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ORIGINAL ARTICLE

Novel polymerase chain reaction primers for the specific detection of bacterial copper P-type ATPases gene sequences in environmental isolates and metagenomic DNA

R. De la Iglesia[1,2], D. Valenzuela-Heredia[1], J.P. Pavissich[1], S. Freyhoffer[1], S. Andrade[2], J.A. Correa[2] and B. González[1,3]

1 Departamento de Genética Molecular y Microbiología and Millennium Nucleus on Microbial Ecology and Environmental Microbiology and Biotechnology, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile
2 Departamento de Ecología and Center for Advanced Studies in Ecology and Biodiversity, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile
3 Facultad de Ingeniería y Ciencia, Universidad Adolfo Ibáñez, Santiago, Chile

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ATPases, bacterial communities, copper resistance, metagenomic DNA, PCR primer pair.

Correspondence
Bernardo González, Facultad de Ingeniería y Ciencia, Universidad Adolfo Ibáñez, 7941169 Santiago, Chile. E-mail: bernardo.gonzalez@uai.cl

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Abstract

Aims: In the last decades, the worldwide increase in copper wastes release by industrial activities like mining has driven environmental metal contents to toxic levels. For this reason, the study of the biological copper-resistance mechanisms in natural environments is important. Therefore, an appropriate molecular tool for the detection and tracking of copper-resistance genes was developed.

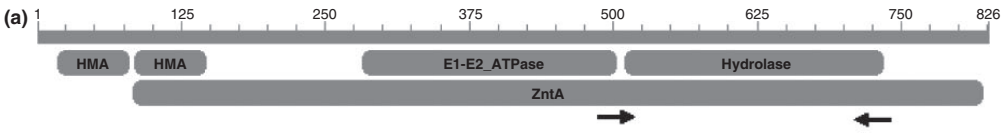
Methods and Results: In this work, we designed a PCR primer pair to specifically detect copper P-type ATPases gene sequences. These PCR primers were tested in bacterial isolates and metagenomic DNA from intertidal marine environments impacted by copper pollution. As well, T-RFLP fingerprinting of these gene sequences was used to compare the genetic composition of such genes in microbial communities, in normal and copper-polluted coastal environments. New copper P-type ATPases gene sequences were found, and a high degree of change in the genetic composition because of copper exposure was also determined.

Conclusions: This PCR based method is useful to track bacterial copper-resistance gene sequences in the environment.

Significance and Impact of the Study: This study is the first to report the design and use of a PCR primer pair as a molecular marker to track bacterial copper-resistance determinants, providing an excellent tool for long-term analysis of environmental communities exposed to metal pollution.

Introduction

Copper is an essential micronutrient for all living organisms; however, high levels of copper may become toxic (Halliwell and Gutteridge 1984) and, thus, cellular copper homeostasis is tightly regulated and several mechanisms that prevent heavy metal intoxication have been described. In micro-organisms, resistance to metals is mediated by a variety of systems (reviewed in Silver and Phung 1996; Nies 1999; Bruins *et al.* 2000). Efflux systems by P-type cation-transporting ATPases represent the largest known category of resistance systems and are often highly specific for the metal(s) they export (Nies and Silver 1995; Argüello *et al.* 2007). P-type ATPases are a group of ubiquitous membrane proteins, classified in eight super families, which share an ATP-binding motif GDGXNDXP, and a conserved DKTGT motif containing an aspartate residue that is phosphorylated during a catalytic cycle (Palmgren and Axelsen 1998). Copper P-type ATPases (Fig. 1) are part of the IB group of heavy metal



(b)

	F	S	K	G	D	T	L	I	V	D	K	T	G	T	L	T	E	G	K	P	K	L	T
1. copA *R. leguminosarum* (Q9X5V3) IB1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
2. copA *S. meliloti* (Q9X5X3) IB1	L	R	D	A	.	V	V	A	.	.	.	.	.	.	.	.	K	.	R	.	E	.	.
3. copA *B. subtillis* (O32220) IB1	T	H	R	L	.	.	I	V	L	.	.	.	.	.	V	.	N	.	.	.	R	.	.
4. copA *E. coli* (Q59385) IB1	A	.	T	L	.	.	V	V	F	.	.	.	.	.	.	.	.	.	.	.	Q	V	V
5. copB *E. hirae* (P05425) IB3	A	N	D	L	.	V	I	M	L	.	.	.	.	.	.	.	Q	.	.	F	T	V	.
6. pacS *S. elongatus* PCC 7942 (P37279) IB1	A	Q	T	I	Q	.	V	.	L	.	.	.	.	.	.	.	Q	.	Q	.	S	V	.
7. silP *S. typhimurium* (Q9ZHC7) IB1	L	E	.	V	.	.	.	V	.	.	.	.	.	.	.	.	.	.	S	.	T	V	.
8. copB *A. fulgidus* DSM4304 (O30085) IB3	A	K	D	L	Q	A	V	.	F	.	.	.	.	.	.	.	.	.	R	F	G	V	.
9. cadA *H. pylori* (Q59465) IB2	L	T	Q	A	K	S	I	A	F	.	.	.	.	.	.	.	K	.	V	F	.	V	.
10. cadA *S. aureus* (pI258) (P20021) IB2	L	G	A	I	K	.	V	A	F	.	.	.	.	.	.	.	K	.	V	.	V	V	.
11. cadA *L. monocytogenes* (Q60048) IB2	I	G	G	L	K	A	I	A	F	.	.	.	.	.	.	.	K	.	V	.	V	V	.
12. DC1	A	E	.	V	T	H	.	.	T	.	.	.	.	.	.	.	.	.	.	.	S	V	V
13. DC2	L	R	E	A	.	V	I	A	L	.	.	.	.	.	.	.	K	.	.	.	E	.	.
14. DC3	M	E	.	I	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S
15. NI RS1	A	.	.	I	.	A	V	V	F	.	.	.	.	.	.	.	Q	.	.	.	.	T	V
16. I MS1	L	T	S	V	G	V	V	A	L	.	.	.	.	.	V	.	.	.	R	.	E	.	.
17. I MS2	L	N	D	V	E	V	.	V	.	.	.	.	.	.	.	.	.	.	.	.	A	V	E
18. I MS3	.	E	.	V	.	.	V	V	.	.	.	.	.	.	.	.	.	.	R	.	E	.	V
19. I MS4	L	R	R	V	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	S	.	E
20. I MS5	M	R	.	V	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V
21. I MS6	A	R	W	T	P	–	W	W	.	.	.	.	.	.	.	.	.	.	H	.	E	.	V
22. I RS1	L	Q	E	A	K	V	I	A	L	.	.	.	.	.	.	.	K	.	.	.	E	.	.
23. I RS2	L	.	E	V	.	L	I	A	F	.	.	.	.	.	.	.	.	.	R	.	V	.	S
24. NI RS2	P	A	H	L	K	A	I	A	L	.	.	.	.	.	.	.	.	.	R	.	V	V	V
25. NI RS3	L	Q	G	V	.	V	I	A	F	.	.	.	.	.	.	.	.	.	.	.	E	.	S
26. BI1	Q	–	–	–	R	A	N	V	F	.	.	.	.	.	.	.	H	.	T	.	R	.	.
27. BI2	–	–	–	–	–	–	P	N	Q	.	.	.	.	.	.	.	.	H	S	.	G	T	S
28. BI3	Q	P	R	P	R	.	.	M	.	.	.	.	.	.	.	.	.	.	R	.	V	F	H
29. BI4	L	K	D	A	Q	V	V	A	.	.	.	.	.	.	.	.	.	.	R	.	R	.	.
30. BI5	–	–	–	–	–	–	P	N	Q	.	.	.	.	.	.	.	.	H	S	.	G	T	S
31. BI6	–	–	–	–	–	–	P	N	Q	.	.	.	.	.	.	.	.	H	S	.	G	T	S

	L	I	D	E	L	R	S	K	G	A	I	I	–	A	M	A	G	D	G	V	N	D	A
1. copA *R. leguminosarum* (Q9X5V3) IB1	.	.	.	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.
2. copA *S. meliloti* (Q9X5X3) IB1	A	V	R	K	.	.	Q	G	.	R	S	V	–	.	F	I	.	.	.	I	.	.	.
3. copA *B.subtillis* (O32220) IB1	E	.	A	R	.	Q	K	E	.	R	Q	T	–	.	.	V	.	.	.	I	.	.	.
4. copA *E. coli* (Q59385) IB1	A	.	K	H	.	Q	.	E	.	R	Q	V	–	.	.	V	.	.	.	I	.	.	.
5. copB *E. hirae* (P05425) IB3	I	V	Q	R	Y	L	D	Q	.	K	K	V	–	I	.	V	.	.	.	I	.	.	.
6. pacS *S. elongatus* PCC 7942 (P37279) IB1	Q	V	A	Q	.	Q	.	R	.	Q	V	V	–	.	.	V	.	.	.	I	.	.	.
7. silP *S. typhimurium* (Q9ZHC7) IB1	V	.	T	R	.	K	A	S	.	H	V	V	–	.	.	.	.	.	.	.	.	.	.
8. copB *A. fulgidus* DSM4304 (O30085) IB3	K	V	K	.	.	E	V	Q	Q	K	Y	V	T	.	.	V	.	.	.	.	.	.	.
9. cadA *H. pylori* (Q59465) IB2	V	F	K	T	F	K	E	R	Y	K	A	P	A	I	F	V	.	.	.	I	.	.	.
10. cadA *S. aureus* (pI258) (P20021) IB2	Y	.	K	K	M	Q	.	E	Y	D	N	V	–	.	.	I	.	.	.	.	.	.	.
11. cadA *L. monocytogenes* (Q60048) IB2	Y	.	K	Q	.	K	I	N	F	G	K	V	–	.	.	V	.	.	.	I	.	.	.
12. DC1	I	V	N	.	.	K	D	.	.	Y	R	V	–	T	.	.	.	.	.	I	.	.	.
13. DC2	A	L	Q	R	.	.	A	S	.	R	K	L	–	.	F	V	.	.	.	I	.	.	.
14. DC3	V	V	K	.	.	.	N	A	.	R	.	V	–	.	.	.	.	.	.	I	.	.	S
15. NI RS1	Y	.	V	Q	.	Q	Q	Q	Y	K	S	V	–	.	.	V	.	.	.	I	.	.	.
16. I MS1	A	L	.	Q	.	.	.	G	.	Q	K	.	–	.	F	V	.	.	.	I	.	.	.
17. I MS2	E	V	E	R	M	Q	K	E	.	K	.	V	–	.	.	.	.	.	.	I	.	.	.
18. I MS3	V	V	R	.	.	Q	A	N	.	.	.	V	–	.	.	.	.	.	.	I	.	.	.
19. I MS4	V	V	E	R	.	L	E	E	.	R	F	V	–	.	.	.	.	.	.	I	.	.	.
20. I MS5	V	V	K	Q	.	Q	D	E	.	R	.	V	–	.	.	.	.	.	.	I	.	.	.
21. I MS6	V	V	R	Q	.	Q	G	E	.	K	V	V	–	.	.	.	.	.	.	I	.	.	.
22. I RS1	A	V	K	.	.	Q	A	E	.	V	K	V	–	.	F	V	.	.	.	I	.	.	.
23. I RS2	A	L	E	T	F	S	R	D	.	T	R	.	–	V	F	V	.	.	.	I	.	.	.
24. NI RS2	A	V	E	N	.	V	D	R	Y	G	S	V	–	.	.	V	.	.	.	I	.	.	.
25. NI RS3	V	L	K	T	.	Q	A	S	.	N	K	V	–	.	F	V	.	.	.	I	.	.	.
26. BI1	R	E	H	Q	.	Q	Q	H	D	P	A	V	–	.	.	.	.	.	.	I	.	Q	P
27. BI2	H	P	K	.	T	N	N	G	Q	R	S	K	R	P	F	F	.	.	.	I	.	.	.
28. BI3	–	–	–	–	–	–	–	–	D	C	T	R	–	S	Q	W	K	.	.	I	.	.	R
29. BI4	A	V	R	–	–	–	–	–	–	–	–	–	–	–	–	R	.	.	.	I	.	.	.
30. BI5	H	P	K	.	T	N	N	G	Q	R	S	K	R	P	F	F	.	.	.	I	.	.	.
31. BI6	H	P	K	.	T	N	N	G	Q	R	S	K	R	P	F	F	.	.	.	I	.	.	.

Figure 1 (a) Schematic representation of P_{IB}-type ATPases. Arrows indicate the relative position of the copA primer pair. HMA: Heavy metal–associated domain. ZntA: P-Type ATPases family. Not to scale. (b) Alignment of described sequences and sequences from environmental isolates and metagenomic DNA libraries. Boxes indicate the conserved DKTGT and GDGXNDX domain. Dots indicate the same amino acid. Sequence on top corresponds to consensus sequence.

transport ATPases and have been detected in many organisms (see as examples, Petersen and Moller 2000; Solioz and Stoyanov 2003). However, the presence of copper-resistance genetic determinants such as *copA*-like genes (Petersen and Moller 2000; Adaikkalam and Swarup 2005) in environmental microbial samples is practically

unknown. This is mainly because of the unavailability of molecular tools for profiling such gene sequences. In this work, we designed a polymerase chain reaction (PCR) primer pair to target copper P_{IB}-type ATPases gene sequences, and tested it in environmental isolates and metagenomic DNA of intertidal marine samples from copper polluted areas. With this molecular marker, we detected novel copper P_{IB}-type ATPases gene sequences and successfully applied the terminal restriction fragment length polymorphism (T-RFLP) molecular fingerprinting technique. The high specificity of the designed primers to detect the P_{IB}-type ATPases gene provides a reliable tool to track and compare microbial copper resistance in diverse environments.

Materials and methods

Design of PCR primers for the amplification of copper P_{IB}-type ATPases gene sequences

DNA sequences were obtained from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov) and compared using the VECTOR NTI 8 software (Invitrogen, Carlsbad, CA, USA). One cluster composed of five copper P_{IB}-type ATPases gene sequences showing high similarity (ranging from 88 to 35% based on amino acids) was initially selected for primer design. This gene cluster included genes from *Corynebacterium glutamicum* (BAF53086), *Corynebacterium jeikeium* (CAI37602), *Rubrobacter xylanophilus* (ABG03112), *Desulfovibrio vulgaris* (AAS96797) and *Deinococcus geothermalis* (ABF44026). After the detection of the most conserved region between these sequences, one primer pair: copAUF (5′-GGT GCT GAT CAT CGC CTG-3′) and copAUR (5′-GGG CGT CGT TGA TAC CGT-3′), amplifying a sequence of about 750 bp, was selected after an *in silico* test for copper P_{IB}-type ATPases gene detection against the NCBI database, using the BLAST algorithm (Altschul *et al.* 1997). PCR conditions were optimized using DNA from copper-resistant bacterial isolates obtained from copper polluted rocky shore samples. The PCR mixture (final volume 25 μl) and conditions have been described elsewhere (Pavissich *et al.* 2010). PCR tests were run in a Perkin-Elmer 2400 thermal cycler, and amplification products were visualized by electrophoresis on 1% agarose gels stained with SYBR safe (SYBR Green DNA-binding dye; Invitrogen).

Sediments and rock sampling

Samples used in this study were selected within the framework of a broader study covering several intertidal environments affected by severe copper enrichment (Ramírez *et al.* 2005; Andrade *et al.* 2006; Morán *et al.* 2008). Three different environments were selected for primer pair testing: mining-waste discharge canal sediments, intertidal rock samples and marine sediments (MS). The area selected for the study was a tailing deposit that operated from 1975 to 1989, which received more than 130 000 million metric tones of untreated copper mining wastes that persist to the present. Sediment samples (labelled as DC) were taken from the outlet of Canal Palito (the discharge canal) as described elsewhere (Pavissich *et al.* 2010). Intertidal rock samples of about 20 cm^2 of exposed surface were collected during low tide using a hammer and a chisel, in two different sites along the intertidal rocky shore near the copper enriched area: Palito (26°15·8′S; 70°40·6′W) located at <200 m to the south of the discharge canal, defined as an impacted site (I-RS), and Pan de Azúcar (26°8·2′S; 70°39·3′W), 15 km north from the copper-impacted area, defined as nonimpacted site (NI-RS) because it is placed outside the influence of the mine tailings affecting Chañaral Bay (Andrade *et al.* 2006). In each site, six different rock samples were taken and stored in 50-ml sterile plastic polypropylene tubes. MS were collected in two different sandy beaches near Chañaral: La Lancha, an artificial beach formed mainly by untreated copper mine tailings (Correa *et al.* 1999), selected as a copper-impacted marine sediment site (I-MS), and Playa Blanca, located inside the Pan de Azúcar National Park, selected as nonimpacted site (NI-MS), because of its normal copper levels (Ramírez *et al.* 2005). In each MS site, four 2-m depth sediment cores separated by 50 m were taken during low tide using a drill. Pore water samples were collected from each core. Samples for DNA extraction (100 g in 50 ml sterile polypropylene tubes) were taken from each core at 0·1, 0·5, 1 and 2 m depth. All samples were stored in the dark at 4°C and rapidly transported to the laboratory for analyses.

Isolation and characterization of bacterial strains

Copper-resistant strains were isolated from the surface of intertidal rock samples exposed to copper. These samples were sonicated for 10 min in 1× TBE (Tris-borate EDTA) buffer (89 mmol l^{-1} Tris, 89 mmol l^{-1} boric acid, 2 mmol l^{-1} EDTA, pH 8), using an ultrasound bath (Tru-Sweep™, 50/60 Hz; Crest, Trenton, NJ, USA). The rock surface extracts were serially diluted, spread onto R2A agar plates and incubated for 4 days at 30°C. Bacterial colonies were purified after two transfers to R2A agar plates. A minimal inhibitory concentration test (MIC) was performed in a range between 0·25 to 4 mmol l^{-1}, using a 100 mmol l^{-1} copper sulfate stock solution and minimal salts vitamins pyruvate medium (Teitzel and Parsek 2003). DNA from bacterial isolates that showed

copper resistance (growth at a concentration higher than 0·5 mmol l^{-1} copper, with *Escherichia coli* DH5α as a noncopper resistant reference strain) was obtained using thermal lyses (Ausubel *et al.* 1994). The copper-resistant strains were ribotyped by restriction fragment length polymorphisms (RFLP) analysis of the 16S rRNA gene sequence PCR product obtained from the respective genomic DNA, using primers 8F and 1492R described by Lane (1991), digested with the *Msp*I or *Hha*I endonucleases. PCRs and amplification conditions were as previously reported (Pavissich *et al.* 2010). One clone of each ribotype group was selected for further identification through 16S rRNA gene sequencing.

DNA extraction

UltraClean® soil DNA isolation kit (Mo Bio Laboratories Inc., Solana Beach, CA, USA) was used to extract the total community DNA from 1 g of DC or MS samples, according to the protocol provided by the manufacturer. Intertidal rock samples were sonicated in 20 ml 1× TBE buffer (89 mmol l^{-1} Tris, 89 mmol l^{-1} boric acid, 2 mmol l^{-1} EDTA, pH 8) in an ultrasound bath (Tru-Sweep™, 50/60 Hz; Crest), for 10 min in the same plastic tubes used for transport. The liquid was then collected in sterile 50-ml polycarbonate centrifuge tubes (Nalgene Company, Rochester, NY, USA), centrifuged for 15 min at 18 000 ***g*** and the pellet re-suspended in 200 μl of 1× TBE buffer. A modified version of the method for DNA extraction described by Ausubel *et al.* (1994) was used for intertidal rock samples. Briefly, 400 μl of CTAB (cetyl trimethyl ammonium bromide) solution (1% CTAB, 0·75 mol l^{-1} NaCl, 50 mmol l^{-1} Tris pH 8, 10 mmol l^{-1} EDTA) and 3 μl of proteinase K (100 μg ml^{-1}) were added and the mixture was incubated at 60°C for 1 h. After addition of 2% SDS, the mixture was incubated for 1 h at 60°C, extracted three times with 1 ml of phenol–chloroform–isoamylic alcohol (25 : 24 : 1) and centrifuged at 18 000 ***g*** for 1 min. Phenol was removed by extraction with 1 ml chloroform and centrifuged at 18 000 ***g*** for 1 min. Total community DNA from the aqueous phase was precipitated with 1·5 volumes of ethanol and left overnight at −20°C. Pellets were collected by centrifugation at 18 000 ***g*** for 30 min, washed with 70% ethanol, centrifuged for 15 min at 18 000 ***g***, vacuum dried and re-suspended in 50 μl sterile MilliQ water (Millipore, Bedford, MA). DNA integrity and concentration was estimated by electrophoresis on 1% agarose gels stained with SYBR safe (Invitrogen).

DNA sequence analysis

P-type copper ATPases and 16S rRNA gene sequences PCR products were cloned into the TOPO-TA cloning vector (Promega Corp., Madison, WI, USA), using the manufacturer's procedure. In the case of P-type copper ATPases clone libraries, clones were grouped into phylotypes by RFLP using the *Alu*I endonuclease. One representative clone for each phylotype was sequenced. Selected clones were sequenced (Macrogen, Seoul, Korea), and sequence analyses were carried out using MEGA 4 software (Tamura *et al.* 2007). Sequences were aligned using the multiple alignment algorithms CLUSTALW (Thompson *et al.* 1994). Clone library coverage was determined using the formula $C = 1 - (n_1/N)$, where n_1 is the number of unique phylotypes, and N is the library size (Kemp and Aller 2004). Dendrograms for P-type copper ATPases were constructed using the neighbour-joining method with a bootstrap test of 1000 iterations, including only sequences of P_{IB}-type ATPases with biochemical evidence of function. GenBank accession numbers for the sequences obtained in this work are EU489611–EU489630.

Terminal restriction fragment length polymorphisms (T-RFLP) analysis

For T-RFLP analysis, the primer *cop*AUF was labelled at the 5′ end with the FAM fluorochrome. The PCRs and conditions were the same as described previously, but in a final volume of 50 μl. An *in silico* endonuclease restriction site analysis indicated that *Alu*I was the best enzyme to discriminate among different copper P_{IB}-type ATPases sequences. Each PCR product was digested with 20 U of *Alu*I in a total volume of 20 μl at 37°C for 3 h. Digested PCR products were mixed with 3 mol l^{-1} sodium acetate, pH 5·2 (0·1 vol) and ethanol (2·5 vol), incubated for 1 h at −80°C to precipitate DNA, centrifuged for 15 min at 18 000 ***g*** at 4°C, washed with 70% (v/v) ethanol, centrifuged for 15 min at 18 000 ***g*** and vacuum dried. A total of 40 ng of digestion product from each sample was analysed. DNA fragments were separated by capillary electrophoresis using a Perkin-Elmer ABI Prism 310 sequencer and fragment sizes were determined using the internal standard ROX 500 as reference. Analysis of the *Alu*I T-RFLP profiles, nonmetric multidimensional scaling (NMDS), and the analyses of similarity (ANOSIM) to examine the statistical significance of grouping was carried out as described elsewhere (Clarke 1993; Rees *et al.* 2004; Pavissich *et al.* 2010). Briefly, each raw data set consisted of peaks reflecting the size of terminal restriction fragments (T-RFs), measured in nucleotides, and the area of each peak measured in fluorescence units. The T-RF areas were analysed with the multivariate statistical software Primer 5 (Primer-E Ltd, Plymouth, UK), and NMDS analyses were used to group data according to their similarity as described by Clarke (1993) and Rees *et al.* (2004). ANOSIM were carried out to examine the

Table 1 Mismatches analysis of the copAUF and copAUR primer sequences with: (A) sequences used to generate the primer pair (bold) and with well characterized sequences. (B) *In silico* detected copper P-type ATPases and (C) *copA* gene sequences from bacterial isolates

A

Organism	Gene name	Gene product	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
			GGTGCTGATCATCGCCTG	**ACGGTATCAACGACGCCC**
Deinococcus geothermalis DSM 11300 (CP000358)	Dgeo_2592	Heavy metal P-type ATPase	GGTGCTGATCAT**T**GCCTG	ACGG**C**ATCAACGA**T**GCCC
Corynebacterium glutamicum R (AP009044)	cgR_0125	Hypothetical protein	**C**GTGCTGATCATCGCCTG	ACGGT**G**TCAA**T**GACGCCC
Corynebacterium jeikeium k411 (CR931997)	copB	Copper-exporting ATPase	GGTGCTG**G**TCATCGCCTG	ACGGT**G**TCAA**T**GACGCCC
Rubrobacter xylanophilus DSM 9941 (CP000386)	Rxyl_0133	P-type copper transporter	GGTGCT**C**ATCATCGCCTG	ACGG**C**ATCAACGACGCCC
Desulfovibrio vulgaris subsp. vulgaris (AE017285)	DVU2324	Copper-translocating P-type ATPase	GGT**CA**TG**G**TCATCGCCTG	ACGGTATCAACGACGCCC
Escherichia coli K-12 (NC_000913)	copA	Copper transporter	GGT**A**CTGATT**A**TT**G**CCTG	ACGG**C**AT**T**AACGACGC**G**C
Salmonella typhimurium (AF067954)	silP	Cation transporting P-type ATPase	GGT**C**CTGATT**A**TT**G**CCTG	ACGGT**G**T**G**AATGAT**G**CCC
Bacillus subtilis (NC_000964)	ycnJ	Copper exporting protein	GGTTCT**TG**TCATT**G**CCTG	ACGG**A**AT**T**AACGAT**G**C**G**C
Enterococcus hiriae (L13292)	copB	Copper transporting ATPase	**C**GTG**TT**CATCAT**T**GC**T**TG	ACGG**C**ATCAA**T**GAT**G**C**G**C
Helicobacter pylori (NC_000915)	cadA	Cadmium-transporting P-type ATPase	GG**ATTT**A**A**TCATCGC**AG**G	AT**G**G**C**ATCAA**T**GACGC**T**C
Saccharomyces cerevisiae S288C (AAB64451)	Ccc2p	Cation transporting ATPase	**T**GT**TG**TCATC**G**TCGC**A**TG	AT**G**G**C**AT**T**AACGACGC**T**C

B

Organism	Gene name	Gene product	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
Streptomyces avermitilis MA-4680 (BA000030)	*cop*A	Copper transporting P-type ATPase	GGTGCTGATCATCGCCTG	ACGG**GG**TCAACGACGC**GG**
Nocardioides sp. JS614 (CP000509)	Noca_3746	Copper transporting P-type ATPase	GGTGCTGATCATCGCCTG	ACGG**CG**TCAACGACGC**G**C
Mycobacterium ulcerans Agy99 (CP000325)	*ctp*V	Cation transporting P-type ATPase	GGTGCTGATCATCGCCTG	ACGG**CG**TCAACGACGCCC
Bifidobacterium longum NCC2705 (AE014295)	*sil*P	Copper transporting ATPase	**T**GTGCTGATCATCGCCTG	ACGG**C**ATCAACGACGCCC
Synechococcus sp. JA-2-3B′ (CP000240)	CYB_2346	Copper transporting P-type ATPase	GGTGCTGATCATCGCCTG	AT**G**G**C**AT**T**AACGAT**G**CT**C**
Synechocystis sp. PCC 6803 (BA000022)	sll1920	Cation transporting P-type ATPase	**A**GTGCTGATCATCGCCTG	AT**G**G**C**AT**T**AACGAT**G**CT**C**
Deinococcus geothermalis DSM 11300 (CP000358)	Dgeo_2592	Heavy metal P-type ATPase	GGTGCTGATCATCGCCTG	ACGG**C**ATCAACGA**T**GCCC
Symbiobacterium thermophilum IAM 14863 (AP006840)	STH2616	Copper transporting P-type ATPase	GGTGCTG**G**TCATCGCCTG	ACGGC**A**TCAACGACGC**G**C
Ruegeria pomeroyi DSS-3 (CP000031)	SPO0794	Copper-translocating P-type ATPase	GGTGCTGATCATCGCCTG	AT**G**G**C**ATCAACGAT**G**C**G**C
Sinorhizobium meliloti 1021(AL591985)	*atc*U2	Copper transporting P-type ATPase	GGTGCTGATCATCGCCTG	ACGG**C**ATCAA**T**GAT**G**C**G**C
Bradyrhizobium sp. ORS278 (CU234118)	*act*P	Copper-translocating P-type ATPase	**C**GTGCTGATCATCGCCTG	ACGG**CG**TCAACGACGC**G**C
Novosphingobium aromaticivorans DSM 12444 (CP000248)	Saro_2142	Heavy metal P-type ATPase	**T**GTGCTGATCATCGCCTG	ACGGT**G**T**G**AACGAT**G**CCC
Methylibium petroleiphilum PM1 (CP000555)	*cop*F	HMA domain P-type ATPase	**C**GTGCTGATCATCGCCTG	ACGG**C**ATCAACGAT**G**C**A**C
Acidovorax sp. JS42 (CP000539)	Ajs_2710	Heavy metal P-type ATPase	**C**GTGCTGATCATCGCCTG	AT**G**G**C**ATCAACGAT**G**C**G**C
Enterobacter sp. 638 (CP000653)	Ent638_0962	Copper-translocating P-type ATPase	GGTGCTGATCATCGCCTG	ACGG**C**ATCAACGAT**G**C**A**C
Shewanella sp. MR-7 (CP000444)	Shewmr7_2654	Copper-translocating P-type ATPase	**C**GTGCTGATCATCGCCTG	ACGGTATCAACGAT**G**C**A**C
Chromohalobacter salexigens DSM 3043 (CP000285)	Csal_3006	Copper-translocating P-type ATPase	**C**GTGCTGATCATCGCCTG	AT**G**G**C**ATCAACGACGC**G**C
Hahella chejuensis KCTC 2396 (CP000155)	HCH_06735	Copper-translocating P-type ATPase	**C**GTGCTGATCATCGCCTG	ACGG**CG**TCAATGACGC**T**C
Desulfovibrio vulgaris (CP000527)	Dvul_0934	Heavy metal P-type ATPase	GGTG**A**TG**G**TCAT**A**GCCTG	ACGGTATCAACGACGCCC

Table 1 (Continued)

Isolate	*copA* sequence	Gene product	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
BI-1	*Rubrobacter xylanophilus* DSM 9941 (CP000386)	P-type copper transporter	GGTGCTC**AT**CATCGCCTG	ACGG**C**ATCAACGACGCCC
BI-2	*Erythrobacter litoralis* HTCC2594 (CP000157)	Copper/silver transporting P-type ATPase	GGT**C**CTGATCATCGC**TT**G	ACGG**G**ATCAACGA**TG**C**T**C
BI-3	*Pseudomonas mendocina* ymp (CP000680)	Copper transporting P-type ATPase	GGTGCT**AA**TCATC**A**T**GG**TG	ACGG**C**ATCAACGA**TG**C**G**C
BI-4	*Acidovorax* sp. JS42 (CP000539)	Heavy metal P-type ATPase	**C**GTGCTGATCATC**TG**CCTG	ACGG**C**ATCAACGA**TG**C**G**C
BI-5	*E. litoralis* HTCC2594 (CP000157)	Copper/silver transporting P-type ATPase	GGT**C**CTGATCATCGC**TT**G	ACGG**G**ATCAACGA**TG**C**T**C
BI-6	*E. litoralis* HTCC2594 (CP000157)	Copper/silver transporting P-type ATPase	GGT**C**CTGATCATCGC**TT**G	ACGG**G**ATCAACGA**TG**C**T**C

statistical significance of grouping (Clarke 1993; Rees *et al.* 2004). The output statistics, *R* values for pair-wise comparisons among groups, ranges from 0 when there is no separation of the community structure because of the factor analysed to 1 when total separation takes place (Clarke 1993). This statistics allows establishing a hierarchy of the factors under study by sorting them according to the *R* values as follows: $R > 0{\cdot}5$ accounts for high differences, whereas differences between 0·25 and 0·5 are considered moderate.

Results

Design of PCR primer pair targeting copper P-type ATPases (copA-like) sequences

Sequences encoding the *copA* bacterial gene were aligned and compared by cluster analysis to determine groups of sequences with high internal similarity (data not shown). A cluster with five highly similar *copA* gene sequences was then selected for primer pair design; copAU-F aligns at the 1419 position and the copAU-R primer aligns at the 2159 position, using the *E. coli* K-12 *copA* gene (NC_000913) as a reference. The forward and reverse primer pair sequences have 1–3, and 1–2 mismatches, respectively, with respect to the five bacterial *cop*A sequences used to generate it (Table 1A, bolded sequences). The number of mismatches only slightly increased when *copA* gene sequences from other microbial models were analysed (Table 1A). When this primer pair sequence was blasted against the GenBank database, P_{IB}-type ATPases gene sequences related to copper resistance were found for organisms belonging to the *Actinobacteria*, *Cyanobacteria*, *Deinococcus-Thermus*, *Firmicutes* phyla and the α-, *β*-, *γ*- and *δ*-proteobacterial classes (Table 1B). All these sequences showed a high similarity with this primer pair, with 1–3 mismatches for the forward primer and 1–4 for the reverse primer, and gave a predicted amplification product of 733–766 bp, thus indicating that this primer pair was potentially useful to detect a larger range of *copA*-like gene sequences.

Detection of copper P-type ATPases gene sequences in copper-resistant environmental bacterial isolates

Sixteen copper-resistant bacterial strains (MIC > 0·5 mmol l^{-1}), isolated from intertidal rock samples exposed or not to copper pollution and belonging to different ribotype groups, were tested for the presence of P_{IB}-type ATPases gene sequences with the copA primer pair. Six strains, named BI-1 to BI-6, gave a unique PCR product of the expected size (*c.* 750 bp). The sequences of the copA PCR products showed 63–78% amino acid identity

with reported copper P_{IB}-type ATPases sequences and had 1–3 mismatches with the primer pair (Table 1C). These six new sequences displayed the conserved motifs for phosphorylation and ATP binding (Fig. 1b), indicating they correspond to P-type ATPases.

Detection of copper P_{IB}-type ATPases gene sequences in metagenomic DNA

Metagenomic DNA from sediments of a copper mining-waste discharge canal (DC), copper-impacted beach sediments (I-MS) and copper-impacted (I-RS) and nonimpacted (NI-RS) intertidal rocky shore biofilms were used to test copA primer pair performance. A unique PCR product of the expected size was obtained in the four samples. *copA* gene sequence libraries were prepared using the corresponding PCR products. The RFLP analysis of 30 clones from each library allowed the detection of three, six, three and two unique phylotypes for the DC, I-MS, I-RS and the NI-RS samples, respectively. Clone library coverage ranged between 80 and 90%. Although these values are low for diversity studies, they are high enough to test the use of the designed primers in environmental DNA samples. Sequence analysis of the unique clones showed that all the sequences displayed the conserved motifs for phosphorylation and ATP binding for P-type ATPases (Fig. 1b), further confirming the specificity of this primer pair. When these environmental sequences were aligned with P_{IB}-type ATPases sequences with reported biochemical function, two major clusters were observed (Fig. 2). One is composed of clone sequences DC1, I-MS2, DC3, I-MS3, I-MS4, I-MS5 and I-MS6. Clone sequences DC3, I-MS3, I-MS4, I-MS5 and I-MS6 form a distinct group of sequences inside this cluster, which does not include any of the well-characterized sequences analysed. The second cluster is composed of sequences DC2, I-RS1, I-MS1, I-RS2, I-RS3 and NI-RS1 (Fig. 2).

Copper-impacted and nonimpacted environments show different copper P_{IB}-type ATPases genes composition in the bacterial community

To test the use of the copA primer pair as a tool for functional community fingerprinting analysis, two different environments, MS and intertidal rocks exposed to copper pollution, were selected for study using the T-RFLP technique. Richness values (defined as the number of terminal restriction fragments, T-RFs) for the epilithic bacterial community from copper polluted and nonpolluted rocks were essentially the same (*c.* 30 operational taxonomic units). Whereas rock samples from an impacted area had a more homogeneous distribution of the relative abundances of the T-RFs, with a slight dominance in relative abundance of a T-RF of 450 bp (Fig. 3a, upper panel), rock samples from nonimpacted areas showed a dominance of one T-RF of 300 bp, and the presence of several, low abundance T-RFs (Fig. 3a, lower panel). MS were also analysed by *copA* gene sequences T-RFLP profiling. In this case, samples from different depths were taken from two sandy beaches impacted or not by copper. copA T-RFLP profiles from MS (Fig. 4a) had a higher number of T-RFs than those from rock surface samples. Furthermore, pristine sediments showed

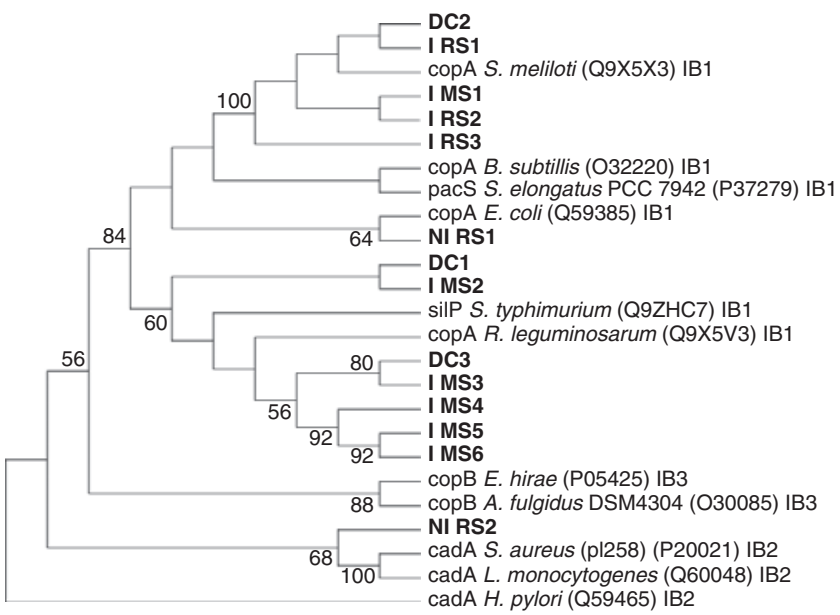


Figure 2 Dendrogram showing the similarity of different P_{IB}-type ATPases, constructed using the neighbour-joining method. Bold names correspond to sequences found in this work.

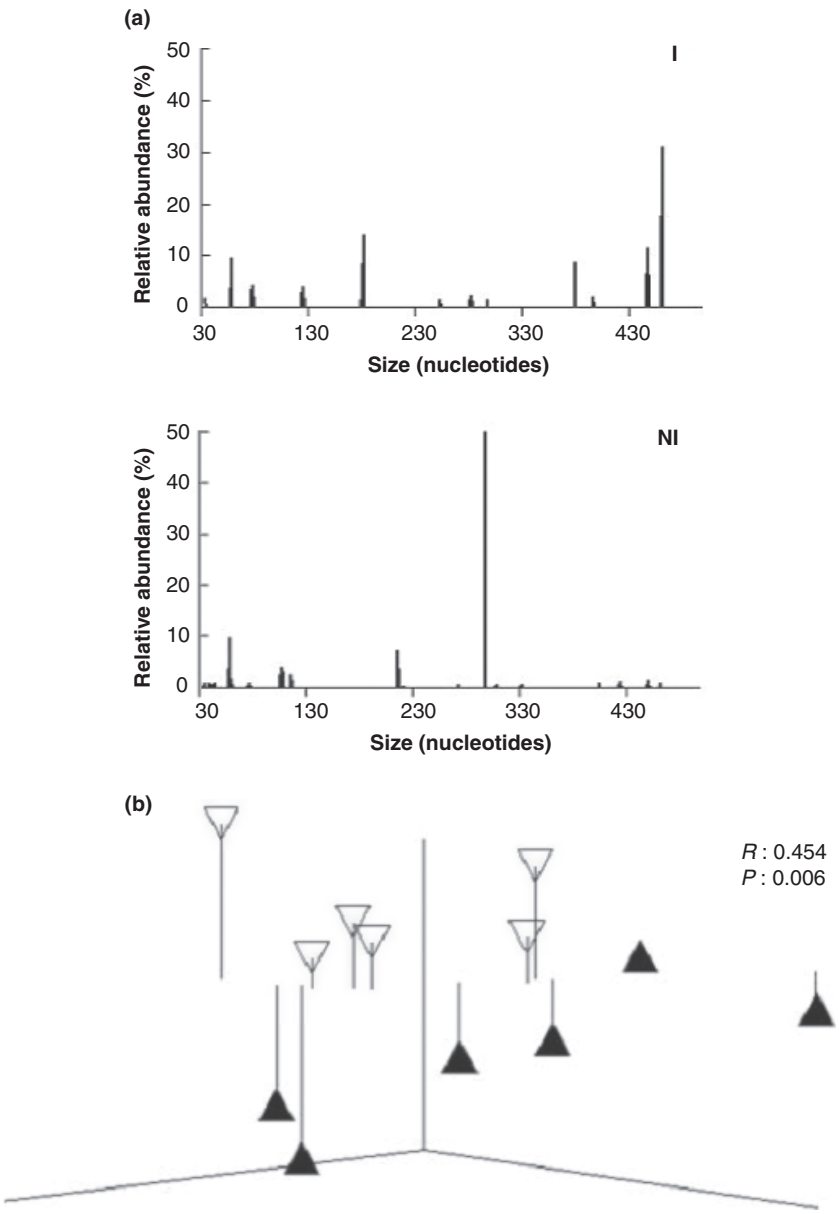


Figure 3 (a) T-RFLP profiles of impacted (I) and nonimpacted (NI) rock surface bacterial communities, using the copA-labelled primer. (b) Nonmetric multidimensional scaling plot showing the grouping of I (closed triangles) and NI (open triangles) T-RFLP profiles. Each triangle corresponds to each of the six samples. *R* and *P* values correspond to an analyses of similarity analysis by copper factor. Bars in each triangle indicate the position in the vertical axis.

a higher number of *cop*A sequences. Only small differences among T-RFLP profiles were observed in impacted samples taken at different depths (Fig. 4, left). In contrast, the nonimpacted samples showed a variation in *copA* gene structure with depth, showing a significant increase in the abundance of T-RFs of 42 bp (from 11 to 24%), 45 bp (from 4 to 19%) and 78 bp (from 10 to 17%), and a significant decrease of a T-RF of 222 bp (from 16 to 3%) (Fig. 4, right).

Discussion

In this study, we developed and used a new molecular tool for the detection of genes involved in copper resistance. A PCR primer set was designed and tested for the detection of P-type like genes, using both bacterial isolates and metagenomic DNA. All P-Type ATPases protein sequences displayed a similar structure (Fig. 1): two heavy metal–associated domains, an ATPase domain and a hydrolase domain (Argüello *et al.* 2007). There are two conserved sequences in the hydrolase domain shared by all P-type ATPases: the DKTGT phosphorylation motif and the GDGXNDXP ATP-binding motif (Argüello *et al.* 2007) (Fig. 1). The nondegenerated PCR primer pair designed in this study targeted a region that includes both conserved sequences in the hydrolase domain. Six copper-resistant bacterial isolates tested in this study were negative for copA PCR amplification. It should be stressed

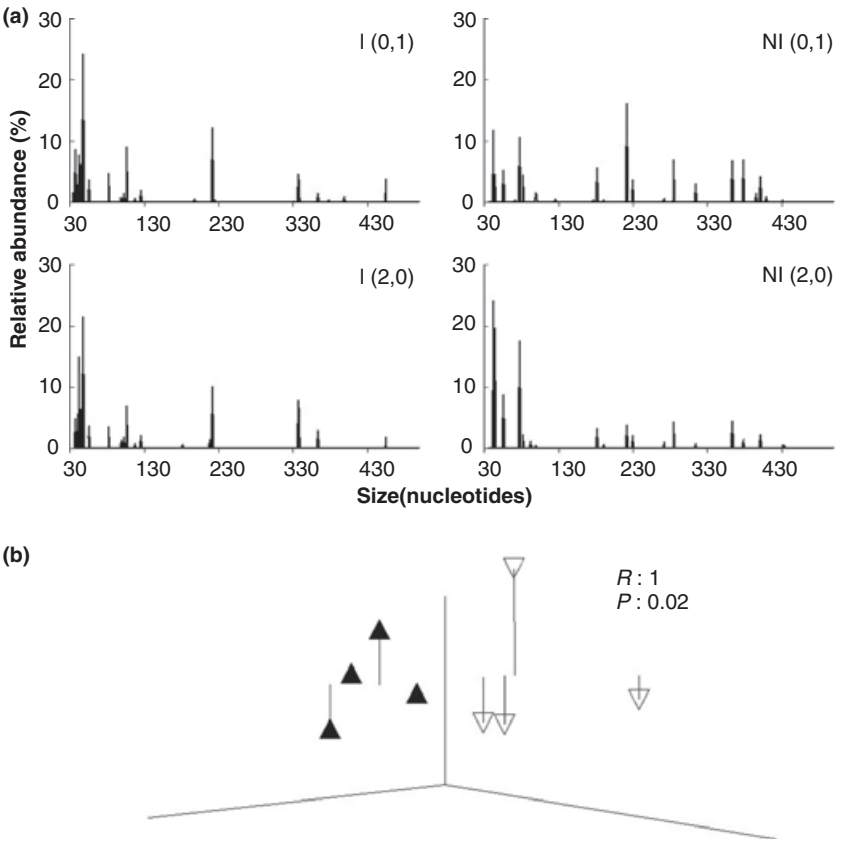


Figure 4 (a) T-RFLP profiles of impacted (I) and nonimpacted (NI) marine sediments bacterial communities, using the copA-labelled primers. Numbers indicate the depth of the sample. (b) Nonmetric multidimensional scaling plot showing the grouping of I (closed triangles) and NI (open triangles) T-RFLP profiles. Each triangle corresponds to one depth. *R* and *P* values correspond to an analyses of similarity analysis by copper factor. Bars in each triangle indicate the position in the vertical axis.

that any mismatch in one of the primers, especially in the 3′ end, could prevent amplification and therefore a negative amplification does not always means absence of a *copA* sequence. One possible explanation for the absence of PCR product is that this primer pair does not target their P_{IB}-type ATPases sequences. An alternative and more suitable explanation is the presence of other copper-resistance genetic determinants in those isolates, like copper-chelating ligands, periplasmic chelating proteins or biofilm formation (Moffett and Brand 1996; Kotrba *et al.* 1999; Gordon *et al.* 2000) that would not be targeted by the designed primers.

All environmental DNA tested gave amplification products whose sequence analysis confirmed them as P_{IB}-type ATPases (Fig. 1b). When these environmental sequences were aligned with P_{IB}-type ATPases sequences with reported biochemical function, two major clusters were observed (Fig. 2). One is composed of clone sequences DC1, I-MS2, DC3, I-MS3, I-MS4, I-MS5 and I-MS6. DC1 and I-MS2 clone sequences were close to *sil*P gene of *Salmonella typhimurium*, a gene related to silver and copper resistance (Gupta *et al.* 1999) and to the copper-resistance *copA* gene sequence of *Rhizobium leguminosarum* (Reeve *et al.* 2002). Clone sequences DC3, I-MS3, I-MS4, I-MS5 and I-MS6 form a distinct group of sequences, which does not include any of the well-characterized sequences analysed, but it is related with *copA* gene sequence of *Rh. leguminosarum* (Reeve *et al.* 2002). The second cluster is composed of sequences DC2, I-RS1, I-MS1, I-RS2, I-RS3 and NI-RS1. NI-RS1 clone sequence shows a good level of similarity with *E. coli copA* gene, a Cu(I)-translocating P-type ATPases related to copper homeostasis (Petersen and Moller 2000) and copper resistance (Fan *et al.* 2001). I-RS1, I-RS2, I-RS3, DC2 and I-MS1 clone sequences showed similarity with *copA* gene of *Sinorhizobium meliloti*, a gene related with copper resistance in low-pH copper induced toxicity (Reeve *et al.* 2002). One of these environmental sequences (NI-RS2) presented similarity to cadmium P-type ATPases. These results could indicate that this primer pair may also detect cadmium-resistance sequences. An alternative explanation would come from the ability of such pumps to transport cations other than cadmium (Argüello *et al.* 2007). In that case, it is possible that the detected sequence related to cadmium-type ATPase may also be a copper P-type ATPase.

The use of this primer pair for community profiling analysis of intertidal rocks and MS indicated that copper pollution increases the abundance of *copA* gene sequences already present in pristine environments (Figs 3b and 4b). This may be explained by a selection of genes involved in

copper resistance that are normally present at a low abundance in the community. The observation of a high diversity of genes encoding for copper adaptation in pristine environments is in agreement with a previous study using a *copA* gene-based primer pair, but designed only for proteobacterial sequences (Lejon *et al.* 2007).

In the case of MS, the large differences observed in the *copA* gene sequence composition of the bacterial communities from exposed and nonexposed sediments are probably because of the copper content, although differences in total nitrogen or phosphorus contents may also affect the functional structure of the community (R. De la Iglesia, S. Andrade, J. Correa and B. González, unpublished data). The effect of copper and other factors in taxonomic and functional structure of bacterial communities has been previously reported for other copper-polluted environments (De la Iglesia *et al.* 2006; Lejon *et al.* 2007; Morán *et al.* 2008). Copper-impacted MS do not display a change in composition according with depth as it was observed for nonimpacted MS (Fig. 4a). That result is explained by the presence of high copper levels along all the sediment profile, in which case a high selection pressure for copper-tolerant communities is expected, causing a more stable community across the depth profile.

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