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Abundance and diversity of copper resistance genes *cus*A and *cop*A in microbial communities in relation to the impact of copper on Chilean marine sediments

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ABSTRACT

Microorganisms have developed copper-resistance mechanisms in order to survive in contaminated environments. The abundance of the copper-resistance genes *cus*A and *cop*A, encoding respectively for a Resistance Cell Nodulation protein and for a P-type ATP-ase pump, was assessed in copper and non-copper-impacted Chilean marine sediment cores by the use of molecular tools. We demonstrated that number of *cop*A and *cus*A genes per bacterial cell was higher in the contaminated sediment, and that *cop*A gene was more abundant than *cus*A gene in the impacted sediment. The molecular phylogeny of the two copper-resistance genes was studied and reveals an impact of copper on the genetic composition of *cop*A and *cus*A genes.

1. Introduction

Copper is an essential trace element and serves as co-factor for numerous enzymes (Brown et al., 1997). Copper at high cytoplasmic concentrations can compete with other metals for their binding sites in proteins, leading to dysfunctional proteins (Kershaw et al., 2005). Moreover, Cu(I) present in cells can react with hydrogen peroxide, resulting in the production of hydroxyl radicals which damage lipids, DNA and other molecules (Harrison et al., 2000). Previous publications have already described modifications of microbial communities as being a consequence of copper contamination in soils (Ranjard et al., 2006), and in freshwater (Konstantinidis et al., 2003) or marine sediments (Gillan et al., 2005). In microorganisms, resistance to copper is mainly mediated by three different systems: the efflux ATP-ase pump CopA able to extrude copper ions (Rensing et al., 2000); the chemo-osmotic copper extrusion cus system, encoding especially for the CusA protein, belonging to the Resistance, Nodulation and cell Division (RND) family responsible for heavy metal export (HME–RND) (Piddock, 2006); and the periplasmic pco system, only present on plasmids and conferring high resistance to copper (Brown et al., 1995). The *pco* system encodes especially for PcoA, a multi-copper oxidase protein responsible for oxidation of Cu(I) in the periplasmic space.

Most studies concerning the field of copper-resistance genes have been carried out on pure cultures from microorganisms belonging to *Bacteria* phylum such as *Acidithiobacillus ferrooxidans* (Navarro et al., 2009), *Shewanella oneidensis* (Toes et al., 2008), *Escherichia coli* (Outten et al., 2001), or to *Archaea* such as *Sulfolobus solfataricus* (Villafane et al., 2009).

Only a few studies have been dedicated to the investigation of copper-resistance genes in impacted environments (Lejon et al., 2007; De la Iglesia et al., 2010) and describe preferentially the genetic structure of copper-resistance genes in relation to the increase in copper concentrations. To our knowledge, no publication has yet investigated the abundance of copper-resistance genes in environmental samples. Determination of the abundance of copper-resistance genes in copper-contaminated environments will give important information about the mechanisms used by microorganisms in such environments and about which mechanisms are preferentially present in impacted environments.

In this work, to better understand microbial copper-resistance genes in impacted sediments, the abundance and diversity of *cop*A and *cus*A genes in contaminated and uncontaminated Chilean marine sediments were studied.

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2. Materials and methods

2.1. Sediment sampling

Between 1938 and 1975, the Chanaral coastline received approximately 150 million tons of raw Cu mining flotation tailings from the mining activities of the copper mine El Salvador. In 1976, the tailing discharge point was moved 10 km north of Chanaral Bay to Caleta Palito, releasing more than 130 million tons of waste up to 1989. Two 25 cm-long sediment cores (10 cm diameter) were collected in October 2009 from 2 sites presenting different concentrations of copper (Fig. 1). The first site was located at the edge the Palito canal (and named Palito (26°15.8′S; 70°40.6′W)), while the second one was a reference site that was not copper-contaminated, and was located on an intertidal site on the beach of Flamenco (26°34.4′; 70°41.3) (Fig. 1).

The cores collected were used for porewater extraction, solid phase metal analysis. The first 10 cm section of each core was cut into 2 cm slices, and the remaining section of each core into 5 cm slices. Subsamples (1 g wet weight) of each slice were used directly for nucleic acid extraction, or frozen at −80 °C until subsequent molecular analysis. About 50 g of wet sediment subsamples were transferred into an acid-cleaned and nitrogen-purged plastic centrifuge bottle, and centrifuged at 8000 rpm for 30 min. Overlying water was removed from each centrifuge bottle and the remaining porewater was vacuum-filtered through a 0.45 μm polycarbonate membrane in a glove box purged with nitrogen. The collected porewater was then acidified with 0.5 ml of ultrapure HNO_3 for metal analysis.

2.2. Trace metal analysis: total and available metal concentration

Concentrations of heavy metals were determined by using inductively coupled plasma–atomic emission spectroscopy (ICP-AES; Varian Vista Pro, axial view) for Cu, Pb and Zn and by inductively coupled plasma–mass spectrometry (ICP-MS, X Series Thermo elemental) for Cd. The spectrometers were calibrated using standard solutions, and blank corrections were applied when necessary. The total contents of elements in the sediments were determined after the following total mineralization. Briefly, dried sediments were attacked first by 10 ml of a concentrated HF solution for 2 h at boiling point, then, after evaporation of these acids, by 10 ml of a freshly-prepared HNO_3/HCl mixture (1/2 v:v) to eliminate the remaining solid grains at boiling point. The solutions recovered were afterwards diluted in a known volume of ultrapure water (Milli Q plus) and analyzed using ICP-AES and ICP-MS. The Simultaneously Extracted Metals (SEM: Cd, Cu, Pb and Zn) were measured in the 50.0 ml aliquot removed from the sediment extract by the same technique ICP-AES or ICP-MS. These metals are weakly associated with the sediments, and are removed in a cold weak acid treatment (HCl 1 M) (Ankley et al., 1993); they can provide a proxy for potential metal bio-availability within the environmental community (Cooper and Morse, 1998; Simpson et al., 2000).

2.3. Determination of other auxiliary parameters

The pH and Eh were determined as a function of depth in the sediments by means of a glass microelectrode for pH and a platinum electrode for Eh. These indicator electrodes were both combined with an Ag/AgCl reference electrode with a potential equal to +0.22 V versus a hydrogen normal electrode (HNE). The electrodes were introduced inside holes (capped with rubber during the sampling operation) that were present all along the Perspex tube and corresponded to determined sediment depths. The results were read only after reaching the potential stability. Concentrations of dissolved Na^+ in porewater was determined using inductively coupled plasma-atomic emission spectroscopy (ICP-AES;

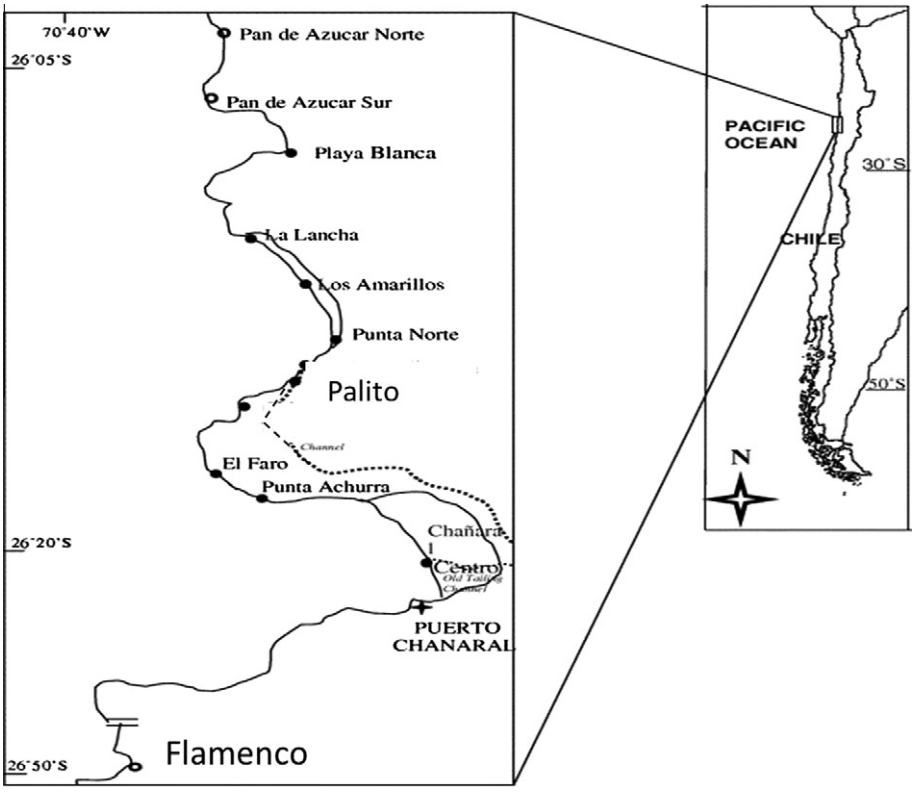

Fig. 1. Map of the area studied and location of sampling stations Palito and Flamenco.

Varian VistaPro, axial view). The concentrations of total organic carbon (TOC) was analyzed with a "TOC Shimadzu 5050" carbon analyzer (TOC = total carbon (TC) − total inorganic carbon (IC)). TOC Shimadzu 5050 calibration was performed using standard TC and IC solutions of 10, 25, 50, 100 mg/l respectively. TOC values were obtained by injecting 100 µl aliquots into the TOC analyzer. Measurement reproductibility was < to 5%. Concentrations of dissolved SO_4^{2-} in pore water was determined using inductively coupled plasma atomic emission spectroscopy (ICP-AES; Varian VistaPro, axial view).

2.4. RNA/DNA extraction and cDNA synthesis

Total nucleic acids were extracted from 2 g of sediment using the RNA® Powersoil kit (Mo-Bio, France) according to the manufacturer's protocol. DNA and RNA were then separated and purified using All Prep DNA/RNA Mini kit (Qiagen, France) based on manufacturer's protocol. All extracts were aliquoted and stored at −80 °C until further processing. 10 µL of extracted DNA was mixed with loading buffer and electrophoresed on 1% [w/v] agarose gel stained with ethidium bromide. The extracted DNA was compared to the amounts of DNA present in the Smart Ladder (Eurogentec, France); images of the gels were analyzed with Quantum-Capt software present on the Quantum-ST4 image acquisition system (Vilber Louma, Deutschland). DNase-treated RNA (0.5 µg) was reverse transcribed with High Capacity cDNA reverse transcription kit (Applied Biosystems, France) with MultiScribe™ Reverse Transcriptase and random primers. RT products were used immediately for PCR amplifications, possible DNA contamination of RNA extracts was checked by PCR amplification of aliquots of RNA without reverse transcription step. No DNA was detected in these controls.

2.5. Design of PCR primers for amplification of the cusA gene

DNA sequences were downloaded from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and aligned with ClustalW2 software. A group of 10 sequences was selected for the design of primers: *Klebsiella pneumoniae* 342 (gi|206575712:4954190-4957339), *E. coli* APECO1 (gi|117622295:585766-588909), *Burkholderia cenocepacia* J2315 (gi|206561868:489316-492522), *Pectobacterium atroscepticum* SCRI1043 (gi|50118965:c1567041-1563904), *Stenotrophomonas maltophilia* K279a (gi|190572091:2461510-2464650), *Pseudoalteromonas haloplanktis* TAC125 (gi|77358982:316904-320020), *Aeromonas hydrophila subsp.hydrophila* ATCC7966 (gi|117617447:3331559-3334687), *Aromatoleum aromaticum* EbN1 (gi|56475432:1275856-1279032), *Colwellia psychrerythraea* 34H (gi|71277742:5150754-5153912), *Alteromonas macleodii "Deep ecotype"* (gi|196154825:349114-352251). Two degenerated primers were created: cusF: 5′ATGCSACVGGYGTTGGCTGG 3′ and cusR: 5′ CCRTTCAGYTCGGCRATRCC 3′ to amplify a sequence of 410 bp. Polymerase chain reaction was carried out in a Perkin Elmer, Gene Amp, PCR System 9700. The selectivity of this primer pair was evaluated using the BLASTN program (Basic Logical Alignment Tool, http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1997) available from GenBank.

The PCR reaction was carried out as follows: 5 min at 95 °C, 30 cycles of 45 s at 95 °C, 45 s at 61 °C, 45 s at 72 °C and a final extension step of 7 min at 72 °C. PCR products were visualized on a 2% (w/v) agarose gel in 0.5 × TAE stained with ethidium bromide.

2.6. Construction of competitive plasmid for quantification of the copA gene

An amplicon of the *cop*A gene (765 bp) was amplified with primers copAUF (5′-GGT GCT GAT CAT CGCCTG-3′) and copAUR (5′-GGG CGT CGT TGA TAC CGT-3′) (De la Iglesia et al., 2010). An internal fragment of 295 bp was deleted using the restriction enzyme *Alu*I according to the manufacturer's protocol (Roche Applied Science, France).

The deleted and intact *cop*A amplicons were cloned into plasmid Pgem-T (Promega, France). Recombinant plasmids (named pCOPAtarg for the plasmid containing an intact *cop*A fragment with a size of 765 bp, and pCOPAcomp for the plasmid containing a deleted competitor *cop*A fragment with a size of 472 bp) were used to transform *E. coli* DH5-α according to the manufacturer's protocol. After growth of transformed cells in LB media at 37 °C for 24 h, plasmids were extracted with Wizard(R) Plus Minipreps DNA (PROMEGA, France) and linearized with *Pst*I (Roche Applied Science, France). Plasmid concentrations were visualized on agarose gel and quantified with Quantum-Capt software present on the Quantum-ST4 image acquisition system (Vilber Louma, Deutschland).

2.7. Construction of competitive plasmid for the quantification of the cusA gene

Competitor DNA was obtained by PCR as described by Ross et al. (1995). A first fragment of 500 bp located downstream from the *cus*A target gene from the *E. coli* strain ED1A was amplified by PCR using primers COMPCUSF: 5′-*atgcsacvggygttggctg*TGAAGGACAAACTGGAAACGC-3′ and COMPCUSR 5′- *ccrttcagytcggcratrcc* TCCTGCCCTTCCAGGGTGAAA-3′. The nucleotides written in italics correspond to the last 15 nucleotides of cusF and cusR primers described in the study; the other 15 nucleotides hybridize with the 5′ and 3′ ends of the 500 bp fragment. The competitor sequence of 540 bp was then obtained by PCR amplification of the 500 bp fragment with the cusF and cusR primers.

The two amplicons were cloned into Pgem-T (Promega, France), used to transform *E. coli* DH5-α, amplified, purified, linearized and quantified as described previously. They were named pCUSAtarg (for plasmid containing the *cus*A target gene of size 410 bp) and pCUSAcomp (for plasmid containing the *cus*A competitor gene of size 540 bp).

2.8. Quantification of cusA and copA genes and statistical analyses

The abundance of *cus*A and *cop*A genes present in DNA extracted from sediment samples was quantified separately by competitive PCR as described: 150 fg of each competitor plasmid was added to serial dilutions (10–625 fg) of the target sequence carried by pCUSAtarg or pCOPAtarg. Similarly, 150 fg of every respective competitor plasmid was added to 10 ng of DNA extracted from the sediments. PCR reactions were carried out in a 50 µl reaction mixture composed of 1X PCR buffer, 0.25 µM concentrations of each primer, 250 µM of dNTP, 2 mM of $MgCl_2$ and 1 U of GoTaq DNA Polymerase (Promega, France). Pixel intensities of PCR products were quantified from digitized gel images. A standard curve was constructed by plotting the log of the pixel intensity of DNA amplified from the serial dilution against the pixel intensity of the amplified competitor ([plasmid with target gene/plasmid with competitor gene]). The amount of target was then determined from the log of the pixel intensity ratio of the target gene from extracted sediment DNA versus the competitor gene DNA. The Kruskal–Wallis test was used to determine whether data sets were significantly different ($p < 0.05$).

2.9. Abundance of the 16S rRNA gene and statistical analyses

The 16S rRNA gene was quantified with real-time PCR (system Chromo4 detector, BIORAD, France) and GoTaq qPCR Master Mix (Promega). Total bacteria were quantified using the primers 63F:

5′CAGGCCTAACACATGCAAGTC-3′ (Marchesi et al., 1998) and BU16S4: 5′-CTGCTGCCTCCCGTAGG-3′ (derived from 341 F, Muyzer et al., 1993) to amplify a 314 bp DNA fragment, as described by Plassart et al. (2008). The PCR was carried out as follows: initial denaturation step (10 min at 95 °C), 40 cycles of denaturation (40 s at 95 °C), annealing (45 s at 64 °C) and elongation (30 s at 72 °C), and a final extension step (7 min at 72 °C).

Melting curve analyses were completed between 60 °C and 100 °C to determine whether there was a detectable primer-dimer contribution to the SYBR I Green fluorescence measurement. The procedures were performed in triplicate. The Kruskal–Wallis test was used to determine whether data sets were significantly different ($p < 0.05$).

2.10. Diversity of copper-resistance genes copA and cusA

*cop*A and *cus*A PCR products from depth 4–6 cm of the 2 sediment cores were cloned into pGEM-T-EASY cloning vector (Promega Corp, Madison, WI, USA) according to the manufacturer's procedure in order to construct 4 clone libraries. For each library, 64 clones were sequenced (GATC, Konstanz, Germany). Sequences were cleaned up, translated into amino acid sequences, and aligned with the multiple alignment algorithms ClustalX (Thompson et al., 1997). A phylogenetic tree was constructed using MEGA 4.0 software (Tamura et al., 2007), and distance-based evolutionary trees were constructed using Kimura's corrected similarity values in the neighbor-joining algorithm of Saitou and Nei (1987). The topography of the branching order within the dendrogram was evaluated by applying the Maximum Parsimony character-based algorithm in parallel, combined with bootstrap analysis, to a round of 1,000 samplings. The sequences obtained in this study have been deposited in the GenBank database under accession No. JX293052 to JX293121.

3. Results

3.1. Physico-chemical parameters

The total and available metal concentrations found in the two sediment cores are described in Table 1. According to the sediment quality criteria for the classification of sediments developed by the Hong Kong SAR Government (ETWB 2002), the Palito edge sediment is highly contaminated as it contains levels of copper (average concentration of 1500 mg/g) that are much higher than the Upper Chemical Excedance Levels (UCELs) assessed in contaminated marine sediment (Cu = 110 mg/g). Copper concentrations for the Flamenco sediment vary between 5 and 6 ppm. This sediment, used as a reference, is not contaminated, having metal concentrations under the Lower Chemical Excedance Levels (LCELs) (Cu = 65 mg/g). The concentrations of available copper remain much higher in the Palito sediment (a mean of around 282 mg/g) than in the Flamenco sediment (a mean of around 1.62 mg/g).

The pH and NaCl concentrations described in our study are similar in both sediment cores for each depth analyzed, but TOC concentration is about 17 times higher in the Palito sediment core (means of 349 and 20.32 mg/g respectively throughout the Palito and Flamenco sediment cores). Eh values indicate that reducing conditions are present only from the depth of 2 cm in the two sediment cores.

3.2. Design of PCR primer set targeting the copper-resistance gene cusA

All the genes belonging to the HME–RND family present several conserved nucleotide motifs; to create our primer sets, we chose two motifs specific to the *cus*A gene. The specificity of the primers was confirmed by the *cus*A clone libraries constructed later in our study (Fig. 2). The cusF and cusR primers contained respectively 3 and 4 degenerated nucleotides for primers that corresponded to 18 and 16 total degeneracies.

Ten *cus*A sequences were selected to design the primers; these sequences were aligned to determine the conserved nucleotides sequences .With the *cus*A gene from *K. pneumoniae* 342 as a reference, cusF aligned at the position 437 bp and cusR aligned at the position 833 bp; an amplicon of approximately 410 bp was obtained. To quantify the abundance of the *cus*A gene in the environment by competitive PCR (cPCR), a competitor plasmid was created containing the *cus*A competitor sequence.

3.3. Amplification efficiency of the different plasmids used for molecular quantification of the cusA and copA genes by competitive PCR

The amplification efficiency of the four plasmids (pCUSAtarg, pCOPAtarg, pCUSAcomp and pCOPAcomp) was checked before determining the abundance of *cop*A and *cus*A genes present in the environmental samples. Competitive PCR was performed by mixing the same amounts of competitive and target plasmids DNA for: 18, 22, 26 and 30 cycles. The amplification kinetics of the four amplicons were similar and reached the plateau phase after the same number of phases (30 cycles), confirming the accurate construction of the *cop*A and *cus*A competitors (data not shown).

3.4. Abundance of copper-resistance genes and total bacterial cell in sediments exposed to different copper concentrations

The abundance of *cop*A and *cus*A copper resistance genes was monitored by competitive PCR (Fig. 3). The detection limits for this method were 31 copies for *cus*A and 42 copies for *cop*A. These two genes were detected and quantified throughout the two sediment cores.

For the uncontaminated Flamenco site, *cus*A gene copy numbers were between 1.3×10^7 and 9.8×10^5 copies, whereas for the contaminated site maximum and minimum abundance was 1.8×10^7 and 6.2×10^5 copies. Throughout both sediments, means of

Table 1
Physico–chemical characteristics, total and available copper concentrations according to depth of the sediments of Palito and Flamenco. Values of each parameter are given as mean of triplicates.

Depth (cm)	NaCl (g/L)		TOC (mg/g)		pH		Eh (mV)		Total Cu (mg/g)		Available Cu (mg/g)	
	Flamenco	Palito	Flamenco	Palito	Flamenco	Palito	Flamenco	Palito	Flamenco	Palito	Flamenco	Palito
0–2	28.5	25.2	21.2	349.01	7.70	7.82	37	17	5	1410	0.9	251
2–4	28.5	25.2	21.0	349.01	7.70	7.82	−52	−87	5	1410	0.9	251
4–6	28.4	25.2	21.0	349.01	7.69	7.82	−145	−158	5	1410	0.9	251
6–8	28.3	25.1	20.9	349.01	7.68	7.82	−155	−165	5	1410	0.9	251
8–10	28.3	25.1	20.8	349.01	7.68	7.82	−158	−170	5	1586	0,9	318
10–15	28.3	25.1	20.5	349.05	7.65	7.91	−168	−175	6	1586	2.6	318
15–20	28.2	25.0	20.2	349.05	7.64	7.91	−176	−182	6	1586	2.6	318
20–25	28.1	25.0	20	348.97	7.62	7.85	−182	−190	6	1600	3.3	297

cusA Klebsiella pneumonieae 342	5'	A C G G C A C C G G C G T C G G C T G G 3'
cusA Escherichia coli APEC01	5'	A T G C C A C G G G T G T T G G C T G G 3'
cusA Oligotropha carboxidovorans 0M5	5'	A C G C A A C G G G C G T C G G A T G G 3'
cusA Aromatoleum aromaticum EbN1	5'	A T G C G A C C G G C G T C G G C T G G 3'
cusA Stenotrophomonas ùaltophilia K279a	5'	A T G C G A C A G G A C T G G G C T G G 3'
cusA Burkholderia cenocepacia J2315	5'	A T G C G A C A G G A C T G G G C T G G 3'
cusA Aeromonas hydrophila subsp. hydrophila ATCC	5'	A T G C T A C C G G G G T G G G C T G G 3'
cusA Pseudoalteromonas haloplanktis TAC125	5'	A T G C C A C C G G T G T T G G T T G G 3'
cusA Colwellia psychrerythraea 34H	5'	A T G C G A C A G G T G T T G G T T G G 3'
cusA Alteromonas macleodii "Deep ecotype"	5'	A T G C C A C A G G C G T T G G T T G G 3'
***consensus sequence* : cusF**	**5'**	**A T G C S A C V G G Y G T T G G C T G G 3'**
cusA Klebsiella pneumonieae 342	5'	G G T A T C G C C G A G C T T A A T G G 3'
cusA Escherichia coli APEC01	5'	G G C A T T G C C G A A C T G A A C G G 3'
cusA Oligotropha carboxidovorans 0M5	5'	G G A A T C A C C G A A C T C A A C G G 3'
cusA Aromatoleum aromaticum EbN1	5'	G G G A T C G C C G A A T T G A A C G G 3'
cusA Stenotrophomonas ùaltophilia K279a	5'	G G T A T C G C A G A G T T G G A T G G 3'
cusA Burkholderia cenocepacia J2315	5'	G G T A T C G C G G A A C T G A A C G G 3'
cusA Aeromonas hydrophila subsp. hydrophila ATCC	5'	G G C C T G G C C G A G C T C A A C G G 3'
cusA Pseudoalteromonas haloplanktis TAC125	5'	G G T A T T G C C G A A C T T A A T G G 3'
cusA Colwellia psychrerythraea 34H	5'	G G C A T T G C C G A G C T A A A T G G 3'
cusA Alteromonas macleodii "Deep ecotype"	5'	G G T A T A G C A G A G C T A A A C G G 3'
consensus sequence	5'	G G Y A T Y G C C G A R C T G A A Y G G 3'
Deduced sequence of : cusR	**5'**	**C C R T T C A G Y T C G G C R A T R C C 3'**

Fig. 2. Alignment of the selected bacterial *cus*A gene sequences downloaded from GENBANK and design of specific cusF and cusR primers.

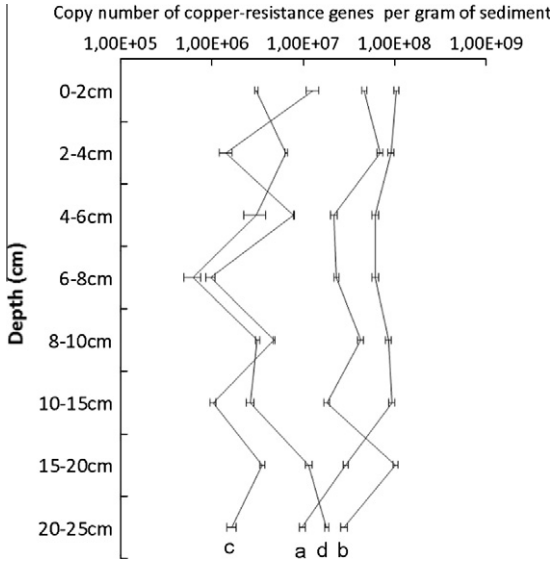

Fig. 3. Abundance of *cop*A and *cus*A genes per gram of sediment according to depth in Palito and Flamenco cores (a) *cop*A Flamenco sediment core; (b) *cop*A Palito sediment core; (c) *cus*A Flamenco sediment core; and (d) *cus*A Palito sediment core. Values are given as mean standard deviation of triplicates.

6.07×10^6 and 4.25×10^6 *cus*A copy numbers were found respectively for the Palito and Flamenco cores. No statistically significant difference was observed between the two sites (Kruskal–Wallis test; p-value > 0.05).

Concerning the *cop*A gene copy numbers, maxima and minima were 1.05×10^8 and 9.75×10^6 for the Flamenco sediment core, whereas those values were $4.68 \times 10^7$and 1.81×10^7 for the Palito sediment core. Mean averages of 4.41×10^7 and 6.72×10^7 *cop*A copies were found respectively for the Palito and Flamenco sediments. Overall, the abundance of the *cop*A gene along all the Flamenco sediment core was higher than in the contaminated site (except at the two deepest depths), but this result was not statistically significant (p-value > 0.05).

The abundance of total bacterial cell was determined by qPCR for all depths in both sediments by assuming that the average number of 16S rRNA gene copies per cell was 3.6 (Klappenbach et al., 2001), (Fig. 4). This abundance differed very little in the uncontaminated sediment whatever the depth; indeed, maximum and minimum bacterial cells were 1.52×10^9 and 9.78×10^8. For the Palito sediment core, total bacterial cell abundance varied between a minimum of 1.18×10^8 and a maximum of 9.33×10^8. Total bacterial cell abundance was always statistically higher at all depths of the Flamenco sediment site (p-value = 0.0007).

3.5. Ratio of copA/16S rRNA and cusA/16S rRNA

Ratios of both these copper-resistance gene copy numbers versus 16S rRNA copy numbers were calculated for both sites (Fig. 5).

The minimum and maximum percentages of *cop*A/16S rRNA copy numbers were 1.4% and 5.8% for the Palito sediment, and 0.23% and 2% for the Flamenco site. A mean of 3.41% was calculated throughout the Palito sediment core, while the mean was 1.48% in the Flamenco sediment site. The *cop*A/16SrRNA ratio was higher at each depth in the Palito sediment core (p-value = 0.0045).

In the Palito sediment core, the *cus*A/16S rRNA ratio was significantly higher than in the Flamenco core (p-value = 0.0045), except at depth 0–2 cm. The minimum and maximum percentages of *cus*A/16S rRNA copy numbers for the Palito sediment core were 0.14% and 0.9%, and for the Flamenco sediment core were 0.018% and 0.2%. The means were 0.40% and 0.09% for the Palito and Flamenco sediment cores respectively.

Overall, the relative abundance of the two copper-resistance genes was higher in the contaminated site. Moreover, the *cop*A gene was more abundant than the *cus*A gene for all depths (in oxic and anoxic conditions) and for both sites (p-value = 0.0007).

3.6. Impact of copper on the genetic structures of CusA and CopA

To investigate the diversity of *cus*A and *cop*A genes, four clone libraries were constructed from total DNA from depth 4–6 cm of each core. A total of 64 clones from each library were sequenced.

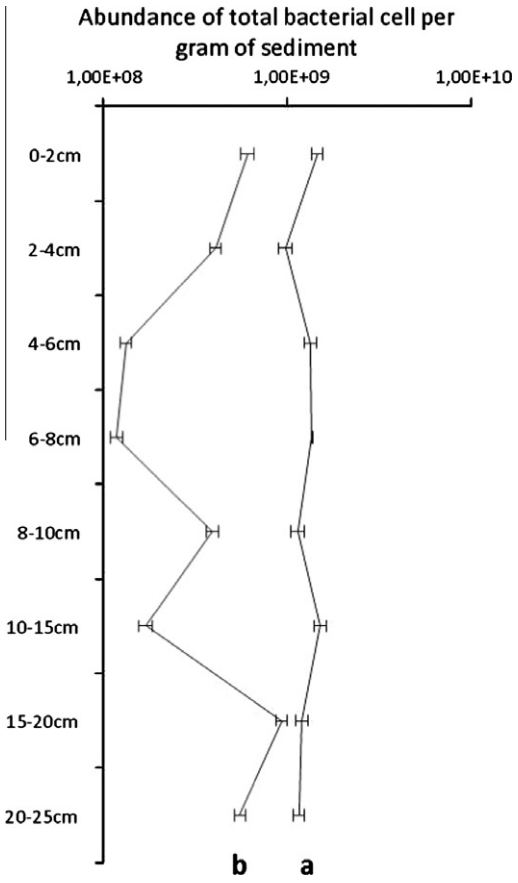

Fig. 4. Abundance of total bacterial cell per gram of sediment according to depth in Flamenco (a) and Palito sediment (b). Values are given as mean standard deviation of triplicates.

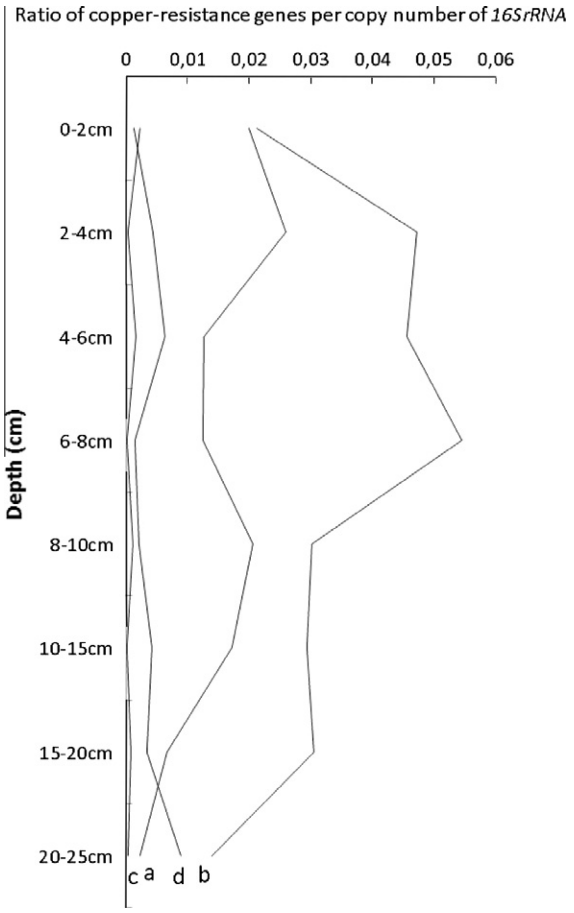

Fig. 5. Comparison of ratios of copy number *cusA*/16S rRNA and *copA*/16S rRNA according to depth for Palito and Flamenco: (a) *copA*/16S rRNA Flamenco sediment core; (b) *copA*/16S rRNA Palito sediment core; (c) *cusA*/16S rRNA Flamenco sediment core; and (d) *cusA*/16S rRNA Palito sediment core.

For the four clone libraries analyzed, rarefaction analyses were performed using PAST software (http://folk.uio.no/ohammer/past/), and a plateau phase was observed for each one, except for the Palito *cus*A clone library, demonstrating that a sufficient number of clones was analyzed (data not shown). The Shannon diversity index was calculated for the four clone libraries and was 1.705 and 0.57 for Flamenco and Palito *cus*A, and 1.303 and 1.578 for Flamenco and Palito *cop*A clone libraries, indicating a relatively low complexity for those communities.

Concerning the *cus*A gene library, the clones were grouped in 4 different clusters (Fig. 6). Clones from the uncontaminated sediment were divided into 2 clusters, while those from the contaminated sites were divided into 3 clusters.

Most of the clones from Flamenco (85% of the total clones) were grouped in Cluster I, which was composed of 7 OTUs; a minority group of 2 OTUs, representing 15% of the total clones, was located in Cluster III.

For the Palito core sediment, 6 OTUs were encountered. One of them was in the majority, representing 86% of the total clones, and was located in Cluster IV. Two OTUs, representing 6% of the total, were found in Cluster II. The three remaining OTUs were grouped in Cluster I and represented 8% of the total clones. The clones present in Clusters I and II defined 2 new CusA protein sequence clusters.

Concerning the *cop*A gene cloning and sequencing results (Fig. 7), 8 OTUs were found in the Palito sediment core and 12 OTUs in the Flamenco core. All the clones analyzed here were present in the three Clusters.

For the Flamenco sediment core, 10 OTUs, representing 25% of the total clones, were found in Cluster I. One OTU, encountered in Cluster II, represented 11% of the total clones. The remaining OTU (clone *cop*A Flamenco1) present in Cluster III, was in the majority, representing 64% of the total clones.

4. Discussion

The main goal of our work was to study and compare the abundance and diversity of two copper-resistance genes in metagenomic DNA extracted from uncontaminated and contaminated Chilean marine sediments. To analyze *cop*A copper-resistance genes, we used non-degenerated copAUF and copAUR primers designed by De la Iglesia et al. (2010). To amplify the *cus*A copper-resistance gene, two primers (cusF and cusR) were designed. Primers were created by aligning sequences of *cus*A genes originated from numerous different bacterial species, to detect the gene in a broad taxonomic group. It is generally accepted that degenerated primers, unlike non-degenerated primers, enable a higher diversity of genes to be amplified. The specificity of the primers was confirmed with the two clone libraries constructed from the non-contaminated and contaminated sites. Such specificity allowed us to quantify the amounts of *cus*A gene from contaminated and uncontaminated sediments, and to compare them with the copy numbers of 16S rRNA.

In order to quantify the total abundance of bacteria in heavy metal sediments, several studies have developed techniques such as DAPI counts (Gillan et al., 2005), the FISH method (Margesin et al., 2011) or the MPN cultivation technique. Nevertheless, to our knowledge, only a few studies have quantified the abundance

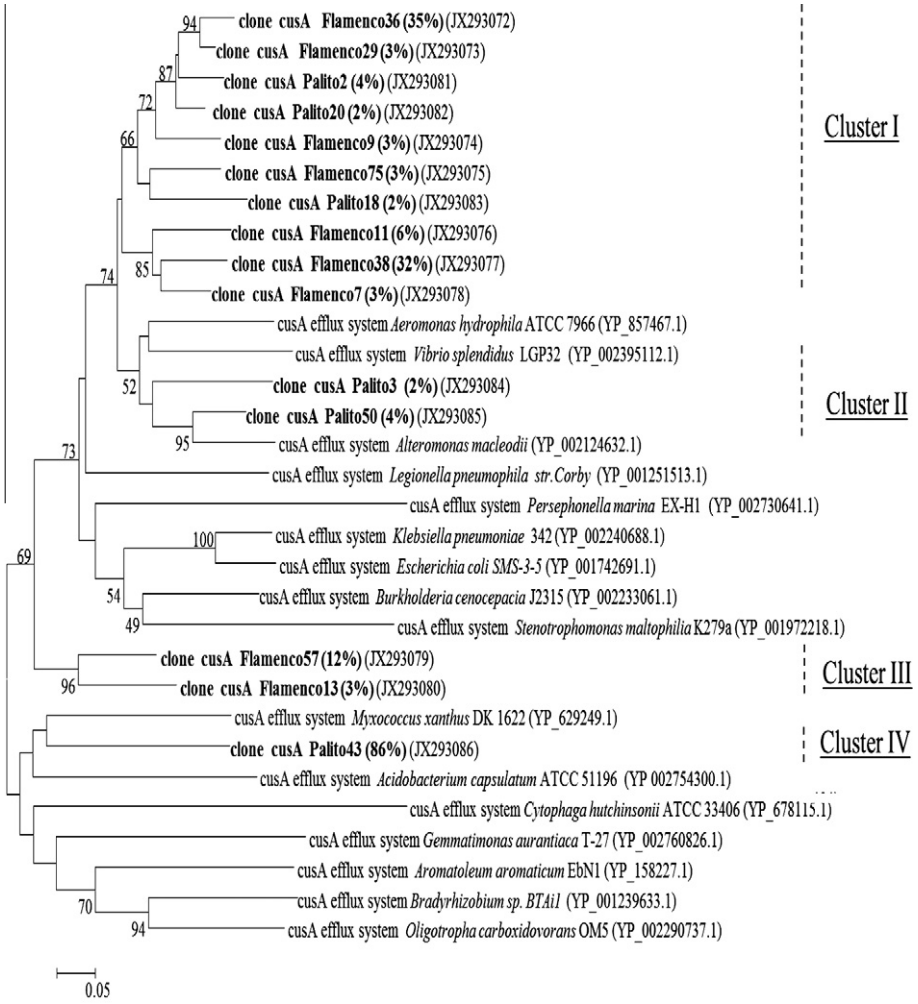

Fig. 6. Molecular phylogenetic analysis of CusA protein sequences obtained from cloning of PCR products of *cus*A gene. The phylogenetic tree was generated by the neighbor-joining method (the maximum-parsimony analysis gave very similar results). Bootstrap values of over 50% are shown at the nodes. The distance scale is the corrected proportion of amino acid changes (0.05 substitutions per amino acid).

of total bacteria by qPCR in a heavy-metal-contaminated sediment (Quillet et al., 2011). Results of quantification by qPCR revealed a significant statistical decrease (p-value = 0.0007) in the abundance of total bacteria when copper concentrations increased. Surprisingly, others previous studies dealing with abundance of total microorganisms in copper environments related no correlation between abundance of total bacteria and copper (Ranjard et al., 2006; Gillan et al., 2005). But despite high copper concentrations, microorganisms are still present in the Palito sediment, in the same proportions encountered in other heavy-metal-impacted sediments (Gillan et al., 2005; Quillet et al., 2011). The presence of this quantity of total bacteria is explained by the adaptation of microorganisms to copper contamination. This adaptation to highly impacted environments could be due in part to microorganisms developing different metal resistance mechanisms, such as bioaccumulation, biotransformation, or the expression of resistance genes (Diaz-Ravina and Baath, 1996).

In relation with the abundance of microorganisms resistant to copper contamination in those sediments, the abundance of copper-resistance genes *cop*A and *cus*A was determined by competitive Q-PCR. The presence of the *pcoA* copper-resistance gene, which encodes for a multi-copper oxidase protein of a third system involved in copper resistance, was tested by PCR, as described by Trajanovska et al. (1997) but was not detected.

Competitive PCR is an accurate, robust (Zentilin and Giacca, 2007) and sensitive method, and has already been used to quantify heavy-metal-resistance genes in impacted environments (Oger et al., 2001). Competitive PCR for was chosen because the size of the amplicon copA (765 bp) is superior to the maximal size required for qPCR real time (450 pb) (Zentilin and Giacca, 2007). As because *cop*A gene was quantified with competitive PCR using this technique, we decided also to quantify *cus*A gene (410 pb) with the same technique cPCR instead of qPCR real time to avoid different bias of quantifications between the two genes. The results obtained from amplification kinetics of the four plasmids created enabled copper-resistance genes in impacted environments to be quantified precisely. Our results are expressed in numbers of copies of copper-resistance genes (*cop*A or *cus*A) per gram of sediment. Obviously, this result depends on the number of copies of those genes present on the genomes of microorganisms. Genome sequences of hundreds of microorganisms available on GenBank show that if one copper-resistance gene is present on one genome, generally only one copy is encountered, with the exception of certain microorganisms such as *A. ferrooxidans* (Navarro et al., 2009), that can possess up to three copies of *cop*A and *cus*A genes.

Analysis of the different ratios determined for *cop*A/16S rRNA and *cus*A/16S rRNA in both sediments and for all depths, shows that copper-resistance genes per bacterial cell are more abundant

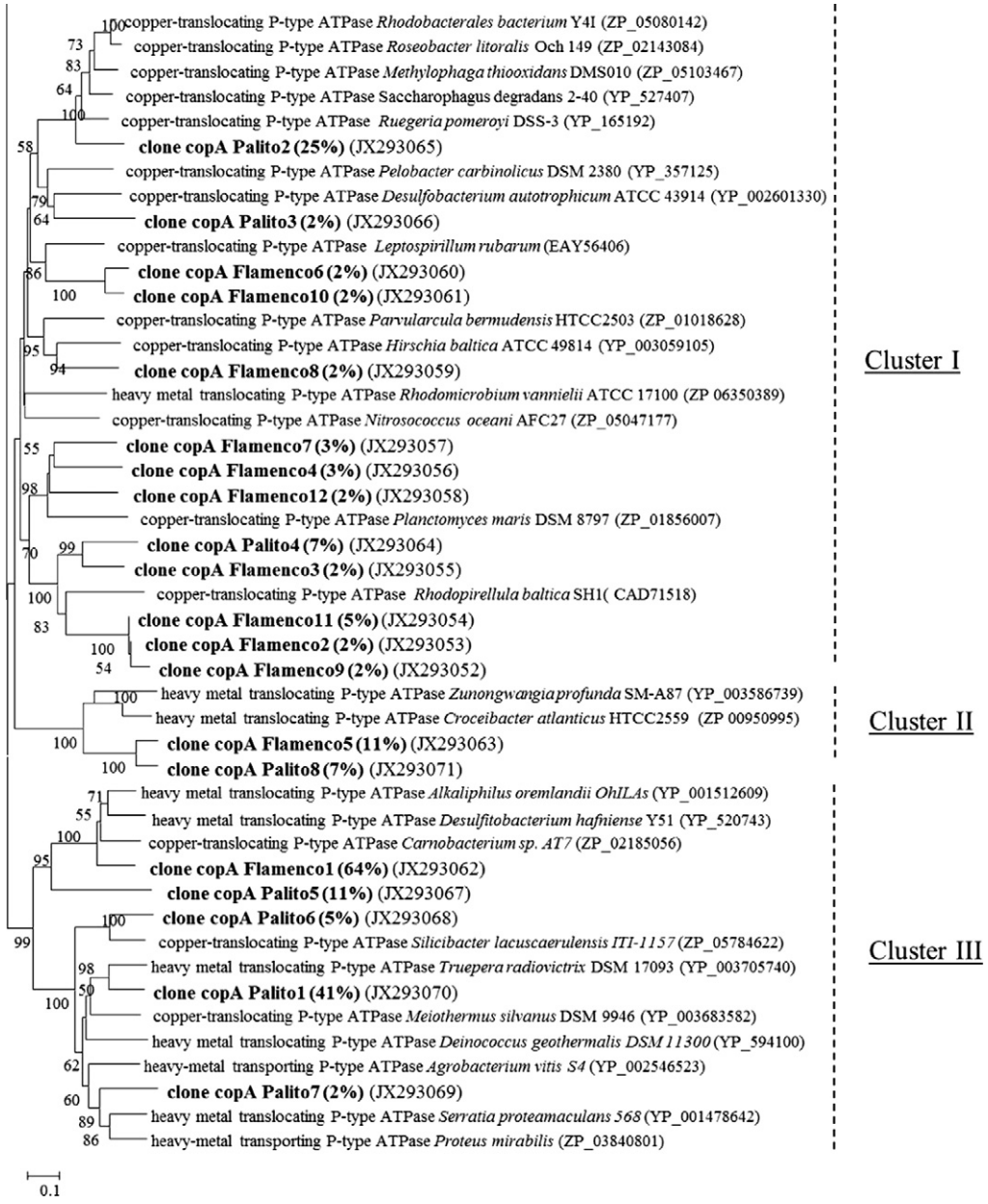

Fig. 7. Molecular phylogenetic analysis of CopA protein sequences deduced from cloning of PCR products of *cop*A gene. The phylogenetic tree was generated by the neighbor-joining method (the maximum-parsimony analysis gave very similar results). Bootstrap values of over 50% are shown at the nodes. The distance scale is the corrected proportion of amino acid changes (0.1 substitutions per amino acid).

in the contaminated site than in the uncontaminated site for almost all the depths tested. This rise confirms a possible adaptation of the microbial communities impacted by copper and suggests that both genes are involved in the copper-resistance of those communities at high levels of copper contamination.

Increases in the abundance of heavy-metal-resistance genes with the augmentation of the heavy-metal concentration in sediments have already been reported for other heavy-metal-resistance genes such as *cad*A (Oger et al., 2003) and *mer*A (Ramond et al., 2008), encoding ATP-ase efflux pumps responsible for the transport of respectively, cadmium and mercury, out of the cell.

The number of *cop*A genes per bacterial cell is significantly higher for all depths tested for Palito and Flamenco sediment cores than the number of *cus*A genes. This result thus suggests that microorganisms show a preference for *cop*A rather than *cus*A when labile copper concentration is high. To our knowledge, the expression of heavy-metal-resistance genes has only been studied on pure cultures (Outten et al., 2001). The results have concluded that in *E. coli, cop*A was expressed under low, medium and high copper stress in aerobic and also anaerobic conditions, whereas *cus*A was only expressed at high concentrations of copper in aerobic and anaerobic conditions. Nevertheless, the expression of *cus*A was preferentially induced in aerobic and anaerobic conditions tested during copper stress for *S. oneidensis* (Toes et al., 2008). To try to better understand the expression of copper-resistance genes in microbial communities *in situ*, we also extracted RNA from all samples but failed to detect the presence of *cop*A and *cus*A from the cDNA obtained. These results could be explained by putting forward the hypothesis that most bacteria inhabiting those environments are considered to be either dead or dormant, as was shown in marine sediment by Luna et al. (2002). A second hypothesis would be that the microorganisms present in the Palito sediment core have adapted to such high levels of contamination by using other, unknown, mechanisms and do not need to express

the copper-resistance genes. The last hypothesis would be that the RNA expression of those genes is low and under the detection limit of our technique.

Indeed, resistance and tolerance of microorganisms to copper is not mediated by those copper-resistance genes alone. Previous studies have demonstrated that copper could be sequestrated by metallotioneins (Blindauer et al., 2002), transported by metallochaperones (Rosenzweig, 2001). Furthermore, microorganisms can also bioaccumulate copper (Albarracin et al., 2010), or reduce their bioavailability by producing sulfide (Martins et al., 2009) or iron oxides (Takematsu, 1979). They can also develop biosorption processes (Andreazza et al., 2011) or produce long filaments of inorganic poly-(P) to sequestrate metals (Orell et al., 2010).

The higher abundance of *cop*A gene can also be partly explained because PIB-type ATP-ases can be horizontally transferred from bacteria inhabiting contaminated soils (Martinez et al., 2006), whereas such horizontal transfer has not been proved for other types of copper-resistance genes. Nevertheless, it appears surprising here that the *cop*A gene is more abundant than the *cus*A gene in the impacted Palito sediment core. Indeed, CopA requires ATP for heavy metal efflux outside the membrane cell whereas CusA is an energy-free mechanism and only requires an antiport system (Rensing and Grass, 2003; Nies, 2003).

To our knowledge, it is the first time that the diversity of the *cus*A gene has been studied in a copper-contaminated environment. Analysis of *cus*A cloned sequences in the two sediment cores reveals that the distribution of *cus*A sequences is affected by the augmentation of copper concentrations. Indeed, the rise of copper concentrations has modified the richness of CusA amino acid sequences. OTU *cus*A clone Palito43 is highly dominant in the contaminated site demonstrating a selection caused by copper enrichment in sediment. This sequence is relatively close to the CusA found in *Myxococcus xanthus* DK1622 (Goldman et al., 2006). Clones from Cluster II were assigned to *A. macleodii* (Raguenes et al., 1996). The results of *cus*A clone libraries also revealed the presence of two new clusters (I and II) that are not affiliated to already-known sequences of CusA. Cluster II is specific to Palito copper-contaminated sediment.

Analysis of the phylogenetic tree of CopA amino acid sequences reveals that, as was seen for CusA amino acid sequences, sequence richness decreases when copper concentrations increase. One OTU (CopA clone Palito 1) is in majoritary in the contaminated site and close to the CopA amino acid sequence present in *Truepera radioctivrix* DSM 17093. The most abundant clone found in the Flamenco sediment core (CopA clone Flamenco1) was affiliated to the CopA sequence found in *Carnobacteium* sp. AT7. The results of clone libraries revealed no common OTU between the two environments and thus an important modification of the genetic structure of the *cop*A gene.

Results indicate that copper changes the diversity of copper P-ATPases and also of the CusA RND type protein. These modifications could be explained by the fact that the new proteins found in the contaminated site would be more efficient in extruding copper, and allow bacteria to become resistant to high concentrations of copper. Similar results showing a decrease in the richness of copper-resistance genes were obtained from other contaminated sediments (De La Iglesia et al., 2010; Pavissich et al., 2010) and soil microcosms (Lejon et al., 2007).

5. Conclusion

Our microbial investigations in Chilean marine sediments, impacted or not by copper, based on molecular studies, allowed us to quantify, for the first time, the abundance of two major copper-resistance genes *cop*A and *cus*A. Our results show that *cop*A is the most abundant gene present in the impacted sediment, whether in oxic or anoxic conditions. An increase in copper also modifies the genetic structure of *cus*A and *cop*A genes. Further experiments are being undertaken to study the evolution of this amino acids change, and the expression of both copper-resistance genes in microcosms presenting a gradient of copper-concentration.

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References

Albarracin, V., Alonso-Vega, P., Trujillo, M., Amoroso, M., Abate, C., 2010. Amycolatopsis tucumanensis sp. nov., a copper-resistant actinobacterium isolated from polluted sediments. Int. J. Syst. Evol. Microbiol. 60, 397–401.

Altschul, S., Madden, T., Schäffer, A., Zhang, J., Zhang, Z., Miller, W., Lipman, D., 1997. Gapped BLAST PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Andreazza, R., Okeke, B., Pieniz, S., Camargo, F., 2011. Characterization of copper-resistant rhizosphere bacteria from avena sativa plantago lanceolata for copper bioreduction biosorption. Biol. Trace Element Res., 1–9.

Ankley, G., Mattson, V., Leonard, E., West, C., Bennett, J., 1993. Predicting the acute toxicity of copper in freshwater sediments: evaluation of the role of acid-volatile sulfide. Environ. Toxicol. Chem. 12, 315–320.

Blindauer, C., Harrison, M., Robinson, A., Parkinson, J., Bowness, P., Sadler, P., Robinson, N., 2002. Multiple bacteria encode metallothioneins SmtA-like zinc fingers. Mol. Microbiol. 45, 1421–1432.

Brown, N., Barrett, S., Camakaris, J., Lee, B., Rouch, D., 1995. Molecular genetics transport analysis of the copper-resistance determinant pco from *Escherichia coli* plasmid pRJ1004. Mol. Microbiol. 17, 1153–1166.

Brown, D., Qin, K., Herms, J., Madlung, A., Manson, J., Strome, R., Fraser, P., Kruck, T., Von Bohlen, A., Schulz-Schaeffer, W., 1997. The cellular prion protein binds. Nature 390, 685.

Cooper, D., Morse, J., 1998. Extractability of metal sulfide minerals in acidic solutions: application to environmental studies of trace metal contamination within anoxic sediments. Environ. Sci. Technol. 32, 1076–1078.

De la Iglesia, R., Valenzuela-Heredia, D., Pavissich, J., Freyhoffer, S., Andrrade, S., Correa, J., González, B., 2010. Novel polymerase chain reaction primers for the specific detection of bacterial copper P-type ATPases gene sequences in environmental isolates metagenomic DNA. Lett. Appl. Microbiol. 50, 552–562.

Diaz-Ravina, M., Baath, E., 1996. Development of metal tolerance in soil bacterial communities exposed to experimentally increased metal levels. Appl. Environ. Microbiol. 62, 2970–2977.

Gillan, D., Danis, B., Pernet, P., Joly, G., Dubois, P., 2005. Structure of sediment-associated microbial communities along a heavy-metal contamination gradient in the marine environment. Appl. Environ. Microbiol. 71, 679–690.

Goldman, B., Nierman, W., Kaiser, D., Slater, S., Durkin, A., Eisen, J., Ronning, C., Barbazuk, W., Blanchard, M., Field, C., 2006. Evolution of sensory complexity recorded in a myxobacterial genome. Proc. Nat. Acad. Sci. 103, 15200.

Harrison, M., Jones, C., Solioz, M., Dameron, C., 2000. Intracellular copper routing: the role of copper chaperones. Trends Biochem. Sci. 25, 29–32.

Kershaw, C., Brown, N., Constantinidou, C., Patel, M., Hobman, J., 2005. The expression profile of *Escherichia coli* K-12 in response to minimal, optimal excess copper concentrations. Microbiology 151, 1187–1198.

Klappenbach, J., Saxman, P., Cole, J., Schmidt, T., 2001. Rrndb: the ribosomal RNA operon copy number database. Nucleic Acids Res. 29, 181–184.

Konstantinidis, K., Isaacs, N., Fett, J., Simpson, S., Long, D., Marsh, T., 2003. Microbial diversity resistance to copper in metal-contaminated lake sediment. Microb. Ecol. 45, 191–202.

Lejon, D., Nowak, V., Bouko, S., Pascault, N., Mougel, C., Martins, J., Ranjard, L., 2007. Fingerprinting diversity of bacterial copA genes in response to soil types, soil organic status copper contamination. FEMS Microbiol. Ecol. 61, 424–437.

Luna, G., Manini, E., Danovaro, R., 2002. Large fraction of dead inactive bacteria in coastal marine sediments: comparison of protocols for determination ecological significance. Appl. Environ. Microbiol. 68, 3509–3513.

Marchesi, J., Sato, T., Weightman, A., Martin, T., Fry, J., Hiom, S., Wade, W., 1998. Design evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl. Environ. Microbiol. 64, 795–799.

Margesin, R., Plaza, G., Kasenbacher, S., 2011. Characterization of bacterial communities at heavy-metal-contaminated sites. Chemosphere.

Martinez, R., Wang, Y., Raimondo, M., Coombs, J., Barkay, T., Sobecky, P., 2006. Horizontal gene transfer of PIB-type ATPases among bacteria isolated from radionuclide-metal-contaminated subsurface soils. Appl. Environ. Microbiol. 72, 3111–3118.

Martins, M., Faleiro, M., Barros, R., Veríssimo, A., Barreiros, M., Costa, M., 2009. Characterization activity studies of highly heavy metal resistant sulphate-reducing bacteria to be used in acid mine drainage decontamination. J. Hazard. Mater. 166, 706–713.

Muyzer, G., De Waal, E., Uitterlinden, A., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700.

Navarro, C., Orellana, L., Mauriaca, C., Jerez, C., 2009. Transcriptional functional studies of *Acidithiobacillus ferrooxidans* genes related to survival in the presence of copper. Appl. Environ. Microbiol. 75, 6102–6109.

Nies, D., 2003. Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol. Rev. 27, 313–339.

Oger, C., Berthe, T., Quillet, L., Barray, S., Chiffoleau, J., Petit, F., 2001. Estimation of the abundance of the cadmium resistance gene cadA in microbial communities in polluted estuary water. Res. Microbiol. 152, 671–678.

Oger, C., Mahillon, J., Petit, F., 2003. Distribution diversity of a cadmium resistance cadA determinant occurrence of IS257 insertion sequences in Staphylococcal bacteria isolated from a contaminated estuary Seine, France. FEMS Microbiol. Ecol. 43, 173–183.

Orell, A., Navarro, C., Arancibia, R., Mobarec, J., Jerez, C., 2010. Life in blue: copper resistance mechanisms of bacteria Archaea used in industrial biomining of minerals. Biotechnol. Adv. 28, 839–848.

Outten, F., Huffman, D., Hale, J., O'Halloran, T., 2001. The independent cue cussystems confer copper tolerance during aerobic anaerobic growth in *Escherichia coli*. J. Biol. Chem. 276, 30670.

Pavissich, J., Silva, M., González, B., 2010. Sulfate reduction, molecular diversity, copper amendment effects in bacterial communities enriched from sediments exposed to copper mining residues. Environ. Toxicol. Chem. 29, 256–264.

Piddock, L., 2006. Multidrug-resistance efflux pumps not just for resistance. Nat. Rev. Microbiol. 4, 629–636.

Plassart, P., Akpa Vinceslas, M., Gangneux, C., Mercier, A., Barray, S., Laval, K., 2008. Molecular functional responses of soil microbial communities under grassl restoration. Agric. Ecosyst. Environ. 127, 286–293.

Quillet, L., Besaury, L., Popova, M., Paissé, S., Deloffre, J., Ouddane, B., 2011. Abundance, diversity activity of sulfate-reducing prokaryotes in heavy metal-contaminated sediment from a salt marsh in the Medway Estuary UK. Marine Biotechnol. pp. 1–19.

Raguenes, G., Pignet, P., Gauthier, G., Peres, A., Christen, R., Rougeaux, H., Barbier, G., Guezennec, J., 1996. Description of a new polymer-secreting bacterium from a deep-sea hydrothermal vent, *Alteromonas macleodii* subsp. fijiensis, preliminary characterization of the polymer. Appl. Environ. Microbiol. 62, 67–73.

Ramond, J., Berthe, T., Lafite, R., Deloffre, J., Ouddane, B., Petit, F., 2008. Relationships between hydrosedimentary processes occurrence of mercury-resistant bacteria merA in estuary mudflats Seine, France. Mar. Pollut. Bull. 56, 1168–1176.

Ranjard, L., Echairi, A., Nowak, V., Lejon, D., Nouaïm, R., Chaussod, R., 2006. Field microcosm experiments to evaluate the effects of agricultural Cu treatment on the density genetic structure of microbial communities in two different soils. FEMS Microbiol. Ecol. 58, 303–315.

Rensing, C., Grass, G., 2003. *Escherichia coli* mechanisms of copper homeostasis in a changing environment. FEMS Microbiol. Rev. 27, 197–213.

Rensing, C., Fan, B., Sharma, R., Mitra, B., Rosen, B., 2000. CopA: an *Escherichia coli* Cu I-translocating P-type ATPase. Proc. Nat. Acad. Sci. 97, 652.

Rosenzweig, A., 2001. Copper delivery by metallochaperone proteins. Acc. Chem. Res. 34, 119–128.

Ross, R., Kleiz, R., Reske-Kunz, A., 1995. A method for rapid generation of competitive stard molecules for RT-PCR avoiding the problem of competitor/probe cross-reactions. Genome Res. 4, 371–375.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Simpson, S., Apte, S., Batley, G., 2000. Effect of short-term resuspension events on the oxidation of cadmium, lead, zinc sulfide phases in anoxic estuarine sediments. Environ. Sci. Technol. 34, 4533–4537.

Takematsu, N., 1979. Sorption of transition metals on manganese iron oxides, silicate minerals. J. Oceanogr. 35, 36–42.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis MEGA software version 4.0. Mol. Biol. Evol. 24, 1596–1599.

Thompson, J., Gibson, T., Plewniak, F., Jeanmougin, F., Higgins, D., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

Toes, A., Daleke, M., Kuenen, J., Muyzer, G., 2008. Expression of copA cusA in Shewanella during copper stress. Microbiology 154, 2709–2718.

Trajanovska, S., Britz, M., Bhave, M., 1997. Detection of heavy metal ion resistance genes in gram-positive gram-negative bacteria isolated from a lead-contaminated site. Biodegradation 8, 113–124.

Villafane, A., Voskoboynik, Y., Cuebas, M., Ruhl, I., Bini, E., 2009. Response to excess copper in the hyperthermophile *Sulfolobus solfataricus* strain 98/2. Biochem. Biophys. Res. Commun. 385, 67–71.

Zentilin, L., Giacca, M., 2007. Competitive PCR for precise nucleic acid quantification. Nat. Protoc. 2, 2092–2104.