

Mini-symposium

Cupriavidus metallidurans:
a unique β -proteobacterium,
appearing where you least expect it.

A status of 30 years dedicated research

Mol, Belgium, April 21-23, 2008

Hosted by the SCK•CEN
The Belgian Nuclear Research Centre
Co-sponsored by the BSM
Belgian Society for Microbiology



SCK•CEN
Boeretang 200
BE-2400 Mol
Belgium
<http://www.sckcen.be>

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Introduction

Cupriavidus metallidurans is a β -proteobacterium which can be found in some of the harshest environments. Already 30 years of dedicated and collaborated research has unravelled many of *C. metallidurans* unique properties. Besides being an excellent survivor and a model system for heavy metal response, *C. metallidurans* harbours a number of interesting characteristics of which some are already economically or ecologically exploited.

This 2008 workshop on *C. metallidurans* aims to offer a forum, where milestones from the past, basic fundamental research, innovative applications and emerging new research can be discussed. We hope that everyone, who is involved or interested in *C. metallidurans* investigation, will be able to communicate his or hers ideas, work or achievements.

Welcome at the SCK•CEN in MOL (Belgium)!

Programme: Mini-symposium *Cupriavidus metallidurans*

21-April-2008

- 18h00 **Welcome, Registration and buffet**
- 19h50 Introduction
Max Mergeay
- 20h00 *Cupriavidus metallidurans* strain CH34: Evolution of a metal-resistant bacterium
Dietrich Nies

22-April-2008

Chairmen: Jacques Mahillon

- 9h00 The genome annotation of *Cupriavidus metallidurans*: status report and first insights
Paul janssen
- 9h30 *Cupriavidus metallidurans* CH34 genomic islands and environmental adaptation
Max Mergeay
- 10h10 ACLAME: a database for the classification of the prokaryotic Mobilome
Ariane Toussaint
- 10h40 **Coffee Break**

Chairwomen: Nathalie verbruggen

- 11h00 *Cupriavidus metallidurans* CH34: from ecology to applications
Ludo Diels
- 11h30 Probing the metal-responsive MerR regulator complement of *Cupriavidus metallidurans* using a broad host range promoter probe plasmid
Jon Hobman
- 12h00 Lead resistance in *Cupriavidus metallidurans* CH34 – regulation of gene expression and lead(II)-selectivity
Daniel van der Lelie
- 12h30 *Cupriavidus metallidurans* CH34: metal response a transcriptomic overview
Pieter Monsieurs
- 13h00 **Sandwich lunch + poster session**

Chairmen: Nicolas Glansdorff

- 14h00 *Cupriavidus metallidurans* CH34 resistance to copper: a differential proteomic analysis using a gel free approach
Baptiste Leroy
- 14h30 The cop genes of *Cupriavidus metallidurans* CH34 pMOL30 plasmid are responsible for two kinds of functions: active resistance to copper and protection around the minimal inhibitory concentration
Sébastien Van Aelst
- 15h00 Characterization of CopH and CzcE from *Cupriavidus metallidurans* CH34 revealed unusual modes of copper binding
Jacques Covès
- 15h40 **Coffee Break**

Chairwomen: Martine Cuillel

- 16h00 Structural and metal-binding studies of CopK, a periplasmic protein involved in copper resistance in *Cupriavidus metallidurans*
Beate Bersch
- 16h30 Characterization of the Sil system from *Cupriavidus metallidurans* CH34
Guy Vandebussche
- 17h00 Relevance of the four Pb(II)/Zn(II)/Cd(II) P-type ATPases for cadmium, cobalt or zinc detoxification in *Cupriavidus metallidurans* CH34
Judith Scherer
- 17h30 β -proteobacterial and not archaeal ammonia oxidizing bacteria restore nitrification in a zinc contaminated soil
Jelle Mertens
- 19h00 **Conference dinner**

23-April-2008

Chairmen: Jos Vanderleyden

- 9h00 The use of a microarray to investigate microbial weathering of basalt by *Cupriavidus metallidurans* CH34
Charles Cockell
- 9h30 Study of the acetone metabolic pathway in *Cupriavidus metallidurans* CH34
Caroline Rosier
- 10h00 The Quorum Sensing system of *Cupriavidus metallidurans* CH34:
organisation, function and regulation
Hugo Moors
- 10h30 **Coffee Break**

Chairwomen: Ariane Toussaint

- 10h50 Horizontal gene transfer by broad-host-range plasmids: lessons from *Cupriavidus metallidurans* CH34
Eva Top
- 11h20 *Cupriavidus metallidurans* CH34 in space
Natalie Leys
- 12h00 Cupro-resistant bacteria from Katanga: soil dwellers and endophytic species
Corinne Vander Wauven
- 12h30 **Hot lunch in restaurant**

Chairwomen: Tatiana Vallaey

- 14h00 "Nano-toxicity" of CdTe quantum dots towards bacteria
Christophe Merlin
- 14h30 Chemical forms of selenium accumulated by *Cupriavidus metallidurans* CH34
exposed to selenite and selenate
Geraldine Sarret
- 15h00 Bio-Arsenic: from Ancient Genes (and Proteins) to Modern Geocycles and
Global Pollution
Simon Silver
- 15h30 Panel discussion: Perspectives and future in *Cupriavidus metallidurans* research
Panel: Max Mergeay, Dietrich H. Nies, Simon Silver, Rob Van Houdt
Moderator: Natalie Leys
- 16h00 **Symposium end**

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ABSTRACTS

Oral presentations

***Cupriavidus metallidurans* strain CH34: Evolution of a metal-resistant bacterium**

Dietrich H. Nies, Judith Scheerer, Torsten von Rozicki, Doreen Koch, Doreen Munkelt, Grit Rehbein, Antje Legazki, Gregor Grass and Cornelia Große
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Evolution is a difficult problem to tackle because we cannot test our theories directly in an experiment. To define “metal resistance” as a first step, the metal-resistant bacterium *Cupriavidus metallidurans* strain CH34 is compared to the moderately metal-tolerant bacterium *Escherichia coli*. RND (resistance, nodulation, cell division) systems for metal detoxification are numerous in *C. metallidurans* while *E. coli* contains only one. RND systems form a first line of defense against superfluous metal concentrations. Evidence from several RND systems indicates that RND systems *in vivo* protect the periplasm against heavy metal cations. CDF (cation diffusion facilitators) and P-type ATPases transport toxic metals from the cytoplasm across the cytoplasmic membrane, forwarding them to the RND systems. Contribution of at least one of the two chromosomal Zn/Cd-exporting CPx-type ATPases of *C. metallidurans* is essential for full cadmium resistance, but not for full zinc resistance. Three groups of CDF proteins can be differentiated. Each group contains transporters with a broad substrate specificity, which is respectively centered around the central substrates Fe(II), Zn(II) or Co(II). The function of CDF proteins is essential to obtain cobalt resistance in *C. metallidurans*, which demonstrates that RND proteins are unable to detoxify substrates from the cytoplasm, but gather them in the periplasm instead.

Taking it all together, *C. metallidurans* has more and more sophisticated metal resistance determinants than the “control” *E. coli*.

Signatures of evolution are:

- (i) a reservoir of metal resistance genes in a kind of “ecological metagenome”,
- (ii) accumulation of these genes in the genome of *C. metallidurans*,
- (iii) usage of multiple back-up systems for metal resistance functions in a mix of inducible, constitutively expressed and silent operons.

Plasmids and transposons were obviously needed to acquire these genes by horizontal gene transfer and to swap them between plasmids, chromosomes and different operons. Although not completely clear, a fascinating picture arises how the past of *C. metallidurans* might have looked like.

The genome annotation of *Cupriavidus metallidurans*: status report and first insights

Paul Janssen¹, Rob Van Houdt¹, Hugo Moors¹, Sébastien Monchy^{1,2}, Pieter Monsieurs¹, Claudine Médigue³ and Max Mergeay¹

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The genome of *C. metallidurans* consists of four replicons: a 3.93 Mb chromosome (CHR), a 2.58 Mb large megaplasmid (MPL) and two smaller plasmids pMOL28 and pMOL30 that are 171 and 234 kb in size, respectively. The genes of the two plasmids were earlier annotated and analysed for expression under various heavy metal conditions (1). Since the completed DNA sequence of the two large replicons is available from GenBank, we decided to annotate these replicons by importing their DNA sequences into the MaGe annotation platform (2). This system offers a set of graphical interfaces (with connections to other data repositories) allowing biologists to perform relevant expert annotation.

In a first annotation phase, gene structures and other features were automatically predicted by a variety of methods and algorithms, and genes were functionally annotated by similarity searches in protein sequence databases. This phase also provided prediction of protein localisation and enzymatic functions. In a second stage, automatically generated data was browsed for additional manual annotation based on various lines of evidence. Importantly, the data generated by the automated and manual annotations were stored away into relational databases using MySQL, allowing easy export of data into multiple formats and the extensive exploration of all results through complex querying.

From the 3,815 CHR and 2,531 MPL genes predicted *in silico*, we manually annotated 3,770 (99%) and 1,261 (50%) genes (and their products), respectively. Most of those encoded enzymes (35%) or putative enzymes (6%). The second largest group consisted of transporters (14%) followed by regulators (10%). Overall, around one quarter of gene products retained the status of hypothetical (9%) or conserved hypothetical (19%). Remarkably, almost 1,400 proteins appeared to group into 43 clusters of 10 or more members, with 4 clusters holding more than 110 members. These large paralogous families represent GGDEF/EAL domain proteins involved in signal transduction (N=138), extra-cytoplasmic solute receptors (BugT family) (N=123), LysR-type transcriptional regulators (N=121), and ABC-transporter related proteins (N=110).

Currently, the genome sequences of 626 bacterial species, including four *Cupriavidus* species that were isolated from diverse biotopes and which have different life styles, are available (3, 4). This opens up a range of opportunities for comparative genomics. A special feature of the MaGe system is the use of genome syntenic maps allowing easy detection of genome rearrangements, thereby giving clues about possible gene transfer events and the presence of mobile genetic elements. In addition, the MaGe Explore function provides precalculated orthology data leading to interesting observations in terms of evolution, metabolic capacity, physiology, and biochemical pathway organisation.

References:

- (1) Monchy *et al.* 2007. J Bact 189:7417-7425.
- (2) Vallenet *et al.* 2006. Nucl Ac Res 34:53-65; (<https://www.genoscope.cns.fr/agc/mage/>)
- (3) The Genomes On Line Database at <http://www.genomesonline.org/>
- (4) NCBI/Genbank depository at <http://www.ncbi.nih.gov/>

ACLAME: A database for the classification of the prokaryotic mobilome.

Raphael Leplae, Gipsi Lima-Mendez and Ariane Toussaint

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Prokaryotic Mobile Genetic Elements (MGEs) are central players in mobilizing genes, whether within a given genome (intra-cellular mobility) or between bacterial cells (inter-cellular mobility). Traditionally, MGEs have been classified as either bacteriophages, plasmids or transposons, a classification that becomes more and more obsolete as many chimerical elements are identified, which share features of more than one of those families. Prokaryotic MGEs can often be subdivided into a series of complex functional modules (see 2 for phages). Based on this 'functional module' principle, we have built the ACLAME database of prokaryotic MGEs. The database currently contains 457 phage genomes and 1109 plasmids. Proteins encoded by these elements have been classified automatically into families (similar sequences and same function) using the Search [1] and MCL [2] algorithms. Many families have been annotated manually using the GeneOntology (GO) [3] and the Phage Ontology (PhiGO) [4]. Using the information available in the ACLAME database, advanced analysis can be performed such as the construction of a reticulate classification of the phage population using graph theory (see abstract of G. Lima-Mendez, J. van Helden, A. Toussaint and R. Leplae: Reticulate representation of evolutionary and functional relationships between phage genomes). An heuristic prediction tool called Prophinder [5] has been developed for accurate prophage detection in bacterial genomes. A BLASTP interface allows searching similar protein sequences in ACLAME and therefore provides a simple solution to help MGE annotations process.

The ACLAME database and the associated tools are accessible via a web interface: <http://aclame.ulb.ac.be>. An open annotation system is available, allowing the community contributing to the annotation process.

References:

1. Pearson W, Lipman D: Improved tools for biological sequence comparison. *Proc Natl Acad Sci U S A* 1988, 85(8):2444-2448.
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Cupriavidus metallidurans CH34 genomic islands and environmental adaptation

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The facultative chemoautolithotrophic β -proteobacterium *Cupriavidus metallidurans* CH34 (formerly *Ralstonia metallidurans*) is equipped to overcome acute environmental stresses and to survive in oligotrophic mineral environments. Besides two megareplicons (chromosome (CHR) 3.9 Mb and megaplasmid (MPL) 2.6 Mb), *C. metallidurans* CH34 contains a high amount of various Mobile Genetic Elements (MGEs), including plasmids, transposons, genomic islands and IS elements and was intensively studied for its resistance to at least 15 heavy metal cations or oxyanions, mainly governed by genes located on the plasmids pMOL28 (171 kb) and pMOL30 (234 kb) (1).

The plasmid-borne resistance genes are located on three islands (two on pMOL30: CMGI-30a and b, and one on pMOL28: CMGI-28a) flanked by IS elements or a mercury transposon (pMOL30) in such way that one extremity is partially deleted to ensure for genetic stability. These three islands contain around 120 metal response or resistance genes further maximising the already substantial tolerance of heavy metals governed by chromosomal and MPL genes.

The chromosome of *C. metallidurans* CH34 contains also seven genomic islands revealed by at least three or more of the following features: lack of synteny with related genomes (*Cupriavidus*, *Ralstonia*, *Burkholderia*), location near tRNA genes, presence of recombinase genes at one extremity, presence of plasmid maintenance, mobilisation or transfer genes, presence of metal resistance or catabolic genes, higher density in IS elements or in unknown genes. CMGI-1 (105kb), the main genomic island of the *C. metallidurans* chromosome is identical to PAGI-2C found in *P. aeruginosa* clone C (2) strains isolated from cystic fibrosis patients. This island containing some lead resistance genes that were also found in metal resistance transposons of *B. xenovorans* and other betaproteobacteria. CMGI-7, a small island, contains genes for the resistance to arsenic oxyanions. CMGI-2 and CMGI-3 belong to the family of catabolic transposon Tn4371 of *R. oxalatica* (3). CMGI-3 carries genes involved in degradation of benzene, toluene and xylene. CMGI-2 and CMGI-3 carry all the genes involved in chemolithoautotrophic growth: these genes are flanked by IS elements and especially IS1071, often encountered in catabolic plasmids. This lets think to a rather recent capture of the genes allowing the growth in presence of H₂ and CO₂. A curious feature of the genomic islands of *C. metallidurans* is the systematic inactivation (and therefore the stabilisation) of key mobility genes by new kinds of MGEs as TnCme2, a four genes transposon containing *gspA* (a gene encoding a major protein of type II secretion pathway) or genetic structures made by triads of XerD-like recombinase genes.

In conclusion, the expert annotation of the *C. metallidurans* genome clearly allowed to put in evidence the unexpected diversity of MGEs in soil bacteria and other bacteria adapted to harsh and industrial environments. The evaluation of the effective diversity of MGEs is a key element in preventing undesired gene dissemination in confined environments as the ISS (International space station), the polar scientific stations, the space stations and later the inhabited planetary stations.

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- (1) Monchy, S., et al. Plasmids pMOL28 and pMOL30 of *Cupriavidus metallidurans* are specialised in the maximal viable response to heavy metals. *J.Bacteriol.*,2007,189,7417-7425
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Cupriavidus metallidurans: from ecology to applications

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Cupriavidus metallidurans is a β -proteobacterium isolated from a metal contaminated decantation basin. Further investigations showed the strain bearing many special survival systems which allowed that strain not only to live under chemolithotrophic conditions, but also in harsh environmental situations as heavy metal pollutions. Often this strain was grouped as an extremophile. As several related strains could be isolated from different environments there was no doubt anymore about the specific ecological niche in which the hydrogen bacteria could live, emerge and survive.

The presence of several large plasmids and different mercury transposons certainly are interesting tools to allow the strain to exchange specific genes and to assemble specific pathways and heavy metals resistance concepts.

The way the strain is reacting on heavy metals where metals play the role of inducer of the metal resistance operons allowed us to construct several biosensors, now patented under the name BIOMET[®]. The sensors were based on the combination of regulatory genes with *lux* genes. In this concept the sensors are producing light in relation to the presence of available metals even in solid matrices. The fact that the strain can survive in harsh environments make them to so called positive sensors in the way they produce more light in function of increasing metal availability. At the moment these sensors are about the only ones allowing to detect directly bioavailability of heavy metals in solid environmental matrices as soil, sludge, waste, suspended solids etc.

As *C. metallidurans* is not only sensing the metals but also doing something with it, other applications are also available. The combination of chemiosmotic efflux systems with the production of extracellular polymers bearing functional groups that bind metal ions allow the strains to prevent continuous circulation of metals from the environment to the cytoplasm and vice versa. Finally, a very interesting precipitation process of metal carbonates occurs at the cell wall of the strain.

The metal precipitation process allows the strains to remove metals from industrial effluent. The use of the strains in a moving bed sandfilter allowed to remove metals in a continuous and controlled way from effluents were regeneration was a non line ongoing process contrary to the classical biomass adsorption based processes which always failed commercially due to this regeneration need. The so called MERESAFIN process (as well patented) allowed to control the biomass growth (active system) and removal of metal precipitates from the biomass in a sand washing device allowing to a sludge which contained up to 20% of heavy metals, by far higher than the adsorption processes.

This metal binding and precipitation process allowed us also to produce metal crystals in a controlled way in a contactor or so called and patented BICMER approach. Nowadays, in the new era of nanocrystal developments this idea gains new interested and will be again exploited. Some new ideas concerning future applications will be presented based on the immobilization of metals (with specific catalytic activities and specifications) on membrane surfaces for new catalytic chemical reactor systems necessary in the further development of fine chemicals conversion processes.

Probing the metal-responsive MerR regulator complement of *Ralstonia metallidurans* using a broad host range promoter probe plasmid

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The MerR family of metal-ion responsive transcriptional activators includes the archetype of the family, MerR which responds to Hg, ZntR (Zn), CueR (Cu and Ag), CadR (Cd) and PbrR (Pb). There are 9 known or predicted metal ion responsive MerR family regulators in *C. metallidurans* CH34 (reviewed in Mergeay et al., 2003), those of known function include several that are highly related or identical to the Hg(II) responsive Tn501 MerR (Diels et al., 1985; Brown et al., 2003), the Pb(II) responsive PbrR from the *pbr* operon carried on pMOL30 (Borremans et al., 2001), and a chromosomal PbrR homologue, *pbrR691* (Chen et al., 2005). Other MerR family regulators have been identified by sequence similarity and proximity, or functional linkage, to genes predicted to encode metal-ion resistance proteins, and include CupR, and PbrR₂ (Monchy et al., 2006b).

Here, we report the use of a plasmid-borne β -galactosidase assay to assess metal ion specificity and activity of MerR family activators encoded on the chromosome or endogenous plasmids in *C. metallidurans*, using the broad host range *lacZ* promoter probe plasmid, pMU2385. We used derivatives of pMU2385 containing cloned MerR family promoters as targets for transcriptional activation by MerR family regulators in *C. metallidurans*. We also demonstrate that pMU2385 is suitable for *in trans* assays of cloned MerR family regulators in *C. metallidurans*.

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Heavy metal resistance in *Cupriavidus metallidurans*: towards the reconstruction of regulatory networks

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Bioremediation technologies aim at the use of different types of micro-organisms for cleaning up environments that are contaminated with organic pollutants, pharmaceutical products, or heavy metals. The soil bacterium *Cupriavidus metallidurans* shows great promise in light of these technologies since it contains a significantly high number of metal-resistance genes. Recently, the two plasmids pMOL28 and pMOL30 were annotated and various heavy metal resistance gene clusters were identified like the *cop*, *czc* and *mer* genes. Thanks to progress in the annotation efforts directed to the two larger replicons (chromosome and mega-plasmid) and the availability of whole-genome expression data, it now has become feasible to investigate the full genomic response of *C. metallidurans* when challenged to different heavy metals. Our ultimate goal is the full reconstruction of regulatory networks enabling *C. metallidurans* to overcome metal toxicity.

We have performed a large number of microarray experiments to follow the whole-genome transcriptomic response of *C. metallidurans* when exposed to heavy metals such as zinc, copper, cadmium, and lead. Obtained data suggest that the total number of genes that are up- or down-regulated relates to the level of toxicity exerted by the heavy metal e.g. the more toxic a heavy metal is for *C. metallidurans*, the more severe the transcriptional response appears to be.

In addition, genome-wide expression experiments also show that there is multiple cross-talk at transcriptional level between the different heavy metal responses. Different clusters of co-expressed genes can be identified showing similar expression profiles when exposed to varying combinations of heavy metals. Some genes were only switched on by one particular metal while others were activated by different metals. This suggests that *C. metallidurans* reacts to (heavy) metal toxicity at two separate transcriptional levels corresponding to an initial global response and a secondary metal-specific response.

Our hypothesis is that such a complex transcriptional response must be mediated by a joined action of different transcription factors (TFs) that bind common or variant DNA target sites (i.e. regulatory motifs) near the initiation site of transcription and generally assist or repress RNA polymerase activity. In order to reconstruct these metal-response transcriptional regulatory networks in *C. metallidurans*, we have started to search for regulatory motifs in the promoter regions of co-regulated metal-induced genes and their orthologs. An overview of the different methods that can be used to detect such motifs and preliminary results will be presented.

Lead resistance in *Cupriavidus metallidurans* CH34 – regulation of gene expression and lead(II)-selectivity

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Lead contamination is a serious threat to human health and the environment. *Cupriavidus metallidurans* CH34 is the only bacterium that has been shown to contain a lead-specific resistant pathway so far. The *pbr* operon, located on plasmid pMOL30, combines functions involved in uptake (*pbrUT*), efflux (*pbrA*), and accumulation/sequestration (*pbrBCD*) of Pb^{2+} . All genes in the *pbr* operon (*pbrUTRABCD*) are regulated by PbrR, which mediates Pb^{2+} -inducible transcription. All genes show basic levels of transcription in the absence of Pb^{2+} ; the presence of Pb^{2+} results in a significant induction of *pbrABC*, which constitute the core of the Pb^{2+} resistance. Mutation analysis revealed that inactivation of *pbrB* resulted in a loss of Pb^{2+} resistance, while inactivation of *pbrA*, *pbrC* or *pbrD* did not affect the Pb^{2+} resistance phenotype. However, the presence of an additional *pbrRAC*-like operon on the PAGE-2C island might allow for complementation for the inactivated *pbrA* and *pbrC* genes.

PbrR691 of the *pbrRAC*-like operon has been assigned as a homologue of PbrR; In contrast to PbrR, PbrR691 could be overexpressed and purified from *E. coli*, allowing studying *in vitro* binding of Pb^{2+} . PbrR691 was found to bind Pb^{2+} almost 1,000-fold more selectively over other metal ions. The unprecedented selectivity exhibited by PbrR691 prompted us to study the underlying molecular mechanism.

PbrR691 belongs to the MerR family transcriptional factors that regulate the concentrations of a range of toxic or essential metal ions in bacteria. The prototype is the Hg^{2+} -binding MerR itself that uses three highly conserved Cys residues to selectively bind Hg^{2+} in a proposed trigonal geometry. A sequence alignment indicates that the three Cys residues are conserved as Cys78, Cys113, and Cys122 in PbrR691. It is not a surprise that Cys residues are used to recognize soft Pb^{2+} , but how PbrR691 discriminates other soft metal ions from binding is unclear.

The coordination chemistry of Pb^{2+} is quite unique. The relativistic effect causes contraction of the 6s orbital of Pb^{2+} . As a result, Pb^{2+} exhibits a stereochemically active lone pair that is resistant to engage in binding to ligands. Thus, in low coordinate Pb^{2+} complexes (3- or 4-coordinate), the lone pair likes to be exposed and Pb^{2+} center adopts a hemidirected geometry with all ligands clustered on one side of the metal. We were able to demonstrate that protein folding enforces such a unique geometry which enables PbrR691 to selectively bind Pb^{2+} and exclude other soft metal ions.

***Cupriavidus metallidurans* CH34 resistance to copper: a differential proteomic analysis using a gel free approach**

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Cupriavidus metallidurans CH34 is resistant to high concentration of different metal such as zinc, cadmium or copper. Although extensive effort have already been undertaken in order to understand the fine mechanism of copper resistance, some crucial point are still unclear. Genomic and transcriptomic analyses have already highlighted different gene possibly related to the response to copper. Gel based proteomic analyses have also been used and allowed to reveal the important role of different protein such as CopK. Here, we used a gel free differential proteomic approaches in order to identify supplemental proteins implicated in the copper resistance in *Cupriavidus metallidurans* CH34. This approach consists in crude proteome enzymatic digest followed by analysis in liquid chromatography of produced peptides. It allows rapid and large scale proteome mapping and is amenable to relative quantification of proteins. Using this approach several hundred of proteins have been identified and their response to copper stress has been monitored. The principal results of this analysis as their implication in general copper resistance mechanism will be discussed.

The *cop* genes of *Cupriavidus metallidurans* CH34 pMOL30 plasmid are responsible for two kinds of functions: active resistance to copper and protection around the minimal inhibitory concentration

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Cupriavidus metallidurans CH34 carries a cluster of 21 *cop* genes induced by copper and other heavy metals on plasmid pMOL30 : *copVTKMN SRABCDIJGFO LQHEW*. *copVTKMN* and *copLQHEW* genes have no or very few equivalents in the genomic databases. The whole cluster belongs to the CMGI-30a island of pMOL30, is flanked by (deleted) tyrosine recombinase genes and was cloned on a vector giving rise to plasmid pMOL1024. The introduction of pMOL1024 in a plasmid-free derivative of *C. metallidurans* CH34 increased the MIC of the strain to the wild-type level. Mini*Tn5* insertion was used to *in vitro* mutagenize the *cop* genes. Mutants were obtained in 15 genes (*copVTMK SRABD JGF LQE*). Their phenotype was analyzed in two genetic contexts: the plasmids pMOL30 and pMOL1024. Two main phenotypes were observed:

- (i) mutations in *copSRABDJFE* led to sensitivity to Cu(II) and correspond to genes encoding the well documented active detoxification of the cell
- (ii) mutations in *copVTMK* and *copGLQ* were as resistant or even more resistant than their parents except at high copper concentrations (above or equal to MIC) while the wild-type strain displayed a kind of persistor state in the domain 1,5mM to 3mM in cultures preinduced by Cu(II).

The *copB* mutant is particularly interesting in the pMOL1024 context where it is more sensitive to copper than the plasmid –free derivative while in the pMOL30 context the mutation maximizes the resistance to copper resistance except around the MIC. This variation in phenotype reveals the importance of examining the behaviour of the mutants in different genetic backgrounds and different culture conditions.

The role of *copVTKM B GLQ* genes will be investigated in the perspective of the long term static survival in soil environments with bioavailable metal concentrations largely superior to the MIC as it was the case in industrial environments where strain CH34 and other *C. metallidurans* strains have been isolated.

Characterization of CopH and CzcE from *Cupriavidus metallidurans* CH34 revealed unusual modes of copper binding

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CopH [1] and CzcE [2] are two periplasmic proteins involved in copper resistance in *Cupriavidus metallidurans* CH34. Each of them has its own unusual copper binding mode.

Dimeric wild-type CopH contains two high-affinity copper-binding sites, one in each subunit. They are characterized by a distorted octahedral geometry with a 2N 2O coordination sphere in the equatorial plane. Double mutation of the histidine residues previously suspected to be part of the Cu(II)-binding sites does not modify the number of strongly coupled nitrogens demonstrating that the two histidine imidazole groups are not ligand of the metal. A weakly coupled remote nitrogen is also detected that remains present upon mutation of the two histidines. These mutations cause a dramatic decrease of the affinity for copper assigned to a modification of the local structure of the binding sites. We suggest that the two strongly coupled nitrogens are provided by deprotonated backbone nitrogens located in the spatial proximity of the histidine side chains. This unprecedented coordination chemistry should favor the traffic of Cu(II) between CopH and putative partners.

CzcE is encoded by the most distal gene of the *czc* determinant that allows *C. metallidurans* CH34 to modulate its internal concentrations of cobalt, zinc and cadmium by regulation of the expression of the efflux pump CzcCBA. CzcE is a dimeric protein able to specifically bind 4 Cu-equiv per dimer. Spectrophotometry and EPR are indicative of type II copper with typical d-d transitions. Re-oxidation of fully reduced CzcE led to the formation of an air-stable semi-reduced form binding both 2 Cu(I) and 2 Cu(II) ions. The spectroscopic characteristics of the semi-reduced form are different of those of the oxidized one, suggesting a change in the environment of Cu(II). This protein encoded for by a gene transcribed in the presence of zinc is actually specific for copper. This could be an elegant strategy for the bacterium to respond to environmental conditions in which copper is associated to zinc in multi-elemental pollutions. CzcE could be a metal sensor protein and its role should be to trigger the induction of the genes required for copper resistance.

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Structural and metal-binding studies of CopK, a periplasmic protein involved in copper resistance in *Cupriavidus metallidurans*

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CopK is a periplasmic protein found in *Cupriavidus metallidurans*. The corresponding gene belongs to the *cop* operon, situated on the plasmid pMOL30, which comprises 19 *ORFs* coding for proteins involved in copper detoxification (1). The synthesis of CopK is highly induced by copper but its exact function remains to be determined.

The structure and metal-binding properties of CopK were investigated by NMR and mass spectrometry. The well-defined structure shows the presence of two β -sheets in a perpendicular arrangement and an unstructured C-terminal tail. ¹⁵N-relaxation data indicate that *apo*-CopK is dimeric. Numerous experimental observations suggest that the dimer interface is formed by the surface of the C-terminal β -sheet.

Metal-binding to CopK, which contains no cysteine residues, was studied using IMAC chromatography, mass spectrometry and NMR. CopK was found to bind Cu(I), Cu(II) and Ag(I). While binding of Cu(II) appears to be non specific, CopK bound two Cu(I) ions by subunit with nano- and micromolar affinity. Cu(I)-binding induces significant structural modifications as seen in the NMR experiments. Comparison between *apo* and Cu(I)-bound CopK shows that copper-binding induces partial opening of the C-terminal β -strand whereas ¹⁵N-relaxation of the Cu(I)-bound protein clearly demonstrates dissociation of the dimeric *apo* protein due to copper binding.

Charaterization of the Sil system from *Cupriavidus metallidurans* CH34

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Cupriavidus metallidurans CH34 (formerly known as *Ralstonia metallidurans*) is a β -Proteobacterium colonizing industrial sediments, soils or wastes with a high content of heavy metals. The large number of 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.

The tripartite CBA efflux systems driven by proteins of the resistance-nodulation-cell division superfamily (RND) are highly represented in *Cupriavidus metallidurans* CH34 as 12 gene clusters encoding putative HME-RND (RND/Heavy Metal Efflux) were identified in the bacterium genome. These HME-RND systems are composed of a RND-type inner membrane protein (cation/proton antiport protein A), a periplasmic membrane fusion protein (MFP – protein B), and an outer membrane protein (OMF – protein C). Using a proteomic differential approach based on the separation of membrane protein rich fractions by two-dimensional electrophoresis, we have demonstrated the overexpression of at least three uncharacterized resistance protein HME-RND complexes in response to copper or silver, and to nickel or cobalt.

The system Sil, induced by copper or silver, has been chosen for further characterization. The genes *silA*, *silB* and *silC* were cloned and the corresponding proteins were overexpressed in different *E. coli* strains. The proteins were purified by immobilized metal ion affinity chromatography, in the presence of n-dodecyl β -D-maltoside for SilA and SilC. The membrane proteins SilA and SilC were reconstituted in lipid vesicles composed of *E. coli* polar lipid extract. The characterization of the three proteins in terms of structure and function is in progress.

Relevance of the four Pb(II)/Zn(II)/Cd(II) P-type ATPases for cadmium, cobalt or zinc detoxification in *Cupriavidus metallidurans* CH34

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The megaplasmid pMOL30-encoded CzcCBA cation-proton-antiporter complex is one of the most complex and efficient heavy-metal resistance systems known. CzcCBA is essential for survival of *Cupriavidus metallidurans* CH34 at high cobalt, zinc or cadmium concentrations. There is accumulating evidence that this protein complex transports the metals Co(II) and Cd(II) not from the cytoplasm but from the periplasm to the outside. For transport from the cytoplasm to the periplasm other proteins are utilized, e.g. cytoplasmic Co(II) is detoxified by the chromosomally-encoded CDF-transporter DmeF.

The genome of *C. metallidurans* CH34 encodes four genes for Pb(II)/Cd(II)/Zn(II) P-type ATPases. Two are located on the chromosomes (*cadA* and *zntA*) and two on the plasmid pMOL30 (*czcP* and *pbrA*). For Cadmium, studies with strains deleted in one or all genes for these P-type ATPases lead to the conclusion that Cd(II) detoxification is also a two step mechanism. In a first step ions are transported by the ATPases CadA, ZntA, CzcP and PbrA to the periplasm and in a second step the cations are extruded by CzcCBA to the outer medium. Heterologous expression of the ATPase genes in the Cd(II)/Zn(II) sensitive *E. coli* strain GG48 lead to increased growth yield under Cd(II) stress in contrast to a vector only control. Resistance was conferred in following order: *cadA* > *zntA* >> *pbrA* >>> *czcP* ≈ vector control.

For zinc, an alternative mechanism is possible. All Zn(II) transporting P-type ATPases were dispensable for full Zn(II) resistance. Solely the CzcCBA system seemed to have the key role in cellular Zn(II) detoxification. Possibly, CzcCBA is able to “vacuum” all surplus Zn(II) ions from the periplasm and thus, ultimately prevent toxic Zn(II) accumulation in the cytoplasm. In this model the CzcCBA system is preferentially a Zn(II) efflux system with lower affinities for related cations such as Cd(II) or Co(II). Nevertheless, some of the P-type ATPase gene deletion strains were as zinc sensitive as the *czcA* gene deletion strain. Increased growth yield under Zn(II) stress was observed in strain GG48 with heterologous gene expression in the order *czcP* = *zntA* > *cadA* > *pbrA*. In summary, in *C. metallidurans* CH34 cadmium and cobalt detoxification is different from that of zinc.

β -proteobacterial and not archaeal ammonia oxidizing bacteria restore nitrification in a zinc contaminated soil

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Biological ammonia oxidation, the rate-limiting step in nitrification, plays a crucial role in the flow of nitrogen through terrestrial and aquatic ecosystems. In soil, the process has been long-time thought to be entirely mediated by discrete clades of β -proteobacteria (ammonia oxidizing bacteria; AOB). However, recently ammonia-oxidizing Crenarchaeota (ammonia oxidizing archaea; AOA) have been proposed to be the dominant agents of ammonia oxidation in soils. However, the dynamics of AOB versus AOA and their relative contribution to soil ammonium oxidation as a response to stress and environmental perturbations remain unclear. Using soil samples from an ongoing field study and the *amoA* gene as a molecular marker, we demonstrate that AOB, and not AOA, explain the recovery of nitrification after zinc contamination in an Australian arable soil. The pristine soils showed approximately equal numbers and transcriptional activities of *amoA* of AOB and AOA, yet the latter group was more sensitive to intermediate doses of zinc. At zinc concentrations of 33.7 mmol kg⁻¹, ammonia oxidation was completely inhibited, and both AOB and AOA *amoA* gene numbers and especially transcriptional activities were reduced. The ammonia oxidation process in fields which had received this contamination level was however fully restored after 2 years, concomitant with a restoration of the number of AOB *amoA* gene copies and AOB *amoA* transcriptional activity to levels similar to those for the non-contaminated control soil and the development of a zinc-tolerant AOB community with a composition different from the original community. In contrast, no resilience in AOA *amoA* gene numbers and activity took place after two years and their values were 3 and 10⁴ fold lower than those of AOB, respectively. These findings demonstrate that AOB were more important for nitrification under Zn-stressed soil conditions, as compared to AOA communities. Thus, while recent findings emphasized on the potential dominant role of Archaea in soil-borne ammonia oxidation, our findings demonstrate that a balanced view of both AOB and AOA is needed to understand the biological principle of this critical step in the nitrogen cycle and its response to perturbation.

The use of a microarray to investigate microbial weathering of basalt by *Cupriavidus metallidurans* CH34

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A large area of the earth consists of volcanic rocks, the most common being basalt and rock weathering controls nutrient flux to the biosphere and carbon dioxide levels in the atmosphere. However, our understanding of bacterial rock weathering is limited. To further the insight into microbial weathering, the gene regulation in the multi-metal resistant bacterium *Cupriavidus metallidurans* CH34 in contact with basalt rock was investigated. The total genome DNA microarray of *Cupriavidus metallidurans* CH34 was used to investigate differential gene expression between growth in an iron-rich medium and an iron-limiting medium in the presence of basalt. A number of genes involved in membrane processes, such as permeability and transport were identified as induced by basalt. Also genes involved in osmoregulation were more actively transcribed in the presence of the rock. However, no differential expression of genes involved in siderophore production was detected. This observation is in accordance with physiological data that demonstrated lack of siderophore production by basalt. These preliminary findings of this work are the first to employ microarray techniques to investigate microbial weathering.

Study of the acetone metabolic pathway in *Cupriavidus metallidurans* strain CH34

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In the frame of the European Space Agency experiments named “Microbial Experiments in the Space Station About Gene Expression”, *Cupriavidus metallidurans* CH34 was cultivated twice in the International Space Station (ISS) during 10 days missions (MESSAGE 1 and 2), in parallel with similar control procedures on ground. At the end of these two missions, proteomic analyses revealed the overexpression of different proteins in space, including, the acetone carboxylase enzyme, while its presence in *C. metallidurans* proteome was never shown so far.

To date, a few bacteria with a complete sequenced genome are identified to possess this enzyme, known to be a key enzyme in the bacterial acetone metabolism. The presence of this enzyme, expressed for yet unknown reasons after a 10 days journey inside the ISS, in *C. metallidurans* CH34, suggests its ability to metabolize acetone or similar compounds. Thus, it is of interest to determine

- (i) if *C. metallidurans* is really able to use acetone as a source of carbon and energy, but also isopropanol or N-propanol and
- (ii) which metabolic pathways are induced. Indeed, a complete bacterial acetone metabolic pathway has never been elucidated to date.

In this context, bacterial cultures were realized in minimal salts medium containing either gluconate (control), acetone, isopropanol or N-propanol as carbon source. Moreover, differential proteomic analysis were performed using either gel (2-Dimensional Differential Gel Electrophoresis) and gel-free (2-Dimensional Liquid Chromatography coupled with tandem Mass Spectrometry) approaches. Proteomic analysis reveal a profound modification in the protein expression pattern in presence of acetone. Among the most important overexpressed proteins, the acetone carboxylase shows its three subunits, α , β and γ . This enzyme will be purified and characterized.

Kinetic and proteomic results reveal a similar behaviour of *C. metallidurans* CH34 in presence of acetone and isopropanol. Indeed, in presence of isopropanol, the same clusters of both over and down expressed proteins were observed, indicating a similar metabolic pathway for the consumption of both carbon sources. In contrast, proteomic results obtained in N-propanol culture conditions revealed a distinct modification of *C. metallidurans* proteome, suggesting an independent catabolic pathway for this alcohol.

With the characterization of the different enzymes overexpressed in presence of acetone or isopropanol, we could propose, for the first time, a complete metabolic pathway for the consumption of both compounds by *Cupriavidus metallidurans* CH34.

The Quorum Sensing system of *Cupriavidus metallidurans* CH34: organisation, function and regulation

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Quorum Sensing (QS) is one of the most important chemical languages that bacteria use to coordinate multilateral actions in response to cell density. QS initiates processes that benefit the bacterial population as a whole, rather than the individual bacterial cell. Pathogenic bacteria often use QS-systems for organising and planning their aggressive virulent attacks. Non-pathogenic soil bacteria like *Cupriavidus metallidurans* CH34 can also possess QS-systems. For these bacteria, QS normally contributes to efficient environmental survival. The study of the QS-system and its regulation in *C. metallidurans* CH34 might provide fundamental insights on the organisation and functioning of bacterial populations.

A remarkable resemblance exists between one of the QS-systems of *Ralstonia solanacearum* and *C. metallidurans*. Both micro-organisms contain quasi identical copies of the four phenotypic conversion, *phc*, genes: *phcA*, coding for a LysR transcription factor, *phcB*, a synthase, responsible for formation of the QS-signal molecule, methyl 3-hydroxy hexadecanoate and *phcS/phcR*, two genes coding for a two component signal transduction system.

Besides the *phc* Quorum Sensing genes, *C. metallidurans* CH34 contains also a homologue of the two component signal transduction system of the Escherichia coli EHEC bacterium, QseB and QseC. The same homologue pair is found in *C. taiwanensis* but not in *R. solanacearum*. The function of this two component signal transduction system remains until now unresolved.

The first micro-array transcriptome comparison between *C. metallidurans* CH34 Wild type and a *phcA* mutant, a mutant able to sense but unable to react to the cognate QS-signal molecule, indicates that the QS-system of *C. metallidurans* is a straight forward system. In QS mode, two metabolic pathways, rubisco, the *ccb*-genes, and hydrogen oxidation, the *hox*-genes, are upregulated. Also the motility of the Wild type is increased compared to the *phcA* mutant. On the other hand, the homologue of the VsrD-transcription factor in *C. metallidurans* CH34 is downregulated by PhcA.

Swim tests in low concentrated agar solutions support the micro-array observation that motility is increased when QS is switched on in *C. metallidurans* CH34. Also, the small but detectable slower growth rate of the *C. metallidurans* CH34 Wild type suggests a possible metabolic influence of PhcA.

Apparently, gluconate consumption is not affected by QS, since Wild type and *phcA* mutant show a similar gluconate consumption rate.

Bio-assay suggests that the *C. metallidurans phcA* mutant produces very low levels of methyl 3-hydroxy hexadecanoate. This is a strong indication that *phcB* is supervised by the PhcA transcription factor, thus belonging to the QS-regulon.

Horizontal gene transfer by broad-host-range plasmids: lessons from *Cupriavidus metallidurans* CH34

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It is generally accepted today that horizontal gene transfer (HGT) is a strong driving force in the evolution and rapid adaptation of Bacteria and Archaea, including those that infect humans. Among the various mechanisms of HGT, conjugative gene transfer mediated by broad-host-range (BHR) plasmids may well be most important for the exchange of host-beneficial genes between distantly related bacteria. While there is no doubt about the role of HGT in the evolution and adaptation of prokaryotes, the diversity of the plasmids involved, and the rates at which they are exchanged in various ecosystems are poorly understood. For the past 20 years, *Cupriavidus metallidurans* CH34 has been a very useful model recipient strain in HGT studies in at least two different ways:

- (i) in microcosms experiments aimed at understanding the effects of environmental factors on the transfer and fate of BHR plasmids in soil, and
- (ii) as a tool to capture and subsequently characterize diverse novel BHR plasmids from various ecosystems.

To address the first aim, we first established a model system for assaying the intergeneric transmission and expression of cloned genes in microcosms with sterile and nonsterile soil. This consisted of a plasmid-free derivative of *C. metallidurans* CH34 as recipient strain, *E. coli* as donor, plasmids of the IncP-1 group as model BHR plasmids, and the heavy metal resistance genes *czc* from *C. metallidurans* CH34 as model genes. Using this system we demonstrated that genes on non-conjugative plasmids can be mobilized to recipients in soil under specific conditions. The importance of nutrients and selection pressure in the form of heavy metal pollution on the fate of plasmids was emphasized, and the intriguing and controversial topic of retrotransfer was examined. The second important use of *C. metallidurans* CH34 has been as recipient to capture diverse novel mobilizing BHR plasmids from soil, sewage sludge and rhizosphere, based solely on the ability of these plasmids to mobilize an IncQ vector. Sequence analysis of a few of these plasmid genomes revealed low similarity in the backbone gene composition and organization with known plasmids, and illustrates our ignorance of BHR plasmid diversity. In conclusion, *C. metallidurans* CH34 has made a vital contribution to our current understanding of the host range, mobility, and genetic diversity of BHR plasmids in Bacteria.

Cupriavidus metallidurans CH34 in space

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When man goes to space, inevitably microbes hitchhike along, some needed, others unwanted. Microbes are essential for human well-being, for water recycling, oxygen and food production, but can also jeopardise crew health or structural materials and equipment. Therefore spacecrafts and the ultra clean rooms they are assembled in, are routinely monitored for microbial contamination.

Cupriavidus and *Ralstonia* bacteria were found on the floor, air and surfaces of such spacecraft assembly rooms, on surfaces of space robots such as the Mars Odyssey Orbiter prior-to-flight and in ISS cooling water and Shuttle drinking water in-flight. The phenotypic analysis of these isolates showed that they are able to use a wide variety of substrates as carbon sources, including ethanol and acetone, and have accumulated moderate resistances to a variety of physical and chemical antimicrobial agents, including heavy metal cations. Moreover, these resistance properties may be encoded in mobile genetic elements as several large plasmids were detected in these strains. This phenotype is very similar to that of well-known strain *Cupriavidus metallidurans* CH34^T which carries a big collection of heavy metal resistance genes in its genome, especially on its 2 large plasmids pMOL30 and pMOL28.

C. metallidurans CH34^T was taken as model bacterium and its physiological and metabolic response and adaptation of to space flight conditions was investigated. The strain was grown (1) in the International Space Station (ISS) (MESSAGE, MOBILISATION and BASE flight experiments), (2) in the Rotating Wall Vessel (RWV) and on the Random Positioning Machine (RPM) mimicking microgravity on ground, and (3) in simulated space flight radiation conditions on ground. Space flight experiments demand unusual culture conditions and it was clear that, pre-, in- and post-flight incubation conditions are critical and should be controlled, monitored and taken into account as much as possible when comparing space flight with ground grown cells. Via flowcytometry, minor but distinct changes in physiology and metabolism were observed in the cell cultures grown in space flight when compared to correct ground control cultures. Transcriptome analysis using a total genome CH34 DNA-microarray chip showed increased transcription of several genes, some typically involved in oxidative stress response, after a 10-day mission inside the ISS and in simulated ground experiments. Proteome analysis using 2D-gel electrophoresis and HPLC-MS/MS identified in addition, several proteins over-produced in space conditions, including genes involved in acetone metabolism.

Thus the accumulation of resistance mechanisms, coding also for resistance to heavy metals, seems to enable *Cupriavidus* and *Ralstonia* bacteria to survive in harsh oligotrophic man-made environments such as spacecraft and spacecraft assembly rooms. And space flight could alter the physiology and the metabolism of these type bacteria, which may be of importance for the development of microbial monitoring systems for long-term manned space flight.

Bacteria-Plant Associations in the Katangese Copper Belt

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The Katangese Copper Belt (RDC) is one of the most mineralized areas in the world. It contains not only large quantities of copper and cobalt ores but also Zn, Mn, Cd, Cr, Pb, Ag, U... . Mining activities are the source of heavy metal dispersion, resulting in the emergence of plant and bacterial populations adapted to heavy metals. Among these plant species *Haumaniastrum katangense* and *Crepidiorhapon tenuis* were originally found on copper/cobalt anomalies and were then considered as indicators of metal deposits .

Although plants are known to interact with endo- and ecto- microorganisms, the relationship between cuprophytes and bacteria in polluted biotopes remains undocumented. The objective of this work is to assess the contribution of the bacterial flora to the resistance of the plants. In this study we characterize bacteria associated with the copper/cobalt resistant plant species. *H. katangense* and *C. tenuis* plants together with their rhizospheric soil were collected at two sites. Roots, shoots and leaves were surface sterilized and ground separately. Bacterial endophytes were isolated after plating on rich medium with or without Cu (NO₃)₂.

Cuproresistant bacteria were isolated from most of the analyzed plants. Between specimens of the same plant species, total endophytic bacterial count varied from 0 to 10⁷ CFUg⁻¹FW. At 0,8mM Cu, cuproresistant endophytic bacteria represent between 0 - 97 % of the total cultivable endophytic bacteria. This ratio was in general higher than in the rhizosphere. Different bacterial species colonizing roots or leaves have been isolated. Some cuproresistant bacteria have been found in the two plant species although each of them had also a specific endophytic microflora. Some endophytic bacterial strains were hyper-resistant to copper and were able to grow on copper concentrations that were inhibitory for *C. metallidurans* CH34. Their role in the host plant metal-resistance remains to be unraveled. Seeds collected *in situ* from *H. katangense* were free of copper-resistant endophytes. Further research will include re-inoculation of seedlings and growth tests on contaminated soils to investigate the impact of bacterial colonization on copper-resistance of cuprophytes.

Characterization of copper-resistant strains from Katangese sites: prevalence of cop genes

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In regions such as Katanga (DR Congo), extensive mining of the ore deposits, and related industrial activities have increased the number of sites contaminated with copper and other heavy metals. Despite potential interest for bioremediation, very little is known about copper-resistant bacteria thriving in soils with very high contents of copper.

Resistance to copper has been studied in different organisms and several mechanisms, based on efflux and sequestration of the ions, are known in bacteria. Sequestration of copper ions in the periplasm and outer membrane in strains with cop genes confers them increased resistance (Cooksey, 1994). Two proteins, CopA and CopB, are essential to resistance in this mechanism (Cha and Cooksey, 1991). In the multi-resistant model strain *Cupriavidus metallidurans* CH34 (Mergeay et al., 2003), additional copper-induced genes, many of unknown function and reported only in CH34, surround the core genes copABCD (Monchy et al, 2006).

In this work, cupro-resistant strains, able to grow on copper concentrations that are inhibitory for *C. metallidurans* CH34, were isolated from soil samples taken at several locations, mainly mining sites, of the Katangese Copper Belt. These soils are characterized by very high copper contents. Out of this collection, 60 strains were identified and investigated further. They belong to several (gram-negative) phyla, and are representatives of the genera: *Cupriavidus*, *Ralstonia*, *Luteifibra*, *Chitinophaga*, *Stenotrophomonas*, *Sphingomonas* and *Methylobacterium*.

The prevalence of cop-like mechanisms in these highly resistant strain was analysed by PCR amplification of several cop genes. CopA was detected in a majority of the *Cupriavidus* and *Ralstonia* strains (19/20), in *Luteifibra* (5/6), and *Sphingomonas* (7/8). Most of the *Stenotrophomonas* isolates were also copA-positive. Some of the copA-positive strains also had a copK gene, until now only described in *C. metallidurans* CH34 (Monchy et al. 2006). It seems in consequence that some of the strains could rely on an "extensive", CH34-like version of the cop mechanism. On the contrary, *Methylobacterium* strains do not own a copA gene, and never accumulate copper ions. High resistance is clearly achieved in this genus through an unknown mechanism.

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“Nano-toxicity” of CdTe quantum dots towards bacteria

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We are currently exploring the possibility of using customized inorganic nanoprobe to study specific chemical and/or physical properties on biological materials. With this respect, colloidal semiconductor nanocrystals (known as quantum dots, QDs) are very attractive because of their unique optical properties, such as sharp and symmetrical emission spectra, high quantum yield, good photo-stability, and size dependent emission-wavelength tunability (Michalet *et al*, 2005). Additionally, QDs could be surface-functionalized with different ligands that would provide them with a specificity towards a given target.

A series of water-soluble CdTe core QDs, with diameters below 5.0 nm and surface-functionalized with ligands such as thioglycolic acid (TGA) or the tripeptide glutathione (GSH), were synthesized using hydrothermal methods (Qian *et al*, 2006). These fluorescent nanocrystals were used for the labeling of several bacterial species including *Escherichia coli* MG1655, *Shewanella oneidensis* MR-1A, *Cupriavidus metallidurans* CH34, and *Bacillus subtilis* LMG7135^T. Fluorescent microscopy revealed strong differences in labeling from one strain to another with the best results obtained with Gram positive bacteria.

While carrying out these labeling experiments, it was fortuitously noticed that *Shewanella oneidensis* cells exhibited abnormal elongated shapes (e.g., filamentation), thus suggesting a genotoxic effect caused by the QDs. The toxicity of these nanoparticles was therefore investigated on different bacterial species in a comparative study based on growth inhibition. Preliminary results showed that CdTe-based QDs exhibited a toxicity that depends on several factors including the strain used and the surface functionality of the nanocrystals. Because the toxicity was found to vary with time after QDs were redispersed in aqueous solution, a phenomenon involving QDs' breaking down with a release of Cd²⁺ was first assumed. However, further results have demonstrated that *Cupriavidus metallidurans* CH34 and its heavy metal sensitive derivative AE104 did not show significant differences in terms of growth inhibition, suggesting that the main toxicity mechanism associated to these QDs was not solely related to the release of Cd²⁺ (if any) from the nanocrystal.

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Chemical forms of selenium accumulated by *Cupriavidus metallidurans* CH34 exposed to selenite and selenate

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Cupriavidus metallidurans CH34 is able to resist to a variety of metallic divalent cations (Zn^{2+} , Cd^{2+} , Cu^{2+} , Hg^{2+} , Ni^{2+} among others) as well as oxyanions (arsenite, arsenate, chromate, selenite Se^{IV} , and selenate Se^{VI}). Accumulated selenite is reduced to intracellular granules of elemental selenium (Se_0) (Roux et al., 2001, Appl. Environ. Microbiol. 67, 769-773). We have studied the kinetics of selenite and selenate accumulation and used X-ray absorption near edge structure (XANES) spectroscopy to identify the fate of selenate after accumulation and the possible chemical intermediates during the transformation of these two oxyanions (Sarret et al., 2005, Appl. Environ. Microbiol. 71, 2331-2337). When introduced during the lag phase, the presence of selenite increased the duration of this phase. Selenite introduction was followed by a period of slow uptake, during which the bacteria contained Se_0 and alkyl selenide in equivalent proportions. This suggests that two reactions with similar kinetics take place: an assimilatory pathway leading to alkyl selenide and a slow detoxification pathway leading to Se_0 . Subsequently, selenite uptake strongly increased (up to 340 mg Se per g of proteins) and Se_0 was the predominant transformation product, suggesting an activation of selenite transport and reduction systems after several hours of contact. Exposure to selenate did not induce an increase in the lag phase duration, and the bacteria accumulated approximately 25-fold less Se than when exposed to selenite. Se^{IV} was detected as a transient species in the first 12 h after selenate introduction, Se_0 occurred as a minor species, and the major accumulated form was alkyl selenide. This species was further identified as selenomethionine by HPLC (Avoscan et al., 2006, Appl Environ. Microbiol. 72, 6414-6416). Thus, in the present experimental conditions, selenate mostly follows an assimilatory pathway and the reduction pathway is not activated upon selenate exposure. These results show that *C. metallidurans* CH34 may be suitable for the remediation of selenite-, but not selenate-, contaminated environments.

Bio-Arsenic: from Ancient Genes (and Proteins) to Modern Geocycles and Global Pollution

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Here, at a Festschrift celebrating a single bacterial strain *Cupriavidus metallidurans* CH34, renowned for its multiple systems for resistances to many toxic inorganic metal compounds, it is useful to consider one (arsenic resistance) that raises broad global questions and provides a paradigm model for others. By *in silico* sequence analysis of protein products based on DNA sequencing, and with extensive environmental and biochemical studies, one can make several conclusions:

- (1) Arsenic resistance is ancient, probably predating the split of prokaryotes into bacteria and Archaea and certainly predating an oxygen atmosphere generated by cyanobacteria.
- (2) Genes for arsenic resistance and metabolism are very widely found, basically in all bacterial genomes with 2000 or more genes and more frequently found (for example) than those for tryptophan synthesis (as one can get tryptophan from organic nutrients synthesized by other microbes, but arsenic polluted environments are ubiquitous).
- (3) The central basis for arsenic resistance is the familiar “*ars* operon” consisting of a core of three genes, *arsRBC*, plus additional genes in some but not all cases.
- (4) For some essential functions, the basic gene (and function) was “invented” by early evolution more than once, so that completely unrelated sequence and structure clades carry out very similar physiology and chemistry.

This is best demonstrated for the ArsC (ACR2) arsenate reductase enzyme and for the ArsB (ACR3) membrane transport proteins. Three unrelated clades (“trees”) for small intracellular arsenate reductase enzymes are based on multiple cysteine redox chemistry and are coupled biochemically to thioredoxin (in one well-studied example in *Staphylococcus*) or to glutaredoxin (with two separate clades including plasmids in *E. coli* and chromosomal determinants in fungi). The distribution among bacteria is varied, and the *Pseudomonas aeruginosa* genome for example has both thioredoxin- and glutaredoxin-coupled arsenate reductases). The two clades of (ArsB) membrane protein arsenite efflux systems include larger proteins of both Gram-negative and –positive bacteria that either function alone coupled to energy from the membrane potential with an additional large protein, ArsA, as an efflux ATPase. A second class of smaller membrane protein efflux pumps is found in both bacteria and in fungi. Almost invariably, the *ars* operons are regulated by ArsR repressor proteins of the large class of “winged helix” dimeric transcriptional repressors that are homologous to other proteins functioning for example in cadmium resistance. The small ArsD protein is currently thought to function as an oxyanion “chaperone” delivering arsenate to the large ArsA subunit, thus protecting the cytoplasm and activating the ArsA ATPase. In addition to central *ars* operon genes (*arsRDABC*), some microbes have additional genes including *arsH*, which appears to produce a redox flavoprotein of unknown function and *arsM* which is the central enzyme in converting inorganic arsenic to mono-, di- and trimethylarsenic compounds that can be at the As(III) or As(V) redox level. While arsenate is thought to invariably be taken up by the cells

through transport systems designed for the closely related phosphate oxyanions, arsenite is taken up – and reaches targets of toxicity – a different type of membrane carrier protein, an aquaglyceroporin, called GlyF (for glycerol facilitator in *E. coli*) or AqpS in eukaryotic cells. Finally, bacteria have invented and evolved the very ancient membrane surface respiratory arsenate reductases and arsenite oxidases, which are dimeric redox enzymes, based on completely different chemistry from the intracellular ArsC proteins. The respiratory arsenite oxidase can be thought of as “eating” arsenic through an electron transport chain coupled to oxygen consumption while the arsenite reductase “breaths” arsenite as an alternative electronic acceptor. Both enzyme dimers contain the large Mo-pterin and [Fe-S] cage containing primary subunit plus unrelated secondary smaller [Fe-S] subunits. It is been sensibly argued that respiratory arsenate reductase is an ancient enzyme, functioning in early electron transport chains prior to the availability of O₂ as a terminal electron acceptor.

ABSTRACTS

Poster presentations

Extracytoplasmic function (ECF) sigma factors from *Cupriavidus metallidurans* CH34

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Bacterial sigma factors play a major role in regulation of transcription initiation by providing promotor specificity to the RNA polymerase. A subfamily of RpoD-like proteins, the extracytoplasmic function (ECF) sigma factors, are involved in adaption to changing environmental conditions. Genomic sequencing of the highly heavy metal resistant bacterium *Cupriavidus metallidurans* CH34 revealed 17 putative sigma factors; 11 of them could be assigned to the ECF-subfamily including CnrH which has already been characterized as the regulator of the cobalt/nickel-resistance-determinant *cnr* located on megaplasmid pMOL28.

A correlation between the high number of ECF-sigma factor genes and the outstanding heavy metal resistance of this bacterium could be possible. Therefore a screening for further ECF-sigma factors that might be involved in regulation of metal homeostasis was performed. The transcription levels of the ECF-sigma factor genes after incubation with different heavy metal cations was determined by using real-time-PCR. Three ECF-sigma factor genes that showed similarity to the *Escherichia coli* FecI sigma factor involved in iron acquisition were up regulated by nickel and copper cations indicating a possible function in iron/nickel/copper homeostasis. Furthermore several deletion mutants of the ECF genes showed an altered resistance towards different heavy metals suggesting an involvement in heavy metal homeostasis. Transcriptional analysis showed that all ECF genes were co-transcribed with putative membrane proteins indicating a possible regulation through an anti-sigma factor.

The ABC-transporter AtmA is involved in nickel/cobalt resistance of *Cupriavidus metallidurans* CH34

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ATP binding cassette (ABC) transporters constitute a large family of proteins which transport substrates by hydrolysis of ATP. In eukaryotes they have been found in various cellular membranes and have been demonstrated to recognize a broad spectrum of substrates, e.g. fatty acids, peptides and hydrophobic compounds. Bacterial ABC transporters are frequently involved in the efflux of antibiotics or in the uptake of nutrients.

AtmA is an ABC transporter encoded by chromosome 1 of *Cupriavidus metallidurans*. Sequence alignments showed high similarity to yeast mitochondrial ABC transporter Atm1p, which belongs to the MDR/TAP subfamily of ABC transporters. This protein plays an essential role in the biogenesis of cytosolic/nuclear iron-sulfur proteins and in cellular iron homeostasis of yeast.

The physiological effect of a *ATM1* gene deletion in yeast could not be complemented by expressing the *atmA* gene of *C. metallidurans*, in contrast to the related proteins Stal from *Arabidopsis thaliana* or the human proteins ABCB7 and ABCB6. However, a gene deletion of *atmA* in *C. metallidurans* CH34 and in its plasmid free derivative strain AE104 resulted in reduced resistance against Co(II) or Ni(II) compared to the parental strains. The observed decrease of nickel or cobalt resistance in strain CH34 was surprising because this strain contains the RND-driven outer membrane efflux system CnrCBA as well as the cytoplasmic membrane efflux systems CnrT and DmeF for Ni(II)/Co(II) detoxification. This strain also harbors the efficient Co(II) efflux system CzcCBA.

The *atmA* gene was successfully overexpressed in an *Escherichia coli* host by fermentation and the protein was purified to homogeneity. Metal dependent ATPase-activity of the purified protein was determined by a colorimetric assay. This suggests that AtmA is stimulated by nickel or cobalt cations.

Moreover, heterologous expression of the *atmA* gene in *E. coli* resulted in increased nickel or cobalt resistance, indicating a functional AtmA protein.

In conclusion, AtmA is probably a further factor of cobalt/nickel resistance in *C. metallidurans*.

Biofilm dynamics of a linuron-degrading multispecies consortium consisting primarily of β -proteobacteria as a response to different nutrient conditions

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Being the predominant mode of bacterial life in the environment, biofilms play a crucial role in degradation of organic contaminants. Variations in nutrient status might impact the structure and composition of pollutant biodegrading biofilms. This would be especially the case for pollutant-degrading consortia in which the activity depends on the synergistic interactions between the members. However, studies on the effects of changing nutrient status conditions on biofilms of organic pollutant-degrading consortia are scarce. Linuron-degrading consortia consist primarily of β -proteobacteria such as *Variovorax* and *Comamonas*. We examined whether the degradation activity of a linuron-degrading consortium biofilm was affected when (i) exposed to carbon and/or nitrogen sources in addition to linuron and (ii) exposed to changes in nutrient regime (non-selective, N- and/or C-starvation). Effects on biodegradation activity were related to biofilm structure and/or composition. In the used consortium, *Variovorax* sp. WDL1 transforms linuron to 3,4-DCA and *N,O*-DMHA. 3,4-DCA and *N,O*-DMHA are excreted and support growth of the second and third member *Comamonas testosteroni* WDL7 and the α -proteobacterium *Hyphomicrobium sulfonivorans* WDL6, respectively. Biofilms were cultivated in flow cells and analyzed with CLSM. In the presence of C-sources as citrate and TSB in the feed in addition to linuron, the efficiency of linuron degradation was reduced to 50% and 80% depending on the concentration of the additional C-source. Moreover, it affected degradation of 3,4-DCA formed from linuron. Also under conditions in which a linuron-degrading consortium biofilm was intermittently fed with a N and/or C- starved non-selective medium, a negative effect on the linuron-degrading activity of the biofilm was observed when feed with linuron was restored . In all cases, the decreased performance of the biofilm could be related to changes in biofilm structure and composition. Interestingly, each condition resulted into a particular biofilm structure and composition. Our results show that pesticide-degrading consortia, organized in biofilms, and their pesticide-degrading activity will strongly depend on the nutrient conditions of the moment and that probably, the ideal biofilm configuration observed under selective conditions will not be existing in real-life environmental conditions where mixtures of C/N-sources exist and alternative periods of feast and famine can be expected.

Pesticide-primed soils inhabited by linuron-degrading β -proteobacteria for bioaugmentation of on-farm bioremediation systems treating pesticide-contaminated waste water

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On-farm biofilters are used for the treatment of pesticide-contaminated waste water produced by farmers during cleaning/filling of their spray equipment. The biodegradation activity in biofilters can be increased by introducing pesticide-degrading bacteria but lab-cultivated inoculants do not always survive long in a new biotope. This study investigates whether bioaugmentation using pesticide-primed soils with pesticide-degrading activity can be applied for stimulating pesticide biodegradation in biofilters. We examined whether the pesticide-degrading population in the soil survives and performs its ability in the biofilter after inoculation and after successive conditions of stress expected to occur in real systems, i.e, a stop in pesticide-supply, a cold period, a drought period and the addition of a pesticide cocktail. Linuron was used as a model-pesticide while a long-term linuron treated soil was used as pesticide-primed soil. An earlier study showed that the linuron-degrading population in this soil consisted of β -proteobacteria such as *Variovorax*, *Cupriavidus* and *Comamonas*. Lab-scale biofilters were inoculated with either the linuron-primed soil or a reference soil without pesticide-degrading activity. The change in pesticide-degrading activity in the filters was monitored by means of ¹⁴C-mineralisation experiments performed on regularly taken samples.

The biofilter inoculated with the linuron-primed soil immediately acquired the ability of degrading linuron in contrast with the biofilter inoculated with the control soil. The degrading activity was maintained for almost a year and application of linuron resulted into an increased size of the linuron-degrading population. The cold period and the presence of a pesticide cocktail did neither affect the degrading capacity nor the size of the degrading community. The stop in pesticide supply and the drought period resulted into a decrease in size of the linuron-degrading population but when standard conditions were re-established, resilience of the linuron-degrading population towards the original size was observed. The biofilter inoculated with the non-primed soil unexpectedly developed a pesticide-degrading activity but only after an extended period of pesticide supply. Moreover, this activity showed less resistance towards stress periods.

We conclude that inoculation of biofilters with the appropriate pesticide-primed soil is a cheap and labour-extensive method for increasing the pesticide-degrading activity in on-farm biofilters systems.

Identification of enzymes involved in linuron and 3,4-dichloroaniline degradation in the linuron-degrading β -proteobacterium *Variovorax* sp. WDL1: a proteomic approach

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Phenylureas are commonly applied herbicides in agriculture and their extensive use has resulted into important environmental contamination. Members of the genus *Variovorax* are often isolated as the main linuron degraders from linuron-treated agricultural soils and seem to fulfill a crucial role in linuron degradation in soil. The main bacterial degradation route of linuron is initiated with a direct hydrolysis of the amide bond resulting in the metabolites 3,4-dichloroaniline (3,4-DCA) and *N,O*-dimethylhydroxylamine (*N,O*-DMHA). Further degradation of 3,4-DCA can proceed via different possible routes which include transformation of this product to catechol-based moieties. Knowledge about the catabolic genes and enzymes involved in linuron hydrolysis and further mineralization of 3,4-DCA is however scarce. We initiated research on the linuron/3,4-DCA catabolic genes and proteins of the linuron-degrading β -proteobacterium *Variovorax* sp. WDL1 by means of a differential proteomic approach. Differential protein expression analysis of *Variovorax* sp. WDL1 grown in a heterotrophic medium in the presence and absence of linuron or 3,4-DCA was conducted using 2-D SDS-PAGE and selected up- and down-regulated proteins were identified with NanoLC-ESI-MS/MS. In the 3,4-DCA-supplemented culture, the up-regulation of several components of earlier described multicomponent aniline dioxygenases (AD) was observed. The different components of this putative (di)chloroaniline dioxygenase were phylogenetically related to AD-like components of aniline-degrading *Proteobacteria*. The data indicated that possibly multiple versions of the AD complex are expressed. Unfortunately, several interesting protein spots which were up-regulated in the linuron- and/or 3,4-DCA-supplemented cultures compared to the control cultures did not show reliable or significant similarity to known protein functions. Currently, a similar analysis on *Variovorax* sp. WDL1 mutants impaired in linuron and/or 3,4-DCA degradation is performed to provide evidence on the participation of those proteins in linuron/3,4-DCA degradation. Moreover, we initiated the genetic identification of the AD-complex in strain WDL1.