Ralstonia metallidurans, a bacterium specifically adapted to toxic metals: towards a catalogue of metal-responsive genes

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Abstract

Ralstonia metallidurans, formerly known as Alcaligenes eutrophus and thereafter as Ralstonia eutropha, is a β-Proteobacterium colonizing industrial sediments, soils or wastes with a high content of heavy metals. The type strain CH34 carries two large plasmids (pMOL28 and pMOL30) bearing a variety of genes for metal resistance. A chronological overview describes the progress made in the knowledge of the plasmid-borne metal resistance mechanisms, the genetics of R. metallidurans CH34 and its taxonomy, and the applications of this strain in the fields of environmental remediation and microbial ecology. Recently, the sequence draft of the genome of R. metallidurans has become available. This allowed a comparison of these preliminary data with the published genome data of the plant pathogen Ralstonia solanacearum, which harbors a megaplasmid (of 2.1 Mb) carrying some metal resistance genes that are similar to those found in R. metallidurans CH34. In addition, a first inventory of metal resistance genes and operons across these two organisms could be made. This inventory, which partly relied on the use of proteomic approaches, revealed the presence of numerous loci not only on the large plasmids pMOL28 and pMOL30 but also on the chromosome. It suggests that metal-resistant Ralstonia, through evolution, are particularly well adapted to the harsh environments typically created by extreme anthropogenic situations or biotopes.

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Keywords: Ralstonia metallidurans; Heavy metal; Resistance gene; P1-ATPase; Genome; Proteomics; HME-RND efflux system; Plasmid; Mobile genetic element; Regulation

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1. Introduction: *Ralstonia metallidurans*: general description and historical overview

1.1. Strain CH34

Metal-resistant bacteria [1], formerly known as *Alcaligenes eutrophus* [2], then *Ralstonia* sp., *Ralstonia eutropha* or *R. eutropha*-like and finally *Ralstonia metallidurans* [3], carry plasmid-borne *czc* (resistance to cadmium, zinc and cobalt) [2,4], *ncc* (nickel, cobalt and cadmium) [5] or *cnr* (cobalt and nickel) [6] cation efflux metal resistance operons. Members of this genus are mostly found in industrial and polluted biotopes, prevailing in metallurgical wastes. The first strain of this group (and still the reference strain), *R. metallidurans* CH34, was found in the late 1970s in sediments of a decantation basin of a zinc factory in Belgium, near Liège [1]. The strain was found to be highly resistant to Zn(II), Cd(II), and Co(II) and later to Ni(II), Cu(II), CrO$_2$$^2^-$, Hg(II) and Pb(II). Mutants lacking the resistance to Zn(II), Cd(II) and Co(II) were easily obtained among survivors at 37°C, the maximum temperature where growth could be observed [1,2]. This suggested a possible curing of plasmid-borne genetic determinants for the resistance to these metals. Transfer of metal resistance markers to other strains supported this view [7-9]. The strain proved also to be accessible to genetic analysis and to act as a good recipient of foreign genes in plasmid-mediated mobilization of chromosomal genes between different bacteria [10]. As a facultative chemolithotrophic bacterium able to grow aerobically in the presence of H$_2$ and CO$_2$, strain CH34 was shown to possess two hydrogenases, one soluble and one particle-bound, which at that time was considered typical for *A. eutrophus* [2]. The taxonomic proximity between metal-resistant strains such as CH34 and the *A. eutrophus* hydrogenotrophs was further supported by a variety of genetic and phenotypic tests. Yet, as discussed below, the taxonomic status of *A. eutrophus* and related strains [11,12] was recently revised, and the bacteria were eventually reclassified in the genus *Ralstonia* [13].

1.2. Plasmids

The plasmid-borne character of the resistance to Cd(II), Zn(II), Co(II) and Ni(II) in strain CH34 was demonstrated via appropriate curing and transfer experiments: resistance to Zn(II), Cd(II) and Co(II) was linked to the plasmid pMOL30 (~250 kb) while resistance to Co(II) and Ni(II) as well as resistance to chromate was linked to plasmid pMOL28 (180 kb) [14]. In addition, both plasmids were shown to encode resistance to Ti(IV) and Hg(II), the latter being encoded by the two mercury transposons Tn4378 (pMOL28) and Tn4380 (pMOL30) [15]. The Czc"- mutants that were obtained among the survivors at 37°C were a first indication of the mutator phenotype that was
further described in the strain CH34 and other R. metal-
lidurans strains [14,16,17].

1.3. Overview of metal resistance research

As far as the resistance to heavy metals is concerned, the
first studies focused on the czc locus (resistance to Cd(II),
Zn(II) and Co(II)) located on pMOL30 [4,18–21], to the
mercury transposons Tn4378 (pMOL28) and Tn4380
(pMOL30) [15], to the resistance to Ni(II), and then to
the corresponding cnr locus on pMOL28 [6,22–24]. Resis-
tance to chromate was also studied early on [25,26]. In
the related strain R. metallidurans 31A (formerly known as
Alcaligenes xylosoxidans 31A or sometimes as Achromo-
bacter xylosoxidans 31A) [3,12], ncc-mediated resistance to
Ni(II) was found to be very high with a minimum inhibi-
tory concentration (MIC) value of 40 mM (the MIC
value for Ni(II) conferred by the cnr genetic determinant
in strain CH34 is not higher than 2.5 mM) [5]. Of all the
metals for which plasmid-borne resistance of heterotrophic
bacteria has been reported, such a high level of resistance
was only observed for Ni(II) [27]. A similar high level of resis-
tance to Ni(II) has been described in the strain Arthro-
bacter ilicis that was isolated in nickel-rich sediments of
a metallurgical factory in Tyrol, Austria [28].

Other plasmid-borne determinants of R. metallidurans
were described much later, including the pbr locus (resis-
tance to lead) [29] and the cop locus (resistance to copper)
[30], both of which are located on pMOL30. In the same
period of the late 1990s, fundamental studies on the
czc operon [31–33], the cnrYXH regulatory region of
pMOL28 [34,35], the chr chromate resistance operons
(located chromosomally and on pMOL28) [36], and the
nickel resistance determinant nreB of R. metallidurans
31A (the third route to resistance to Ni(II) besides cnr
and ncc) [5,37,38] were undertaken. Although not directly
linked to metal resistance as such, various additional re-
ports on siderophores and the oxidative stress response
point to other aspects of metal metabolism in R. metalli-
durans [39–45].

1.4. Microbial ecology and environmental biotechnology

The knowledge about the mechanisms of resistance to
heavy metals and especially their regulation stimulated
further work in microbial ecology [46–51] and in environ-
mental biotechnology [52]. Industrial applications of met-
al-resistant bacteria and their genes include the use of
biosensors for monitoring the concentration of heavy metals
in a variety of substrates and soils [53–58], the develop-
ment of bioreactors to remove heavy metals from polluted
effluents or soils [59,60], and bioaugmentation [61] and
phytoremediation [62] (Y. Wang, W. Hai, J. Vander Ley-
den and M. Mergeay, unpublished). A recombinant strain
of R. metallidurans CH34, expressing a metallothionein
gene, was successfully used to immobilize metals in soil,
allowing revegetation [63,64]. The ncc-nre nickel resistance
system of R. metallidurans 31A was efficiently expressed in
the endophytic bacteria Burkholderia cepacia L.S.2.4 and
Herbaspirillum seropedicae LMG2284. The heterologous
expression of ncc-nre-encoded nickel resistance was ac-
companied by nickel removal from the culture medium.
This capacity to remove nickel through sequestration or
bioprecipitation processes and consequently lowering the
free nickel concentration offers interesting benefits for
these endophytic bacteria and their host plants. Lupinus
luteus L, when grown on a nickel-enriched substrate and
inoculated with B. cepacia L.S.2.4::ncc-nre, showed a sig-
nificant increase (30%) of the nickel concentration in the
roots, whereas the nickel concentration in the shoots re-
mained comparable with that of the control plants [62].

1.5. Genetic tools and features

Conjugation experiments, the isolation and character-
ization of R-prime plasmids [10,65], the construction of
a cosmid library [39], and the development of electropora-
tion [66] allowed the circularity of the chromosome to be
established and various loci to be mapped [10,16,65,67,68].
Genetic and physical maps of the pMOL28 plasmid and
its conjugation-proficient derivative pMOL50 were pro-
duced [14]. The formation of R-prime plasmids by retro-
transfer and the capture of small plasmids was also ob-
served and studied in these conjugation studies [69,70].
Finally, several mobile genetic elements present in the
CH34 genome were trapped on appropriate positive selec-
tion vectors and characterized [34,71,72] (M. Hassan, per-
sonal communication).

1.6. From the genome of R. metallidurans towards an
inventory of metal-relevant functions

Recently, the sequencing of the genome of R. metalli-
durans CH34 was carried out by the Joint Genome Insti-
tute in California (http://www.jgi.doe.gov/JGI_microbial/
html/ralstonia/ralston_homepage.html) and a first draft
of the sequence was released in 2000. The available se-
quence still contains some gaps, some of them being closed
thanks to the joint efforts of our laboratories (ftp://ftp.
genome.bnl.gov/pub/Sequences/Rmetallidurans/). Yet it al-
ready allows making a first inventory of functions related
to the resistance to heavy metals as well as to the adapta-
tion to harsh biotopes.

Previous reviews have addressed the plasmid-borne re-
stance to heavy metals as well as the biology and the
environmental applications of R. metallidurans [17,52,73–
83]. In the present review, we want to take the opportunity
to combine the availability of genome sequence data and
the first results of proteomic approaches to generate an
inventory of the genes and protein functions involved in
bacterial adaptation and resistance to heavy metals. Although we already had a fairly good view of the plas-
mid-borne functions, chromosomal genes look far more diversified and abundant than first expected. This tentative inventory is preceded by an update on the taxonomic and genomics situation of \textit{R. metallidurans} with special reference to other well-known species of the genus \textit{Ralstonia}: the hydrogenotroph \textit{R. eutropha} and the plant pathogen \textit{R. solanacearum}. Thereafter the following themes will be addressed in the inventory:

- the major families of metal resistance mechanisms: the RND (resistance–nodulation–cell division) [84–86] and CDF (cation diffusion facilitation) detoxification systems, the efflux P1-ATPases, and the systems focusing more specifically on mercury, copper, chromate, or arsenic/antimony oxyanions
- the pMOL28- and pMOL30-borne genes involved in metal resistance with special emphasis on the resistance to copper and to lead
- finally, a special section will be devoted to the regulatory genes involved in metal resistance.

2. Taxonomy of the genus \textit{Ralstonia} with emphasis on the relationships between metal-resistant species and phytopathogenic species

The need for a taxonomic reassessment came when it was clear that \textit{A. eutrophus} could no longer be clustered with \textit{Alcaligenes faecalis} [13] and had to be transferred to the genus \textit{Ralstonia}. The metal-resistant hydrogenotrophs such as strain CH34 proved to be closely related to clinical isolates known as ‘CDC-IVc2’ that were later renamed \textit{Ralstonia paucula} [87]. A first molecular study of metal-resistant strains confirmed a narrow clustering of metal-resistant strains isolated from polluted or industrial environments [12]. Finally, a polyphasic study based on 16s rRNA, whole cell protein profiling, fatty acid analysis, and other data made it possible to divide the bacteria carrying \textit{czc} or \textit{ncc} genes over three \textit{Ralstonia} species: \textit{R. metallidurans}, \textit{R. basilensis} and \textit{R. campinensis} [3]. Fig. 1 shows a dendrogram of the \textit{Ralstonia} genus and the taxonomic relationship between the two species for which extensive genomic data are available: \textit{R. solanacearum} [88] and \textit{R. metallidurans} (a draft of the genome has been released by the Joint Genome Institute). \textit{R. solanacearum} is a plant pathogen: most isolates of \textit{R. solanacearum} are specialized in the colonization and the infection of plants and may be considered to colonize plant-bound niches. The genus \textit{Ralstonia} is remarkably diverse in terms of ecological niches and specializations or capabilities: plant pathogenesis (\textit{R. solanacearum}), nodulation and nitrogen fixation in tropical legumes (\textit{R. taiwanensis}) [89], nosocomial infections (\textit{R. pickettii}, \textit{gilardi}, \textit{paucula}) [87].
hydrogenotrophy (R. eutropha), colonization of metal-rich biotopes (R. metallidurans, campinensis, basilensis), degradation of recalcitrant aromatic compounds and man-made chemicals (R. oxalaticus, eutropha, basilensis), and the synthesis of polyhydroxylalkanoates (R. eutropha, oxalaticus, metallidurans). This diversity of functions, specializations, and range of biotopes in the genus Ralstonia illustrates why it was originally assigned to the large bacterial group of Proteobacteria (in reference to the Greek god Proteus, famous for his diversity of shapes). In the same perspective, natural genetic engineering is clearly at stake to promote the functional diversity in this genus.

3. Genomics of the genus Ralstonia

3.1. Main features of the genome of R. solanacearum and R. metallidurans

The genome of R. solanacearum is made up of two large replicons: a 3.5-Mb chromosome and a 2.1-Mb megaplasmid [88]. Pathogenesis is linked with large clusters of genes that are located on both replicons.

It is interesting to compare this situation with the genome organization of the soil-dwelling hydrogenotroph R. eutropha H16. In this bacterium, pulse field electrophoresis studies have indeed shown the presence of two major replicons of 4.1 Mb and 2.9 Mb, and of a 0.44-Mb megaplasmid that contains the genetic determinants for hydrogenotrophy and enables anaerobic growth at the expense of nitrate [90]. These genetic determinants are chromosome-encoded in R. metallidurans CH34 as they are present in the plasmid-free derivative of R. metallidurans CH34 [2,16]. Reversely, the 0.44-Mb megaplasmid (pHG1) of R. eutropha H16 does not appear to contain any genetic determinants for heavy metal resistance genes (E. Schwartz, personal communication). Conjugal mapping using two different conjugal plasmids (pULB113, an RP4::miniMu derivative, and pMOL50, a derivative of pMOL28) able to mobilize chromosomal determinants showed that these determinants are linked to other housekeeping gene determinants on the chromosome [16]. It is not yet known if the genome of R. metallidurans contains a structure like the megaplasmid of R. solanacearum, or more generally two major replicons as is the case for both R. solanacearum G1000 and R. eutropha H16 [88,90].

Strain CH34 contains two large plasmids of 180 and ∼ 250 kb [14,75] that are especially rich in metal resistance genes and in open reading frames (ORFs) similar to well-known metal resistance genes. Within the scope of this review, we will refer to the chromosome of R. metallidurans CH34 for all genes and functions that are not located on the plasmids pMOL28 and pMOL30, and this without prejudice of the possible presence of two major replicons as in R. solanacearum or R. eutropha.

The genomes of R. solanacearum and R. metallidurans apparently share a large number of orthologous sequences (Table 1). In addition, the major replicons of R. solanacearum contain a number of genes that display substantial sequence similarity with genes of other bacteria (Table 1): the 3.5-Mb chromosome of R. solanacearum contains up to 65% of genes displaying good identity with those of R. metallidurans while this percentage drops to 30% in the megaplasmid. These figures are much higher than the figures obtained in the comparison of R. solanacearum with the available genomes of other β- and γ-Proteobacteria (Table 1).

3.2. Inventory of metal resistance genes or gene equivalents in R. solanacearum and R. metallidurans

The genome of R. solanacearum has been extensively tested for the presence of equivalents of known metal resistance genes and especially those represented in the genome of R. metallidurans. This search was carried out by looking at ars- (arsenate, arsenate), chr- (chromate), cop- (Cu(II)), czc- (Cd(II),Zn(II),Co(II)), cnr- (Co(II),Ni(II)), mer- (Hg(II) and organomercurials), pbr- (Pb(II)), and sil- (Ag(I)) like genes as well as at genes encoding P-ATPases, although it is not always easy to define those involved in metal resistance. The main observation is that most of the putative metal resistance genes are linked to the megaplasmid of R. solanacearum as reported in Fig. 2. On the chromosome, there are two loci governed by a merR-like regulator: only one locus has recognizable structural genes for metal transport (P-ATPase); there are also five arsR/arsC paralogues but none of them can be associated with a metal resistance function. The chromosome contains an arsR-arsC (ArsC stands for reductase of arsenate to arsenite) cluster (as the megaplasmid) but at present there is no indication for a possible relationship with resistance to arsenicals.

Fig. 2 reports the localization of the metal resistance genes and of the IS elements on the R. solanacearum megaplasmid. This localization may give some important clues on the genetic mobility of metal resistance genes and their possible involvement in horizontal gene transfer events. The map of the R. solanacearum megaplasmid shows the presence of many clusters of putative metal resistance genes.

Table 1

| Distribution of R. solanacearum proteins conserved in other organisms |
|--------------------------|-----------------------|
| Chromosome               | Megaplasmid           |
| Ralstonia metallidurans  | 2148                  | 661                     |
| Neisseria meningitidis   | 856                   | 124                     |
| Xylella fastidiosa       | 792                   | 137                     |
| Pseudomonas aeruginosa   | 1558                  | 398                     |
| Escherichia coli         | 1133                  | 322                     |
| Unknown proteins         | 315                   | 433                     |

The number of orthologous genes in various organisms was determined for each R. solanacearum protein having 35% or more sequence identity over at least 80% of its total length and an expected value of BLASTP below 10^-6.
genes that are ordered as they are observed on chromosomal contigs of *R. metallidurans* and much less as they are observed on the large plasmids pMOL28 and pMOL30. Fig. 2 indicates the clusters of metal resistance genes of *R. solanacearum* that have an equivalent with the same putative gene order in *R. metallidurans*. As an example, the order of the *cop* genes is similar to that observed in *R. metallidurans* (see below).

There are also four clusters with *czc*-like genes (HME-RND efflux according to the nomenclature proposed in another paper in this issue [84]: RND stands for resistance–nodulation–cell division [86] and HME for the class of RND that is specialized in efflux of heavy metal cations [91]) with four paralogues for *czcA*/*silA*. The number of putative HME-RND genes in *R. solanacearum* is higher than for *B. cepacia*, *Pseudomonas aeruginosa* or *Escherichia coli* but much less than was observed in *R. metallidurans* as shown in the next section.

4. HME-RND gene clusters in *R. metallidurans*

At least 12 (putative) genes encoding a HME-RND [86] efflux system (CzcA orthologues and paralogues) are present on the genome of *R. metallidurans*: one in pMOL28 (*cnrA*), three on pMOL30, and eight on the chromosome (Fig. 3). This high number of (putative) CzcA paralogues and of corresponding gene clusters or operons supports the specialization of *R. metallidurans* in the adaptation to high concentrations of heavy metals, although it is not yet possible to assign functions or metal specificities and concentration ranges for most of them.
### Table 1: R. metallidurans gene clusters containing czcA paralogues or orthologues

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Contig or gene</th>
<th>Location</th>
<th>Cluster czc</th>
<th>RND class</th>
<th>Phenotypes &amp; observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. metallidurans</td>
<td>R. metallidurans 663</td>
<td>chrom.</td>
<td>czcA</td>
<td>Resistance Cd, Zn, Co</td>
<td></td>
</tr>
<tr>
<td>R. solanacearum</td>
<td>R. solanacearum 280+465</td>
<td>pMOL30</td>
<td>czcA</td>
<td>HME1</td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>P. aeruginosa 280+465</td>
<td>RSp493</td>
<td>czcA</td>
<td>Cd, Zn</td>
<td></td>
</tr>
<tr>
<td>L. pneumophila</td>
<td>L. pneumophila 720</td>
<td>helh</td>
<td>czcA</td>
<td>Cd, Zn</td>
<td></td>
</tr>
<tr>
<td>P. fluorescens</td>
<td>P. fluorescens 720</td>
<td>czcT</td>
<td>czcA</td>
<td>Cd, Zn</td>
<td></td>
</tr>
<tr>
<td>A. caligenes sp.</td>
<td>A. caligenes sp. 720</td>
<td>czcC</td>
<td>czcA</td>
<td>Cd, Zn</td>
<td></td>
</tr>
<tr>
<td>X. campestris</td>
<td>X. campestris 720</td>
<td>czcC</td>
<td>czcA</td>
<td>Cd, Zn</td>
<td></td>
</tr>
</tbody>
</table>

### Diagram 1: General phylogeny of czcA paralogues

Fig. 4 shows the general phylogeny of czcA paralogues taking into account czcA orthologues in various bacteria. This high number of paralogues is a strong argument in favor of the long history of adaptation of R. metallidurans to metal-rich biotopes. In HME1, besides the putative orthologue of R. solanacearum and other duly assigned orthologues from other bacteria, one recognizes the well-known plasmid-borne czc operon and a putative chromosomal counterpart (fused contigs 280–465, data deposited in ftp://ftp.genome.bnl.gov/pub/Sequences/Rmetallidurans/) with which no phenotype or any other characteristic is associated.

The HME2 group (nickel detoxification systems) includes the pMOL28 cnr operon and the pTOM9 ncc operon of R. metallidurans 31A [4], but also a pMOL30-borne cluster that is probably not functional, at least as far as nickel resistance is concerned, because no nickel resistance is associated with pMOL30.

The HME3 type a and b groups are not yet assigned to any function: according to Nies [84], HME3a czcA paralogues are closely related and might process monovalent cations. Two of them belong to the same contig 647.

![Diagram 1: General phylogeny of czcA paralogues](image-url)
the Tn4371 family (biphenyl transposon) [92,93]. An element of this family is also present in the chromosome of R. solanacearum [88,94].

The HME4 group includes two operons that show genotypic and phenotypic similarity with known RND systems involved in copper and silver detoxification: cus in E. coli [95] and sil in a Salmonella typhimurium plasmid [96]: a cus-like operon was found on contig 619 and a sil-like cluster was present on plasmid pMOL30. Both systems were at least partially revealed by proteomics in R. metallidurans. Challenging with silver helped to reveal CusB and CusC. Challenging with copper revealed a polypeptide corresponding to the pMOL30 silB gene (V. Auquier, G. Vandenbussche and J.-M. Ruyschaert, personal communication), CusB and CusC (Fig. 3, Table 2). Proteomics appears to be a promising tool in the detection of B and C components of RND systems. An interesting challenge will reside in the choice of appropriate inducers to reveal HME3 type a and b proteins.

5. The P1-ATPases

P1-type ATPases are ancient proteins found in all organisms [97,98]. An average of two to three genes encoding P-type ATPases are currently found in fully sequenced genomes. For instance, two are found in E. coli, three in Listeria species, Synechocystis, Bacillus subtilis or Helicobacter pylori, five in Mycobacterium tuberculosis. Three genes are found in R. solanacearum, one on the megaplasmid (Fig. 2) and two on the chromosome. In contrast, the R. metallidurans genome contains up to 11 putative genes encoding P-type ATPases among which at least eight metal-transporting P1-ATPases (also called CPx-ATPases)
one gene sequence being incomplete and located on contigs 331 and 304). This resembles the situation for the RND system of metal detoxification. Fig. 5 shows the various clusters where these (putative) genes are located.

5.1. Prediction of structural characteristics of R. metallidurans P1-ATPases

Structure predictions suggest that the seven complete (or eight) putative P1-ATPases of R. metallidurans share the classic features of canonical ATPases (see [84,95,98,99]). More specifically they display the following features:

1. one or more heavy metal binding sites at the amino-terminal end (see Table 3)
2. a conserved intramembranous CPx motif (x = C, H or S)
3. a conserved dipeptide HP in the second cytoplasmic domain
4. a unique number and topology of the membrane-spanning domains [100]. Following the polar amino-terminus, the polypeptide chain was predicted to cross the membrane four times before the first cytoplasmic domain (except for the chromosomal CadA (contig 649) and a CadA-like ATPase belonging to contig 692 (pMOL30) [29], copF: pMOL30, annotation of sequence AJ278983 and the corresponding fragment on contig 709).

Table 2
Proteomics in R. metallidurans

<table>
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<th>Location or localization</th>
<th>Amount of isoforms</th>
<th><em>M</em> (kDa)</th>
<th><em>pI</em></th>
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The table shows proteins that are induced or repressed in presence of heavy metals. + or – signs indicate relative induction or repression. Proteins were detected in 2D PAGE [118].

Fig. 5. Genetic organization of the clusters containing genes encoding (putative) efflux P1-ATPases in R. metallidurans CH34. The cadA-like gene belongs to contig 692 (see also Fig. 7). Chromosomal cadA cluster; proposed annotation for the gene cluster close to the cadA ATPase of contig 649. PAGI-2(C) loci; proposed annotation partially according to the sequence published by Larbig et al. [104]. cupA: contig 710. pbrA: contig 692 (pMOL30) [29]. copF: pMOL30, annotation of sequence AJ278983 and the corresponding fragment on contig 709.
5.2. Phylogeny

Phylogenetic approaches demonstrated that the eight genes grouped within three functionally, structurally, and phylogenetically distinct clusters [98,101–103]. One of those, harbored by the PAGI-2(C) genomic island [104], belongs to a separate cluster of ATPases presenting some similarities with FixI proteins, but for which metal specificity has not yet been determined. This ATPase is in fact part of a locus which also includes an arrNR two-component regulatory operon, four genes whose products display similarities to cytochrome oxidases, and a cutE homologue. Three out of the seven P-type ATPase sequences found in the R. metallidurans genome encode transport proteins belonging to the copper/silver kind of transporters (Fig. 6): one lies on the chromosome (contig 710) and is provisionally called cupA, the other is the pMOL30-borne copF (copper resistance), and the third is still incomplete. The proteins corresponding to the first two genes belonged to distinct branches of the Cu/Ag type of transporters: copF had no counterpart in the genome of the related R. solanacearum, but is rather phylogenetically linked with siiP, a plasmid-borne S. typhimurium ATPase involved in silver resistance [96], and to a lesser extent with the Enterococcus hirae copB ATPase involved in copper export [105,106]. The three corresponding proteins share an extended CXXC motif in which the number of amino acids between the two cysteine has been increased to three (conserved as YF residues 31–94, ARYRIENMD), residues 104–167, TVLAIKMD* (contig 649).

Chromosomal CadA (contig 692)

1. residues 21–55(*), HGRGHDHDSKHLDSSHSNLHGHVGKLDHDAH
2. residues 92–154, AAYRIEMDCPTFEETLIRNKIGMAGVAAFLDNLQVLTVHTLDSLPVKALDSGMAEPL

CupA chromosomal putative Cu-ATPase (contig 710)

1. residues 13–77, WRLPVEGMTCSACVRRVETALKVPVGHDVAVNLATEQATLQADSPEVLNAAKADAGYDVPR
2. residues 80–145, IELNADMTACGNKVRERALAVPGVDAQQNLATERATTVGLGADVQATLAAYEYGATP

CopF (pMOL30 copper resistance cluster copKTSRABCDIGFLH), residues 10–34(*), HTHSAQGGGSPQDAHDHSAHAGH

CadA-like (unknown phenotype or function) (contig 692)

1. residues 13–77, WRLPVEGMTCSACVRRVETALKVPVGHDVAVNLATEQATLQADSPEVLNAAKADAGYDVPR
2. residues 80–145, IELNADMTACGNKVRERALAVPGVDAQQNLATERATTVGLGADVQATLAAYEYGATP

Relevant C, H and EE residues are underlined.

The metal binding sites can be composed of a conserved motif (about 60 residues long) or a histidine-rich amino-terminus (marked with an asterisk) [148] or both.

The real function of the chromosomal cupA ATPase is not known but could resemble the detoxification function of the chromosomal E. coli CupA ATPase (see [107,108]). The CupA denomination is used in reference to the chromosomal E. coli CupA ATPase and to avoid confusion with the CupA periplasmic proteins (encoded in the copSRABCD gene clusters that are found on both plasmid pMOL30 and chromosome of R. metallidurans). The possible eighth (incomplete) ATPase (contigs 304 and 331) might also belong to the Cu/Ag family.

The four remaining ATPase genes of R. metallidurans appeared to be related to the Cd Zn Hg Pb transport P1-ATPases. Among these, a cadA-like gene located downstream of the czc locus (resistance to Co(II), Zn(II) and Cd(II)) of pMOL30 grouped with the Zn/Cd ATPases encountered in R. capsulatus and Caulobacter crescentus, and, to a lesser extent, with the Cd ATPases from diverse Firmicutes. This cadA-like gene does not seem to belong to an operon: flanking genes do not give any clue. A separate cluster grouped around a P-type ATPase of P. aeruginosa. This branch comprised the lead ATPase pbrA of R. metallidurans CH34 [29] and the proteins corresponding to a pbrA-like gene located on the PAGI genomic island [104], and to a chromosomal cadA (contig 649). cadA insertion mutants made in the plasmid-free derivative of R. metallidurans CH34 resulted in increased sensitivity to thallium, Cd(II) and lead (T. Vallaees, M. Mergeay and A. Bossus, unpublished). In the presence of pMOL30, the same kind of mutants displayed only a slight sensitivity to Cd(II). This suggests that another pMOL30 locus complements the increased sensitivity to heavy ions and that the pMOL30 czc system alone does not confer the maximal resistance to Cd(II) (T. Vallaees, M. Mergeay and A. Bossus, unpublished).
5.3. Metal fixation motifs

Table 3 reports putative metal binding motifs for six ATPases of *R. metallidurans*. The CXXE motifs rather than CXXC motifs were found in all *R. metallidurans* P-type ATPases belonging to the PbrA type of transporters (PbrA, chromosomal CadA and PbrA-like of the PAGI-2(C) genomic island). This motif was present as part of a fully conserved MDCXXEELXR motif which could be repeated up to three times in the protein sequence as is the case in the *pbrA*-like gene of the PAGI-2(C) island. The chromosomal CadA harbored both a CXXE and a histidine-rich metal fixation motif. Conversely the chromosomal copper ATPase harbored the canonical HMA motif CXXC, in two close copies in the N-terminal part of the protein sequence. Similarly, a copy of the so-called extended CXXXC site was found in CopF, in addition to a histidine-rich type of metal binding motif.

6. CDF, CHR and ars-like genes

CDF stands for cation diffusion facilitator: CzcD encoded by the pMOL30 czc operon is the first prokaryotic representative of this family to have been described [19, 85, 109, 110].

Two paralogues of CzcD are represented in contigs 705 and 708 of the *R. metallidurans* draft sequence but do not seem to be integrated in metal responses. Inside the czc cluster, *czcD* is integrated in an operon *czcDRSE* that is involved in the regulation of the resistance to Zn(II), Cd(II) and Co(II), with *czcR* and *S* as classic representa-
tives of the two-component sensor/activator system (see below). CzcD is involved in direct detoxification and in the regulation of another metal efflux system [84].

ChrA proteins characterize the CHR family of transporters [111]. There are two ChrA orthologues: one is chromosome-bound (cluster chrBAF) [112], the other is pMOL28 plasmid-borne (cluster chrIBACEF) [25,112]. In contig 665, a third putative chrA is tightly linked to two regulation genes that warrant further investigation. A fourth chrA (putative paralogue) was seen in contig 393 and could not yet be assigned to a metal-processing function.

Both chr clusters govern the resistance to chromate at different toxicity levels [36]. Outside these resistance mechanisms, one putative gene encoding a protein that is similar to the Pseudomonas chrB gene is also present in contig 628.

*R. metallidurans* does not appear to display an otherwise anticipated resistance to arsenic oxyanions [84]. However, contig 596 contains a peculiar assembly of chr-like genes in the order *arsR*→*arsR*→*arsC*→*arsB*→*arsC*→*arsH*. The latter gene, *arsH*, was first found in a defective transposon of a *Yersinia enterocolitica* plasmid conferring resistance to arsenite and arsenate [113], later in *Acidithiobacillus ferrooxidans* [114].

The striking feature of the *R. metallidurans* *ars* assembly is the presence of three adjacent genes encoding a putative arsenate reductase activity. The corresponding phylogeny (data not shown) indicates that the three putative reductases are quite distinct. Maybe, if they are functional they would correspond to different oxyanionic substrates. It would be interesting to see if non-arsenic oxyanions would reveal one of these putative reductases via proteomics.

7. Large plasmids and mobile genetic elements of *R. metallidurans*: content in heavy metal resistance genes

*R. metallidurans* strains often harbor conjugative plasmids with metal resistance genes: the frequency of self-transfer is sometimes very high [52] but their host range seems to be limited to some species of the *Ralstonia* genus: *R. metallidurans*, *R. eutropha*, *R. oxalatica*, *R. basiilensis*, and *R. campinensis* ([52], M. Mergey, A. Sadouk and J. Gerits, unpublished). The markers that are known to be transferable are essentially those that are linked to the determinants of Zn(II) and Cd(II) (*czcC, czcB, czcA*, *czcF*, *czcE*, *czcD*, *czcR*), Ni(II) (*nccA, nccB, nccC, nccD*), and copper (*cop*) resistance. As far as pMOL28 and pMOL30 from *R. metallidurans* CH34 are concerned, they are not self-transferable (or only at a very low frequency, e.g. as is the case for pMOL28) but they can be efficiently mobilized by such ubiquitous plasmids as RP4 or other IncP/P1 plasmids [15,115]. Mobilization processes, based on the formation of cointegrates after transposition, and the subsequent homologous recombination to resolve the cointegrates in replicons, made it possible to demonstrate the transposition activity of the mercury transposons Tn4378 (pMOL28) and Tn4380 (pMOL30) [15].

The sequence of pMOL28 was obtained from the JGI draft sequence of *R. metallidurans*. In pMOL28, metal resistance genes are confined in a rather compact region of the plasmid including the mercury transposon Tn4378 (merRTPADE), at least six ORFs involved in the resistance to chromate (chrI chrIBACEF) [36], and the cnrYXHCBAntrT cluster involved in the resistance to Ni(II) and Co(II) (Fig. 7) (for *cnrT*, see [84]). This ensemble received the name of ‘serpentine regulon’ referring to the presence of nickel, chromate and mercury in serpentine-rich sediments that are thought to be a potential reservoir of metal-resistant strains [77,116].

Genetic and sequencing data on resistance to Cd(II), Zn(II) and Co(II) (*czcC*) [20,32], resistance to Pb(II) [29], and resistance to Cu(II) [30] helped to assign contigs 653, 692, and part of 709 to pMOL30. These contigs also contain some other putative genes for metal resistance with unknown functions. Contigs 663 (with *czcE*) and 692 (with *pbr*) are linked (see ftp://ftp genome.bnl.gov/pub/Sequences/Metallidurans/). Fig. 7 shows the various clusters of metal resistance genes that are associated with pMOL30, including the mercury transposon Tn4380 [15].

In pMOL30, according to the sequence data currently available and the present annotation and functional analysis (e.g. via mutational analysis, transcriptomics by real-time reverse transcription polymerase chain reaction [30] or proteomics; see Table 2) [117,118], four functional clusters have been identified: the *czc* cluster (*czcNICODERSE*), allowing plasmid-borne resistance to Zn(II), Cd(II) and Co(II), the *pbr* cluster (*pbrTRABCD*, confering resistance to lead), the *cop* cluster (*copTKSRABC-DIGFLL*, confering resistance to copper), and the mercury transposon Tn4380 (merRTPADE). This transposon was mapped on pMOL30 by classic genetic methods (deletion mapping, restriction mapping, etc.) but did not appear as such in the JGI draft of the *R. metallidurans* CH34 genomic sequence. Both mercury transposons Tn4378 (pMOL28) and Tn4380 (pMOL30) carry *merE* ORFs [119]. These *merE* ORFs, just like the corresponding MerE polypeptides of Tn501 and Tn21 transposons, contain the LTCPCHL motif. Another likely non-functional *mer* cluster is located near *czc* as well as a paralogue of *mgtC* (magnesium transport, Q. Dong, personal communication). Other clusters (located on contig 709: Fig. 7) that must be mentioned are a putative *nclencr* cluster with no adjacent regulation genes (and which probably does not confer nickel resistance) and a putative *sil* cluster, again with no adjacent regulation genes but that does seem to respond to the presence of copper in a proteomics assay (V. Auquier, G. Vandenbussche and J.-M. Ruyschaert, personal communication).

Perhaps the most surprising feature of *R. metallidurans*
mobile genetic elements was only recently put forward by findings in a study of *P. aeruginosa* nosocomial infections (a major cause of morbidity in cystic fibrosis patients) as it was found that the *R. metallidurans* genome actually contains a large genomic island PAGI-2(C) conserved at 100% in these *P. aeruginosa* strains [104]. This observation strongly suggests a close contact and gene transfer between *P. aeruginosa* and *R. metallidurans*, very likely in (polluted) soil environments. Coexistence of both genera has been observed before in a zinc-desertified area [11]. *P. aeruginosa* isolates have also been isolated from river sediment that are strongly polluted by heavy metals [120]. The PAGI-2(C) genomic island (Fig. 7) contains many genes involved in the biogenesis of cytochromes or hemopores. Yet it also contains a *pbrA*-like gene cluster and a mercury gene cluster that could be functional.

8. Proteomic approaches in *R. metallidurans*

More recent annotations of the genome estimate the number of candidate protein-encoding gene models at 6530. The next logical step that follows genome sequencing is shifting from DNA sequence analysis and gene structure prediction to the experimental identification and confirmation of the proposed gene products at the cellular level, i.e. by transcriptome and proteome analyses. These include studies on possible post-transcriptional and post-translational modifications of the gene products, their regulation of expression depending on changing environmental conditions, especially in the presence of heavy metals, and functional assignments and additional studies on the possible interactions between gene products within the cell. The first functional proteomic analysis corresponding...
to a global analysis of the *R. metallidurans* proteome has been initiated in our laboratories as a prelude for the large-scale study of heavy metal response. Current proteomic analysis is based on two-dimensional gel electrophoresis (2-DE) and on mass spectrometry analysis [121, 122]. The main challenge of heavy metal proteomics is the detection of membrane proteins, which are overrepresented in all the mechanisms conferring metal resistance. In this context, a soluble and membrane protein 2-DE database is being constructed. This work shows the importance of the proteomic approach for genome analyses through (i) the identification of previously undetected ORFs, (ii) the identification of proteins not represented by the draft genome sequence, and (iii) the characterization of protein interactions, for instance between gene products originating from two different contigs [118].

Moreover, the first differential proteomic analysis applied to *R. metallidurans* revealed new plasmid-borne proteins as well as a series of chromosomal gene clusters involved in the response to high concentrations of heavy metals. Table 2 shows a selection of proteins that were induced or repressed in the presence of metals, especially Cu(II). The specificity of the protein response has been studied by comparison with other metals such as Ag(I), Zn(II), and Ni(II). The copper concentration used (0.8 mM) is a typical restrictive concentration at which only the pMOL30-bearing strains can grow: it is not surprising that most of the induced proteins are plasmid-borne as the regulator CopR (see Sections 9 and 11.1), the membrane-bound proteins CopB, and the periplasmic CopC and CopK proteins (as described below). The proteomic approach also reveals the presence of different isoforms of CopC, CopK, and CopB. The function of these isoforms remains unknown and is currently being studied in our laboratories. The expression of chromosomally derived periplasmic proteins with unknown functions was also modulated in the presence of Cu(II), i.e. CupX (induced) and Cuf (repressed). Table 2 also shows the presence of heat shock and oxidative stress proteins. These proteins are used as reference points on the 2-DE gel landscape.

9. Plasmid-borne copper resistance genes in *R. metallidurans*

Resistance to copper is found on the large plasmid pMOL30 and is transmissible by mobilization of pMOL30 to a plasmid-free derivative of *R. metallidurans* CH34. The presence of pMOL30 increases the MIC to copper by a factor of two to three in the transconjugants. Plasmid-borne resistance to copper was cloned and sequenced (accession number: AJ278983). Genomic analysis shows that both the chromosome of *R. metallidurans* and pMOL30 share the six genes *copSR-copABCD* likely to correspond to a basic resistance mechanism [107] that is also observed in a plasmid (provided with genes
E. coli strain found in the feces of copper-fed pigs [107,123,124], and in plasmids of Pseudomonas syringae strains isolated after copper-based antifungal treatments of crop vegetables in California [125–129]. Genes of P. syringae variety tomato have the order copABCD-copRS [126,130], which is similar to the pco operon structure where the transcription of both the cop/pcoABCD structural genes and the two-component copRS regulatory genes [131,132] is in the same orientation. In contrast, the transcription of regulatory and structural genes is divergent in the Ralstonia genus (Fig. 8). As is shown in Fig. 9 for copC taken as a representative example of the six genes, the copSRABCD genes of R. metallidurans (chromosomally and on plasmid pMOL30) and their R. solanacearum counterparts are phylogenetically tightly clustered. Intriguingly, the copSR-copABCD genes of pMOL30 are included in a larger cluster of genes that respond to copper: copTKSRABCDIFGLH (Figs. 7 and 8). Transcriptomic approaches have also shown that these 13 genes are transcribed in the presence of copper [30]. A differential proteomic approach also shows plasmid-borne proteins corresponding to periplasmic CopK and CopC, outer membrane-bound CopB, and cytoplasmic CopR (Table 2). In this respect, CopT is probably a paralogue of PbrT [29], seemingly an importer of lead as described in Section 10, and CopK is revealed as a new periplasmic copper binding protein [117] (Table 2) while CopF is a P1-ATPase and CopG is likely to be involved in the expression of CopF.

As shown in Fig. 9, the phyletic position of CopI branches far away with respect to the periplasmic CopC. Its function is unknown although both proteins appear to be related to Cu-containing oxidoreductases.

CopH shares identity with the putative gene product of cze [32] but the function of both proteins is still unknown. This is also the case for the putative membrane protein CopL.

It is difficult to understand the function of such complex protein assemblies induced by copper. Yet, two already known mechanisms seem to be involved: the efflux of cytoplasmic copper (thiol pool) that is mediated by a P1-ATPase (CopF) and the detoxification of periplasmic copper via the copSR-copABCD-mediated efflux (by analogy with what was observed or deduced in the E. coli pco system [107] and the P. syringae cop system). It is to be recalled that the chromosome also contains functional copSR-copABCD genes as well as a genetic determinant for a putative Cu P1-ATPase, provisionally called copA (see Section 5.2).

The combinatory and regulatory integration (as seen for the large gene cluster in pMOL30) of different resistance mechanisms that otherwise are not genetically linked (as is the case on the chromosome) may explain the higher resistance to copper conferred by the pMOL30 plasmid. On the other hand, the complete deciphering of the copper resistance mechanism should also entail further studies on the isoforms for CopC, CopB and CopK, and the elucidation of their possible functions (Table 2).

**COP C & I**

Fig. 9. Phylogenetic representation of CopC proteins. Includes CopC of R. metallidurans (chromosomal and plasmid-borne), R. solanacearum, Pseudomonas fluorescens, P. syringae, Xanthomonas campestris, E. coli strain K12, E. coli strain O157:H7, S. typhimurium, Salmonella enterica, Y. pestis, Deinococcus radiodurans, Corynebacterium glutamicum, and CopI of R. metallidurans pMOL30. The same method was used for the phylogenetic analysis of the ATPases and copC. Using the R. metallidurans chromosome and pMOL30 copC (shown in bold) as reference, several copC homologues were found in different organisms. NCBI accession numbers are given in brackets. copC genes were clustered in three different groups: R. solanacearum (shown in bold gray), P. fluorescens, P. syringae, X. campestris for the first group phylogenetically close to R. metallidurans; E. coli strain K12, E. coli strain O157:H7, S. typhimurium, S. enterica, Y. pestis form the second group, and D. radiodurans, C. glutamicum which seems to be phylogenetically apart. R. metallidurans pMOL30 copI is also included in this phylogeny but does not group with the other genes.
Fig. 10. Proposed mechanism for pMOL30 pbr-mediated resistance to lead. Model for the pbrTRABCD operon-encoded Pb resistance of R. metallidurans CH34. The model involves the following proteins: PbrT, which transports Pb into the cytoplasm; PbrA, the Pb efflux ATPase; PbrB and PbrC, the Pb transport-facilitating lipoprotein and its prolipoprotein signal peptidase, respectively; and PbrD, a protein involved in cytoplasmic Pb sequestration. The PbrR protein, which mediates Pb-inducible transcription from its divergent promoter, regulates the expression of the pbr operon (from [29] with permission of the authors).

10. Plasmid-borne genes for resistance to lead in R. metallidurans

Lead resistance was early recognized to be plasmid-borne in R. metallidurans [115], although it was only recently described at the molecular level [29]. However, data about lead resistance have also been reported for Arthrobacter sp. and other Firmicutes that were isolated from the waste site of an Australian factory of lead batteries [133]. Pseudomonas marginalis showing extracellular lead exclusion and Bacillus megaterium demonstrating intracellular cytoplasmic lead accumulation were isolated from other lead-contaminated soils [134]. Pb(II)-resistant strains of Staphylococcus aureus and Citrobacter freundii have also been isolated that accumulated the metal as an intracellular lead phosphate [135], though the molecular mechanism of detoxification remains to be elucidated. Efflux of Pb(II) has also been reported for the CadA ATPase of S. aureus and the ZntA ATPase of E. coli [101,102].

The lead resistance operon, pbr, of R. metallidurans CH34 is unique, in the way that it combines functions involved in uptake, efflux, and accumulation of Pb(II) [29] (Fig. 10). A MerR-like regulator, PbrR, controls transcription of the pbrABCD structural genes from the PbrA promoter (Table 4), with the first gene downstream of the pbr operator/promoter encoding a P-type ATPase, like the zntA operon [136]. The PbrA Pb(II) ATPase is phylogenetically grouped with the CadA-type Cd ATPases and the ZntA Zn/Cd ATPase, and they form a group distinct from the Cu/Ag type ATPases. However, unlike CadA and ZntA, we have previously seen that PbrA possesses two HMA motifs with the amino acid sequence Cys-Pro-Thr-Glu-Glu instead of the consensus sequence Cys-X-X-Cys [29].

In contrast to the cad and znt operons, which both appear to comprise a regulatory gene plus an efflux ATPase only, additional proteins are required for maximal Pb(II) resistance in R. metallidurans CH34. These are PbrT, PbrB, PbrC, and PbrD. The first step in pbr-encoded Pb(II) resistance involves a Pb(II) uptake system encoded by PbrT. Expression of PbrT in the absence of PbrABCD results in Pb(II) hypersensitivity, probably due to increased Pb(II) uptake into the cytoplasm. The result of this Pb(II) uptake would be to reduce the interaction of free Pb(II) with side chains of membrane and periplasmic proteins, which would cause extensive cellular damage. Once Pb(II) has entered the cytoplasm, it can be exported by the PbrA Pb(II) efflux ATPase or be bound by the PbrD protein, which may function as a chaperone for Pb(II). PbrD is not absolutely required for Pb(II) resistance, but cells lacking PbrD show a decreased accumulation of Pb(II) compared to wild-type cells, and this accumulation may protect against free exported Pb(II) and the futile cycle of Pb(II) uptake into the cytoplasm.

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The PbrA Pb(II) efflux ATPase has been shown to be

Table 4

Comparison of the promoter regions of different R. metallidurans CH34 genes that are under control of MerR- and PbrR-like regulators

<table>
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<th>Gene</th>
<th>CH34 Regions</th>
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<td>pbrA</td>
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<td>zntA</td>
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<tr>
<td>zntA</td>
<td>TGA(T)CTATAGTAACTAGAAGGGTT</td>
</tr>
<tr>
<td>pbrA</td>
<td>TGA(T)CTATAGTAACTAGAAGGGTT</td>
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<td>zntA</td>
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<tr>
<td>zntA</td>
<td>TGA(T)CTATAGTAACTAGAAGGGTT</td>
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</table>

As comparison the PzntA (Zn resistance in E. coli) [136] and PmerT (Hg resistance of Ts301) [119] promoters are included. Bold: positions of −35 and −10 regions; inverted repeats are underlined. Cnnn indicates the contig numbers of the newly identified merR-pbrR regulatory genes.
functional and able to compensate for the Pb(II) uptake driven by PbrT. However, for full Pb(II) resistance PbrB and PbrC are required. PbrB and related proteins may be part of a new family of transporters assisting resistance (TAR) proteins. Comparison of PbrB with the EMBL database resulted in 16 hits. However, with the exception of the BcrC protein of Bacillus licheniformis, all others were hypothetical membrane (lip)proteins. The BcrC protein of B. licheniformis encodes a hydrophobic membrane protein that, together with the BcrB membrane protein, functions as membrane components of the bacitracin resistance ABC transporter [137]. Inactivation of BcrC results in bacitracin sensitivity, and inactivation of PbrB results in Pb(II) sensitivity. The PbrB lipoprotein may promote transfer of Pb(II) from the periplasm to the outer membrane. This would result in a decreased Pb(II) uptake by PbrT.

At increased concentrations of Pb(II), metal removal from the solution was observed for strain CH34 during the late exponential phase and the stationary phase, together with a progressive increase of the pH (up to 9). At this increased pH the formation of lead complexes with hydroxide and carbonates will be strongly favored and should play an important role in avoiding re-entry of Pb(II). A similar phenomenon has been described for the cze Cd(II)-Zn(II)-Co(II) resistance system of R. metallidurans CH34 [52].

11. Regulatory genes in the heavy metal resistance gene clusters

Regulatory genes for resistance to heavy metals in Ralstonia are mostly strongly linked to structural genes, often upstream of the structural genes with a divergent transcription orientation or downstream of the structural genes but having the same orientation. By far the most frequent combination of regulatory genes belongs to the large two-component sensor and regulator family using phosphorylation signals. But a few other families are discussed too, including the well-known merR family. The arsRiccadClsmntB family of regulators is also dealt with although the correlation of the arsRC gene clusters observed in R. solanacearum genomes or of the ars cluster of R. metallidurans (contig 596) with an arsenic resistance phenotype has still to be demonstrated. Some details about new families of regulators such as cmrnccYXH and the possibly new family czeI are included in the discussion. Yet there are still quite a lot of genes found in the metal resistance clusters, generally membrane-bound, with a possible regulatory function for which no details or plausible mechanisms are currently available.

11.1. The R and S two-component family

Two-component RS systems are one of the major mechanisms responsible for the coordinated adaptation of living organisms in response to environmental challenges. Each system consists of a sensor kinase, generally an integral membrane protein provided with a signal recognition device, and a response regulator transcription factor, which activates or represses gene expression. In the presence of an external stimulus, e.g. a threshold in extracellular metal concentration, the sensor kinase autophosphorylates at an internal histidine and then transfers the high-energy phosphate group to an aspartyl residue on the response regulator. Two-component systems exist in organisms belonging to all three domains of life, including non-animal Eucarya and Archaea. However, outside the domain of Bacteria, two-component regulatory systems are less abundant and seem to have been acquired by horizontal gene transfer. In bacteria, such systems are usually organized as a pair of adjacent genes included in a large operon. They mediate adaptive responses to a wide spectrum of environmental stimuli and play a crucial role in metabolic processes such as aerobic respiration, osmoregulation, sporulation, phosphate or nitrogen regulation, chemotaxis and virulence (for a review, see [138]).

In many organisms, such as E. coli [139], R. solanacearum [88] and P. aeruginosa [140], genes and/or operons involved in efflux mechanisms are under the control of various regulators, including two-component systems. R. metallidurans contains a high number of such proteins, with 42 sensor kinases and 67 response regulators so far identified. This is more than the number found in the genome of E. coli, i.e. 29 and 37, respectively, and that of R. solanacearum, i.e. 37 and 55, respectively, but less than in the genome of P. aeruginosa, i.e. 55 and 89, respectively. RS-mediated regulation of metal resistance genes is a common feature of operons with RND genes (tricomponent cation/proton antiporter efflux), especially those displaying a CdtII (cze) [32], silver (sil) [96], or copper (cus) [141] resistance phenotype, but are also associated with copl pco-mediated resistance to copper [131]. An association with a putative chrA should also be mentioned (see Section 6).

In R. metallidurans, at least seven two-component systems are associated with genes of the RND family, including five cze-like operons. A large diversity can be observed in terms of operon structure (Fig. 3). For example, some R and S genes are divergently expressed from their structural resistance genes, sometimes the R and S genes constitute an operon where both genes overlap, while they are either located downstream [32] or upstream of the structural resistance genes of cze-like clusters.

Sensor kinases associated with cze-like and cop operon components are highly similar to proteins of homologous systems in various microorganisms, e.g. SilRS in S. typhimurium and CusRS in E. coli, and belong to type IA, which also includes the osmoregulation EnvZ/OmpR system and the KdpD/KdpE potassium transport in E. coli [142] (Fig. 11). The histidine site of phosphorylation is
located in the H-box domain and the kinase domain consists of several conserved consensus motifs. Yet, a few residues are conserved for all the S proteins involved in the regulation of (putative) metal resistance genes (a Phe in position 3 and an Arg in position 50 in Fig. 11a) and are not found in the other tested systems (Fig. 11a).

Similarly, an alignment of their response regulator counterparts demonstrates the presence of highly conserved residues, including not only amino acids known to be involved in the catalytic transfer of the phosphoryl group but also some residues located at their vicinity that may play an important role in the anchoring of both components during this process [143] (Fig. 11b). The observation that these sensors and regulators form distinct clusters in a phylogenetic tree containing all two-component systems identified in *R. metallidurans* further supports their close evolutionary relationship. However, such a comparative phylogenetic analysis of RS systems involved in metal resistance with the corresponding regulated genes suggests that some of them may have evolved independently from each other (data not shown). With this respect, RS systems and their target genes may constitute distinct evolutionary modules.

Neither a metal binding site nor a histidine-rich domain, known to be frequently associated with metal–protein interactions, was found to be conserved in the response regulators or sensor kinases. This suggests that the mechanism by which bacterial cells sense the presence of heavy metals in their environment remains unclear at the protein level. Nevertheless, as far as metal specificity is concerned, it seems that the systems (sensor and kinase) involved in copper resistance (*cop* and *cus*) remain clustered together, which is not the case for the RS genes that are implicated in the regulation of the other HME-RND genes.

At the direct protein level, at least one regulator (CopR in *P. syringae*) has been described and further studied [132]. In *R. metallidurans*, proteomics helped to reveal the plasmid-borne CopR regulator (Table 2). We are confident that additional proteins with similar characteristics will be found in the near future.

### 11.2. The merR family

In *R. solanacearum*, seven *merR* paralogues (of which five are on the chromosome) have been identified. Only two of these, one on the chromosome and one on the megaplasmid, are adjacent to a putative gene for metal resistance, in both cases a P-ATPase. There is no putative cluster conferring mercury resistance in this phytopathogen.

Within the genome of *R. metallidurans* CH34, nine MerR-like regulators have been identified. These regulators may be divided into four major groups based on sequence similarity and functional linkage to structural resistance proteins.

The first group consists of the MerR regulators, of which the corresponding genes are found on pMOL28 (Tn4378), on PAGI-2(C) and close to the *czc* operon, and on pMOL30 (Tn4380). They show close similarity with the *merR* gene product of Tn501 and are always linked to other, structural mercury resistance genes, while the *merR*-like gene close to the *czc* operon is linked to a *merTP* cassette. Owing to the absence of a *merA* gene, the corresponding *merRTP* operon does not encode a functional Hg resistance system. In contrast, the PAGI-2(C) *merR* is linked to a *merTPA* Hg resistance cassette and could encode a functional Hg resistance system; the pMOL28 *merR* is the regulator for the Tn4378-encoded Hg resistance (*merRTPAD* operon). Interestingly, the four MerR proteins of Tn4378 (pMOL28), Tn4380 (pMOL30) [15] (Fig. 7) and Tn501 [144] are identical and recognize identical PmerT promoters. This opens the possibility for fine-tuning the gene expression between the two major Hg resistance determinants located on the mercury resistance transposons of strain CH34.

The second group of regulators consists of PbrR, the regulator of the CH34 lead resistance operon that is located on pMOL30, and two regulators found on PAGI-2(C) and the chromosomal contig 710. As for the *pbrTRABCD* operon, the *pbrR*-like gene of PAGI-2(C) is linked to a heavy metal efflux ATPase, most similar to a Cd efflux ATPase, and a *pbcC*-like gene (Fig. 5).

The *pbrR*-like gene on contig 710 was not linked to any structural heavy metal resistance gene. However, we cannot rule out that this regulator is involved in heavy metal resistance, since *zntR* [136] and *cueR* [145] are physically distant on the chromosome from their cognate promoters.

A second *merR*-like gene of contig 710 is linked to a Cu ATPase-type gene. This MerR-like regulator is more similar to *ZntR* (Fig. 12), and forms the third group. The corresponding gene is located downstream of *cupA* (see Figs. 5 and 6, and Table 3). However, the ATPase and the MerR-like regulator form one unit that is transcribed from a promoter, *PcupA* (contig 710), that has the properties of a typical MerR-recognized promoter (Table 4).

The Mer-like regulators of contigs 518 and 671 form the last group. These two regulators are not physically linked to any structural heavy metal resistance gene, and their involvement in heavy metal resistance might be questionable.

### 11.3. The arsR family

*arsR/cadC* paralogues are represented in both *R. metallidurans* and *R. solanacearum*. In this latter bacterium, three of them are associated with the chromosome, while three are located on the megaplasmid. On each *R. solanacearum* replicon, an association of (putative) *arsR-arsC* is observed (see Fig. 2 for the *arsRC* of the megaplasmid). In *R. metallidurans*, only one complete *arsR* sensu stricto is observed in the intriguing putative *ars* cluster described above. Eight other ‘*arsR*’ paralogues were identified that
Fig. 11. a: Multiple sequence alignments of putative S genes from two-component sensor/kinase regulator systems. Amino acid alignment of the C-terminal part of sensor kinases belonging to the corresponding czc and cze-like operons. This includes the sequence of two other members of the type Ia family [142], i.e. EnvZ and KdpD. Only the sequence downstream of the L237 residue in EnvZ and the corresponding sequence of other sensor kinases are shown. The histidine box is black underlined as well as the downstream X region and the positive residues located at the extremity of the X region. Various conserved domains, i.e. N, G1, F, G2, G3, are also gray underlined. b: Multiple sequence alignments of putative R genes from two-component sensor/kinase regulator systems. Amino acid alignment of the N-terminal part of the regulator belonging to the corresponding czc and cze-like operons. This includes the sequence of two other members of the OmpR family, i.e. OmpR and KdpE. Only the sequence up to the E111 residue in OmpR and the corresponding sequence of other response regulators are shown. Conserved (gray arrows) and variable (gray dotted line) residues of loops 1–5 and helix 1 supposed to be involved in the interaction surface of response regulators of the OmpR family with their sensor counterpart [143] are boxed. Black arrows point to catalytic residues.
belong to the \textit{cadC} branch \cite{146}: two are located on the PAGI-2(C) genomic island (Fig. 7), one of them being associated with the \textit{pbrA}-like gene (see also Fig. 5). Another \textit{cadC}-like gene is adjacent to the (mutated) HME3b-RND operon located on contig 711 (see Fig. 3). Therefore, this would be an example of a new category of metal resistance genes regulated by the family \textit{arsR}.

11.4. The \textit{cnrYXH} family

RND-mediated resistance to Ni(II) has been reported in \textit{R. metallidurans} plasmids pMOL28 (strain CH34) \cite{6,24} and pTOM9 (strain 31A) \cite{5} and is controlled by the gene clusters \textit{cnrYXHCBAT} and \textit{nccYXHCBAN}, respectively. In both gene clusters, \textit{nccY}, \textit{nccX}, and \textit{nccH} are considered regulatory genes, of which the latter gene, \textit{cnrH}, controls a \(\sigma\) factor of RNA polymerase belonging to the ECF (environmental control factor) network. Mutations in \textit{cnrY} were found to derepress the expression of resistance to Ni(II) and Co(II). Two phenotypes were associated with this derepression: the MIC of Ni(II) and Co(II) increases from 2.5 mM to 8 mM, and from 5 to 10 mM respectively. The \textit{CnrY} mutants were also resistant to Zn(II) and can be positively selected through this phenotype \cite{6,34,72}. Mutants in \textit{cnrX} are also constitutive but they did not display a higher MIC to Ni(II) or Co(II) \cite{6}.

Detailed molecular studies showed the presence of at least two promoters that are recognized by \textit{CnrH}: a promoter located upstream of \textit{cnrY} \cite{34,35}, and another one located at the end of \textit{cnrX}, a few bases upstream of the start codon of \textit{cnrH} (\textit{cnrHp}) \cite{34}. The former promoter, \textit{cnrYp}, controls the transcription of \textit{cnrYXH} mainly in the absence of Ni(II): yet no transcription of \textit{cnrCBA} is observed in such conditions, suggesting that the transcription from \textit{cnrYp} results in the synthesis of a complex of closely interacting proteins (\textit{CnrY}, \textit{CnrX} and \textit{CnrH}) attached to the inner membrane, allowing no access of the \(\sigma_{ECF}\) factor \textit{CnrH} to the transcription apparatus. In the presence of Ni(II), there is evidence for transcription from the \textit{cnrHp} promoter that extends to the structural \textit{cnrCBA} genes and allows the expression of full resistance to Ni(II) and Co(II). This active transcription from \textit{cnrHp} seems to go together with decreased transcription from \textit{cnrYp}: membrane proteins \textit{CnrY} and \textit{CnrX} are no longer produced and repression is no longer possible. Phenotypically, \textit{CnrY} acts as a repressor although it is a membrane-bound protein with no direct genotropic effect. There is also an indication that transcription starts from a site between \textit{cnrH} and \textit{cnrC} \cite{35} but the most active transcription in the presence of otherwise toxic concentrations of Ni(II) clearly occurs from \textit{cnrHp} \cite{34}. The protein–protein interaction between \textit{CnrY} and \textit{CnrH} was confirmed using the BacterioMatch Two-Hybrid system from Stratagene (S. Taghavi, unpublished results). In order to show these interactions it was necessary to add a linker, consisting of [\text{Gly-Gly-Gly-Ser}]\textsubscript{2} \text{U} between the 3’ end of the bacteriophage \textit{\lambda}C repressor and the \textit{cnrY} coding region. In addition, \textit{cnrY} (the predicted anti-\(\sigma\) factor) was modified by replacing two Phe residues of the predicted transmembrane region by Glu residues in order to destroy this transmembrane domain, allowing access of the two-hybrid complex to the tran-
scription apparatus. The mutation resulted in a functional λcl–CnrY hybrid protein. In addition, a RNA polymerase–cnrH translational fusion was constructed. Introduction of both constructs in the appropriate E. coli reporter strain resulted in ampicillin resistance plus β-galactosidase activity. This phenotype was not observed for the control experiments with strains carrying vector sequences only or with constructs that lacked the CnrY or CnrH fusion proteins. This clearly demonstrates the interaction between CnrY and CnrH and supports the model of an anti-σECP factor, CnrY, controlling the activity of CnrH, the σECP factor.

This mode of regulation is unique among metal resistance operons and so far has only been observed in the related plasmid-borne nce resistance operon (strain R. metallidurans 31A).

11.5. Other regulatory genes

There are, in particular for the intricate plasmid-borne clusters, still a few genes with unknown function or which have been given a putative regulatory function.

The genes czcN, nccN and copL encode membrane proteins that are likely to be involved in regulation although their precise role remains enigmatic. nccN mutants decreased the full resistance to nickel in R. metallidurans 31A [5].

czl is involved in the regulation of plasmid-borne Czc-mediated resistance to Zn(II), Cd(II) and Co(II). A paralogue of czl might also act as a regulator in a small cluster containing a czcC-like gene and the proposed cadA gene encoding a P1-ATPase (contig 649; Fig. 5).

12. Concluding remarks

The present inventory of genes and operons that are involved in the resistance to heavy metals in R. metallidurans suggests that the genome of this bacterium contains more genes related to this kind of function than any other genome sequenced before. This is a striking notion when we look at the underlying genetic determinants for HME-RND efflux systems, the P1-ATPases, and the plasmid-mediated heavy metal resistance. It also supports the reflection from the molecular taxonomy data that R. metallidurans is specialized in the adaptation to heavy metals or to biotopes that are either occasionally or constitutively rich in heavy metals. It may therefore be postulated that the greater part of this specialization took place during a period largely before the development of anthropogenic activities. At the present time, R. metallidurans CH34 and its related strains are found almost exclusively in anthropogenic, industrial biotopes mostly bound to the wastes of metallurgical activities. The colonization by R. metallidurans and related bacteria of such biotopes may be three centuries old if we refer to the European Industrial Revolution that was determinant or even a few millennia old if we refer to the very first developments of metallurgical industry (copper, tin, mercury, etc.). In this respect, one wonders what the ‘natural’ biotopes of R. metallidurans could be [62]. A possible clue towards volcanic biotopes could derive from the fact that R. metallidurans strains are often hydrogenotrophs, and that serpentine rocks and dusts are rich in heavy metals and derive from the hydration of eruptive olivine rocks. This hydration releases measurable amounts of hydrogen that could help to support the growth of hydrogenotrophs at the expense of atmospheric CO₂ and air [147].

This kind of speculation should mainly attract the attention to the necessity to look at the microbial ecology and taxonomy of harsh environments or any sites where the selection pressure would enhance the diversity and the expression of metal resistance genes.

The present catalogue of genes will surely be improved with the completion of the genome. The closure of the genome will be essential to evaluate the genetic linkage between metal resistance determinants and mobile genetic elements (plasmids, transposons, IS elements, genomic islands) and the role of mobile genetic elements in the emergence or in the acquisition of genetic determinants for metal resistance and processing. It will also help to check the presence, in R. metallidurans, of a second large replicon as observed in R. solanacearum and in R. eutropha. In this respect, it is remarkable that, in R. solanacearum, most of the (putative) metal resistance genetic determinants are located on the megaplasmid and not on the chromosome. Importantly, with four HME-RND systems, R. solanacearum appears to be only second to R. metallidurans in diversity of metal resistance genes.

The catalogue points to the overlooked importance of chromosomal genes (with respect to the situation observed in the plant pathogen R. solanacearum – and very likely in R. eutropha [90]), and within the context of this review, the term ‘chromosomal’ actually refers to the genetic support of genes that are carried in R. solanacearum by the chromosome and the 2.1-Mb megaplasmid [88]). Yet the importance of ‘ordinary’ plasmids in R. metallidurans should not be underestimated, for at least three reasons:

- pMOL28 and mainly pMOL30 have a uniquely high amount of metal resistance genes in comparison to all currently known plasmids from other bacteria
- they are remarkable for the composition of metal resistance gene clusters: those contain equivalents of chromosomal operons flanked by additional genes
- finally, R. metallidurans plasmids carry various genes for which no real equivalents are known in other genomes, such as is copK (Table 2), cnrY and some other genes from the pbr, cop, cnr and czc plasmid-borne loci.

The present catalogue of genes points mainly to cadmium, cobalt, zinc, copper, and chromate at the plasmid and chromosomal levels, to mercury, nickel, and lead at the plasmid level, and to silver – at least at the chromosomal
level. As far as other metals are concerned, the presence of some plasmids mediates an increased tolerance to thallium but this metal does not seem to induce plasmid-borne genes. Yet, chromosomal lux-based gene fusions that are specifically induced by thallium have been reported in *R. metallidurans* CH34 and AB2 (P. Corbisier and A. Bossus, personal communication).

As this inventory mainly relies on the comparison of already known genes or mechanisms either in *Ralstonia* genomes or in other genomes, genuine novel genes or functions may be easily overlooked. Proteomics is of invaluable help in this respect: it helps to assign functions to putative genes and it helps to detect new proteins, functions and metal signatures or domains. Consequently, the future of such catalogues lies in the combination of proteomics and functional genomics. It would give a non-empirical basis for the further development of biotechnological tools (including protein engineering by site-directed mutagenesis, etc.) to detect, concentrate, process or remove heavy metals, including the heavy radionuclides, from waste storage or disposal sites.

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