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Metal and antibiotic resistance of bacteria isolated from the Baltic Sea

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Summary. The resistance of 49 strains of bacteria isolated from surface Baltic Sea waters to 11 antibiotics was analyzed and the resistance of selected strains to three metal ions (Ni^{2+}, Mn^{2+}, Zn^{2+}) was tested. Most isolates belonged to Gammaproteobacteria (78 %), while Alphaproteobacteria (8 %), Actinobacteria (10 %), and Bacteroidetes (4 %) were less abundant. Even though previous reports suggested relationships between resistance and the presence of plasmids or the ability to produce pigments, no compelling evidence for such relationships was obtained for the strains isolated in this work. In particular, strains resistant to multiple antibiotics did not carry plasmids more frequently than sensitive strains. A relation between resistance and the four aminoglycosides tested (gentamycin, kanamycin, neomycin, and streptomycin), but not to spectinomycin, was demonstrated. This observation is of interest given that spectinomycin is not always classified as an aminoglycoside because it lacks a traditional sugar moiety. Statistical analysis indicated relationships between resistance to some antibiotics (ampicillin and erythromycin, chloramphenicol and erythromycin, chloramphenicol and tetracycline, erythromycin and tetracycline), suggesting the linkage of resistance genes for antibiotics belonging to different classes. The effects of $NiSO_4$, $ZnCl_2$ and $MnCl_2$ on various media suggested that the composition of Marine Broth might result in low concentrations of Mn^{2+} due to chemical interactions that potentially lead to precipitation. [**Int Microbiol** 2012; 15(3):131-139]

Keywords: antibiotic resistance · metal resistance · marine bacteria · pigmentation · plasmids · Baltic Sea

Introduction

Bacterial resistance to antibiotics is an extensively investigated phenomenon of considerable medical importance [6]. Resistant bacteria are common in the natural environment, especially in aquatic habitats [9,10] and even in habitats that seem unlikely to have been exposed to anthropogenic antibiotics [33]. However, the indiscriminate use of antibiotics leads to water contamination (with concentrations ranging from 1 to 10^3 mg/l [10,16]), which can promote higher abundances of resistant bacteria in marine microbial ecosystems [12,23]. This is mainly due to the selection and dissemination of antibiotic-resistant organisms. Antibiotic and metal resistance of bacteria may be related through the linkage of genetic determinants and shared resistance mechanisms [2,12]. High frequencies of bacteria resistant to antibiotics

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can be viewed as indicative of environmental pollution [22,39], and aquatic systems are considered to be major reservoirs of resistance genes, important for their maintenance, mixing, and mobilization [36,38,39].

The distribution of antibiotic-resistant bacteria in freshwater environments has been addressed in many studies [9,10], while others concern free-living antibiotic-resistant bacteria in the marine environment. Available data indicate diverse patterns of antibiotic resistance—including multiple drug resistance—in bacteria isolated from seawater, marine sediments, and beach sand [3,5,7,8,13–15,17–20,24,29]. Diverse patterns have been observed even for closely related bacteria (e.g., *Vibrio* spp. [21] or *Staphylococcus* spp. [34]), isolated from very close geographical areas during the same season. In previous reports, pigmented bacteria have been observed to be more frequently resistant to antibiotics [5,7,17,18,20,29] and/or metal ions [5,20].

In this work, 49 bacterial strains isolated from surface water collected in the Bay of Gdansk (southern Baltic Sea) were characterized, including their resistance to 11 antibiotics. Five of these antibiotics were aminoglycosides: gentamycin (GEN), kanamycin (KAN), neomycin (NEO), spectinomycin (SPC), streptomycin (STR); the other six belonged to different classes: ampicillin (AMP), chloramphenicol (CAM), erythromycin (ERY), nalidixic acid (NAL), rifampicin (RIF), tetracycline (TET). In addition, the pigmentation status of the strains, the presence of plasmids, and the resistance of selected strains to three metal ions, Ni^{2+}, Mn^{2+}, and Zn^{2+}, was investigated. Our approach was based on cultivation, although we are aware that only a small fraction (perhaps < 1 %) of bacteria present in a given environment can be cultivated. Nonetheless, the presence of plasmids, colony morphology and the antibiotic resistance of isolates assigned to specific phylogenetic groups can only be investigated using a cultivation approach.

Materials and methods

Sampling and culture conditions. Water samples close to the coast of the Bay of Gdansk, southern Baltic Sea, were collected in April, August and October 2005. The samples were diluted in sterile sea water and spread on Marine Broth (MB; Difco, Sparks, MD, USA) plates. Bacteria were cultured at 30 ºC. The growth of all isolates was also tested on: salt-free LB (Difco) plates, Mueller-Hinton (Difco) plates (supplemented or not with NaCl, at a final concentration of 0.7 %), ZoBell plates (peptone 5 g, yeast extract 1 g, $FePO_4 \cdot 4H_2O$ 0.01 g, agar 15 g, aged Baltic sea water 750 ml, distilled water 250 ml, pH adjusted to 7.6), and R2A (Difco) plates.

Identification of isolates. The isolates were initially identified based on colony morphology, and then their 16S rRNA gene sequence, with gene amplification carried out using previously published primers [11]: 1492R (5′-GGT TAC CTT GTT ACG ACT T), with 27S-F (5′-CAA GAG TTT GAT CCT GGC TCA G). The amplification products (about 1.5 kb) were first analyzed using *Alu*I, *Bsu*RI (*Hae*III), and *Hin*6I (*Hha*I), as described previously [37]. The 16S rRNA gene sequences (about 1360 nucleotides) were assembled from two readouts (from forward and reverse primers), and compared to sequences in GenBank using nucleotide blast (blastn) [http://blast.ncbi.nlm.nih.gov] and a non-redundant database (posted on 23 June 2011). To obtain a phylogenetic tree, the sequences were aligned using Mothur [32] with default settings and the 16S rRNA SILVA alignment database [28]. Columns with gaps were removed. The tree was obtained using RAxML [35] with the GTR+Γ model (found optimal with jModelTest [27] according to the Akaike Information Criterion). The sequences were assigned to species using the highest-scoring sequence for which species information was available when sequence similarity was above 97 %. The 16S rRNA gene sequences obtained in this work were submitted to GenBank under accession numbers JQ012948 to JQ012996.

Isolation of plasmid DNA. A commercial kit (Plasmid MiniKit, A&A Biotechnology, Gdynia, Poland), based on alkaline lysis and the binding of DNA on silica membranes, was used to isolate plasmid DNA. We used *Escherichia coli* strain DH10B carrying a plasmid pRK2 to verify that the kit allows extraction of plasmids up to 60,099 bp [25]. The presence of plasmids was assessed using agarose gel electrophoresis, and plasmid sizes were estimated using a supercoiled DNA ladder (Invitrogen, Grand Island, NY, USA).

Analysis of pigmentation status, and antibiotic and metal ion resistance. Minimal inhibitory concentration (MIC) for antibiotics of clinical isolates was initially determined using the disk method with Mueller-Hinton medium [4]. This is a standard method because it is low in substances that affect resistance to sulfonamide and trimethoprim and allows the growth of many pathogenic bacteria. However, many of the Baltic isolates grew very poorly (6 strains) or not at all (14 strains) on this medium, even when supplemented with NaCl (14 and 1 isolate, respectively; not shown). Accordingly, a more exact approach was used to determine MIC, i.e., the culture was spread on plates suitable for the cultivation of these isolates (MB plates) and containing various antibiotic concentrations.

As mentioned above, the strains were tested for resistance to 11 antibiotics. All of the antibiotics were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Ten µl of bacterial culture (A_{600} = 0.3) were spread onto MB plates containing 0, 5, 10, 20, 50, 75 and 100 µl/ml of a given antibiotic. In all experiments, the plates were incubated at 30 °C for 24 h. The same conditions were used to assess the production of pigments. In several strains, the effects of lower incubation temperatures (for example, 20 °C) were examined, but under these conditions the bacteria grew very slowly even though colonies of pigmented bacteria remained colored. However, extremely low (4 °C) or high (37 °C) temperatures often led to a loss of pigmentation (data not shown). The resistance of the 14 selected strains to metal ions was tested also using the dilution method on MB plates, with 0, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 2.5, 5, 10, 20 and 40 mM $NiSO_4$, $MnCl_2$, or $ZnCl_2$. In addition, the effect of the medium on the resistance patterns of four selected strains was examined using salt-free LB medium. Two plasmid-free non-pigmented laboratory strains of marine bacteria, *Vibrio fischeri* MJ1 [30] and *Photobacterium leiognathi* 721 [1], were used as controls for the quality of plate preparation in all experiments. All experiments were independently repeated three times. All statistical analyses were done using the R package (http://www.r-project.org/). A significance level of 0.01 was used with the Bonferroni correction (B) and the less conservative Benjamini-Hochberg correction (BH) for multiple tests.

Results and Discussion

A high diversity of resistance patterns to 11 antibiotics was determined in the 49 strains isolated from Baltic surface waters (Fig. 1). In Fig. 1, the numbers close to the internal branches indicate bootstrap support (based on 1000 pseudoreplicates); they were underlined when the branch was significantly longer than zero according to the weighted least-squares likelihood ratio test [31]. The phylogenetic composition of our isolates might have influenced the patterns observed, if a particular phylogenetic group had a higher intrinsic resistance to antibiotics. A similar caveat is appropriate for any study based on a cultivation approach. Both the principal components analysis (PCA; Fig. 2) and Kendall's tau rank correlation test indicated a relation between resistance and four aminoglycoside antibiotics, but not with SPC, which is not always classified as an aminoglycoside antibiotic because it lacks a traditional sugar moiety. These results suggest that while GEN, KAN, NEO, and STR share resistance mechanisms, the mechanism of resistance to SPC in these isolates is different. The six correlation coefficients for four aminoglycosides were all significant (B, BH $P < 0.001$).

The PCA (Fig. 2) also suggested possible relationships between AMP and ERY resistance, which was significant according to Kendall's tau (B, BH $P < 0.001$), and between AMP and TET (BH $P < 0.01$, but B $p > 0.05$). The other possible relationships suggested by PCA involved CAM, ERY, NAL, RIF, and TET (Fig. 2). For five antibiotics, there were 10 pairs to consider. Five of these correlations were significant with BH, but only three with both the B and BH corrections (for CAM-ERY, CAM-TET, ERY-TET B, BH $P < 0.01$; for CAM-NAL and ERY-RIF BH $P > 0.01$, but B $P > 0.05$; for other pairs: BH, B $P > 0.05$, with the exception of CAM-RIF BH $P = 0.016$, B $P > 0.05$). These results suggest linkages between resistance genes for antibiotics belonging to different classes.

While multi-antibiotic resistance was frequent among Baltic isolates, a wide range of patterns was observed (Figs. 1 and 2): from weak resistance to only two antibiotics (strain number 29; strain numbers follow the order on the tree in Fig. 1) to strong resistance to all tested antibiotics except rifampicin (strain number 45). For clinical isolates, strain resistance or susceptibility is determined based on taxonomically-specific MIC values determined using standard medium [4]. In this work, environmental strains and a medium suitable for their growth were used. Based on an arbitrary, but reasonable MIC value (a strain was considered sensitive when growth was totally inhibited at antibiotic concentrations of 20 μg ml/l concentration or lower, and resistant otherwise), the mean number of antibiotics to which our isolates were resistant was 5.6. The highest frequency of resistant strains was observed for TET (88%), followed by NAL (71 %), STR (69 %), KAN and AMP (both 65 %). This is a very different pattern from the one reported previously for isolates from beach sand and seawater in the Southern Baltic [19], Eastern Mediterranean [13], Atlantic [24], and Indian Ocean [29], where resistance to TET is apparently rare while resistance to β-lactams seems to be more prevalent.

Nineteen (39 %) isolates carried plasmids, with molecular sizes ranging from 3 to 8 kb. Of those, 15 (31 %) formed pigmented colonies on MB plates. Two thirds of these strains formed pigmented colonies on the other media tested, while six Pseudomonadales and one Vibrionales strain produced pigment during growth on other media (data not shown). On MB plates, the mean and range of the number of antibiotics to which a given strain was resistant was similar for plasmid-containing (mean: 6.1, range: 2–10, n =19) and plasmid-less bacteria (mean: 5.2, range: 2–9, n = 30), and for pigmented (mean: 6.1, range: 3–10, n = 14) and non-pigmented bacteria (mean: 5.3, range: 2–9, n = 35). In general, there was no significant positive relation between antibiotic resistance and the presence of plasmids or pigmentation on MB plates according to a one-sided Wilcoxon test at the 0.01 level, with the exception of KAN and pigmentation (B, BH $P = 0.0036$). High values of the test statistic were obtained also for GEN, NAL, NEO (all: BH $P = 0.016$, B $P > 0.05$), and STR (BH $P = 0.024$, B $P > 0.05$).

We speculate that, if pigment production were related to resistance, both should be observable on the same medium. However, we also asked whether the ability to produce pigments on any of the test media (Table 1) was related to resistance. In this case, the highest value of the Wilcoxon test statistic was obtained for NAL (B, BH $P = 0.017$; for all others B, BH $P > 0.05$). Thus, in summary, our results did not provide compelling evidence that pigmentation was related to antibiotic resistance in the analyzed strains; nor was there any apparent relationship for 14 selected strains (representing different species) between resistance to Ni^{2+}, Mn^{2+}, Zn^{2+} (Fig. 1) and the presence of plasmids or pigmentation. In order to test whether the medium influenced the metal resistance patterns, four Baltic isolates (number 8, 31, 46, 49) able

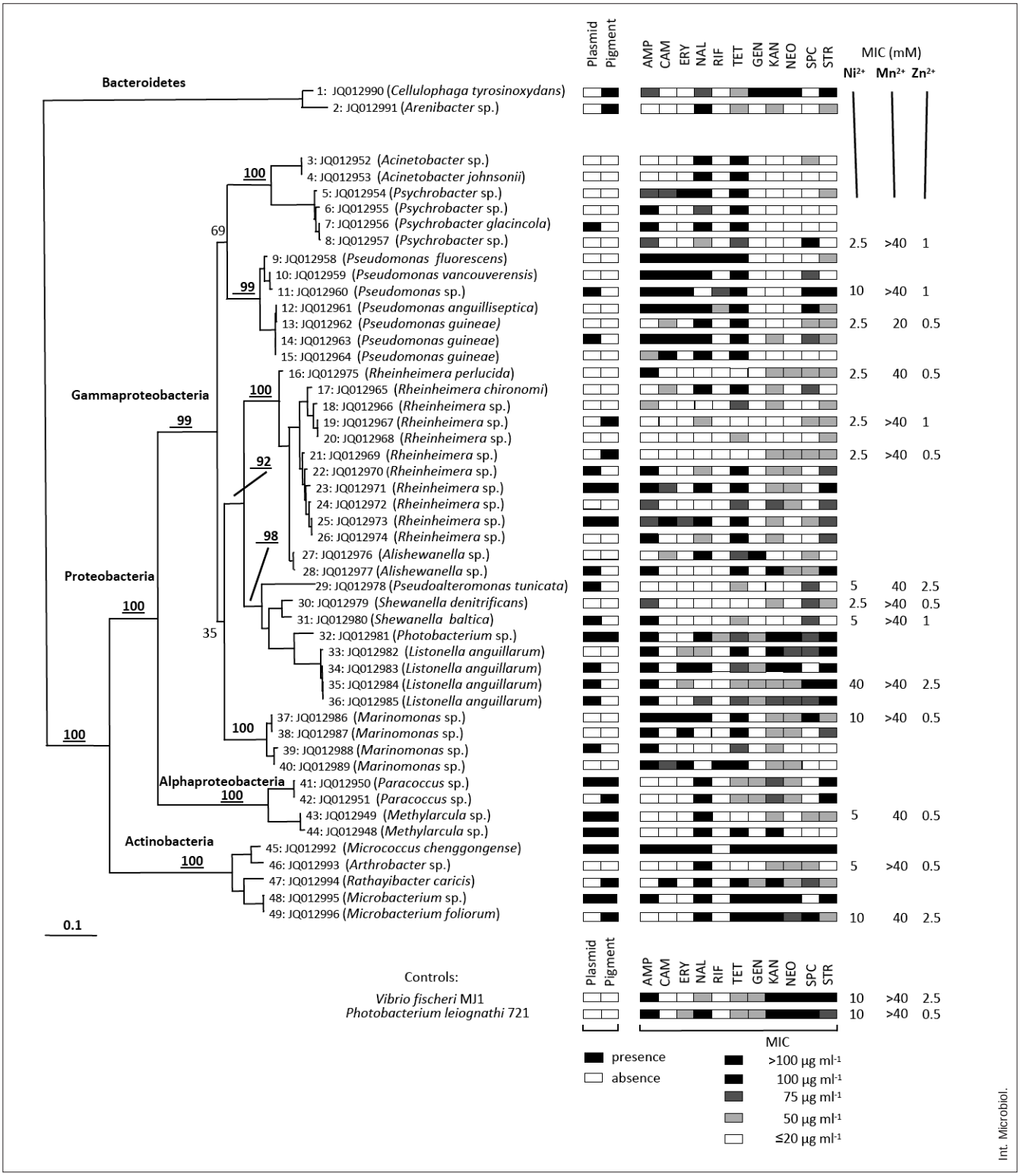

Fig. 1. Maximum likelihood tree of 49 Baltic Sea isolates, based on their 16S rRNA gene sequences. The accession number of the 16S rDNA sequence is shown, with the closest species identified in GenBank using blastn in parentheses. Presence/absence of plasmids and pigment on Marine Broth plates is indicated by black/white squares for all isolates and control strains. Minimal inhibitory concentrations (MIC) for six antibiotics belonging to different classes: ampicillin (AMP), chloramphenicol (CAM), erythromycin (ERY), nalidixic acid (NAL), rifampicin (RIF), tetracycline (TET), and five aminoglycoside antibiotics: gentamycin (GEN), kanamycin (KAN), neomycin (NEO), spectinomycin (SPC), streptomycin (STR) are indicated by gray-scale squares. The scale is given at the bottom of the figure. The MIC of three metal ions was investigated only for 14 selected strains.

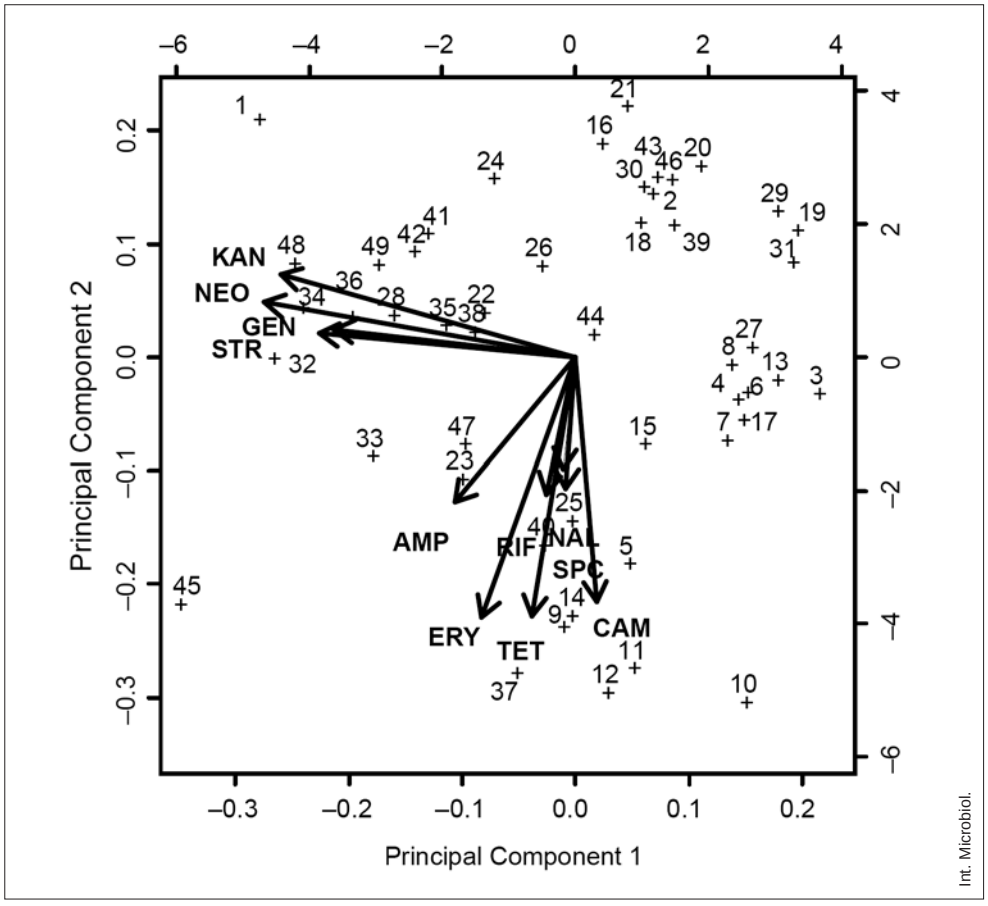

Fig. 2. Principal component analysis of antibiotic resistance in 49 Baltic Sea isolates. The arrows show the directions corresponding to variables in the coordinate system given by the first two principal components. The variables are the ranked (ties were averaged) and normalized minimal inhibitory concentrations (MIC) for six antibiotics belonging to different classes: ampicillin (AMP), chloramphenicol (CAM), erythromycin (ERY), nalidixic acid (NAL), rifampicin (RIF), tetracycline (TET), and five aminoglycoside antibiotics: gentamycin (GEN), kanamycin (KAN), neomycin (NEO), spectinomycin (SPC), streptomycin (STR). Longer arrows pointing in similar directions (smaller angles) correspond to stronger relationships, perpendicular arrows correspond to weaker relationships.

to grow on salt-free LB medium were further investigated. The effects of $NiSO_4$ and $ZnCl_2$ on these strains were similar to those observed on the MB plates. However, the MIC values for $MnCl_2$ (5–20 mM) were considerably lower than those obtained on MB plates, which in every case were at least 40 mM for these four strains and indeed all the remaining strains with the exception of strain number 13, for which the MIC was 20 mM. This finding might be explained by a similar effect of the composition of the MB as reported for surface marine waters, in which low concentrations of Mn^{2+} due to chemical interactions may result in precipitation [26].

The main conclusion of our work is that although there may be links between resistance determinants to various antimicrobial agents (as previously observed, e.g. [8]), there is no obvious relation between resistance and the presence of plasmids or pigmentation. While these findings could reflect the limitations of the method used to detect plasmids, a similar approach was previously employed to detect large plasmids (e.g.. [29]), and in previous studies we have used this method to successfully extract plasmids from a wide range of bacteria, including very large plasmids (>100 kb). Here, a 60-kb plasmid was used to test the quality of the extraction procedure. Of course, resistance plasmids are not the only possible determinants of antibiotic resistance, and it is possible that chromosomal-dependent resistance, perhaps transferred by transducing bacteriophages, plays an important role in marine bacteria.

It has been suggested that pigmented bacteria should be more resistant to antibiotics than non-pigmented strains

Table 1. Pigmentation status of the isolates grown on different media, with additional information on the sea water sample (location and date) from which a given strain was isolated

		Media					
Strain number	Isolate	MB¶	MH¶	MH with 0.7% NaCl	LB	ZoBell	R2A
1	IOMB 403[d,i]	+, P	–	+/–, P	+/–, P	+, P	+/–, P
2	IOMB 394[c,i]	+, P	–	–	–	+/–, P	+/–, P
3	IOMB 292[f,h]	+	+	+	+, P	+	+
4	IOMB 308[f,h]	+	+, P	+, P	+, P	+	+
5	IOMB 189[d,g]	+	+/–, P	+, P	+, P	+	+
6	IOMB 390[b,I]	+	+	+	+	+	+/–
7	IOMB 393[b,i]	+	+, P	+, P	+, P	+	+/–
8	IOMB 296[f,h]	+	+, P	+, P	+, P	+	+
9	IOMB 205[d,g]	+	+, P	+, P	+, P	+, P	+, P
10	IOMB A42[d,i]	+	+	+	+	+	+
11	IOMB 242[a,g]	+	+	+	+	+	+
12	IOMB 182[d,g]	+	+	+	+	+	+
13	IOMB 195[d,g]	+	+/–	+/–	+	+/–	+/–
14	IOMB 351[e, i]	+	+, P	+, P	+, P	+/–	+/–
15	IOMB 389[b,i]	+	+	+	+	+	+
16	IOMB 235[b,g]	+	–	+/–	+/–	+	–
17	IOMB 239[a,g]	+	–	+	–	+	+
18	IOMB 208[d,g]	+	+	+	+	+	+/–
19	IOMB 238[a,g]	+, P	+, P	+, P	+, P	+, P	+/–
20	IOMB 262[a,g]	+	+	+	+	+	–
21	IOMB 193[d,g]	+, P	–	+/–	–	+/–	–
22	IOMB A10[d,i]	+	+	+	+	+/–	–
23	IOMB 309[f,h]	+, P	+, P	+, P	+, P	+, P	+/–, P
24	IOMB 325[f,h]	+	–	+/–	+/–	+	+/–
25	IOMB 347[e,i]	+, P	+	+	+	+	+
26	IOMB 400[d,i]	+	–	+	+	+	+/–
27	IOMB 199[d,g]	+	+/–	+/–	–	+	–
28	IOMB 197[d,g]	+	–	+/–	–	+	–
29	IOMB 301[f,h]	+	–	+/–	–	+	–
30	IOMB 364[b,i]	+	–	+	+/–	+	+/–

(*Continued on next page*)

Table 1. (*Continued*) Pigmentation status of the isolates grown on different media, with additional information on the sea water sample (location and date) from which a given strain was isolated

		Media					
Strain number	Isolate	MB[¶]	MH[¶]	MH with 0.7% NaCl	LB	ZoBell	R2A
31	IOMB 300[f,h]	+	+	+	+	+	+
32	IOMB 384[b,i]	+, P	+, P	+, P	+, P	+, P	–
33	IOMB 329[e,j]	+	+, P	+, P	+, P	+	–
34	IOMB 370[b,i]	+	+	+	+	+	–
35	IOMB 379[b,i]	+	+	+	+	+	–
36	IOMB 376[b,j]	+	+	+	+	+	–
37	IOMB 413[d,i]	+	+	+	+	+/–	+/–
38	IOMB 397[d,i]	+	+/–	+	+	+/–	+/–
39	IOMB 406[d,i]	+	+	+	+	+/–	–
40	IOMB 207[d,g]	+	+	+	+	+	–
41	IOMB 228[b,g]	+, P	–	+, P	+, P	+/–, P	+/–, P
42	IOMB 231[b,g]	+, P	–	+, P	+, P	+/–, P	+/–, P
43	IOMB 369[b,i]	+, P	–	+/–	+/–	+, P	+, P
44	IOMB 204[d,g]	+, P	–	+/–	–	+/–, P	–
45	IOMB 402[d,i]	+, P	+, P	+/–, P	+, P	+, P	+/–, P
46	IOMB 206[d,g]	+	+/–	+/–	+/–	+/–	+/–
47	IOMB 359[e,j]	+, P	+/–, P	+/–, P	+/–, P	+/–, P	+/–, P
48	IOMB 371[b,i]	+, P	+, P	+/–, P	+, P	+, P	+, P
49	IOMB 203[d,g]	+, P	+, P	+, P	+, P	+, P	+, P

[¶]MB, Marine broth; MH, Mueller-Hinton.
[a]54°20′14″ N, 19°13′54″ E; [b]54°30′07″ N, 18°33′34″ E; [c]54°30′08″ N, 18°33′34″ E; [d]54°30′56″ N, 18°33′05″ E; [e]54°31′09″ N, 18°33′36″ E; [f]54°33′01″ N, 18°39′46″ E.
[g]April 1, 2005; [h]August 1, 2005; [i]October 1, 2005.
+, good growth; +/–, poor growth; –, no growth; P, pigment production.

[5,7,17,18,20], and a similar relationship has been claimed for metal resistance [5,20]. Our findings offer little support for either claim. Apart from the obvious fact that the relationships observed for strains isolated in one region do not necessarily hold true for those isolated from another, it should be noted that previously reported differences [17] in antibiotic-resistance between pigmented and non-pigmented bacteria were only minor. Perhaps more importantly, in most of those studies, the species diversity of the isolates was not investigated. In one report in which species assignment was attempted [5], the overwhelming majority of pigmented isolates (10 strains out of 11) belonged to only one genus (*Flavobacterium*, one other pigmented isolate was assigned to *Xanthomonas*). The 15 strains pigmented on MB isolated in this work (Fig. 1) were not equally distributed among the phylogenetic groups. Although most of the isolates belonged to Gammaproteobacteria (38/49 or 77 %), only five were pigmented on MB (13 %), compared with 90 % (10/11) of the isolates of other groups. Four of these five pigmented Gammaproteobacteria could be assigned to one genus (*Rheinheimera*). However, a number

of our Gammaproteobacteria isolates were able to produce pigment on other media. We conclude that previous claims that antibiotic- and metal ions-resistance occurs with higher frequencies in pigmented strains may have reflected the higher frequencies of pigmentation on a particular medium among isolates belonging to a particular species (or genus) rather than among the pigmented strains *per se*.

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Competing interests. None declared.

References

1. Ast JC, Dunlap PV (2004) Phylogenetic analysis of the *lux* operon distinguishes two evolutionarily distinct clades of *Photobacterium leiognathi*. Arch Microbiol 181:352-361
2. Baker-Austin C, Wright MS, Stepanauskas R, McArthur JV (2006) Co-selection of antibiotic and metal resistance. Trends Microbiol 14:176-182
3. Baya AN, Brayton PR, Brown VL, Grimes DJ, Russek-Cohen E, Colwell RR (1986) Coincident plasmids and antimicrobial resistance in marine bacteria isolated from polluted and unpolluted Atlantic Ocean samples. Appl Environ Microbiol 51:1285-1292
4. Clinical and Laboratory Standards Institute (2012) Performance standards for antimicrobial susceptibility testing; twenty-second informational supplement. CLSI Document M100-S22. Clinical and Laboratory Standards Institute, Wayne, PA, USA
5. De Souza MJ, Nair S, LokaBharathi PAL, Chandramohan D (2006) Metal and antibiotic-resistance in psychrotrophic bacteria from Antarctic marine waters. Ecotoxicology 15:379-384
6. Goldstein FW (2007) Combating resistance in a challenging, changing environment. Clin Microbiol Infect 13:2-6
7. Hermansson M, Jones GW, Kjelleberg S (1987) Frequency of antibiotic and heavy metal resistance, pigmentation, and plasmids in bacteria of the marine air-water interface. Appl Environ Microbiol 53:2338-2342
8. Kim S-J, Ogo M, Oh M-J, Suzuki S (2012) Occurrence of tetracycline resistant bacteria and *tet*(M) gene in seawater from Korean coast. In: Kawaguchi M, Misaki K, Sato H, Yokokawa T, Itai T, Nguyen TM, Ono J, Tanabe S (eds) Interdisciplinary studies on environmental chemistry—Environmental pollution and ecotoxicology. TERRAPUB, Tokyo, pp 367-375
9. Kümmerer K (2009) Antibiotics in the aquatic environment—a review. Part I. Chemosphere 75:417-434
10. Kümmerer K (2009) Antibiotics in the aquatic environment – a review. Part II. Chemosphere 75: 435-441
11. Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. John Wiley & Sons, London, pp 115-175
12. Martinez JL (2009) Environmental pollution by antibiotics and by antibiotic resistance determinants. Environ Pollut 157:2893-2902
13. Matyar F (2012) Antibiotic and heavy metal resistance in bacteria isolated from the Eastern Mediterranean Sea coast. Bull Environ Contam Toxicol 89:551-556
14. Matyar F, Kaya A, Dinçer S (2008) Antibacterial agents and heavy metal resistance in Gram-negative bacteria isolated from seawater, shrimp and sediment in Iskenderun Bay, Turkey. Sci Total Environ 407:279-285
15. Miller RV, Gammon K, Day MJ (2009) Antibiotic resistance among bacteria isolated from seawater and penguin fecal samples collected near Palmer station, Antarctica. Can J Microbiol 55:37-45
16. Minh TB, Leung HW, Loi IH et al (2009) Antibiotics in the Hong Kong metropolitan area: ubiquitous distribution and fate in Victoria Harbour. Mar Poll Bull 58:1052-1062
17. Mudryk ZJ (2005) Occurrence and distribution antibiotic resistance of heterotrophic bacteria isolated from a marine beach. Mar Pollut Bull 50:80-86
18. Mudryk ZJ, Skórczewski P (1998) Antibiotic resistance in marine neustonic and planktonic bacteria isolated from the Gdansk Deep. Oceanologia 40:123-134
19. Mudryk ZJ, Perlinski P, Skórczewski P (2010) Detection of antibiotic resistant bacteria inhabiting the sand of non-recreational marine beach. Mar Pollut Bull 60:207-214
20. Nair S, Chandramohan D, LokaBharathi PA (2003) Differential sensitivity of pigmented and non-pigmented marine bacteria to metals and antibiotics. Water Res 26:431-434
21. Neela FA, Nonaka L, Suzuki S (2007) The diversity of multi-drug resistance profiles in tetracycline-resistant *Vibrio* species isolated from coastal sediments and seawater. J Microbiol 45:64-68
22. Nithya C, Pandian SK (2010) Isolation of heterotrophic bacteria from Palk Bay sediments showing heavy metal tolerance and antibiotic production. Microbiol Res 165:578-593
23. Nogales B, Lanfranconi MP, Piña-Villalonga JM, Bosch R (2011) Anthropogenic perturbations in marine microbial communities. FEMS Microbiology Rev 35:275-298
24. Oliveira AJFC, Franca PT, Pinto AB (2010) Antimicrobial resistance of heterotrophic marine bacteria isolated from seawater and sands of recreational beaches with different organic pollution levels in southeastern Brazil: evidences of resistance dissemination. Environ Monit Assess 169:375-384
25. Pansegrau W, Lanka E, Barth PT, et al. (1994) Complete nucleotide sequence of Birmingham IncPα plasmids: Compilation and comparative analysis. J Mol Biol 239:623-663
26. Pohl C, Löffler A, Hennings U (2004) A sediment trap flux study for trace metals under seasonal aspects in the stratified Baltic Sea (Gotland Basin; 57° 19.20′ N; 20° 03.00′ E). Mar Chem 84:143-160
27. Posada D (2008) jModelTest: Phylogenetic model averaging. Mol Biol Evol 25:1253-1256
28. Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, Glöckner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res 35:7188-7196
29. Ramesh S, Manivasagan P, Ashokkumar S, Rajaram G, Mayavu P (2010) Plasmid profiling and multiple antibiotic resistance of heterotrophic bacteria isolated from Muthupettai Mangrove environment, southeast coast of India. Curr Res Bacteriol 3:227-237

30. Ruby EG, Nealson KH (1976) Symbiotic association of *Photobacterium fischeri* with the marine luminous fish *Monocentris japonica*: a model of symbiosis based on bacterial studies. Biol Bull 151:574-586
31. Sanjuan R, Wróbel B (2005) Weighted least-squares likelihood ratio test for branch testing in phylogenies reconstructed from distance measures. Syst Biol 54:218-229
32. Schloss PD, Westcott SL, Ryabin T, et al. (2009) Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537-7541
33. Séveno NA, Kallifidas D, Smalla K, van Elsas JD, Collard J-M, Karagouni AD, Wellington EMH (2002) Occurrence and reservoirs of antibiotic resistance genes in the environment. Rev Med Microbiol 13:15-27
34. Soge OO, Meschke JS, No DB, Roberts MC (2009) Characterization of methicillin-resistant *Staphylococcus aureus* and methicillin-resistant coagulase-negative *Staphylococcus* spp. isolated from US West Coast public marine beaches. J Antimicrob Chemother 64:1148-1155
35. Stamatakis A (2006) RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22:2688-2690
36. Taylor NGH, Verner-Jeffreys DW, Baker-Austin C (2011) Aquatic systems: maintaining, mixing and mobilising antimicrobial resistance? Trends Ecol Evol 26:278-284
37. Vaneechoutte M, Dijkshoorn L, Tjernberg I, Elaichouni A, de Vos G, Claeys P, Verschraegen G (1995) Identification of *Acinetobacter* genomic species by amplified ribosomal DNA restriction analysis. J Clin Microbiol 33:11-15
38. Wright GD (2007) The antibiotic resistome: the nexus of chemical and genetic diversity. Nat Rev Microbiol 5:175-186
39. Zhang XX, Zhang T, Fang HHP (2009) Antibiotic resistance genes in water environment. Appl Microbiol Biotechnol 82:397-414