanella</i> sp. ice-oil-417	99		1			
FJ546071, <i>Shewanella</i> sp. S16-5-4	99			1			
DQ667116, <i>Shewanella</i> sp. E9	99			1			
BAC <sup>l</sup>	EU090718, <i>Flavobacteriaceae</i> bacterium #8	98	1				
ACT <sup>m</sup>	DQ831966, <i>Arthrobacter</i> sp. I34	99			1		
	EF093124, <i>Arthrobacter</i> sp. VTT E-052916	100		1			
	EU305672, <i>Citricoccus</i> sp. FS24	98		1			
FIR <sup>n</sup>	GQ404998, <i>Bacillus</i> sp. SRM0904	99				1	
	AB491184, <i>Oceanobacillus picturae</i>	98				2	
	EU135677, <i>Planococcus</i> sp. YIM C738	98				1	
NA <sup>o</sup>	EF572840, Uncultured bacterium clone S23939	99	1				
	FJ456806, Uncultured bacterium clone	100				1	

<sup>a</sup> Accession Number <sup>b</sup> Sequence Homology <sup>c</sup> *A. joubini* <sup>d</sup> *H. pilosa* <sup>e</sup> *L. nobilis*

<sup>f</sup> *M. hanitschi* <sup>g</sup> *P. glaberrima* <sup>h</sup> Alphaproteobacteria <sup>i</sup> Gammaproteobacteria

<sup>l</sup> CF group of *Bacteroidetes* <sup>m</sup> Actinobacteria <sup>n</sup> Firmicutes <sup>o</sup> not assigned

to more than one antibiotic (Table 3). Among them the 33% appeared resistant to only two antibiotics. In particular, the 74% of non-pigmented resistant strains were resistant to more than one antibiotic, whereas the corresponding result for pigmented strains was 57%. Altogether, a high percentage of isolates exhibited resistance toward O/129 (71%), tetracycline (42%) and nalidixic acid (25%). Any P<sup>+</sup> isolate was resistant to gentamicin. Among the 69 P<sup>-</sup> strains, the 68% showed resistance to more than one antibiotic and 30% were resistant to two drugs (Table 4). The 65% of non-pigmented P<sup>-</sup> strains were resistant to more than one antibiotic, while the corresponding result for pigmented P<sup>-</sup> strains

**Table 3.** Antibiotic resistance frequencies in P<sup>+</sup> strains. The number of P<sup>+</sup> strains *per* sponge species is indicated in brackets.

Antibiotic	Number of resistant isolates from the sponge					Total number	Resistant strains (%)
	Hp <sup>a</sup> (22)	Aj <sup>b</sup> (4)	Ln <sup>c</sup> (25)	Mh <sup>d</sup> (13)	Pg <sup>e</sup> (5)		
Penicillin G	6	3	1	1	3	14	20
Polymixyn B	11	0	2	0	1	14	20
Chloramphenicol	1	2	0	0	0	3	4
Tobramycin	10	0	2	0	0	12	17
Tetracycline	6	3	9	8	3	29	42
Ampicillin	0	2	0	0	0	2	3
Nalidixic Acid	9	2	3	2	1	17	25
Vibriostatic O/129	8	3	22	13	3	49	71
Gentamicin	0	0	0	0	0	0	0
Kanamycin	3	0	0	0	0	3	4
Neomycin	3	0	4	3	0	10	14

<sup>a</sup> *H. pilosa*   <sup>b</sup> *A. joubini*   <sup>c</sup> *L. nobilis*   <sup>d</sup> *M. hanitschi*   <sup>e</sup> *P. glaberrima*

**Table 4.** Antibiotic resistance frequencies in P<sup>-</sup> strains. The number of P<sup>-</sup> strains *per* sponge species is indicated in brackets.

Antibiotic	Number of resistant isolates from the sponge					Total number	Resistant strains (%)
	Hp <sup>a</sup> (54)	Aj <sup>b</sup> (32)	Ln <sup>c</sup> (67)	Mh <sup>d</sup> (34)	Pg <sup>e</sup> (41)		
Penicillin G	2	10	8	1	0	21	30
Polymixyn	0	11	5	2	1	19	28
Chloramphenicol	0	0	1	1	0	2	3
Tobramycin	0	3	2	2	1	8	12
Tetracycline	3	17	10	6	2	38	55
Ampicillin	0	0	1	1	0	2	3
Nalidixic Acid	0	2	0	4	2	8	12
Vibriostatic O/129	4	20	23	11	0	58	84
Gentamicin	0	0	1	1	0	2	3
Kanamycin	0	1	0	0	0	1	1
Neomycin	0	1	4	2	0	7	10

<sup>a</sup> *H. pilosa*   <sup>b</sup> *A. joubini*   <sup>c</sup> *L. nobilis*   <sup>d</sup> *M. hanitschi*   <sup>e</sup> *P. glaberrima*

was 80%. A high percentage of P<sup>-</sup> isolates exhibited resistance toward O/129 (84%), tetracycline (55%), penicillin (30%) and polymixyn (28%).

#### 4. Discussion

In the present study a culture-dependent approach was successfully applied in order to increase the knowledge on the bacterial community inhabiting the Antarctic marine environment. Data derived from the taxonomic characterization of the 297 isolates from the sponge specimens allowed the identification of 108 distinct OTUs belonging to the

phyla *Proteobacteria* (Alpha and Gamma subclasses), *Firmicutes*, *Actinobacteria* and *Bacteroidetes*, confirming previous observations [16]. *Gammaproteobacteria* resulted numerically dominant among bacterial isolates. Such bacteria are consistently found in association with marine invertebrates, mainly in sponges [12], but also oysters [17, 18] and corals [19]. Overall, the 23% of the bacterial isolates showed distinct plasmid bands on agarose gel. Such percentage is consistent with previous reports on bacteria isolated from Antarctica. In particular, Kobori et al. [10] found an overall 20% frequency of occurrence of plasmid-harboring Antarctic bacteria when considering different matrices, both biotic (benthic or ice-associated animals) and abiotic (sea-ice, seawater and sediments). More recently, Michaud et al. [11] found the 14% of plasmid-harboring bacteria among isolates from seawater. Conversely, studies which were carried out in marine environments different from Antarctica generally found much higher percentages of plasmid-harboring bacteria. Hermansson et al. [9] reported a frequency value of up to 52% in bacteria isolated from air-water interface. Glassman and McNicol [6] found that the 46% of the estuarine bacteria from sediment and water column in the Chesapeake Bay carried plasmids. Simon et al. [8] isolated 58 marine luminous bacteria mainly from the Mediterranean and Red Seas and found that the 43% of them harboured plasmids. The comparison of results obtained for human-impacted and pristine sites have been occasionally reported. Hada and Sizemore [7], examining marine *Vibrio* species in the Gulf of Mexico, found that the incidence of plasmids was higher in oil field regions (35%) than in the control area (23%). Moreover, a survey for plasmids in a freshwater environment (South Wales River) reported a plasmid incidence of 10% in unpolluted sites versus 15% in polluted ones [8]. At this regard, bacterial strains used in the present study have been obtained from a highly pristine environment and the percentage of plasmid-harboring bacteria is comparable with those reported for control areas by the authors above. The presence of plasmid molecules in strains belonging to the same and/or different species could be linked to the exchange of genetic information, even among different taxonomic levels [11]. The ability of bacteria to adapt to different environmental conditions and respond quickly to selective pressure is attributed to the acquisition of new genes through horizontal gene transfer, rather than genetic changes due to point mutations [20]. In fact, microorganisms develop resistance in the growing presence of toxic compounds in the environment [21]. The most common resistance is to metals and antibiotics, which can be a result of bio-essentiality or abuse of the metals, and/or antibiotics. In this work, we evaluated the resistance of P<sup>+</sup> strains to 11 different antibiotics. The highest incidence of antibiotic resistance occurred in the presence of the vibriostatic agent O/129 (71%) and tetracycline (42%), whereas no resistance was observed towards gentamicin. Antibiotic resistance was not expected at so high frequency as tested bacteria are from an environment where pollution from both chemicals and drugs is quite scarce. So, it is possible that acquired antibiotic resistance traits could become widespread even in environments where both the exposure to antibiotics and the anthropic pressure are minimal. However, the percentages of resistant strains observed in this work are in contrast with those reported by Kobori et al. [10] and De Souza et al. [2], who found a highest resistance to ampicillin and penicillin in strains isolated from Antarctica. These results could be linked to the existence of different mechanisms of resistance adopted by marine bacteria toward the tested antimicrobials. Briefly, no obvious relationship between

the plasmid presence and antibiotic resistance was found. The presence of P<sup>+</sup> antibiotic-resistant strains initially suggested a possible relationship between the presence of plasmid molecules and resistance. Unfortunately, resistance to antibiotics was also found in strains P<sup>-</sup> at a similar extent. However, the attribution of particular characteristics or specific features of these extrachromosomal molecules is very difficult to determine. Baya et al. [22] showed that the frequency of plasmid and resistance to antibiotics and/or toxic chemicals increases in marine bacteria isolated from contaminated waters. The authors were not able to demonstrate a direct relationship between the presence of plasmids and the observed phenotype. Although the frequency of antibiotic-resistant strains is a widespread phenomenon, the P<sup>+</sup> strains isolated from Antarctic sponges in this study did not show a high resistance against the tested antibiotics. This finding may be related to the particular environmental conditions of Antarctica, which is characterized, as it is well known, by a limited human impact. Conversely, the highest incidence of antibiotic resistant strains has been registered in areas subjected to pollution phenomena [23]. Finally, we can conclude that plasmids are ubiquitous elements even within the bacterial communities associated with Antarctic sponges. Function and evolution of such molecules in natural communities are however still poorly understood and need a deeper investigation.

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