Transcriptomic and proteomic analyses of the pMOL30-encoded copper resistance in *Cupriavidus metallidurans* strain CH34

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The four replicons of *Cupriavidus metallidurans* CH34 (the genome sequence was provided by the US Department of Energy–University of California Joint Genome Institute) contain two gene clusters putatively encoding periplasmic resistance to copper, with an arrangement of genes resembling that of the *copSRABCD* locus on the 2.1 Mb megaplasmid (MPL) of *Ralstonia solanacearum*, a closely related plant pathogen. One of the *copSRABCD* clusters was located on the 2.6 Mb MPL, while the second was found on the pMOL30 (234 kb) plasmid as part of a larger group of genes involved in copper resistance, spanning 17 857 bp in total. In this region, 19 ORFs (*copVTMKNSRABCDIJGFLQHE*) were identified based on the sequencing of a fragment cloned in an IncW vector, on the preliminary annotation by the Joint Genome Institute, and by using transcriptomic and proteomic data. When introduced into plasmid-cured derivatives of *C. metallidurans* CH34, the *cop* locus was able to restore the wild-type MIC, albeit with a biphasic survival curve, with respect to applied Cu(II) concentration. Quantitative-PCR data showed that the 19 ORFs were induced from 2- to 1159-fold when cells were challenged with elevated Cu(II) concentrations. Microarray data showed that the genes that were most induced after a Cu(II) challenge of 0.1 mM belonged to the pMOL30 *cop* cluster. Megaplasmodic *cop* genes were also induced, but at a much lower level, with the exception of the highly expressed MPL *copD*. Proteomic data allowed direct observation on two-dimensional gel electrophoresis, and via mass spectrometry, of pMOL30 CopK, CopR, CopS, CopA, CopB and CopC proteins. Individual *cop* gene expression depended on both the Cu(II) concentration and the exposure time, suggesting a sequential scheme in the resistance process, involving genes such as *copK* and *copT* in an initial phase, while other genes, such as *copH*, seem to be involved in a late response phase. A concentration of 0.4 mM Cu(II) was the highest to induce maximal expression of most *cop* genes.

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Abbreviations: 2-DE, two-dimensional electrophoresis; IOD, integrated optical density; MALDI-TOF, matrix-associated laser desorption ionization time-of-flight; MPL, megaplasmid.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are AJ278983 (copper resistance operon of *C. metallidurans*) and NC_006466 (plasmid pMOL30).

Four supplementary tables are available with the online version of this paper.

INTRODUCTION

Cupriavidus metallidurans CH34 (Vandamme & Coenye, 2004; Vaneechoutte *et al.*, 2004), a facultative hydrogenotroph, is a metal-resistant bacterium isolated from the sludge of a zinc decantation tank in Belgium that was polluted with high concentrations of several heavy metals, and it is regarded as the prime representative for metal-resistant cupriavidi, which have the peculiar property of being able to colonize and adapt to industrial biotopes (Brim *et al.*, 1999; Diels & Mergeay, 1990; Goris *et al.*, 2001; Mergeay *et al.*, 1978, 1985). The *C. metallidurans* type strain CH34 contains two megareplicons [one chromosome of 3.9 Mb, and one megaplasmid (MPL) of 2.6 Mb], and two large plasmids, pMOL28 (171 kb) and pMOL30 (234 kb), harbouring a variety of genes conferring resistance to Cd(II), Zn(II), Co(II), Ni(II), Cr(VI), Hg(II), Pb(II), Ag(I) and Cu(II) (Grass *et al.*, 2000; Juhnke *et al.*, 2002; Mergeay *et al.*, 1985; Taghavi *et al.*, 1997). The Joint Genome Institute (JGI) in California has recently sequenced the genome of this organism, and the draft sequence is now available at http://genome.jgi-psf.org/draft_microbes/ralme/ralme.home.html. The large number of as yet uncharacterized genes or ORFs thought to play a role in metal resistance and/or detoxification makes strain CH34 a model of choice for the investigation of heavy-metal resistance mechanisms (Mergeay, 2000; Mergeay *et al.*, 2003; Nies, 2000, 2003).

Copper is an essential trace element for life, but becomes toxic at high concentrations. High levels of copper are often found in areas such as mine tailings or industrial environments, both of which are commonly colonized by *C. metallidurans* strains (Diels & Mergeay, 1990; Mergeay, 2000).

Resistance mechanisms against high copper concentrations (e.g. far above the quantities required for growth and metabolism) have been found on the *Escherichia coli* plasmid pRJ1004 (Lee *et al.*, 2002; Rensing & Grass, 2003; Tetaz & Luke, 1983), and in *Pseudomonas syringae* pv. *tomato* (Bender & Cooksey, 1987; Cha & Cooksey, 1991; Mellano & Cooksey, 1988), in which the copper-resistance locus is restricted to two adjacent operons harboured by the pPT23D plasmid. In *P. syringae* pv. *tomato*, the *copABCD* operon encodes the structural resistance genes, and is transcribed from a copper-inducible promoter controlled by the two-component regulatory module *copRS* (Mills *et al.*, 1994). In *E. coli*, the plasmid-borne *pcoABCD pcoSR pcoE* locus is involved in periplasmic copper handling (Brown *et al.*, 1992; Lee *et al.*, 2002). The additional gene *pcoE* encodes a peptide involved in the periplasmic binding of copper ions, and is regulated by a separate copper-regulated promoter (Rensing & Grass, 2003). A similar cluster has been described in the large virulence plasmid pLVPF of *Klebsiella pneumoniae* CG43 (Chen *et al.*, 2004) and in R478 (IncH) of *Serratia marcescens* (Gilmour *et al.*, 2004). In *P. syringae*, chromosomal orthologues of the plasmid-borne *copABCD* locus have been identified, suggesting that functional cross-talk occurs between chromosomal- and plasmid-encoded loci to counteract environmental toxicity

(Lim & Cooksey, 1993). Chromosomal orthologues of *pco*/*copABCD* and *pco*/*copRS* have been found in an increasing variety of genomes.

In *C. metallidurans*, curing and conjugation experiments have shown that plasmid pMOL30 is involved in copper resistance (Corbisier, 1997; Diels *et al.*, 1989). A catalogue of heavy-metal resistance genes of *C. metallidurans* (Mergeay *et al.*, 2003), based on the JGI draft sequence, lists the presence of *copSRABCD* genes on the CH34 2.6 Mb MPL, and on pMOL30. These genes are also present on the MPL of *Ralstonia solanacearum* (Salanoubat *et al.*, 2002). The catalogue further lists other chromosomal genes that could be involved in copper resistance and homeostasis, such as the *cus* (Nies, 2003; Rensing & Grass, 2003) genes, and a gene encoding a copper ATPase, *CupA* (Mergeay *et al.*, 2003; Monchy *et al.*, 2006) (locus *cupC cupA cupR*). This paper focuses on the description of the pMOL30-borne copper-resistance locus in *C. metallidurans* CH34, and reports the identification of 19 putative ORFs, all of which are involved in the cellular copper response. The expression of these genes was analysed at the transcriptomic and proteomic levels by quantitative PCR and microarray, and by two-dimensional electrophoresis (2-DE) and MS, respectively.

METHODS

Strains and culture conditions. Tris mineral salts medium (Mergeay *et al.*, 1985; Schlegel *et al.*, 1961), supplemented with 0.2% (w/v) gluconate, was used to grow *C. metallidurans* CH34 and its derivatives. $\text{Cu}(\text{NO}_3)_2$ was added at concentrations ranging from 0.1 to 1.2 mM. *C. metallidurans* cultures were grown at 28 °C on a rotary shaker at 200 r.p.m. Plates were made by adding 1.5% (w/v) minimal agar (Difco) to the medium. Antibiotic-resistant bacteria were selected by addition of tetracycline at 20 µg ml⁻¹.

E. coli strains were grown in 869 medium (Mergeay *et al.*, 1985), supplemented when necessary with the appropriate antibiotics (tetracycline, 20 µg ml⁻¹; ampicillin, 100 µg ml⁻¹). All cultures were incubated at 37 °C.

Cloning of the copper determinant of pMOL30. The *cop* resistance determinant was cloned from *C. metallidurans* CH34 using a pMOL30 cosmid library constructed in pLAFR3, a pSa (IncW) derivative (Staskawicz *et al.*, 1987), as specified previously (Borremans *et al.*, 2001). Standard molecular cloning techniques, electroporation procedures, and plasmid extraction protocols were used. The *C. metallidurans* CH34 cosmid library was introduced by electroporation into *E. coli* HB101, and transformant clones were selected in 869 medium supplemented with 20 µg tetracycline ml⁻¹. The cosmid library was then transferred into the plasmid-free heavy-metal-sensitive derivative of CH34, designated AE104, by three-parental mating with an *E. coli* strain carrying pRK2013 (a broad-host-range mobilizing plasmid that is unable to self replicate in *C. metallidurans*) as helper strain (Figurski & Helinski, 1979). Copper-resistant transconjugants were selected on Tris mineral salts (with gluconate as a carbon source) agar supplemented with 0.6 mM $\text{Cu}(\text{NO}_3)_2$ and 20 µg tetracycline ml⁻¹. MIC values of the isolated clones were determined as described below. Recombinant pLAFR3 cosmids carrying copper-resistance determinants from CH34 were isolated from recipient AE104 cells using standard plasmid-extraction procedures, and electroporated back into *E. coli* HB101. Expression of the cloned copper-resistance genes in *E. coli* transformants was tested

by plating selected clones onto 869 agar supplemented with 20 µg tetracycline ml⁻¹ and copper concentrations ranging from 0 to 20 mM. Restriction patterns of the selected cosmids were determined by digestion with *Bam*HI, *Pst*II, *Not*I, *Hind*III and *Eco*RI, according to the instructions provided by the manufacturer (Boehringer Mannheim). Cosmids that expressed the best copper-resistance phenotype in AE104 were submitted for nucleotide sequence determination of the corresponding insert.

Sequence determination. Subcloning, primer walking, and cycle sequencing strategies were used for sequence determination of selected inserts. The GCG software (Genetics Computer Group) was used for sequence assembly. The sequence of the copper-resistance determinant of *C. metallidurans* CH34 has been submitted to GenBank under accession no. AJ278983. Sequence datasets were compared using standard similarity search programs. FASTA and BLAST subroutines (Pearson & Lipman, 1988) were also used to search for related gene sequences encoding copper-resistance genes in the GenBank database, and in the *C. metallidurans* genome. The CLUSTAL W program was used for multiple sequence alignments. The PHYLIP package was used for dendrogram construction, using neighbour-joining, and bootstrap values were estimated with 100 replications. Identification of ORFs was performed using the GCG software. The DNA sequence of plasmid pMOL30 has been submitted to GenBank under accession no. NC_006466.

PCR determination of copper-resistance genes located on *Sau*3AI partial fragments. Primers were designed for the selective amplification of putative ORFs, as determined by sequence analysis of cloned inserts, using the GCG software. Cosmids responsible for partial or full restoration of the copper-resistant phenotype in strain AE104 were extracted from the corresponding *E. coli* transformants, and submitted for PCR amplification with primer pairs designed for the different putative ORFs. Amplification of target ORFs was performed using a GeneAmp thermocycler (Perkin Elmer). Each 50 µl reaction mixture contained 5 µl PCR buffer (Promega), 2 µl 50 mM MgSO₄, 1.5 µl dNTPs (10 mM each in stock solution), and 1 U *Taq* DNA polymerase (Platinum *Taq* DNA Polymerase High Fidelity; Invitrogen).

Measurements of Cu(II)-mediated gene induction by microarrays

Spotting. The publicly available genome draft of *C. metallidurans* (http://genome.jgi-psf.org/draft_microbes/ralme/ralme.home.html; released in September 2003 and December 2004) was used to design 60-mer amino-modified oligonucleotides corresponding to 6205 ORFs. The oligonucleotides were synthesized at Eurogentec (see Supplementary Table S1, available with the online version of this paper), and then resuspended at the correct concentration in the spotting buffer (3 × SSC), and spotted onto a glass slide (UltraGAPS; Corning) using the MicroGrid (BioRobotics). The *C. metallidurans* library was spotted in duplicate spots on each slide. The spotted slides were then cross-linked, and presoaked in the presoaking solutions from the Pronto kit (Corning-Promega).

Labelling and hybridization. Metal-induced bacterial cultures were grown for total RNA extraction using the Qiagen RNeasy Midi kit. The quality control of total RNA was monitored using the BioAnalyser 2100 (Agilent). A 10 µg quantity of high-quality total RNA was then reverse-transcribed by random priming, according to the instructions provided with the Pronto kit (Corning-Promega), and labelled by incorporation of Cy3-dCTP or Cy5-dCTP nucleotides (Amersham Biosciences). Labelled cDNAs derived from the different bacterial cultures were resuspended in the Universal Hybridization Buffer provided in the Pronto kit, then mixed, and added to the spotted slide for hybridization overnight at 42 °C. The slide was washed according to the washing protocol of the Pronto kit.

Scanning and analysis. The array was scanned with a laser scanner at 532 and 635 nm (Genepix4100A; Axon Instruments). Image analysis was performed with Genepix Pro software. Spot intensities were measured, and artifacts due to the circularity, shape and background were removed. Statistical analyses were performed using S-PLUS software, and printTipLoess normalization was applied to the different slides, then the mean of ratios (R/G) and *P* value were calculated using the significance analysis of microarrays test. Modulated genes were selected based on their *P* value (<0.05) and mean (<-2 or >+2) fold change.

Measurements of Cu(II)-mediated gene induction by real-time quantitative PCR. Liquid cultures were grown at 30 °C in Tris mineral salts (with gluconate as a carbon source) medium, as described above, and shaken at 120 r.p.m. on an orbital shaker until the OD₆₆₀ reached 0.3 (early exponential phase). Aliquots (10 ml) of the cultures were then transferred into Falcon tubes, and challenged with Cu(NO₃)₂ concentrations ranging from 0 (control) to 1.2 mM. Cultures were incubated at 30 °C for 5, 30 and 60 min. Total RNA was extracted from control and induced cultures using the RNeasy Midi system (Qiagen), according to the manufacturer's instructions. Residual DNA was eliminated from the extracts by the addition of 3 U RQ1 RNase-free DNase (Promega), followed by incubation at 37 °C for 30 min. Extracted RNAs were stored at -80 °C until use. Reverse transcription of target mRNA was performed with random hexanucleotides using the Reverse Transcription kit (Applied Biosystems). Quantitative PCR was then conducted on the resulting cDNA, using specific primer sets designed for all the ORFs that were identified on the pMOL30 cloned fragment carrying resistance to copper. Primer design was performed using PrimerExpress software (Applied Biosystems) (see Supplementary Table S2 for primers). Quantitative PCR, using the same procedure as above, was also conducted on the MPL-encoded genes *copR* and *copC*, which are part of the *copSRABCD* cluster. Flanking ORFs and 16S rRNA operons were also tested for induction, and were used as controls. All the quantitative PCRs were done in quadruplicate, and a standard deviation was calculated each time.

Quantitative PCR was performed with Sybr Green (Sybr Green PCR Master Mix; Applied Biosystems), while fluorescence was continuously monitored by the sequence detection system 5700 (Applied Biosystems). Data were analysed by normalization against 16S rRNA levels as an internal control. Fold induction is relative to the control condition [0 mM Cu(NO₃)₂]. After addition of 0.1, 0.2, 0.4, 0.6 and 0.8 mM Cu(NO₃)₂, the 16S rRNA levels were 1.16 ± 0.15, 0.77 ± 0.06, 1.4 ± 0.1, 1.36 ± 0.05 and 0.94 ± 0.05, respectively.

Proteomic analysis of the copper response in *C. metallidurans* CH34

Cell culture. Liquid cultures were grown at 30 °C in Tris mineral salts liquid medium, as described above, and shaken at 120 r.p.m. on an orbital shaker until the OD₆₆₀ reached 0.3 (early exponential phase). Small aliquots (0.5 ml) of the precultures were then used to inoculate a second set of flasks containing 25 ml medium, and they were challenged with Cu(NO₃)₂ concentrations corresponding to 0 (control) or 0.8 mM. Aliquots from exponential- and stationary-phase cultures were collected at OD₆₆₀ values of 0.5 and 1.0, respectively, and then centrifuged at room temperature at 6000 r.p.m. (3200 g) for 10 min. Pellets were stored frozen at -80 °C immediately after centrifugation.

Sample preparation. The bacterial pellets were pipette-resuspended in lysis buffer [8 M urea, 4 % w/v, CHAPS, 40 mM Tris, 0.2 % v/v, Pharnalyte 3-10, 2 mM tributyl phosphine (Bio-Rad) and a quarter tablet ml⁻¹ of complete mini EDTA-free protease inhibitor cocktail (Roche)], and further lysed for 1 min in an Elma Transsonic 450/H

sonicator. The samples were incubated at room temperature with 0.1 mg ml⁻¹ DNase and RNase (Roche Diagnostics) until they were no longer viscous, and were then centrifuged at 45 000 r.p.m. (110 000 g) at 4 °C for 1 h.

Periplasmic proteins were isolated using the chloroform method (Ames *et al.*, 1984). After centrifugation, the cell pellet was resuspended in a minimum culture medium by using a vortex, followed by the addition, with rapid stirring, of 1 ml chloroform. Samples were incubated for 15 min at room temperature, followed by the addition of 10 ml 0.01 M Tris/HCl, pH 8.0. The suspension was centrifuged for 30 min at 13 000 g. Proteins localized in the periplasm were recovered in the supernatant (upper liquid phase). Residual bacterial components were localized between the water phase and the chloroform phase.

Protein concentrations were measured by the Bradford method, using the Bio-Rad Protein Assay kit, with bovine gamma globulin as a protein standard. Supernatants were stored at -20 °C.

Analysis of protein expression. 2-DE was performed, as previously described (Noel-Georis *et al.*, 2004), with a Bio-Rad DodecaCell apparatus, using IPG strips (18 cm), characterized by narrow pH gradients of 4.5–5.5, 5–6, 4–7 and 6–11. The protein spots were visualized by staining the gels (20 × 20 cm) with silver nitrate. Protein patterns within the gels were analysed as digitized images using a high-resolution scanner, in combination with the molecular analysis software PDQuest (Bio-Rad). The amount of protein in a spot was estimated by background-corrected optical density, integrated over all pixels in the spot, and expressed as integrated optical density (IOD). Correction of differences in total staining intensity between different two-dimensional images was done by expressing the protein amount in a spot as the percentage of the individual spot IOD per total IOD of all the spots in the picture (%IOD). Proteins induced by copper were identified by MS analysis. Peptide mapping and MS/MS sequencing were performed either on a Micromass matrix-associated laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer, or on a nanoESI QTOF mass spectrometer (Micromass), as previously described (Noel-Georis *et al.*, 2004).

Intact cell MS. After centrifugation [at 30 °C, 5000 r.p.m. (2500 g) for 5 min], bacteria were resuspended in 200 µl water (Milli-Q), and spotted onto the MALDI-TOF plate (2 µl per sample). The samples were then dried at 37 °C for 1 h. After this step, 2 µl matrix [saturated solution of α -cyano-4-hydroxycinnamic acid (Sigma) in water, acetonitrile and methanol (1:1:1), containing 0.1% (v/v) formic acid and 0.01 M 18-crown-6] was added, and the samples were dried at room temperature. MS measurements were performed on a M@LDI LR (Micromass) laser desorption/ionization instrument equipped with a nitrogen UV laser (λ 337 nm). The mass spectrometer was used in the positive-ion detection mode using an acceleration voltage of +15 kV, and set at linear mode. Each experiment was performed in triplicate.

Phenotypic analysis of copper resistance by viable counts. Viable counts were determined by plating 10–20 µl droplets of appropriate dilutions on plates supplemented with Cu(NO₃)₂, at concentrations ranging from 0.1 to 6.4 mM. Two separate cultures were used for the MIC determination. MICs were estimated from graphs in which log₁₀ of percentiles of survival were reported as a function of the log₂ of the copper concentration (Mergeay, 1995).

Software used for structural and phylogenetic analysis. Prediction of the presence of signal peptide was performed using the software signalP 3.0, available at <http://www.cbs.dtu.dk>. Calculations of protein isoelectric point and molecular mass were done using software available at http://www.expasy.org/tools/pi_tool.html. The

prediction for the localization of protein in the cellular compartment was performed using PSORT, available at <http://psort.nibb.ac.jp>. Determination of putative transmembrane helix and protein secondary structures were respectively performed using TMHMM, available at <http://www.cbs.dtu.dk>, and PHD, available at <http://npsa-pbil.ibcp.fr>. Phylogenetic representations were performed using the PHYLIP package.

RESULTS

The copper-resistance phenotype is linked to the pMOL30 plasmid

We analysed the phenotypes of four *C. metallidurans* reference strains: the wild-type CH34 strain carrying both plasmids pMOL28 and pMOL30, AE128 carrying pMOL30 only, AE126 carrying pMOL28 only, and AE104, as the plasmid-free derivative strain (Mergeay *et al.*, 1985). CH34 and AE128 showed the same MIC for Cu(II) (Fig. 1), which was estimated to be between two and three times higher than the MIC value of strains lacking pMOL30 (AE126 and AE104). This difference in MIC value was less than that observed for other metal-resistance phenotypes linked to pMOL30 and related *C. metallidurans* plasmids. For cadmium (*czc* in pMOL30), nickel (*cnr* in pMOL28), zinc (*czc* in pMOL30) and cobalt (*czc* in pMOL30), ratios of 4, 4, 33 and 133 have been observed, respectively, while a ratio of 30–40 has been observed for nickel with *ncc* in pTOM9 (Schmidt & Schlegel, 1994).

A 16.6 kb fragment of pMOL30 is involved in Cu(II) resistance

A pMOL30 cosmid library was constructed in the broad-host-range cosmid pLAFR3. Cosmids harbouring large

Fig. 1. Plasmid-linked copper-resistance phenotype in *C. metallidurans*, assessed by viable counts in response to Cu(II) challenge. The graph shows the percentage viability of *C. metallidurans* CH34 (◆; wild-type), AE126 (△; carrying pMOL28 only), AE128 (○; carrying pMOL30 only), AE1744 (■; carrying a vector with the *cop* cluster) and AE104 (□; plasmid-free derivative), after addition of increasing copper concentrations. Viability of 100% corresponds to the number of colonies that grew on minimal medium, without the addition of copper. The results are means ± SD (*n* = 3).

pMOL30 fragments, with a mean size of 20 kb, obtained after partial digestion with *Sau3AI*, were introduced into strain AE104, the plasmid-free derivative. Transformants were tested for their copper-resistance phenotype. Restriction analysis of the cosmids of copper-resistant transformants showed that strains carrying either cosmid pMOL1023 or pMOL1024 gave the highest resistance to copper. The insert of pMOL1024 was sequenced, and revealed the presence of ORFs with similarities to genes known to be involved in copper resistance in other bacteria such as *copSRABCD* and *copF*, which encodes a P-type copper-efflux ATPase (Legatzki *et al.*, 2003; Mergeay *et al.*, 2003). The strain containing pMOL1023 was slightly less resistant to Cu(II), and lacked part of a gene that displays identity to *czcE*, a periplasmic protein of the *czc* [Cd(II), Zn(II) and Co(II) resistance] operon of *C. metallidurans* CH34 (Grosse *et al.*, 2004). Due to its presumed role in copper resistance, this gene was tentatively called *copH*. Although pMOL1024 appeared as the most effective construct for copper resistance when introduced in AE104, viable counts for AE104(pMOL1024) showed a biphasic pattern of inhibition with increasing copper concentrations. A decrease in viability was initially observed at a low concentration, but the final MIC was the same as that for the wild type (Fig. 1).

Up to 19 ORFs are included in the copper-resistance cluster

Sequencing of the copper-resistance region of pMOL30 resulted in the preliminary identification of a cluster of 19 putative ORFs flanked by genes called *resU* (determining a putative site-specific recombinase) and *caiA* (determining a putative acyl-CoA dehydrogenase). The description of these 19 genes and ORFs (Fig. 2), designated *copVTMKNRSABCDIJGFLQHE*, is as follows.

copV (350 bp), *copM* (407 bp), *copN* (491 bp), *copQ* (245 bp) and *copE* (632 bp): these five putative ORFs do

not have any equivalent in current databases. No putative function could be assigned to any of them. The putative corresponding proteins CopM and CopQ harbour a leader peptide.

copT (761 bp): the corresponding protein CopT was so called because of its close homology with a part of PbrT, a protein encoded on the pMOL30 *pbr* operon (Borremans *et al.*, 2001). Although it contains the cytochrome *c* domain of PbrT, CopT does not have the transmembrane domain that is believed to be involved in metal transport from the periplasm to the cytoplasm (Borremans *et al.*, 2001).

copK (281 bp): the corresponding CopK protein contains a methionine-rich motif, MXMX₇MXM, on its C-terminal side. In total, of its 94 aa (73 in the mature form), this protein has seven methionines, two histidines, six glutamic acids, but no cysteine residues.

copS (1388 bp) and *copR* (683 bp): CopR and CopS are members of the two-component family of sensor regulators (Mergeay *et al.*, 2003; Mills *et al.*, 1994). *C. metallidurans* *copS* and *copR* (present on both the pMOL30 plasmid and the MPL), and their counterpart in *R. solanacearum* (present on the MPL), are located just upstream of *copABCD*, and transcribed in the opposite direction (Fig. 2). This divergent transcription contrasts with the gene organization in the γ -proteobacteria, where the order *copABCDcopRS* has been observed (*pcoABCDRSE* in *E. coli*).

copA (1841 bp): the corresponding CopA protein is a homologue of *P. syringae* CopA, and of PcoA from the *E. coli* plasmid pRJ1004. CopA is present in both the cytoplasm and the periplasm and is thought to be a multi-copper oxidase [able to oxidize Cu(I) to Cu(II)] (Rensing & Grass, 2003). It contains an MGGM motif that is repeated five times (two MGGM and two MAGM motifs have been observed in *P. syringae* CopA). The *P. syringae* CopA is able to bind 11 Cu(II) ions (Cha & Cooksey, 1991). This protein has a leader peptide with two arginines, which are

Fig. 2. Organization of the *cop* clusters of *C. metallidurans*. The figure shows the *cop* clusters of pMOL30 and the MPL, with the corresponding intergenic regions expressed in base pairs. Putative transcriptional coupling is shown by dumb-bell symbols, where applicable. The putative metal-binding motifs (methionine- and cysteine-rich regions) of the copper-resistance proteins encoded by the pMOL30 *cop* cluster of genes are also shown. The proteins corresponding to genes marked with an asterisk contain a methionine-rich motif, and are probably involved in periplasmic detoxification, whereas the genes marked with a + carry a cysteine-rich motif (CXCC or CXXC), and are probably involved in cytoplasmic detoxification.

Cu(II) suggests a detoxification mechanism that involves both ions.

copD (914 bp): the CopD protein is composed of eight transmembrane α -helices that probably form a channel involved in loading the cytoplasmic CopA with Cu(II) (Rensing & Grass, 2003).

copI (473 bp): the CopI protein has homology with oxidoreductase proteins. A *copI* orthologue, called *copC*, is found in *Aeromonas veronii*, downstream of a *copAB* region (probable orthologues of *C. metallidurans*/*R. solanacearum* *copAB*), and involved in copper resistance (Francki *et al.*, 2000). The *copAB copI* association in *A. veronii* suggests that *copI* may play a role in the complex periplasmic detoxification/transport process of copper mediated by the *copABCD* genes. The CopI protein also contains an MEHEIM motif similar to the motif MXHXXM found 10 times on CopB (Table 1).

copG (410 bp): the putative protein CopG contains a cysteine-rich motif, CXCC.

copF (2414 bp): encodes the putative P1-type ATPase CopF. This protein would be located in the inner membrane, with transmembrane α -helices probably forming a

The CopB methionine-rich motifs (MXHXXMXXM) are from *C. metallidurans* (YP_145681) (pMOL30 and MPL), *P. syringae* (BAA85308), *R. solanacearum* (NP_522218), *Azotobacter vinelandii* (ZP_00415272), *Xy. fastidiosa* (NP_778351), *Xa. axonopodis* (AAM38474), *Xa. campestris* (B36868), *B. pertussis* (NP_881854), *B. paraptetussis* (NP_886244), *N. aromaticivorans* (ZP_00303380), *E. coli* (CAA58526) and *P. putida* (AAN67817). pMOL30 CopB possesses the largest motif, with 10 copies of the motif, compared with the other bacteria, where the motif is present between one and four times only. CopI has also been included in the alignment because this protein contains an MXHXXM motif.

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cation channel, and contain an N-terminal histidine-rich metal-binding domain (HEHSAHGGASPGDHDHDAHHDH-SHAGH) (Mergeay *et al.*, 2003; Monchy *et al.*, 2006). This P1-type ATPase was also predicted to contain an N-terminal TRASH domain (CX₂₁CXXXC) with a cysteine in position 6, and the motif CSNSC. The TRASH domain is involved in metal trafficking (Ettema *et al.*, 2003). CopF is probably involved in Cu(I) efflux from the cytoplasm to the periplasm. The TRASH domain is also present in copper chaperone proteins and regulators, in the arrangement CX₂CX_{19–22}CX₃C (Ettema *et al.*, 2003). The other putative proteins containing a short cysteine-rich motif (CXXC or CXCC), such as CopT, CopL, CopJ and CopG, do not contain the TRASH motif.

copL (1262 bp): the corresponding CopL contains a cysteine-rich motif, HXCXCC. Possible orthologues of *copL* are found in *Magnetospirillum magnetotacticum* (Magn03007111 and Magn03006554). In this species, the Magn03006554 protein-encoding gene is followed by two genes encoding a copper chaperone protein and a copper-transporting P-type ATPase. A similar organization can be found in the *C. metallidurans* chromosome, represented by *cupC cupA cupR*, where the CupC and CupA proteins would correspond to the copper chaperone and a copper P-ATPase respectively, and CupR would correspond to a *merR*-like regulator (Monchy *et al.*, 2006). The protein CopL is possibly involved in the regulation of the CopF ATPase.

copH (458 bp): the protein CopH shares identity with the gene product of *czcE*, which is located in the *czc* (cadmium, zinc and cobalt resistance) cluster of genes, and is also present on pMOL30.

The transcription of the 19 *cop* genes is induced by copper challenge

Transcription analysis was performed using both microarray (Table 2) and quantitative PCR (Table 3, Fig. 3) for every predicted ORF belonging to the pMOL30 *cop* cluster, as well as for the two flanking genes *caiA* and *resU*.

Transcriptomic analysis using microarrays was performed after the addition of 0.1 mM Cu(NO₃)₂, and 30 min incubation. Total RNA from challenged and unchallenged cultures was hybridized on a microarray containing the 6205 ORFs of *C. metallidurans*. Statistical analysis of microarray data, based on two technical replicates present on the same chip, and two biological replicates on two different chips, showed 236 upregulated genes (3.8 % of the total number of ORFs present on the chip), and 186 downregulated genes (3 % of the total number). Data regarding the *cop* genes are reported in Table 2, and show the expression of the pMOL30 and MPL *cop* genes. The maximal induction was observed for the pMOL30 *copC* (21.6-fold), and the lowest induction was observed for *copH* (2.2-fold). Induction of adjacent ORFs located at both extremities of the copper locus of pMOL30 *caiA* and *resU* was not observed, allowing

the definition of the *cop* locus boundaries. The MPL *copRS copABCD* genes were also induced, but in a much lower range than their pMOL30 counterparts (Table 2), except for *copD*, which was overexpressed at the same level as the pMOL30 *copD* gene. Of the 50 most copper-induced ORFs (fold induction ranging from 21.6 to 3.8), 15 belong to the 19 pMOL30 *cop* genes (7 pMOL30 *cop* genes from the 10 most induced). The other induced genes mainly belong to pMOL30 and either have (except for *czcN* and *silA*) similarity to other hypothetical proteins, or are putatively involved in biosynthesis of polysaccharide and of membrane components.

Quantitative PCR was performed on most of the *cop* genes after incubation of *C. metallidurans* CH34 in presence of several Cu(II) concentrations (0.1, 0.2, 0.4, 0.6 and 0.8 mM) (Table 3), and three exposure times (5, 30 and 60 min) (Fig. 3; see also Supplementary Tables S3 and S4). Maximal significant expression was observed after 30 min incubation at any tested concentration, except for *copH*, which gave a significant maximum expression after 1 h (Supplementary Table S4). Expression was maximal after induction with 0.4 mM copper, and it dropped dramatically when the copper concentration rose to 0.6 mM. At 0.6 mM copper, the viability of the wild type started to decrease (Fig. 1). The group *copTKSRABCDI* was highly induced compared with the group *copJGFL* (Fig. 2, Table 3). Expression analysis indicated that the 19 ORFs located between the boundary genes *caiA* and *resU* were induced by copper, and so are likely to participate in the copper-resistance mechanism, with particularly high expression of the periplasmic *copABCD* genes, in addition to *copK* (94- to 1159-fold). MPL *copC* was also induced but at a much lower level than its pMOL30 counterpart (Table 3). The pMOL30 *copC* had a maximum induction at 30 min, while the MPL *copC* reached its maximum induction (31-fold) at 1 h (Fig. 3; Supplementary Table S4). These observations suggest a late and low activation of the MPL *copSRcopABCD* operon, which is probably involved in detoxification at lower Cu(II) concentrations (< 100 µM).

Six Cop proteins are directly visualized by proteomics

Exponentially growing cells, in the absence or presence of 0.8 mM copper, were analysed by comparative 2-DE (Fig. 4) (Mergeay *et al.*, 2003). Proteins induced by copper were identified by matching 2-DE maps of proteins extracted from copper-challenged cultures with a reference gel containing more than 1000 identified proteins by MS or Edman degradation analysis. In the presence of a high copper concentration near the MIC (0.8 mM), we observed from a total cell extract 10 induced proteins, while only one protein was repressed. Most of the induced proteins were pMOL30-encoded, such as CopR, CopS, CopC, CopK, CopB and CopA. The expression of chromosomal proteins with known functions (GroEL and GroES) and unknown functions [CupX (induced) and Cuf (repressed)] was also modulated by the presence of Cu(II) (Fig. 4). A similar

Table 2. Transcriptomic analysis (microarrays) from *C. metallidurans* CH34 cultures challenged with 0.1 mM Cu(II)

Total RNA extractions were performed after 30 min of 0.1 mM copper induction, and the total RNA was hybridized on a chip together with a total RNA extract from a non-copper-challenged culture. The table displays the rank of the genes by fold induction (Fold ind.) within all the *C. metallidurans* ORFs. The results represent the values obtained for two technical replicates present on the same chip, and two biological replicates (independent cultures), for which SD and *P* value (Student's *t* test) were calculated. The location of the gene within the genome is also shown.

Rank	Gene	Location	Fold ind.	SD	<i>P</i> value
1	<i>copC</i>	pMOL30	21.6	0.10	3.99×10^{-5}
2	<i>copB</i>	pMOL30	20.1	0.06	3.20×10^{-4}
3	<i>copQ</i>	pMOL30	18.1	0.15	1.17×10^{-4}
4	<i>copK</i>	pMOL30	15.0	0.51	2.94×10^{-3}
6	<i>copD</i>	pMOL30	14.1	0.35	1.24×10^{-3}
7	<i>copD</i>	MPL	13.7	0.22	2.26×10^{-2}
8	<i>copR</i>	pMOL30	13.3	0.36	1.38×10^{-3}
10	<i>copA</i>	pMOL30	11.8	0.41	8.08×10^{-3}
11	<i>copS</i>	pMOL30	8.8	0.24	8.69×10^{-4}
30	<i>copN</i>	pMOL30	5.1	0.42	7.64×10^{-3}
34	<i>copM</i>	pMOL30	4.9	0.27	2.75×10^{-3}
39	<i>copF</i>	pMOL30	4.6	0.25	2.70×10^{-3}
40	<i>copT</i>	pMOL30	4.5	0.02	1.04×10^{-2}
41	<i>copI</i>	pMOL30	4.4	0.62	2.49×10^{-2}
46	<i>copJ</i>	pMOL30	4.1	0.23	2.81×10^{-3}
48	<i>copA</i>	MPL	3.9	0.50	1.89×10^{-2}
49	<i>copG</i>	pMOL30	3.8	0.07	2.93×10^{-4}
62	<i>copB</i>	MPL	3.4	0.11	8.89×10^{-4}
68	<i>copR</i>	MPL	3.1	0.50	1.03×10^{-1}
82	<i>copE</i>	pMOL30	2.8	0.51	3.78×10^{-2}
95	<i>copC</i>	MPL	2.6	0.40	2.74×10^{-2}
122	<i>copV</i>	pMOL30	2.4	0.16	4.35×10^{-3}
139	<i>copL</i>	pMOL30	2.3	0.28	1.77×10^{-2}
163	<i>copS</i>	MPL	2.2	0.06	4.34×10^{-3}
168	<i>copH</i>	pMOL30	2.2	0.38	3.90×10^{-2}

protein expression profile was observed from a copper-challenged culture in the stationary phase, except that general stress proteins (GroEL and GroES) were not upregulated. The proteomic approach revealed the presence of different isoforms of CopC, CopK, CopB and CopA (Fig. 4). The precise function of these isoforms remains unknown. Moreover, the presence of the CopA in the 2-DE map was observed only when the total protein extract was dialysed against EDTA; this was probably due to the large amount of copper interacting with this protein. The proteomic analysis from the periplasmic fraction obtained by bacterial chloroform treatment showed that CopC and CopK are periplasmic proteins. The identification of the N-terminal sequence by Edman degradation of CopK and CopC (Fig. 4) confirmed the N-terminal maturation of these proteins when they are isolated from the periplasmic fraction or from the total protein extract. MALDI-TOF analysis, which enabled the display of surface and periplasmic proteins from the intact cells, confirmed the presence of a large amount of CopK, and, to a lesser extent, CopC.

DISCUSSION

In *C. metallidurans* CH34, one cluster of six genes, *copSRABCD*, is located on the smaller megareplicon (a MPL of 2.6 Mb). A second locus composed of nineteen genes and ORFs, *copV copT copM copK copN copSR copABCDI copJGF copL copQ copH copE*, is located on plasmid pMOL30, and is involved in high resistance to copper. In this locus, we discerned the presence of three types of genes, as follows. (1) Genes that have been recognized to be involved in the periplasmic detoxification of copper ions: *copSRABCD* (Cha & Cooksey, 1991; Rensing & Grass, 2003) and, very likely, *copI* (Francki *et al.*, 2000). The cluster *copSRABCD* was, in addition to pMOL30, also found on the MPL of *C. metallidurans*. pMOL30, however, contains some peculiar features, such as the CopB richness in methionine residues (motif MXXMXHXXM). This large motif suggests that the plasmidic *copSRABCD* genes are not merely a copy of the MPL counterpart, but may have a modified function by acting on a higher copper concentration range, or, possibly,

Table 3. Transcriptomic analysis of pMOL30 *cop* genes by quantitative PCR

The values are fold inductions, with respect to the unchallenged condition, for the *cop* genes, the flanking genes *caiA* and *resU*, and the MPL *copC*. Total RNA extraction was performed after 30 min metal induction. The expression level after 30 min was the maximal induction time observed for the genes, except for *copH* (Fig. 4). Expression of the *rrnA* gene (encoding the small-subunit rRNA, e.g.16S rRNA) was used as an internal control to normalize gene expression. The results are means $\pm$ SD ($n=4$).

Gene	Fold induction with Cu(II) at:				
	0.1 mM	0.2 mM	0.4 mM	0.6 mM	0.8 mM
<i>caiA</i>	1.36 $\pm$ 0.2	0.77 $\pm$ 0.1	1.58 $\pm$ 0.2	1.16 $\pm$ 0.4	0.94 $\pm$ 0.6
<i>copT</i>	38.6 $\pm$ 6.4	45.2 $\pm$ 11.1	9 $\pm$ 1.9	3.4 $\pm$ 0.8	1.4 $\pm$ 0.3
<i>copK</i>	158.8 $\pm$ 87.2	66.3 $\pm$ 22.9	165.1 $\pm$ 57.6	6.2 $\pm$ 3.1	2.1 $\pm$ 1.1
<i>copS</i>	54.4 $\pm$ 23.1	40.1 $\pm$ 18.4	50.4 $\pm$ 20.8	6.2 $\pm$ 3.6	1.1 $\pm$ 0.3
<i>copR</i>	51 $\pm$ 9.6	46.1 $\pm$ 15.1	54.5 $\pm$ 10.8	13.5 $\pm$ 3.1	6.2 $\pm$ 1.2
<i>copA</i>	86.1 $\pm$ 14.6	74.6 $\pm$ 10.1	94.4 $\pm$ 21.6	14.2 $\pm$ 3.4	4.6 $\pm$ 1.14
<i>copB</i>	134.5 $\pm$ 40	143.5 $\pm$ 31.7	203.3 $\pm$ 29.7	25.8 $\pm$ 8.9	6.4 $\pm$ 3
<i>copC</i>	278.9 $\pm$ 26.6	438 $\pm$ 52.4	1159 $\pm$ 677.4	31.4 $\pm$ 3.3	2.8 $\pm$ 0.6
<i>copD</i>	97.2 $\pm$ 27.7	67 $\pm$ 16.7	158.5 $\pm$ 40.2	10.5 $\pm$ 2.7	1.1 $\pm$ 0.3
<i>copI</i>	55.8 $\pm$ 11.2	16.9 $\pm$ 1.8	20.6 $\pm$ 1.8	5.5 $\pm$ 0.8	1.6 $\pm$ 0.2
<i>copJ</i>	4.6 $\pm$ 0.7	3.3 $\pm$ 0.6	7.9 $\pm$ 1.2	1.2 $\pm$ 0.2	0.33 $\pm$ 0.07
<i>copG</i>	8.4 $\pm$ 1.4	3 $\pm$ 0.7	11.3 $\pm$ 1.9	1.9 $\pm$ 0.3	0.72 $\pm$ 0.1
<i>copF</i>	10.1 $\pm$ 1.1	12.6 $\pm$ 1.3	22.6 $\pm$ 3	6.9 $\pm$ 2.4	1.6 $\pm$ 0.2
<i>copL</i>	16.8 $\pm$ 3	20.2 $\pm$ 2.9	26.1 $\pm$ 2.1	4.2 $\pm$ 1.4	0.59 $\pm$ 0.2
<i>copH</i>	15.3 $\pm$ 3.3	16.9 $\pm$ 3.4	15.5 $\pm$ 3.1	2.2 $\pm$ 0.4	0.4 $\pm$ 0.1
<i>resU</i>	1.4 $\pm$ 0.4	1.1 $\pm$ 1	2.4 $\pm$ 0.6	2.2 $\pm$ 1.1	0.22 $\pm$ 0.06
<i>copC</i> (MPL)	9.8 $\pm$ 5.4	11.2 $\pm$ 7	6.5 $\pm$ 4.7	3.8 $\pm$ 1.6	1.3 $\pm$ 0.6

by handling other ions, such as Ag(I) (Mergeay *et al.*, 2003). (2) Genes that could be involved in the cytoplasmic detoxification of copper ions, especially *copF*, encoding the CopF efflux P1-ATPase (Mergeay *et al.*, 2003; Rensing *et al.*, 2000), and probably *copL*, encoding a cytosol protein. The three genes *copJ*, *G* and *L*, located close to *copF*, encode proteins with cysteine-rich motifs (CXXC or CXCC) that could be partners of the P-ATPase (CopF). (3) Genes for which no or only very few equivalents have been found in

databases; these include *copK*, *J*, *G* and *H*, and also the five small ORFs *copV*, *M*, *N*, *Q* and *E*, which are overexpressed under Cu(II) challenge, as observed in microarrays. Proteomic data show CopK as a major overexpressed protein in the presence of low (0.1 mM) Cu(II). The first published data of the protein structure analysis of CopK have been released (Tricot *et al.*, 2005). *copH* seems to intervene at a later stage in detoxification, because its maximum expression occurs after 1 h.

Fig. 3. Expression patterns as a function of time after induction with 0.4 mM copper. The fold induction, with respect to the unchallenged condition, is shown for 14 *cop* genes, and the MPL *copC*, after addition of 0.4 mM Cu(II), and three exposure times: 5, 30 and 60 min.

Fig. 4. Copper-induced polypeptides in *C. metallidurans* CH34. (a) 2-DE of total proteins of *C. metallidurans* CH34 grown in the absence (control) and presence of 0.8 mM copper. The circled spots are differentially expressed, and include the isoforms. For unambiguous protein identification, MALDI-TOF results were confirmed by microsequence analysis using the Edman degradation or the ESI MS/MS approach. The genes encoding CopX and CupF are chromosomal. (b) MALDI-TOF mass spectrum of CopA. The shaded parts of the CopA sequence correspond to the ions in the CopA MALDI-TOF mass spectrum. (c) Identification of up- or downregulated proteins by N-terminal sequencing, peptide mass fingerprinting, or tandem MS sequencing. Sequence information generated by Edman degradation (underlined) or tandem MS sequencing is given. When MALDI-TOF MS was used for protein identification, sequence coverage is indicated, as calculated by the ProteinProbe search engine.

The entire cluster of 19 genes and ORFs appears to be highly specialized in the response to high concentrations of Cu(II), and it is unique, as it brings together genes involved in the detoxification of the periplasm and the cytoplasm, with possible coordination between these two processes. Other genes present on pMOL30 could also be involved in the response to copper. Indeed, microarrays indicated that other pMOL30 genes responded to copper challenge at levels similar to those observed for most of pMOL30 *cop* genes.

Besides the pMOL30 plasmid, the *C. metallidurans* genome contains number of genes possibly related to the response to copper. On the MPL, we identified *copSRcopABCD* and *cusCBAX*. On the chromosome, we found a cluster of three genes, *cupG cupA cupR*, involved in the expression of a copper P-type ATPase (CupA) (Monchy *et al.*, 2006).

The pMOL30 copper-resistance cluster of 19 genes and ORFs is unique because it is composed of a large cluster of genes and functions, but it provides only a slight increment in the MIC. This is a major contrast to resistance to zinc (33-fold increase of MIC) and cobalt (133-fold increase) in *C. metallidurans* CH34, mediated by the *czc* cluster of genes in pMOL30, and to nickel in *C. metallidurans* 31A (40-fold increase due to the *ncc* operon). Alternatively, it may be speculated that at this range of copper concentration, any increase in the MIC would require a special and costly genetic investment. The genesis of the mosaic structure of the pMOL30 *cop* cluster could have arisen by various rearrangements, including lateral gene transfer, and duplications of genes, and even motifs (CopB).

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