Chapter 3
Response of *Cupriavidus metallidurans* CH34 to Metals

Pieter Monsieurs, Jon Hobman, Guy Vandenbussche, Max Mergeay and Rob Van Houdt

Abstract *Cupriavidus metallidurans* CH34 displays resistance to a plethora of metals. Its response and underlying genetic determinants are dissected and detailed metal by metal (from arsenic to zinc). An important role for its megaplasmids pMOL28 and pMOL30 is shown, with high level resistance to cadmium, chromate, cobalt, copper, mercury, nickel, lead and zinc mediated by well-known genes for detoxification that are often accompanied by other functions linked to acute or chronic stress. Nevertheless, metal resistance determinants are also found on the chromid (e.g. to chromate, copper and zinc) as well as on a large genomic island integrated in the chromosome (e.g. to cadmium, lead and mercury). Even the core genome participates in certain responses such as to gold or selenium. Next, we summarized the environmental applications, which were developed based on the knowledge gained by studying these different determinants, and in particular biosensors and soil and water bioremediation. Finally, the general transcriptional response of *C. metallidurans* to sixteen different metals supplied at different concentrations (including acute stress) is discussed within the framework of its intricate regulatory network.

Keywords *Cupriavidus metallidurans* · Metal resistance · Genetic determinants · Transcriptional regulation · Biotechnology

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3.1 From a to Z: Arsenic to Zinc

The response of *Cupriavidus metallidurans* to a variety of metals has been examined in different ways and from various perspectives that do not necessarily overlap: such as the association of resistance with mobile genetic elements (MGEs), the possible applications to environmental bioremediation, and gene and protein expression levels. The following section describes, metal by metal, the response of *C. metallidurans* to each of them, which helps to put in perspective interesting biological features. However, it should not be forgotten that various gene clusters are addressing responses to more than one metal (e.g. the *cnr* genes respond to cobalt and nickel and the *czc* genes to zinc, cadmium and cobalt). Rules that may govern microbial cation homeostasis from outside the bacterial cell have been formerly provided and shape the physiological and functional landscape of the present description (Nies 2007).

3.1.1 Arsenic: A Chromosome-Bound Gene Cluster

Eight genes (Rmet_0327-Rmet_0334) are putatively involved in resistance to arsenic. This *arsERIC$_2$BC$_1$HP* cluster appears to be only conserved in *C. metallidurans* and codes for the S-adenosyl-methionine (SAM)-dependent methyltransferase ArsE, the As^{3+}-responsive transcriptional regulator ArsR (Zhang et al. 2009), the lactoylglutathione lyase (glyoxalase family) protein ArsI, the glutaredoxin-coupled arsenate reductase $ArsC_1$ and thioredoxin-coupled arsenate reductase $ArsC_2$, the arsenite efflux pump ArsB, the NADPH-dependent FMN reductase ArsH, and the Major Facilitator Superfamily (MFS) permease ArsP. Several individual *ars* gene homologues are found on the chromosome and the chromid such as a putative arsenate reductase (Rmet_1421), a putative arsenite efflux pump (Rmet_3991) and five *arsR*-like regulators (see Sect. 3.3.1.1). However, their participation in arsenic resistance has not been studied (Zhang et al. 2009). The complete *arsERIC$_2$BC$_1$HP* cluster is induced by As^{3+} and As^{5+}, and to a lesser extent by Pb^{2+}, Zn^{2+}, Se^{4+}, Co^{2+} and Cd^{2+} (Monsieurs et al. 2011). Dissociation of the ArsR-promoter/operator region complex was observed in vitro for artificially high concentrations of As^{3+}, Bi^{2+}, Co^{2+}, Cu^{2+} and Ni^{2+} (1 mM), and Cd^{2+}, Pb^{2+} and Zn^{2+} (10 mM) (Zhang et al. 2009) (see Sect. 3.3.1.1). The SAM-dependent methyltransferase ArsE neither contains the characteristics nor the conserved amino acid residues of bacterial and eukaryotic As^{3+} SAM-methyltransferases (Ajees et al. 2012) that can detoxify via volatile arsines (Qin et al. 2006; Yuan et al. 2008). Thus, it appears that *C. metallidurans* CH34 mainly processes toxic levels of As^{3+} and As^{5+}, via initial reduction to As^{3+}, by efflux.

3.1.2 Cadmium, A Combination of Various Efflux Systems

Cadmium was one of the first metals for which plasmid-borne resistance was found in *C. metallidurans* CH34 (Mergeay et al. 1978b). Resistance was associated with the megaplasmid pMOL30, which increased the minimal inhibitory concentration (MIC) of $CdCl_2$ fourfold compared to the plasmid-less derivative AE104 (Mergeay et al. 1985). The resistance genes are located in a 25-gene cluster, part of the genomic island CMGI-30a, that is highly conserved in all *C. metallidurans* strains (Van Houdt et al. 2012) and other strains like *R. pickettii* 12J (see Chap. 2). This cluster encodes three different efflux mechanisms comprising the cation diffusion facilitator (CDF) CzcD (Nies 1992) and the P_{IB4}-type ATPase CzcP (Scherer and Nies 2009), which transport ions from the cytoplasm to the periplasm, and the HME-RND (heavy metal Resistance-Nodulation-Division)-driven system CzcCBA, which transports Cd^{2+} from the periplasm to the outside of the cell. Deletion of *czcB* or *czcA* resulted in the complete loss of efflux and in Cd^{2+}, Co^{2+} and Zn^{2+} sensitivity (Nies et al. 1987, 1989b; Nies 1995; Nies and Silver 1989). In contrast, deletion of the *czcC* gene resulted in a decreased cadmium and cobalt efflux and corresponding sensitive phenotype, while zinc efflux and resistance were unaffected. Next to these efflux systems, the gene cluster codes for the two-component regulatory system CzcRS, the regulatory protein CzcI (Diels et al. 1995a; Grosse et al. 1999), the periplasmic protein CzcE (Zoropogui et al. 2008; Grosse et al. 2004) (see volume II), the membrane-bound isoprenylcysteine carboxyl methyltransferase CzcN (Grosse et al. 1999), the MgtC-like ATPase CzcM and the CopQ-like protein CzcJ (see Sect. 3.1.5.5). These pMOL30-located determinants contribute to cadmium resistance together with the P_{IB2}-type ATPases ZntA (chromid), CadA (genomic island CMGI-1 on the chromosome) and PbrA (pMOL30), the chromosomally-encoded CDF proteins DmeF and FieF, and an uncharacterized pMOL30 gene product, thereby increasing the MIC of Cd^{2+} from 82 nM to 236 μM (Scherer and Nies 2009; Monchy et al. 2006b; Legatzki et al. 2003b). Unlike zinc resistance, the four $P_{IB2/4}$-type ATPases contribute more to cadmium resistance than CzcA. While CadA contributes the most, CzcP is not able to mediate much resistance when being the sole ATPase (Scherer and Nies 2009). However, CzcP has a much higher transport rate compared to the three P_{IB2}-type ATPases. Concerning the three CDF proteins, DmeF and FieF contribute little to cadmium resistance, while the contribution of CzcD is similar to that of CzcP, not being effective alone but enhancing the detoxification mediated by other systems (Scherer and Nies 2009). Therefore, both CzcP and CzcD could function as a rapid cadmium (and zinc) exporter before these metals are able to exert their toxic impact (Scherer and Nies 2009; Nies 2013). Finally, the difference in cadmium resistance between deletion of the above-mentioned determinants in AE128 (carrying only pMOL30) and AE104 (plasmid-less) points towards the involvement of an additional and yet uncharacterized pMOL30 gene product (Scherer and Nies 2009).

A higher MIC of cadmium was observed for *C. metallidurans* CH34 and NE12 compared to NA1, NA2 and NA4 (Mijnendonckx et al. 2013). Interestingly, the

latter three do not contain the *pbrR*$_2$ *cadA pbrC*$_2$ cluster located on the genomic island CMGI-1 (Van Houdt et al. 2012), which is consistent with the important role of CadA. In addition, in contrast to CH34, (at least) NA1, NA4 and NE12 carry a functional chromosomally-encoded HME-RND system ($CzcC_2B_2A_2$) that is highly similar to the pMOL30-encoded CzcCBA (see Chap. 2), which is again consistent with a lesser role for CzcA (or CzcA-like proteins) in cadmium resistance. However, varying global genomic and genetic contexts can affect the interaction patterns and phenotypic compensations between the involved proteins, which can be further studied using these or other *C. metallidurans* strains (see Chap. 2).

Finally, studying the effect of cadmium on *C. metallidurans* has also revealed unexpected properties of its staphyloferrin-B siderophore (formerly alcaligin E) encoded by the chromosomal Rmet_1109-Rmet_1118 genes (Gilis et al. 1996; Munzinger et al. 1999). Gilis et al. (1998) observed that the growth of a staphyloferrin B-deficient (*aleB*) CH34 derivative in the presence of cadmium was markedly stimulated by addition of staphyloferrin-B. Furthermore, staphyloferrin-B was shown to interact with cadmium, thereby decreasing its bioavailability and toxicity. The latter suggests that staphyloferrin-B, besides its function in supplying iron to the cell, may provide protection against heavy metal toxicity (Gilis et al. 1996, 1998; also reviewed in Schalk et al. 2011).

3.1.3 Chromate: More Genes Than Previously Expected

Genes involved in chromate resistance are located on plasmid pMOL28 (more precisely CMGI-28a, see Chap. 2) and on the chromid. The *chrIA*$_1$*B*$_1$*CEF*$_1$ (Rmet_6204-Rmet_6199) cluster on pMOL28 codes for the regulatory proteins ChrI and $ChrF_1$, the chromate efflux pump $ChrA_1$, the putative transcriptional activator $ChrB_2$, the Fe-containing superoxide dismutase-like protein ChrC, which appears to have limited activity (Roux and Coves 2002; Juhnke et al. 2002), and ChrE with sequence similarity to members of the rhodanese superfamily (cleavage of chromium-glutathione complexes) (Juhnke et al. 2002). The cluster on the chromid also codes for the chromate efflux pump $ChrA_2$ (Rmet_3865), the transcriptional repressor $ChrF_2$ (Rmet_3864) and the putative transcriptional activator $ChrB_2$ (Rmet_3866) (Juhnke et al. 2002). Both $ChrA_1$ and $ChrA_2$ belong to the CHR2 subgroup (Diaz-Perez et al. 2007) of the CHR transporter family (first described by Nies et al. 1998).

It became apparent that the pMOL28 cluster contains an additional five genes. The extension of the pMOL28 chromate cluster was based on their strong induction by chromate (Monsieurs et al. 2011), their synteny in the genomes of other bacteria including *Burkholderia pseudomallei*, *Methylobacterium* sp. 4-46 and *Arthrobacter* sp. FB24, and the proteomic response and qRT-PCR analysis of *Arthrobacter* sp. FB24 to chromate (Henne et al. 2009a, b). Accordingly, these genes immediately downstream of the chromate-regulated operon (Rmet_6199 through Rmet_6204) were named *chrO* (Rmet_6198), *chrN* (Rmet_6197), *chrP* (Rmet_6196, encoding a

MFS permease with 10 transmembrane α-helical segments), *chrL* (Rmet_6195, encoding a putative membrane protein), and *chrK* (Rmet_6194, with a WD40/YVTN repeat-like-containing domain) (Henne et al. 2009a).

As a structural analogue of sulphate, chromate enters the cell through sulphate uptake systems (Ohtake et al. 1987) and decreased uptake because of repressed sulphate uptake systems largely contributes to chromate resistance (Juhnke et al. 2002; Nies et al. 1989a). The induction by chromate of genes involved in sulphate and sulphite metabolism (*cysGNDHUIVB*; Rmet_2811-Rmet_2815), in cysteine biosynthesis and (thio)sulphate transport (*cysTWAB*; Rmet_1376-Rmet_1379), and in the formation of the thio-ester binding in acetyl coenzyme A synthesis (Rmet_0607-Rmet_0611) also illustrated the tight relationship between the chromate response and the sulphur metabolism (Monsieurs et al. 2011).

3.1.4 Cobalt: A Substrate of Zinc and Nickel Efflux Systems

Resistance to cobalt was already detected during the early characterization of CH34 (Mergeay et al. 1978a, 1985) and was attributed together with nickel resistance to the *cnr* determinant on pMOL28 (Grass et al. 2000; Liesegang et al. 1993; Siddiqui et al. 1989; Tibazarwa et al. 2000) and together with cadmium and zinc to the *czc* determinant on pMOL30 (Nies et al. 1987; Nies and Silver 1989; Monchy et al. 2007). Resistance to cobalt is also mediated by the *ncc* determinant in other strains such as *C. metallidurans* 31A [carried by plasmid pTOM8 and pTOM9 (Schmidt and Schlegel 1994; Schmidt et al. 1991)], *C. metallidurans* KT02 and strains isolated from New-Caledonia (Schmidt et al. 1991; Stoppel and Schlegel 1995). These genes are very similar to their *cnr* counterpart (see Sect. 3.1.9) but confer a much higher level of resistance to nickel and cobalt. The cluster also contains *nccN*, orthologous to *czcN*, which codes for a putative protein-S-isoprenylcysteine O-methyltransferase possibly involved in C-terminal protein amino acid methylation.

Both HME-RND-driven efflux systems (CzcCBA and CnrCBA) depend on the CDF protein DmeF, which is the most important factor for cobalt resistance (Scherer and Nies 2009; Nies 2013; Munkelt et al. 2004). In contrast to the three P_{IB2}-type ATPases (ZntA, CadA, PbrA), the P_{IB4}-type ATPase CzcP is able to transport cobalt. The contribution of CzcP is similar to that of the CDF protein CzcD, not being effective alone but enhancing the detoxification mediated by other systems (Scherer and Nies 2009; Nies 2013).

In addition, deletion of *atmA* (Rmet_0391) coding for an ATP-binding cassette transporter decreased resistance to cobalt and nickel. AtmA is homologous to Atm1p in the yeast *Saccharomyces cerevisiae*, which is involved in the transport of iron-sulphur cluster-containing proteins. Since AtmA does not transport cobalt or nickel, it is likely to be involved in the transport of compounds required to repair the damage done by periplasmic cobalt or nickel ions (Mikolay and Nies 2009).

3.1.5 Copper: A Large Gene Cluster on pMOL30

3.1.5.1 The *C. metallidurans* CH34 Genomic Landscape of Copper Resistance and Homeostasis Genes

Plasmid-encoded copper resistance was rapidly detected in *C. metallidurans* CH34 (Mergeay et al. 1978b). However, the difference in the MIC between the parental strain and its plasmid-less derivative AE104 is only a factor of 2 to 3, despite the large number of pMOL30 genes that are induced by copper (Monchy et al. 2007, 2006a). However, genome sequencing and additional physiological studies have shown that counterparts of these pMOL30 genes are also present on the chromosome and on the chromid.

3.1.5.2 The Structure of the pMOL30 *cop* Resistance Gene Cluster

The pMOL30 genomic island CMGI-30b contains a 33-gene region that is almost completely induced by Cu^{2+} (Fig. 3.1; Tables 3.1 and 3.2) (Monchy et al. 2006a, 2007; Monsieurs et al. 2011; Mergeay et al. 2009). Within this region, the *cop* gene cluster is unique in respect to its complexity compared to other copper resistance mechanisms described so far. The 21 *cop* genes (Table 3.1), starting with *copV* (Rmet_6105) and ending with *copW* (Rmet_6124), are flanked by two partially deleted genes coding for tyrosine-based site-specific recombinases (Rmet_6103 and *resU*) that may have joined two small genomic islands with components identifiable in other MGEs (Van Houdt et al. 2009, 2013; Ryan et al. 2009). Next to *resU* (Rmet_6125), four copper-induced genes are apparently not directly involved in copper transport or processing but in membrane-related functions with $gtrM_2$ (Rmet_6130) and $gtrB_2$ (Rmet_6128) encoding for a glycosyl transferase, $gtrA_2$ (Rmet_6129) for a bactoprenol-linked glucose translocase, and $ompP_2$ (Rmet_6127) for a porin predicted to form a channel for the diffusion of small hydrophilic molecules (Table 3.2). Orthologs of these four genes, which are located on the genomic island CMGI-30a carrying the *czc* resistance genes (see Chap. 2), are also 100 % conserved in *R. pickettii* 12J as well as in other *C. metallidurans* strains. It could be hypothesized that the *gtrMAB* genes are involved in the

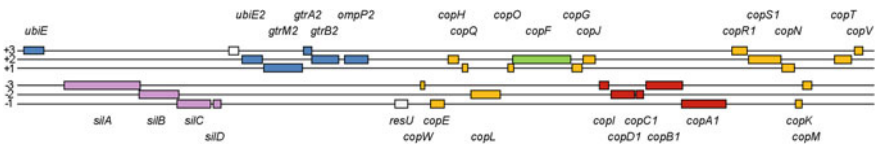

Fig. 3.1 Genomic context of the *cop* cluster on pMOL30 of *C. metallidurans* CH34. Genes coding for the efflux P_I-type ATPase CopF (*green*), the HME-RND-driven efflux system SilCBA (*purple*), the periplasmic detoxification system CopABCDI (*red*), the accessory proteins (*yellow*) and membrane-related functions (*blue*) are shown

Table 3.1 Characteristics of the proteins encoded by the *cop* gene cluster of *C. metallidurans* CH34

Locus tag	Name	AA[a]	Putative function	Localization
Rmet_6105	*copV*	117	Unknown	Cytoplasm
Rmet_6106	*copT*	254[b]	Cytochrome oxidase	Periplasm
Rmet_6107	*copM*	136[b]	Unknown	Periplasm
Rmet_6108	*copK*	94[b]	Effective sequestration Cu^{+} and Cu^{2+}	Periplasm
Rmet_6109	*copN*	164	Unknown	Cytoplasm
Rmet_6110	$copS_1$	463[b]	Histidine kinase	Inner membrane
Rmet_6111	$copR_1$	228	Response regulator	Cytoplasm
Rmet_6112	$copA_1$	614[b]	Multicopper oxidase	Periplasm
Rmet_6113	$copB_1$	495[b]	Cu^{+} storage	Outer membrane
Rmet_6114	$copC_1$	132[b]	Interacts with CopD transporter	Periplasm
Rmet_6115	$copD_1$	305[b]	Transporter (import)	Inner membrane
Rmet_6116	*copI*	158[b]	Copper oxidase	Periplasm
Rmet_6117	*copJ*	174	Participation to the metallic thiol pool	Cytoplasm
Rmet_6118	*copG*	137	Participation to the metallic thiol pool	Cytoplasm
Rmet_6119	*copF*	805[b]	Copper efflux P-ATPase	Inner membrane
Rmet_6382	*copO*	63	Chaperone of CopF	Cytoplasm
Rmet_6120	*copL*	421	Participation to the metallic thiol pool	Cytoplasm
Rmet_6121	*copQ*	78[b]	Highly induced by heavy metals, part of a family of small proteins	Periplasm
Rmet_6122	*copH*	153[b]	Putatively involved in a late stage of the copper response	Periplasm
Rmet_6123	*copE*	211	Unknown but required for maximal expression of copper resistance	Cytoplasm
Rmet_6124	*copW*	69[b]	Unknown, related to CopQ	Periplasm

[a]AA: protein size (number of amino acids)
[b]Size of unprocessed protein, i.e. signal peptide containing form

membrane repair process elicited by metallic stress and that the $OmpP_2$ porin assists the transport of extruded metals to the exterior of the cell. As far as $gtrM_2$ and $gtrB_2$ are concerned, we note that an *Acidiphilium* gene also encoding a glycosyl transferase conferred increased nickel resistance when transferred to an *Escherichia coli* recipient (San Martin-Uriz et al. 2014). This observation suggested the possible relevance of these functions for metal resistance phenotypes. The *silDCBA* (Rmet_6133-6136) cluster, which is mainly induced by Ag^{+} but also by Cu^{2+} (at least *silA* and *silC*), and *ubiE* (Rmet_6137) complete the region of interest for the resistance/response to toxic amounts of copper (Fig. 3.1; Table 3.2).

Table 3.2 Characteristics of the proteins encoded by the copper-induced genes adjacent to the pMOL30 *cop* genes of *C. metallidurans* CH34

Locus tag	Name	AA[a]	Putative function	Localization
Rmet_6127	$ompP_2$	355[b]	Porin predicted to form a channel for the diffusion of small hydrophilic molecules	Outer membrane
Rmet_6128[c]	$gtrB_2$	367	Glycosyltransferase	Inner membrane
Rmet_6129	$gtrA_2$	127	Bactoprenol-linked glucose translocase	Inner membrane
Rmet_6130	$gtrM_2$	541	Glycosyltransferase	Inner membrane
Rmet_6131	$ubiE_2$	289	Methyltransferase	Cytoplasm
Rmet_6132		131	Conserved hypothetical protein	Unknown
Rmet_6133	*silD*	131	Conserved hypothetical protein	Inner membrane
Rmet_6134	*silC*	435[b]	Outer membrane protein of the SilCBA HME-RND efflux system	Outer membrane
Rmet_6135	*silB*	521[b]	Membrane fusion protein of the SilCBA HME-RND efflux system	Periplasm
Rmet_6136	*silA*	1056[b]	Efflux pump of the SilCBA HME-RND efflux system	Inner membrane
Rmet_6137	*ubiE*	290	Methyltransferase	Cytoplasm

[a]AA: protein size (number of amino acids)
[b]Size of unprocessed protein, i.e. signal peptide containing form
[c]Not induced by Cu^{2+}

3.1.5.3 The Three Major Copper Detoxification Systems

The whole pMOL30 copper-induced region codes for three well described copper detoxification systems. The P_{IB1}-type ATPase CopF belongs to the copper efflux subdivision of the P-ATPases (Monchy et al. 2006b; Mergeay et al. 2003) involved in cytoplasmic detoxification. It is assisted by the HME-RND-driven efflux system SilDCBA (Cus-like) (see Sect. 3.1.11) and by the periplasmic copper detoxification system CopABCD. This combination of three different mechanisms of copper resistance is quite frequently observed in a variety of bacteria and linked to mobile genetic elements (Hobman and Crossman 2015). Each system has an additional non-plasmidic counterpart involved in copper detoxification: CupA (*cupRAC;* Rmet_3523-Rmet_3525; chromosome) codes for a P_{IB1}-type ATPase (Wiesemann et al. 2013), *cusDCBAF* (Rmet_5030-Rmet_5034; chromid) encodes a HME-RND-driven efflux system (Auquier 2006; Nies et al. 2006) and $copS_2R_2A_2B_2C_2D_2$ (Rmet_5673-Rmet_5668; chromid) is involved in periplasmic detoxification (Monsieurs et al. 2011).

In order to avoid any confusion over gene or phenotypic designations, Table 3.3 summarizes the closest homologues of the three *C. metallidurans* CH34

Table 3.3 Homologues of *C. metallidurans* CH34 copper resistance proteins

Function	*C. metallidurans*	*E. coli*	*P. syringae*	*E. hirae*
Periplasmic detoxification	$CopA_1B_1C_1D_1$/ $CopA_2B_2C_2D_2$	PcoABCD	CopABCD	
Multicopper oxidase	$CopA_1$/$CopA_2$	PcoA (56 %/56 %) CueO (40 %/38 %)	CopA (55 %/56 %)	
Efflux P_{IB1}-type ATPase	CupA/CopF	CopA (41 %/44 %)		CopA (46 %/41 %) CopB (56 %/57 %)
HME-RND-driven efflux	CusCBA/SilCBA	CusCBA (65 %/66 %)[a]		

The protein sequence similarity is given in parentheses, [a]based on efflux pump (A protein)

detoxification systems in *E. coli*, *Pseudomonas syringae* or *Enterococcus hirae* (i.e. bacteria in which copper homeostasis was extensively studied).

The *C. metallidurans copABCD* genes are homologous to the equivalent *E. coli pco* and *P. syringae cop* genes. The *E. coli pcoABCDRSE* resistance is regulated by the two-component regulatory system PcoRS and contains an additional gene, *pcoE*, encoding a periplasmic copper sponge protein (Zimmermann et al. 2012). The mechanism for *pco* copper resistance is not fully elucidated. Phenotypically, *pco* carrying *E. coli* strains change colour to a dark green/khaki in the presence of excess copper, and there is believed to be a role for pigments/catechol-siderophores in deposition of copper pigments in the cell. The current model for *pco* resistance is that PcoA oxidizes Cu^+ bound to PcoC in the periplasm to less toxic Cu^{2+}. PcoB is predicted to be an outer membrane protein, which is thought to interact with PcoA/C to export copper or to sequestrate oxidized catechol siderophores (Djoko et al. 2008; Rensing and Grass 2003). The role of PcoD is believed to be either to import copper into the cytoplasm, or that PcoC chaperones copper to PcoD which loads it on to periplasmic PcoA which exports it to the periplasm via the twin-arginine translocation pathway (Djoko et al. 2008; Rensing and Grass 2003). Some bacteria may only have the *pcoAB* or *pcoCD* genes. For instance, *Caulobacter crescentus* only uses PcoAB to detoxify copper and deletion results in a 50-fold increased sensitivity (Emeline Lawarée and Jean-Yves Matroule, pers. comm.). There are increasing reports of *pco* and *sil* genes being found together within genomic islands, integrative and conjugative elements, and plasmids [e.g. on the IncH plasmid R478, originally isolated from *Serratia marcescens* (Gilmour et al. 2004), in the chromosome of enterotoxigenic *E. coli* strain H10407 (Crossman et al. 2010) and in *Klebsiella pneumoniae* multiresistance plasmid pUUH239.2 (Sandegren et al. 2012)] (reviewed in Hobman and Crossman 2015).

As mentioned above, the pMOL30-encoded *copSRABCD* genes have counterparts on the chromid, however, there is a major difference in protein sequence between the pMOL30- and chromid-encoded outer membrane protein CopB. The

Fig. 3.2 The CopB motif generated via WebLogo (Crooks et al. 2004) using 434 hits in 119 UniProtKB/TrEMBL sequences. The height of the motif logo represents the bits score, which corresponds to the degree of conservation

pMOL30-encoded CopB contains a 22-fold repeated motif (Fig. 3.2) totalling 44 methionine residues (while the chromid-encoded one only contains 6 methionine residues). This basic motif is observed in other CopB proteins[1] although the number of repeated motifs varies (Fig. 3.3). The whole region ends with a M-[KQ]-M-[KQ] motif and is delimited by (G)-G-S residues especially in the *Cupriavidus* and *Ralstonia* genera. Since it is known that methionine residues are important in binding monovalent copper ions, the observed difference between both CopB proteins may indicate a specialization of the pMOL30-encoded one in the detoxification or neutralization of very high Cu^{+} concentrations.

3.1.5.4 CopK and CopH

Next to the well-identifiable and documented orthologues of *copSRABCD* and *copF*, most of the other *cop* genes, which were first detected in strain CH34 and later in other *C. metallidurans* genomes, remain to be fully deciphered (Table 3.1). They are often absent in most of the conserved synteny blocks involving *cop* genes (and especially those encoding for the three major mechanisms of copper detoxification). It may be hypothesized that such accessory genes are mainly involved in chronic and intense copper stress. Molecular data are currently only available for CopK and CopH.

CopK has been the most intensively studied up to now (Sarret et al. 2010; Chong et al. 2009; Bersch et al. 2008; Monchy et al. 2006a; Tricot et al. 2005). This periplasmic protein, which is one of the most abundant proteins in the periplasmic space during copper challenge (Ruddy Wattiez, pers. comm.), contains seven

[1]ScanProsite (de Castro et al. 2006) detected 119 protein sequences with at least 2 hits for the {M-Q-G-M-D} motif on all UniProtKB/TrEMBL (release 2014_05 of 14-May-2014: 56010222 entries) database sequences.

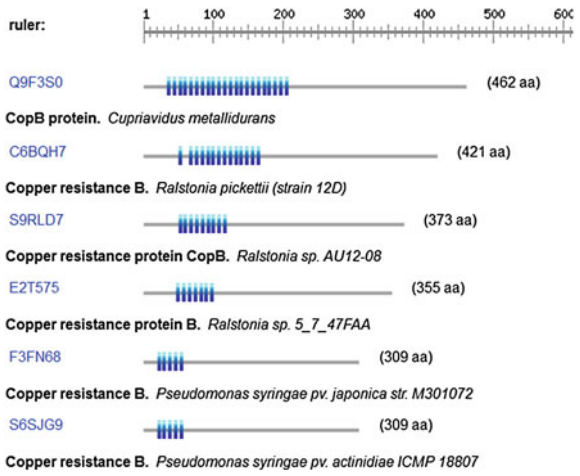

Fig. 3.3 Some examples of CopB proteins with a varying number of motifs (*blue bar*)

methionine residues, five of which appear to be highly conserved, and a tetrathioether-Cu^{+} site (Sarret et al. 2010; Bersch et al. 2008). It is the first protein for which a cooperative binding of Cu^{+} and Cu^{2+} has been observed (Ash et al. 2011; Chong et al. 2009). Binding of Cu^{2+} increased the Cu^{+} affinity of CopK by a factor of 100 and binding of Cu^{+} increased the Cu^{2+} affinity by a factor of at least 10^{6} (Chong et al. 2009). It could be hypothesized that in the presence of high copper levels, CopK strongly chelates the periplasmic copper fraction that could not be timely processed by the ATPase and the periplasmic detoxification system. Therefore, CopK could be seen as an additional copper detoxification system in *C. metallidurans* CH34, especially during chronic and intense metal stress where its sequestration power could prevent saturation of the other systems.

CopH is a paralogue of CzcE, which is involved in the regulation of the *czc* gene cluster (Petit-Haertlein et al. 2010; von Rozycki and Nies 2009; Zoropogui et al. 2008). This protein fixes both Cu^{+} and Cu^{2+} and seems to intervene at a later stage in detoxification because its maximum expression occurs later than for the other *cop* genes (Monchy et al. 2006a). It is a dimeric protein containing one metal-binding site per subunit. These sites have a high affinity for Cu^{2+} but can also bind Zn^{2+} and Ni^{2+}. CopH does not contain any cysteine or methionine residues but contains two histidine residues (Sendra et al. 2006).

3.1.5.5 Additional pMOL30 *cop* Genes: Evidence from Synteny and Gene Structure

As mentioned above, the function of most of the other *cop* genes remains to be fully deciphered (Table 3.1). However, additional hints can be provided by conserved synteny blocks observed during genome annotation. For instance, the *copG*, *copJ* and *copL* genes coding for cysteine-containing cytosolic proteins (CGCC motif in

CopG, ACAACH motif in CopJ and CNCC motif in CopL) (Monchy et al. 2006a) are generally closely linked to a *copF*-like gene coding for a P_{IB1}-type ATPase, suggesting that they could be collectively involved in the removal of Cu^+ from the cytosolic thiol pool (Chong et al. 2009). For instance, an orthologue of *copG*, which codes for a copper-binding protein with a thioredoxin-like fold, was identified to be transcriptionally coupled to a *copF*-like gene in *Vibrio cholerae* (Marrero et al. 2012; Bondarczuk and Piotrowska-Seget 2013). In addition, the *copO* gene is transcriptionally coupled to the *copF* gene and more generally to related genes encoding P-type ATPases of the Ag/Cu family. Most CopO proteins have a predicted length of 63–119 amino acids and contain a C-terminal histidine-rich motif beginning with a methionine residue, suggesting a role as copper chaperone for P-type ATPases. The *copK* gene, which is not frequently observed in conserved synteny blocks of copper resistance genes, is found in $ICE_{Tn4371}6067$ of *Delftia acidovorans* SPH-1 (Ryan et al. 2009; Van Houdt et al. 2009, 2013) and in conserved putative MGEs carrying the *copSRABFICDK* gene cluster found in *Stenotrophomonas maltophilia, Pseudomonas aeruginosa* and *Achromobacter piechaudii* strains. In addition, *copK* genes were also directly identified in some environments via metagenomic analysis (Jia et al. 2013).

Finally, the *copQ* gene belongs to a group of 19 homologous genes encoding for putative small (between 69 and 165 amino acids) stress responsive proteins. All have a distinctive signal peptide and are apparently only found in *Cupriavidus* and *Ralstonia* species (Janssen et al. 2010). Next to *copQ*, also *mmrQ* (*ncc* cluster pMOL30), *czcJ* (*czc* cluster pMOL30) and *mmmQ* (czc_2 cluster chromid) are part of a conserved synteny block together with metal resistance determinants. Ten of these genes were found to be highly induced in response to different heavy metals (Monsieurs et al. 2011) including *copQ* (Monchy et al. 2006a) and *czcJ* (Scherer and Nies 2009), and were even among the most induced ones, suggesting an involvement in acute stress response.

3.1.5.6 Insight in the Phenotype of *cop* Mutants

For 13 of the 21 pMOL30 *cop* genes (*copVTMKSRABDFGQE*), transposon insertion mutants are available in CH34 as well as in AE1744 (a plasmid-less derivative carrying the pMOL30 *cop* gene cluster on a broad host range cosmid) (van Aelst 2008; Monchy et al. 2006a). A slight difference in the MIC of copper between CH34 and AE1744 was observed, probably because of the additional resistance provided by other pMOL30 genes (e.g. the *silCBAD* genes). The transposon mutant library was phenotypically screened through viable counts (Mergeay 1995) in the presence of different copper concentrations (with or without pre-induction with a low copper concentration). In contrast to the MIC of metals, which is measured in liquid medium by scoring visible growth after a fixed incubation time, viable counts allow detection of more nuances in the response (Fig. 3.4). Different phenotypic variations were observed (Fig. 3.4). As expected,

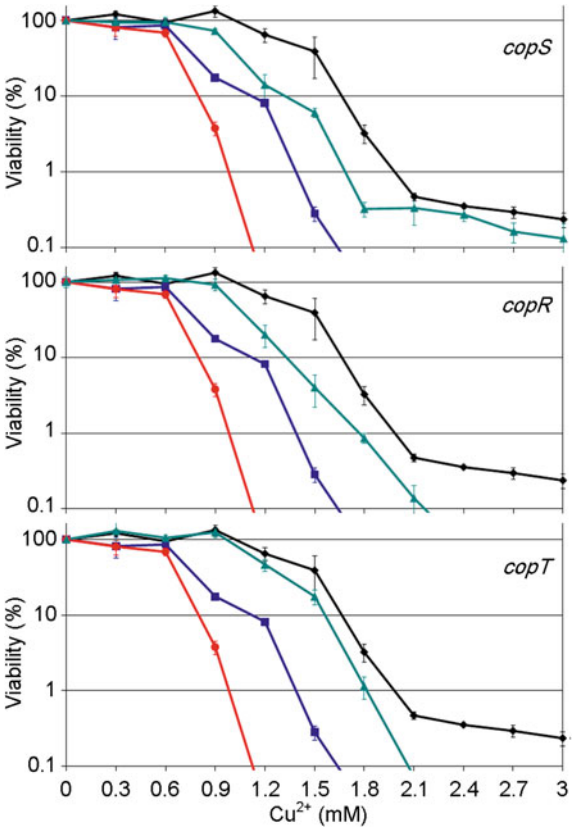

Fig. 3.4 Viability of *C. metallidurans* CH34 (*black diamond*) and its *copS, copR* or *copT* mutant (*green triangle*), AE1744 (*blue square*) and AE104 (*red circle*) on mineral Tris-buffered agar supplemented with different Cu^{2+} concentrations after pre-induction with 0.3 mM Cu^{2+}. Results are expressed as the percentage of viable counts compared with the counts on unsupplemented medium (van Aelst 2008)

cop mutants affected in the known resistance mechanisms (*copRSABCD* and *copF*) are less resistant than CH34 although still more resistant than the plasmid-free derivative AE104. However, at high copper concentrations (above the MIC for CH34), the percentage of survivors varies between different mutants. For instance, there were no CH34 *copR* mutant survivors unlike the *copS* mutant and the parental strain CH34 (Fig. 3.4) (van Aelst 2008). A similar phenotype was observed for the *copG*, *copT* and *copV* genes, which do not belong to one of the archetypical detoxification systems (Fig. 3.4) (van Aelst 2008). This phenotypic variability indicated distinct roles of particular *cop* genes in the response to copper ranging from defining the resistance level and accessory roles not directly affecting viability to surviving concentrations above the MIC. This arsenal may be advantageous to survive acute and chronic metal stress in poor environments while competing with other bacteria for the scarce nutrients.

3.1.6 Gold: An Example of Metal Reduction

C. metallidurans CH34 is able to precipitate gold by reduction of Au^{3+}-complexes to Au^{0}-particles. The process involves the formation of intermediate Au^{+}-S complexes, followed by a slow biochemically-driven reduction and intra- and extracellular deposition of metallic Au particles (Reith et al. 2009). Furthermore, bacterial biofilm communities on gold grains are dominated by *C. metallidurans* (Reith et al. 2006, 2010) and biomineralization of Au in *C. metallidurans* biofilms resulted in Au biominerals that are morphologically analogous to those observed on natural Au grains (Fairbrother et al. 2013).

Transcriptomic analysis indicated that exposure of CH34 to $AuCl_4^-$ strongly induced the *cup* region on the chromosome (*cupRAC*), the Rmet_4682-Rmet_4685 cluster on the chromid and the *cop* region on plasmid pMOL30 (*copVTMKNSRABCDIJGFOLQHEW*) (Reith et al. 2009). However, Wiesemann et al. (2013) demonstrated that plasmids pMOL28 and pMOL30 were neither required for gold resistance nor essential for the biomineralization of metallic gold particles. In addition, although the MerR-like regulator CupR, which acts as a transcriptional activator of the P_{IB1}-type ATPase CupA and the chaperone protein CupC, possesses a much lower affinity for Cu^+ than for Au^+ (Jian et al. 2009), the P_{IB1}-type ATPase CupA only affects copper and not gold resistance (Wiesemann et al. 2013). Furthermore, the *cup* cluster is only induced by $AuCl_4^-$ at concentrations above the MIC (Reith et al. 2009; Jian et al. 2009), while activation of this cluster by sublethal concentrations was only observed for Ag^+, As^{3+} and Pb^{2+} (Monsieurs et al. 2011). The Rmet_4682-Rmet_4685 cluster, which was named *dax* in Monsieurs et al. (2011) and renamed *gig* for "gold-induced genes" in Wiesemann et al. (2013), appeared not to be essential for gold resistance (Wiesemann et al. 2013). This cluster is controlled by the extracytoplasmic function (ECF) sigma factor RpoQ coded by the Rmet_4686-Rmet_4687 operon (Reith et al. 2009; Grosse et al. 2007) (with Rmet_4687 coding for a membrane protein of unknown function that could be a putative antisigma factor). The *rpoQ* gene was induced by $AuCl_4^-$ [50 and 100 μM but not 0.5 and 10 μM (Wiesemann et al. 2013; Monsieurs et al. 2011; Reith et al. 2009)] and Cu^{2+} [300 μM but not 50 μM (Grosse et al. 2007; Wiesemann et al. 2013)]. In addition, the expression of this Rmet_4682-Rmet_4685 cluster is exclusively upregulated by silver based on induction experiments using metal concentrations below the MIC (Monsieurs et al. 2011), while induction with gold only occurs after exposure to gold concentrations above the MIC. Finally, only deletion of the chromid-located $copA_2B_2C_2D_2$ determinant (see Sect. 3.1.5) led to a decrease in gold resistance (Wiesemann et al. 2013).

These results led Wiesemann et al. (2013) to conclude that up till now no gold uptake system has been identified (and putatively is not present) in *C. metallidurans*. Transportation into the cytoplasm is probably limited and reduction into gold nanoparticles occurs in the periplasm putatively by components of the respiratory chain.

3.1.7 Lead: An Interplay Between ATPase, Phosphatase and Peptidase

Lead resistance in *C. metallidurans* CH34 is mainly mediated by the pMOL30-encoded *pbrUTRABCD* (Rmet_5944-Rmet_5949) gene cluster. The *pbr* resistance operon is arranged into two divergently transcribed clusters, one coding for the MerR-family transcriptional regulator PbrR, the putative Pb^{2+} uptake protein PbrT belonging to the ILT (Iron Lead Transporter) family (Debut et al. 2006) and the MFS permease PbrU, the second codes for the P_{IB}-type ATPase PbrA, the undecaprenyl pyrophosphate phosphatase/lipoprotein signal peptidase PbrB/PbrC and the putative intracellular Pb-binding protein PbrD (Hynninen et al. 2009; Hobman et al. 2012; Taghavi et al. 2009; Borremans et al. 2001). The *pbrU* gene, downstream of *pbrT* (Taghavi et al. 2009; Monchy et al. 2007), is inactivated in CH34 by Tn*6049* insertion, but is intact in the other sequenced *C. metallidurans* strains (H1130, HMR-1, NA1, NA4 and NE12A2) (Li et al. 2013; Monsieurs et al. 2013, 2014) (see Chap. 2). The other gene flanking *pbrU*, designated *pbrV*, codes for a putative high affinity Fe^{2+}/Pb^{2+} permease. However, *pbrV* is only full-length in strain HMR-1, while partial in the other strains.

The PbrA ATPase is not specific for Pb^{2+} and also efficiently transports Zn^{2+} and Cd^{2+}. Furthermore, it can be complemented by the P_{IB}-type ATPases ZntA and CadA (Hynninen et al. 2009; Taghavi et al. 2009). The current model for lead resistance proposes that Pb^{2+} induces the expression of PbrABCD as well as ZntA and CadA, which are all able to export Pb^{2+} from the cytoplasm to the periplasm. Interaction with inorganic phosphate groups produced by PbrB-mediated dephoshorylation of undecaprenyl pyrophosphate leads to the formation of insoluble lead phosphate and prevents re-entry in the cytoplasm (Hynninen et al. 2009; Taghavi et al. 2009). PbrC appears not to be essential; however, other CH34 peptidases could putatively complement its function. One of the candidates is $pbrC_2$ located in the $pbrR_2$ *cadA* $pbrC_2$ cluster on CMGI-1 (see Chap. 2). Expression of $pbrC_2$ (and *cadA*) increased when *pbrA*, *pbrB* or *pbrD* were inactivated, probably in response to an increased intracellular Pb^{2+} concentration (Taghavi et al. 2009). Although, only very low levels of cross-regulation of the P_{pbrA} promoter by $PbrR_2$ (putatively regulating *cadA* and $pbrC_2$) and $PbrR_3$ were found (Julian et al. 2009), this indicates a synergistic interaction between both clusters and shows that acquisition of CMGI-1 improved the versatility of *C. metallidurans* CH34 towards metals (Taghavi et al. 2009).

3.1.8 Mercury: Tn*21* Family Transposons

Four mercury resistance operons are present in the genome of CH34. Two identical clusters form the accessory genes of transposons Tn*4378* and Tn*4380* (Rmet_6171-Rmet_6176) located on pMOL28 (in CMGI-28b) and pMOL30 (in

CMGI-30a), respectively (see Chap. 2). A third (partial) operon, *merRTP'*(Rmet_5990-Rmet_5992) is located near the *czc* cluster on pMOL30. The last cluster, *merRTPA* (Rmet_2312-Rmet_2315), is part of the chromosomally located CMGI-1 (Klockgether et al. 2007; Van Houdt et al. 2009; Dressler et al. 1991) (see Chap. 2).

In fact, *mer* genes conferring resistance to mercury are present in all *C. metallidurans* strains and are almost always associated with transposons or genomic islands (as is the case for many other bacteria) (Van Houdt et al. 2009, 2012; Monchy et al. 2007). It is noteworthy that the reported classification of *mer* genes on MGEs has mainly focused on clinical isolates, γ-proteobacteria and a few Firmicutes (Mindlin and Petrova 2013), while the group of mercury resistant MGEs in (metal-resistant) β-proteobacteria and environmental soil bacteria has had less attention (Mindlin and Petrova 2013).

The identical *merRTPADEurf-2* operon structures of Tn*4378* and Tn*4380* are closely related to that of Tn*501* from the *P. aeruginosa* plasmid pVS1. The cluster contains the regulatory genes *merR* and *merD*, four structural genes coding for the cytoplasmic mercuric reductase enzyme MerA, the transport system MerTP and the broad-spectrum mercury transporter MerE (Kiyono et al. 2009; Sone et al. 2013a; b), and *urf-2* coding for an unknown function. All structural proteins carry characteristic cysteine residues that mediate transport across the cytoplasmic membrane, delivery to the MerA active site and reduction (Liebert et al. 1999). All *mer* genes except *merR* are induced by Hg^{2+} and to a lesser extent by Cd^{2+}, Zn^{2+} and Pb^{2+} (Monchy et al. 2007; Monsieurs et al. 2011). In addition, the transposition modules are induced by Hg^{2+} (without an unequivocal discrimination between Tn*4738,* Tn*4380* and Tn*6050*) (Monchy et al. 2007; Monsieurs et al. 2011), which is consistent with the increased transposition frequency of mercury transposons in the presence of mercury (Sherratt et al. 1981; Kitts et al. 1982; Haritha et al. 2009). The *urf-2* gene is not observed outside of the *mer* gene cluster and has not been shown to be directly involved in resistance to mercury. It putatively codes for a signalling diguanylate phosphodiesterase with a typical EAL motif. Furthermore, the 3′ end of *urf-2* overlaps with the 5′ end of *tnpR* in the various Tn*4380* copies of sequenced *C. metallidurans* strains. In transposons of the Tn*21* lineage, a transposition-deficient integron, responsible for the acquisition and expression of antibiotic resistance genes, is inserted into the *urf-2* gene (Kholodii et al. 2003; Essa et al. 2003; Partridge et al. 2001; Liebert et al. 1999; Grinsted et al. 1990).

In strain CH34, the regulatory protein MerD (Champier et al. 2004), the transport proteins MerP (Serre et al. 2004) and MerT (Rossy et al. 2004b), and the MerP-like N-terminal extension of the mercuric reductase MerA (Rossy et al. 2004a) have been investigated. Analysis of MerD showed that although it co-regulates expression of the *mer* operon and shows a high N-terminal sequence similarity to the transcriptional regulator MerR, it does not directly bind the *mer* operator (Champier et al. 2004). Instead, MerD dissociates the Hg-MerR-*mer* operator complex allowing for the synthesis of new apo-MerR that can then bind to the *mer* operator (Silver and Hobman 2007; Champier et al. 2004). For MerP and the MerP-like N-terminal extension of MerA, it was shown that the metal-binding site consists of the highly conserved GMTCXXC sequence found in

metallochaperones and metal-transporting ATPases (Serre et al. 2004; Rossy et al. 2004a). Finally, Rossy et al. (2004b) showed that the cytoplasmic loop of MerT binds and specifically transfers Hg to MerA, in agreement with previous work on the Tn*501* mercury transport system (Morby et al. 1995). The paradigmatic regulatory protein MerR will be discussed in the regulatory section with other *C. metallidurans* regulatory proteins of the same family (Hobman 2007).

C. metallidurans strains harbour a diversity of structural *mer* genes in addition to those identified in strain CH34. For instance, *C. metallidurans* NE12 contains a *merRBTPCADEurf-2* cluster and a a *merRTPFADE* operon associated with a Tn*5053*-family transposon (Hobman et al. 1994) that is highly similar (96 % nucleotide similarity) to Tn*512* identified in *P. aeruginosa* AW54a (Petrovski and Stanisich 2010). In these variant *mer* operons, *merF*, *merE* and *merC* code for additional mercury transporters (Sone et al. 2013a; Wilson et al. 2000), and *merB* codes for an organomercurial lyase that catalyses the demethylation of a wide range of organomercurials (Parks et al. 2009; Melnick and Parkin 2007; Lafrance-Vanasse et al. 2009).

This diversity in *mer* clusters may reflect the specific history of the corresponding strains in various environments [e.g. in the case of *C. metallidurans* NE12 before being trapped in the air filter of the Kennedy Space Center Spacecraft Assembly and Encapsulation Facility II (Newcombe et al. 2008; Mijnendonckx et al. 2013)]. Some of the strains being more anthropized or more exposed to the industrial mercury cycle than others. In the environmental strain *R. pickettii* 12J, the *merTPCADB* cluster, part of a large chromosomal genomic island harbouring many metal resistance genes, contains a non-typical *merB* gene encoding for a putative alkyl-mercury lyase (27 % protein identity with MerB of pSB102 and 55 % protein identity with MerB of *Janthinobacterium* sp. Marseille).

All these observations confirm the presence of mobile genetic elements carrying mercury genes often together with other heavy metal resistance genes concentrated in chimeric genomic islands or plasmids. However, especially in the metallurgic sites where *C. metallidurans* strains were isolated, a selection pressure due to bioavailable mercuric ions is barely visible. Nevertheless, the very toxic mercury, which is much rarer than most of the toxic heavy metals, is subjected to many transformations in soil, water and air including (but not exclusively) strongly anthropized environments and nearby soils or sediments with a high content of other heavy metals (Barkay et al. 2003). The mercury cycle, which is also fed by volcanic (geothermal) releases (Boyd and Barkay 2012), is also evident from the fact that both living and dead mercury-resistant bacteria (including the plasmid-free derivative of *C. metallidurans* CH34) can re-oxidize metallic mercury released by the MerA reductase under anaerobic conditions (Colombo et al. 2014; Barkay et al. 2003).

3.1.9 Nickel: Specific Classes of HME-RND Genes

Nickel shares similar chemical and physical properties with cobalt, its neighbour in the periodic table. It is therefore not surprising to find bacterial resistance determinants that recognize and act upon both metal ions. As described previously in Sect. 3.1.4, this is the case for the inducible HME-RND-driven efflux system CnrCBA encoded by the pMOL28 operon *cnrYXHCBAT* (Rmet_6205-Rmet_6211) (Mergeay et al. 1985; Siddiqui and Schlegel 1987; Liesegang et al. 1993; Grass et al. 2000; Tibazarwa et al. 2000; Nies et al. 2006; Monsieurs et al. 2011). This determinant is mainly induced by Ni^{2+} and Co^{2+} but also by Cu^{2+} and Cd^{2+} (see Sect. 3.3.1.2) (Nies et al. 2006; Monsieurs et al. 2011). The *cnrT* gene, downstream of the *cnrCBA* genes, encodes a putative Ni^{2+} efflux transporter (Nies 2003, 2013) that belongs to the Drug/Metabolite Transporter (DMT) superfamily (see volume II). In addition, CnrT has a C-terminal domain containing 24 histidine residues that could contribute to metal ion binding. Upregulation of *cnrT* transcription was observed in the presence of Ni^{2+}, Cu^{2+} and Cd^{2+} (Monchy et al. 2007; Monsieurs et al. 2011) and the corresponding protein could induce a small degree of nickel resistance (Nies 2003).

The *cnrYXHCBAT* gene cluster on pMOL28 could originate from the acquisition of an *nccYXHCBAN*-like determinant, such as the one present on plasmid pTOM9 from *C. metallidurans* 31A, by horizontal transfer and duplication on the megaplasmid (von Rozycki and Nies 2009). While the *C. metallidurans* 31A *ncc* locus causes a high-level nickel and cobalt resistance, as well as cadmium resistance, when transferred to strain AE104 (Schmidt and Schlegel 1994), the *nccCBA* gene cluster (Rmet_6145-Rmet_6148) located on pMOL30 from *C. metallidurans* CH34 is transcriptionally silent, probably due to the lack of upstream *nccYXH* regulatory genes (Nies et al. 2006; Monsieurs et al. 2011). In addition, the presence of a frameshift in *nccB* would prevent the translation of a functional protein (Monchy et al. 2007). Expression at the protein level of a third HME-RND-driven system, the chromid-encoded NimBAC (Rmet_5682-Rmet_5677), is induced by Ni^{2+} and Co^{2+} (Auquier 2006) and even Cd^{2+} (Nies et al. 2006). However, this efflux system is not functional due to the presence of the insertion sequence element IS*Rme3* in the *nimA* gene (Janssen et al. 2010).

A second nickel resistance locus called *nre* was detected in plasmid pTOM9 from *C. metallidurans* 31A (previously designated *Achromobacter xylosoxidans* 31A) (Schmidt and Schlegel 1994; Grass et al. 2001). Among the four ORFs present in this locus, only the one encoding NreB is specifically induced by Ni^{2+} and necessary to confer low-level nickel resistance in *C. metallidurans* 31A (Grass et al. 2001). When transferred to *E. coli*, NreB reduces Ni^{2+} uptake suggesting that the protein mediates metal ion efflux. The NreB permease is a member of the MFS permeases and possesses a histidine-rich carboxy terminus contributing to maximal nickel resistance (Grass et al. 2001). NreB and its closest homologue NrsD from *Synechocystis* sp. strain PCC 6803 (Garcia-Dominguez et al. 2000) are the only known MFS proteins involved in metal ion transport. In *C. metallidurans* CH34, an

NreB orthologue encoded by Rmet_6144 was identified close to the *ncc* locus in pMOL30. This protein does not possess the characteristic histidine-rich C-terminal domain and no induction or role in nickel resistance has been demonstrated so far (Monsieurs et al. 2011).

As mentioned previously in Sect. 3.1.4, the CDF protein DmeF and the ABC-transporter AtmA are also involved in nickel resistance in *C. metallidurans* CH34 (Munkelt et al. 2004; Mikolay and Nies 2009). AtmA is most likely not an efflux system for cytoplasmic Ni^{2+} and Co^{2+} and could be involved in the transport of compounds required to repair the damage done by periplasmic cobalt or nickel ions (Mikolay and Nies 2009).

3.1.10 *Selenium: Developments About the Chromosomal* dedA *Gene*

In most environments, selenium, an essential oligo-element, predominantly occurs in its oxidized forms, selenate (SeO_4^{2-}, Se^{6+}) and selenite (SeO_3^{2-}, Se^{4+}). These bioavailable oxyanions are soluble and, at elevated concentrations, toxic and mutagenic. Selenite directly promotes oxidative stress through reaction with cellular reduced thiol-containing compounds such as glutathione (Roux et al. 2001). In *C. metallidurans* CH34, the resistance/response to selenium anions (selenite and selenite) is not associated with plasmid-borne functions.

C. metallidurans CH34 is able to resist up to 6 mM selenite and to reduce selenite to elemental red selenium, which accumulates in the cytoplasm as judged from electron microscopy and energy-dispersive X-ray analysis (Roux et al. 2001). Such cytoplasmic storage of an insoluble and non-toxic form of selenium is of interest for bioremediation (Roux et al. 2001; Saiki and Lowe 1987).

In fact, exposure of *C. metallidurans* CH34 cells to selenite induced an adaptation phase followed by a slow and then strongly increased uptake. The first process is balanced by slow assimilation into seleno-L-methionine and slow detoxification to elemental Se, after which selenite transport and reduction are activated (Avoscan et al. 2006). In contrast, exposure to selenate did not induce this adaptation phase and both selenite (transiently) and elemental Se occurred as minor species while organic selenide was the major form accumulated. Therefore, selenate mostly follows an assimilatory pathway and the reduction pathway is not activated upon selenate exposure (Sarret et al. 2005). Consequently, CH34 was not defined as being a good candidate for bioremediation of selenate-contaminated environments (Avoscan et al. 2009).

Ledgham et al. (2005) performed random Tn*5* transposon mutagenesis to isolate *C. metallidurans* CH34 mutants able to resist up to 15 mM selenite. All selenite-resistant mutants contained a Tn*5* insertion into a single chromosomal gene identified as *dedA* (Rmet_2907). The DedA protein family is a highly conserved and ancient family of membrane proteins with representatives in most sequenced

bacterial, archaeal and eukaryotic genomes (Doerrler et al. 2013). Although the functions of the DedA family proteins remain to be fully elucidated, certain bacterial DedA family members have a role in membrane homeostasis and appear to be required to maintain the proton motive force. Mutation of *dedA* affects cell division, temperature sensitivity, membrane lipid composition, envelope-stress response and proton motive force (Doerrler et al. 2013). Furthermore, the *dedA*-family genes are essential in *E. coli* (Boughner and Doerrler 2012), which points towards the presence of other DedA homologues in *C. metallidurans* CH34 (with Rmet_5938 being a possible target). According to Doerrler et al. (2013), a role of DedA in the uptake of selenite is consistent with the occurrence of DedA domains in secondary transporters of the tripartite ATP-independent periplasmic transporter (TRAP-T) family and in transporters displaying a proton symporter or antiporter activity.

3.1.11 Silver: Much More Than a Substrate for Copper-Responsive Genes

C. metallidurans CH34 carries a number of systems that are putatively involved in silver detoxification based on their homologies to known silver resistance systems. The *silDCBA* (Rmet_6133-Rmet_6139) and *cusDCBAF* (Rmet_5030-Rmet_5034) operons, which code for HME-RND-driven efflux systems, are located on pMOL30 (CMGI-30b) and the chromid, respectively (Mergeay et al. 2009; Janssen et al. 2010). The *cupRAC* (Rmet_3523-Rmet_3525) operon codes for a P_{IB1}-type ATPase and is located on the chromosome (Janssen et al. 2010). Expression of all three operons was induced after 30 min exposure to 0.25 μM Ag^+ (Monsieurs et al. 2011). Expression of the Sil and Cus proteins was also induced in *C. metallidurans* after growth in the presence of 1 μM $AgNO_3$ (Auquier 2006; Mergeay et al. 2003) and heterologous expression of SilCBA in *E. coli* GR17 increased the silver resistance of the strain 2.4-fold (Ngonlong Ekendé 2012). Furthermore, the C-terminal domain of SilB is able to bind Ag^+ (Bersch et al. 2011). In addition, eight genes from the pMOL30 *cop* cluster were transcriptionally induced by Ag^+ (Monsieurs et al. 2011; Monchy et al. 2007), including *copK* for which its product binds Ag^+ (Chong et al. 2009), *copC* and *copD* from the periplasmic detoxification system CopABCDI, the *copOFGJ* cluster with *copF* coding for a P_{IB1}-type ATPase, and *copH* coding for a paralogue of CzcE (see also volume II). Deletion of *cupA* as well as *copF* (to a lesser extent) decreased silver resistance, with a double mutant being the most sensitive (Wiesemann et al. 2013). Finally, the expression of the Rmet_4682-Rmet_4685 cluster, which was named *dax* in Monsieurs et al. (2011) and renamed *gig* for "gold-induced genes" in Wiesemann et al. (2013) (see Sect. 3.1.6), is exclusively upregulated by silver based on induction experiments using metal concentrations below the MIC (Monsieurs et al. 2011).

Proteomic data also revealed induction of the chromosomally-encoded AgrR after growth of CH34 in the presence of 1 μM $AgNO_3$ (Auquier 2006). AgrR

(Rmet_1751) is a response regulator and part of a two-component regulatory system with the histidine kinase AgrS (Rmet_1752). They share 58 and 32 % protein sequence similarity with SilR and SilS from *Salmonella enterica* serovar Typhimurium plasmid pMG101, respectively. Their co-localized target codes for an uncharacterized RND-driven efflux system (AgrABC; Rmet_1750-Rmet_1748), which shows characteristics of heavy metal (HME) as well as hydrophilic/amphiphilic compounds (HAE) efflux systems.

3.1.12 Zinc: A Pivotal Role for the RND-Driven System CzcCBA

The MIC of $ZnCl_2$ for *C. metallidurans* CH34 was determined to be 12 mM in Tris minimal medium (Mergeay et al. 1985). This high level of resistance was mainly associated to determinants located on pMOL30. The MIC of Zn^{2+} for the pMOL30-free strains AE104 and AE128 is twenty times lower than for the parental strain CH34 (Mergeay et al. 1985). The major actor is the well characterized inducible HME-RND-driven system CzcCBA (Rmet_5982-Rmet_5980) (Nies et al. 1987; Nies 1992, 1995). As previously described, this system also plays a role in the regulation of cellular cadmium and cobalt concentrations. It is induced by $Zn^{2+} \gg Cd^{2+} > Co^{2+}$ but also to a lesser extent by other cations such as Ni^{2+} and Cu^{2+} (Nies 1992; Nies et al. 2006; Monsieurs et al. 2011; van der Lelie et al. 1997; Legatzki et al. 2003a). Expression of the *czc* system is regulated by the intracellular zinc content. Furthermore, as zinc is the best inducer of *czcCBA*, the encoded protein complex might be mainly a zinc regulation system. For example, constitutive expression of CzcCBA in strain AE104 Δ*zupT* (Rmet_2621, ZupT is a zinc importer) led upon zinc starvation to the disappearance of the CzcA protein and therefore to a decrease of the metal ion efflux (Herzberg et al. 2014a). The three proteins CzcA, CzcB, and CzcC are required for full resistance to Zn^{2+}, Cd^{2+}, and Co^{2+} (Nies et al. 1989b) but CzcA alone is able to mediate a low-level metal ion resistance (Rensing et al. 1997). After reconstitution of CzcA into proteoliposomes, Zn^{2+} was transported with the highest velocity among the three different cations (Goldberg et al. 1999).

CzcCBA is the main efflux system involved in zinc resistance but a functional interplay between this tripartite complex and other transporters from *C. metallidurans* CH34 was observed. The first transporter family consists of proteins from the P-type ATPase family. Four proteins of this family are able to induce zinc resistance in different conditions, more specifically, the three P_{IB2}-type ATPases ZntA (chromid), CadA (CMGI-1 on the chromosome) and PbrA (pMOL30), and the P_{IB4}-type ATPase CzcP (pMOL30) (Scherer and Nies 2009). Heterologous expression of ZntA and CadA in a zinc-sensitive *E. coli* strain increased the resistance to Zn^{2+} and Cd^{2+} (Legatzki et al. 2003b). In strain AE104 (plasmid-less), single gene deletions of *cadA* or *zntA* had only a moderate effect on zinc and

cadmium resistance, but in a double deletion mutant zinc and cadmium resistance decreased 6-fold and 350-fold, respectively (Legatzki et al. 2003b). Neither single nor double gene deletions affected zinc resistance in the presence of CzcCBA. In the absence of CzcCBA and of the three other ATPases, ZntA is responsible for a substantial increase in zinc resistance (Scherer and Nies 2009). Deletion of *zntA* can be partially compensated by at least one of the three other ATPases. CzcP has, however, a different contribution to zinc resistance in comparison to the three P_{IB2}-type ATPases. CzcP transports zinc ions more efficiently than the three P_{IB2}-type ATPases but only when at least one of these proteins is present. Therefore, CzcP seems to play a role as "resistance enhancer" in *C. metallidurans* CH34 (Scherer and Nies 2009).

The second type of transporter that may interfere with the Czc system is represented by proteins of the CDF family. The *czcD* gene (Rmet_5979) is located downstream of the *czcCBA* cluster (Nies and Silver 1995) and mediates a low-level Zn^{2+}, Cd^{2+}, and Co^{2+} resistance when expressed in the absence of the high resistance CzcCBA system (Anton et al. 1999). CzcD is able to functionally substitute the four $P_{IB2/4}$-type ATPases and vice versa (Scherer and Nies 2009). The Zn^{2+} and Cd^{2+} efflux activity of CzcD was demonstrated by heterologous expression in a highly zinc-sensitive *E. coli* strain (Anton et al. 2004). In the presence of CzcCBA, CzcD is not essential for cation efflux in *C. metallidurans* strains (Nies et al. 1989b) but the protein is involved in the regulation of the *czc* determinant (Anton et al. 1999; Nies 1992; Grosse et al. 2004).

DmeF and FieF, two other chromosomally-encoded CDF proteins, predominantly transport Co^{2+} and Fe^{2+}, respectively. Although both proteins have a broad substrate specificity and are, for example, capable of conferring a low-level zinc resistance in a zinc-sensitive *E. coli* strain (Munkelt et al. 2004), disruption of *dmeF* or *fieF* in strains AE128 and AE104 had no influence on the tolerance to Zn^{2+} (Munkelt et al. 2004; Scherer and Nies 2009).

Zinc ions may also interfere with transport systems specific to other cations like the CnrCBA efflux system. For instance, spontaneous mutants derived from strain AE126 (containing only pMOL28) were selected on minimal medium supplemented with 0.6 to 1 mM Zn^{2+} (Collard et al. 1993). These mutants displayed constitutive expression of CnrCBA, resulting in reduced cellular accumulation of Zn^{2+} and increased resistance to zinc, cobalt and nickel (Tibazarwa et al. 2000; Collard et al. 1993)(see Sect. 3.3.1.2). In addition, full expression of the *cnr* genes in strain AE126 carrying only pMOL28 is accompanied by some resistance to zinc and even cadmium.

Finally, these studies about the resistance to zinc were complemented with efforts to make a comprehensive inventory of the zinc (and related metals) uptake systems (Kirsten et al. 2011) and their regulation, with emphasis on the *zupT* gene (Rmet_2621) (Herzberg et al. 2014a; Schmidt et al. 2014), as well as their effect on the cellular zinc pools and repository (Herzberg et al. 2014a, b).

3.1.13 Other Metals: Thallium, Caesium, Palladium and Bismuth

C. metallidurans only showed moderate changes in gene expression after exposure to thallium (around 40 upregulated genes). A surprisingly low number of differentially expressed genes were located on its megaplasmids, especially as it has been observed that the MIC of thallium is drastically reduced in the absence of pMOL28 and pMOL30. The only plasmid gene differentially expressed after thallium exposure was the *copH* gene on pMOL30.

Exposure to caesium resulted in a transcriptional response comparable with that to thallium. Correspondingly, *copH* was one of the only known metal resistance genes that was differentially expressed. This response can partially be explained by the relatively low chemotoxicity of caesium, resulting in a rather high MIC that might be more related to osmotic stress than chemical toxicity.

An even smaller effect was observed at the transcription level after exposure to palladium, resulting in about 10 upregulated genes. Nevertheless, the upregulated genes with palladium included (part of) the *cop* clusters on pMOL30 (including *copH*) and the chromid.

Exposure to bismuth had a more pronounced effect on the transcriptional profile of *C. metallidurans* CH34, resulting in a list of more than 100 differentially expressed genes. The most obvious observation is the high induction of the *ars* operon (on average more than 8-fold), an operon which had already been shown to be activated by a wide range of transition metals (Monsieurs et al. 2011). In addition, next to a large fraction of conserved hypothetical proteins, some metal resistance genes are also upregulated like *czcA*, *czcC* and *zntA*. Furthermore, in a plasmid-free derivative of CH34, mutation of *zntA* increased the sensitivity to bismuth, which is one of the first phenotypes linked to bismuth (Monchy et al. 2006b).

3.2 Applications of the *Cupriavidus* Resistance/Response to Metals

The extensive array of metal efflux/extrusion systems allows *C. metallidurans* to adapt, survive and even thrive in biotopes with a high toxic metal content. Yet surviving chronic and intense metal pollution implies the development of strategies to avoid metal re-entry such as immobilisation or sequestration.

Liquid cultures of *C. metallidurans* grown in the presence of a high levels of cadmium or zinc removed substantial amounts of these metals from the liquid via a process of bioprecipitation initiated at the onset of the stationary phase (Diels et al. 1995a, b, 1996). Crystals of otavite (cadmium carbonate) and hydrozincite (zinc hydrocarbonate: $Zn_5(CO_3)_2(OH)_6$) were observed. This first observation triggered a more thorough analysis of *C. metallidurans* liquid cultures and biofilms with special attention to polysaccharides as nucleation sites for carbonate crystals and as

contributors to the flotation behaviour of slurries containing soil, bacteria and heavy metals (Diels et al. 2009). This led to the development of various applications to efficiently remove metals from effluents, sludge and soils (Diels et al. 2009; Pumpel et al. 2003; Pumpel and Paknikar 2001).

Genetic engineering offered alternative approaches to bioremediation. On the one hand, *C. metallidurans* strains were genetically engineered to enhance their bioremediation potential. For instance, heterologous expression of eukaryotic metallothionein, a cysteine-rich protein that binds heavy metals, enhanced the immobilization capacity of *C. metallidurans* CH34 and conferred a protective effect upon tobacco plant growth in Cd^{2+}-polluted soils (Valls et al. 2000). Another example is the increased potential for mercury bioremediation of contaminated water bodies and industrial wastewater by introduction of the IncP-1β plasmid pTP6 that provides novel and additional *mer* genes (Rojas et al. 2011). On the other hand, *C. metallidurans* genes were introduced and heterologously expressed in endophytes, which assisted their host plant during phytoremediation (Lodewyckx et al. 2001; Taghavi et al. 2001; Weyens et al. 2011). These examples highlight the power of genetic engineering for environmental bioremediation.

Next to these applications, metal-induced resistance genes (mostly located on the megaplasmids from *C. metallidurans* CH34 and *C. basilensis* DS185) allowed the construction of heavy metal whole-cell biosensors[2] that are able to detect the bioavailable concentrations of cadmium, zinc, copper, chromate, cobalt, nickel, thallium, lead or mercury ions after contact with contaminated soils, waste, solids, minerals or ashes (Corbisier et al. 2002; Collard et al. 1994; Vanderlelie et al. 1994; Peitzsch et al. 1998; Nies 2000; Magrisso et al. 2008; Harms 2007). The whole-cell biosensors were constructed by fusing a *luxCDABE* reporter system to a metal-responsive promoter, thereby translating the induction in the presence of a biologically available heavy metal to a measurable bioluminescent signal. In a next step, these whole-cell biosensors were immobilized onto optical fibres and their sensitivity and storage capacity were characterised (Corbisier et al. 1999). Another type of sensor is based on the direct interaction between metal-binding proteins and heavy metal ions. For instance, the capacitance change of the MerR mercury regulatory protein (see Sect. 3.3.1.1) was detected in the presence of femto- to millimolar metal ion concentrations (Corbisier et al. 1999). Recently, a PbrR-based biosensor was also devised to measure lead concentrations (Chiu and Yang 2012).

The *C. metallidurans* biosensors have been efficiently used to measure: (i) the bioavailable metal concentration in solid wastes (such as incinerator fly ash) (Corbisier et al. 1996), soils contaminated by heavy metals (Corbisier et al. 1996; Diels et al. 1999; Almendras et al. 2009), bacteria (Gilis et al. 1998) and photoluminescent nanocrystals (Aboulaich et al. 2012); (ii) the residual concentrations of nickel and lead in soils that had been treated with additives for in situ metal immobilization (Tibazarwa et al. 2001; Geebelen et al. 2003); (iii) the biological

[2]For a general evaluation of the microbial reporters designed to assay metal bioavailability and their possible use in environmental remediation see Magrisso et al. (2008).

accumulation of nickel in specific parts of important agricultural crops such as maize and potato (Tibazarwa et al. 2001); (iv) the runoff from zinc-coated materials and outdoor structures, which is an important source for zinc dispersion in the environment (Heijerick et al. 2002); and (v) the intracellular Pb^{2+} content in human embryonic kidney cells (Chiu and Yang 2012).

Finally, it should be mentioned that metal resistance determinants from *C. metallidurans* CH34 proved to be very versatile tools to study gene dissemination in soil environments (the problematic nature of spreading recombinant genes in the environment) (Top et al. 1990, 1995; Smets et al. 2003), to detect new broad host range plasmids in the rhizosphere or in polluted soils (Top et al. 1994; Van der Auwera et al. 2009; van Elsas et al. 1998), and to evaluate conjugational plasmid transfer between Proteobacteria and Actinobacteria (Margesin and Schinner 1997).

3.3 Lessons Learned from the Transcriptome Response to Metals and Insights into the Regulatory Network

The development of microarrays has opened up a wide area of new opportunities for the field of molecular biology. The availability of microarrays to study gene expression in *C. metallidurans* CH34 has significantly contributed to the identification of new metal resistance regions in its genome. Transcriptome analysis initially focused on the pMOL28- and pMOL30-encoded metal resistance regions as both megaplasmids were known to contain a significant number of metal resistance determinants. At a later stage, the importance of metal resistance regions located on the chromosome and the chromid was recognized (Legatzki et al. 2003b; Grosse et al. 2004; Munkelt et al. 2004; Nies et al. 2006; Monsieurs et al. 2011; Taghavi et al. 2009). A crucial issue in the analysis of transcriptome data is to distinguish differentially expressed genes truly contributing to resistance to a specific metal from those for which the biologically relevant contribution to resistance has not been validated with experimental data (e.g. knock-out mutants). For instance, the transcriptional response of *C. metallidurans* exposed to different individual metals clearly showed an overlapping set of genes being activated, for which it was unclear whether they were contributing to the resistance phenotype or whether they were being activated as a result of crosstalk from other regulators in the highly interconnected transcriptional regulatory network. Moreover, the metal concentration used, and as such the relative toxicity of it, and the time point of analysis are crucial factors in the interpretation of the transcriptomic response. Indeed, when taking the MIC as a measure of relative toxicity, significant differences are observed when comparing gene expression below and above the MIC. Significant differences in gene expression levels were also obtained at 10 min compared to 30 min after metal exposure, suggesting distinct biological responses at both time points.

The first observation on the complex regulatory network and corresponding regulatory proteins underlying heavy metal resistance is more extensively discussed

in the first part of Sect. 3.3. In the second part of Sect. 3.3, the impact of relative metal toxicity and the time-wise evolution of gene expression after exposure to metals are described in detail.

3.3.1 Regulatory Functions

In the most simplistic view, transcriptional regulatory proteins can be split into two different groups: (1) sigma factors that bind to promoter regions and attract an RNA polymerase to start the expression of the corresponding coding regions and (2) regulatory proteins that are responsible for fine-tuning the transcriptional regulation by either enhancing the binding of the sigma factor with a specific region or inhibiting its binding. The regulatory proteins involved in metal resistance are described in Sect. 3.3.1.1, while the large family of sigma factors in *C. metallidurans* CH34 is described in Sect. 3.3.1.2.

3.3.1.1 Regulatory Proteins

Most of the regulatory proteins involved in heavy metal resistance have been identified by the adjacent location of their coding sequences to genes known to be important for heavy metal resistance. Those metal resistance regulatory proteins can largely be subdivided into cytoplasmic regulators, i.e. transcriptional regulators that directly sense the presence of metal ions in the cytoplasm, and two-component regulatory systems, i.e. a regulatory system consisting of a transmembrane sensing protein able to detect metal ions in the periplasm that interacts with the corresponding transcriptional regulatory protein.

MerR-like Regulators

The largest class of cytoplasmic regulators in *C. metallidurans* CH34 involved in heavy metal resistance is the group of MerR-like regulatory proteins, of which the mercuric ion sensing MerR protein is the archetype (Hobman et al. 2005). MerR-like regulators (mostly activators) specifically recognize promoters with an unusually long spacer of 19 or 20 bp between the −10 and −35 regions, and contain cysteine amino acids essential in metal binding and activation of gene expression (Brown et al. 2003; Chen et al. 2007; Helmann et al. 1990; Shewchuk et al. 1989a, b, c). However, it has been suggested that there is some flexibility in the exact binding site recognized by MerR-like regulators since MerR-like activators in *C. metallidurans* CH34 could heterologously activate non-cognate MerR family promoters from other organisms (Julian et al. 2009).

Four mercury resistance operons are present in the CH34 genome (see Sect. 3.1.8). With the exception of the incomplete *mer* operon on pMOL30, each

operon carries a *merR* gene flanked by a mercury reductase gene (*merA*), which reduces the Hg^{2+} ion to volatile Hg(0), together with the auxiliary genes *merP* and *merT* (respectively present in the periplasm and inner membrane for transporting Hg^{2+} into to cytoplasm) (reviewed in Barkay et al. 2003). In most *mer* operons a co-regulator encoded by the *merD* gene is also flanking the *merR* regulatory gene, however, no direct binding of the MerD protein with any of the promoters in the *mer* operon could be detected (Champier et al. 2004).

In the absence of Hg^{2+}, MerR binds as a homodimer to the divergent promoter region of the *merTPAD* operon and the *merR* gene, thereby slightly repressing the transcription of these genes (reviewed in Hobman et al. 2005). However, the MerR regulatory protein is hypersensitive to Hg^{2+}, and upon the presence of Hg^{2+} in the cytoplasm, one Hg^{2+} ion will bind to three essential cysteine residues of the MerR homodimer, two cysteines from one monomer and one from the other (Helmann et al. 1990; Utschig et al. 1995). This will lead to an allosteric underwinding of the promoter DNA thereby aligning the −35 and −10 recognition sites and outweighing the effect of the prolonged spacer between both recognition sites such that RNA polymerase can bind to those sites and activate transcription (Frantz and O'Halloran 1990; Ansari et al. 1992, 1995; Heltzel et al. 1990).

A second subgroup of MerR-like regulators are the PbrR regulatory proteins, of which three copies exist in strain CH34 i.e. the $pbrR_1$ regulator of the *pbr* operon on pMOL30, the $pbrR_2$ on CMGI-1 and the chromosomally-encoded $pbrR_3$. Remarkably and in contrast with the complete *mer* operons, the *pbrR* genes do not always flank the same metal resistance genes. The $pbrR_1$ gene (Rmet_5946) is part of the *pbrUTRABCD* metal resistance region on pMOL30 and is shown to regulate this *pbr* operon (Borremans et al. 2001; Brown et al. 2003), as *pbrRTU* and *pbrABCD* are transcribed via a *merR*-like promoter (Borremans et al. 2001; Taghavi et al. 2009). The $pbrR_2$ gene (Rmet_2302) regulates the flanking efflux ATPase *cadA* gene and a *pbrC* homologue. In contrast, the chromosomally-encoded $PbrR_3$ (Rmet_3456) regulates the chromid-located *zntA* gene encoding for a P_{IB2}-type ATPase (Taghavi et al. 2009). Despite the high homology between all three PbrR regulators, cross-regulation between the three regulatory proteins seems to be rather limited. Indeed, using a β-galactosidase assay where the promoter of the pMOL30 *pbrA* gene was cloned into a reporter plasmid and linked to the *lacZ* gene, Julian et al. (2009) could detect a transcriptional activation in strain CH34 after adding Pb (II). By contrast, no transcriptional activation could be observed under the same conditions when inserting this plasmid carrying the P_{pbrA}-*lacZ* fusion into strain AE104 i.e. a *C. metallidurans* strain lacking pMOL28 and pMOL30. This suggests that transcriptional activation in the first experiment is due to the $pbrR_1$ regulator, since the chromosomally located $pbrR_2$ and $pbrR_3$ are not capable of activating the pMOL30 *pbrA* promoter in a plasmid-free strain. As such, only low cross-talk between the three *pbrR* regulators could be observed at least in the case of the pMOL30 *pbrA* promoter.

The DNA-binding characteristics of $PbrR_1$ have been shown to be similar to the transcriptional activation by MerR proteins, since shortening the internal spacer between the −10 and −35 region in the *pbrA* promoter to 18 bp leads to an

increased expression profile from this promoter even in the absence of Pb^{2+} (Hobman et al. 2012). Similar to other MerR regulatory proteins, it has been shown that specific cysteine amino acids in the $PbrR_1$ regulatory protein (C14, C79 and C134) are essential for Pb^{2+} sensing and activation of the *pbrA* promoter region (Hobman et al. 2012). Additionally, the metal-binding specificity of the $PbrR_2$ protein has been studied in detail, showing that this regulatory protein binds Pb^{2+} a 1000-fold more selective than a wide range of other metal ions (Chen et al. 2005). This specific binding would be the consequence of a specific geometry allowing highly specific binding of Pb^{2+} while preventing the binding with other metal ions (Chen et al. 2007).

A last representative of the MerR-like regulators linked to metal resistance, is the *cupR* gene located on the chromosome. CupR regulates resistance to metal ions by transcriptionally activating the neighbouring *cupA* gene coding for a P_{IB1}-type ATPase and auxiliary chaperone genes like *cupC*. The CupR protein is an ortholog of the GolS regulator in *Salmonella* (Pontel et al. 2007) and the CueR regulatory protein in *E. coli* (Stoyanov and Brown 2003; Stoyanov et al. 2001). CupR in *C. metallidurans* CH34 is important for resistance to monovalent copper (Cu^+), but also seems to have a high affinity for Au^+ (Jian et al. 2009), which is in line with the observation of Changela et al. (2003) who reported a very high sensitivity of the *E. coli* CueR towards Cu^+ as well as Ag^+ and Au^{3+} (Stoyanov et al. 2001; Stoyanov and Brown 2003).

ArsR-like Regulators

Six representatives of the ArsR family of regulatory proteins have been found in *C. metallidurans* CH34, evenly divided over the chromosome and the chromid, however, only one of them (ArsR, Rmet_0333) has been shown to be important for heavy metal resistance and is part of the complete *ars* operon *arsRIC*$_2$*BC*$_1$*HP* (see Sect. 3.1.1). Remarkably, the frequently occurring *arsD* gene, encoding a minor secondary regulatory protein (Silver and Phung 1996), is lacking in the *C. metallidurans* CH34 *ars* operon. Despite upregulation of the *ars* operon being observed in *C. metallidurans* CH34 after exposure to As^{3+} as well as As^{5+}, it has been shown that ArsR could only efficiently bind to the highly toxic arsenite (As^{3+}) but could not bind to the phosphate analogue arsenate (As^{5+}) (Zhang et al. 2009). This can be explained by the fact that when As^{5+} enters the bacterial cell, a small fraction is converted to As^{3+} by the arsenate reductase ArsC. Once ArsR can bind to arsenite, the ArsR repressor dissociates from the promoter region of the *ars* operon containing two arsenate reductases ($ArsC_1$ and $ArsC_2$) able to further convert As^{5+} to As^{3+}, which can then be transported to the exterior of the cell by the ArsB arsenate permease. However, the *ars* operon is not only transcriptionally induced after exposure to arsenite and arsenite, but also to a wide range of other metals like Pb^{2+}, Zn^{2+}, Co^{2+}, Cd^{2+}, and to a lesser extent also Se^{6+}. However, when comparing those expression studies with in vitro binding studies of ArsR with a wide range of metals, the correlation between both observations is not straightforward (Zhang

et al. 2009). Studies using an *arsR* knock-out mutant in other organisms like *Synechocystis* sp. PCC 6803 have shown that those mutants seem to be hypersensitive to metals like Ni^{2+}, Cu^{2+} and Cd^{2+} (Sanchez-Riego et al. 2014).

Two-Component Regulators

In contrast with the one-component regulatory systems such as the MerR-like and ArsR-like regulatory proteins, where the metal ion first needs to enter the cytoplasm before transcriptional activation occurs, two-component systems have the advantage of being able to sense the metal ions in the periplasm. Some of the 56 two-component regulatory systems in *C. metallidurans* CH34 (Janssen et al. 2010) have been shown to be important for heavy metal resistance e.g. the *copRS* (Monchy et al. 2007; Mergeay et al. 2003) and *czcRS* systems (van der Lelie et al. 1997; Nies 1992; Grosse et al. 1999, 2004; Monchy et al. 2007), while for others a link with heavy metal resistance has been suggested e.g. *agrRS* (Mergeay et al. 2003), *hmzRS* (Van Houdt et al. 2009), *zniRS* and *zneRS* (Nies et al. 2006). Despite the fact that two-component systems have been shown to play a crucial role in metal resistance against a wide range of heavy metals (Monchy et al. 2007; Wiesemann et al. 2013), less is known about metal binding affinities and specificities of those two-component regulatory systems. Indeed, as already stated (Hobman et al. 2007), metal responsive cytoplasmic transcription factors are becoming well understood, however, the working mechanisms and binding characteristics of two-component systems, and cross-talk between regulators in heavy metal resistance is less intensively studied.

Most of the regulators of the known metal resistance mechanisms in *C. metallidurans* can be grouped in one of the different classes described above. As mentioned in the introduction of this paragraph, the majority of the regulatory proteins have been identified by adjacency of their coding sequences to known metal resistance clusters. However, some of the regions predicted to be important for heavy metal resistance (e.g. *nim*, *sil* or *hmy*) do not have a regulator in the neighbouring regions. It must be noted that most of those regions do not show differential expression after exposure to a wide range of heavy metals (Monsieurs et al. 2011). Only *nimB* (Rmet_5682) was moderately upregulated after exposure to Cu^{2+} and Co^{2+}, *silA* (Rmet_6136) with Cu^{2+}, *hmyC* (Rmet_4120) with Pb^{2+}, Bi^{2+} and Sr^{2+}, and *hmyB* (Rmet_4121) with Sr^{2+}. For these regions, additional studies will be required to investigate whether they are under control of a specific regulator.

Additionally, it should be noted that the regulatory control of some metal resistance regions needs to be further deciphered. An intriguing example within this context is the transcriptional regulation of the *chr* region, which is responsible for resistance to chromate and present in duplicate in strain CH34 i.e. one copy on pMOL28 and a second one on the chromid. It was already hypothesized that the *chrB* and *chrI* genes are involved in this regulation, suggesting a role in transcriptional activation for *chrB* and a repressive role for *chrI* (Juhnke et al. 2002). However, additional studies are needed to further elucidate this mechanism.

3.3.1.2 Sigma Factors and the Regulation of *cnr/ncc* Nickel Resistance

All bacterial transcriptional processes are initiated by the binding of a sigma factor to the promoter region of a gene. In many cases this transcriptional process is initiated by the housekeeping sigma factor σ^{70}. *C. metallidurans* CH34 has undergone different gene duplication processes including that of the housekeeping sigma factor. Indeed, two RpoD (σ^{70}) copies are present in CH34 i.e. *rpoD1* (Rmet_2606) on the chromosome and *rpoD2* (Rmet_4661) on the chromid (Janssen et al. 2010; von Rozycki and Nies 2009). Comparative genomic analysis suggests that the *rpoD1* gene encodes the native housekeeping factor, while the *rpoD2* gene has been acquired via horizontal gene transfer as it shows good homology with the σ^{70} factor present in *Bordetella* and *Burkholderia* species (Janssen et al. 2010).

In addition to these housekeeping sigma factors, bacteria use alternate sigma factors to fine-tune their transcriptomic profile or to activate subsets of genes in response to a wide range of stresses. As such, *C. metallidurans* CH34 has acquired 18 different sigma factors (compared to 8 in *E. coli*). Even more impressive is the portion of extracytoplasmic function (ECF) sigma factors, as there are up to 11 different ECF sigma factors (Nies 2004; Grosse et al. 2007). This subset of ECF sigma factors is important in the context of metal resistance as they can respond to extracytoplasmic signals. Of these ECF sigma factors, five have been shown to be important for heavy metal resistance and metabolism i.e. RpoI (Rmet_1120) (control of staphyloferrin siderophore biosynthesis), RpoJ (Rmet_4499), RpoK (Rmet_4001), RpoE (Rmet_2425) and CnrH (Rmet_6207).

The latter ECF sigma factor regulates the *cnr* region on megaplasmid pMOL28 and can deal with resistance to cobalt, nickel and zinc (Liesegang et al. 1993; Grass et al. 2000; Tibazarwa et al. 2000). Control of the activity of CnrH occurs via two transmembrane proteins i.e. the anti-sigma factor CnrY and the periplasmic sensor CnrX. In absence of Ni^{2+} and Co^{2+}, the sigma factor CnrH is silenced via the binding of CnrY. However, upon binding of the periplasmic sensor CnrX with Ni^{2+} or Co^{2+} in the periplasm, CnrH is released by CnrY, and the transcription of the *cnr* region can proceed. In a recent paper, Maillard et al. (2014) described the structural interaction between CnrH and CnrY, and identified a hydrophobic knot that is responsible for the release of CnrH from CnrY in the presence of nickel or cobalt. Important to notice within this context is the uncertainty on the promoter regions controlling the *cnr* region. While Grass et al. (2000) suggested the presence of two possible tricistronic transcripts i.e. *cnrYXH* and *cnrCBA*, Tibazarwa et al. (2000) suggested a dicistronic *cnrYX* operon and tetracistronic *cnrHCBA* operon, requiring the promoter of the latter operon to be located inside the *cnrX* gene.

3.3.2 Cross-Regulation

In the most straightforward implementation of transcriptional regulation of heavy metal resistance, each single metal ion would be recognized by one specific sensor

protein, which in turn would activate a dedicated resistance mechanism (Hobman 2007; Hobman et al. 2005, 2007). However, observing the transcriptional response of *C. metallidurans* CH34 when exposed to a wide range of metals and metalloids over time courses has clearly showed that this does not correspond to the biological reality, either on the plasmid (Monchy et al. 2007) or on the chromosome (Monsieurs et al. 2011). Indeed, based on the correlation between gene expression profiles for different individual metals as well as by calculating the number of overlapping genes between different metal responses, a complex interplay between different metal responses was observed. This corresponds with the observation made by Hobman et al. (2007) when exposing *E. coli* to a wide range of metals, thereby stating as conclusion that most of those metal response regulons are not discrete but rather overlap between different metal exposures.

This cross-regulation of gene expression by different metals might be the result of different events. First, it should be noted that many metalloproteins do not bind with their cognate effector metal when faced with mixtures of metals (Waldron and Robinson 2009). This might not be surprising as the chemistry of metal ions makes it such that biological systems may have low metal ion specificity (Hobman et al. 2007). This had already been shown e.g. in *E. coli* where it was shown that CueR could not only bind, but also activate expression in response to Cu^{+}, as well as Ag^{+} and Au^{3+} (Stoyanov et al. 2001; Stoyanov and Brown 2003) and is also illustrated in *C. metallidurans* CH34 for the ArsR regulatory protein that seems to be able to bind a wide range of metals and derepress transcription (Zhang et al. 2009), while the corresponding *ars* operon is only shown to be important for As resistance. Exposing *C. metallidurans* to a specific metal might induce expression of certain gene clusters for which it is not clear whether their gene products contribute to the metal resistance phenotype, or whether it is caused by an erroneous binding of the metal with a metalloprotein.

Secondly, an increase in the concentration of one specific metal in the environment might lead to an elevated concentration in the cytoplasm, thereby influencing the balance of the intracellular metal ion pool (Kaur et al. 2006). This might result in a shift in occupation of metal binding sites by binding of the excessive metal ion to the general metal binding proteins and forcing the trace elements in the cytoplasm to bind with highly specific metal binding proteins, thereby upregulating genes related to specific metal resistance characteristics. Similarly, some toxic metals might have a higher affinity for an occupied essential metal binding site in a protein, and as such displace the essential metal, which would increase the intracellular free metal ion pool. This might for example be the case for the exposure of *C. metallidurans* CH34 to gold. The pMOL30 *cop* cluster was only upregulated at toxic gold concentrations (Reith et al. 2009), which might be explained by occupation of the majority of non-specific metal binding domains by gold causing a shift of copper and other *cop* activating metals to their corresponding highly metal-specific sensors (Monsieurs et al. 2011).

Thirdly, this cross-regulation might be explained by the flexibility of regulatory systems at the transcriptional level to recognize transcription factor binding sites slightly deviating from their consensus site. Indeed, many regulatory proteins

known to be linked to heavy metal resistance are highly conserved i.e. they display a high level of homology. This is, for example, the case for a cluster of CopR-like two-component regulators i.e. the pMOL30-encoded $CopR_1$ and $CzcR_1$, and the chromid-encoded $CopR_2$, $CzcR_2$ and AgrR. All five regulators show a high level of homology at the protein level and are likely to recognize the same type of regulatory motifs. This implies that upon activation these regulatory proteins are not only transcriptionally activating their cognate operons but also other metal resistance operons normally regulated by other regulatory proteins.

Similarly, the sensor proteins of two-component systems, responsible for detecting the presence of metals in two-component regulatory system, might show alike homology as the regulatory proteins. These sensory proteins show flexibility in the corresponding regulatory protein they can activate via phosphorylation. A proof of principle of this type of cross-regulation is the *czc* system in pMOL30. It was shown that upon deletion of the *czcS* gene encoding the histidine kinase, two other homologous histidine kinases were able to take over this function, thereby ensuring the transcriptional activation by the regulatory protein CzcR via binding with the promoter regions of *czcP* and *czcN* (Grosse et al. 1999; Scherer and Nies 2009).

3.3.3 Dose- and Time-Dependency of the Response

In order to allow an objective comparison between different metal ion expression profiles, most data were obtained after metal exposure to similar conditions i.e. an exposure of 30 min to a metal concentration approaching ~50 % of the MIC. Moreover, for most of the metals only one speciation of the metal has been tested. Below, some small-scale results concerning the potential influence of these factors will be discussed.

3.3.3.1 Concentration Effect

As mentioned above, most metal response expression profiles are obtained using one specific metal concentration below the MIC. However, for exposure to Au^{3+}, five different data sets are available: a ten-minute exposure to 10, 50 and 100 μM, and a thirty-minute exposure to 0.5 and 50 μM (Monsieurs et al. 2011; Reith et al. 2009). First of all, a clear effect of the increase in gold concentration can be observed: the total number of upregulated genes at 10 min augments with an increasing concentration of gold i.e. the number of upregulated genes is 53, 127 and 330 for 10, 50 and 100 μM, respectively. A similar trend in differentially upregulated genes can be observed for both concentrations at 30 min (increasing from 59 at 0.5 μM to 136 genes at 50 μM). Moreover, the obtained fold changes increase proportionally to the applied concentration. From a functional point of view, when looking at metal response gene clusters expressed at high gold concentrations (50

and 100 μM) versus lower ones (10 and 0.5 μM), genes related to oxidative stress (e.g. the *ohrR* regulon) and glutaredoxin/glutathione metabolism are exclusively upregulated at high gold concentrations. Additionally, when comparing the different metal responses at the level of functional classes (COGs), a very clear activation of the chaperone proteins (proteins classified in COG class O) is observed with increasing Au^{3+} concentrations: where at 0.5 μM this consisted only of 3 % of all upregulated genes, this percentage increased to 14 % for 100 μM and around 21 % for both observations at 50 μM. Similarly, the increase in the number of upregulated genes is confirmed by the increase of the number of genes related to the transcriptional process, which increased from 1 % at 0.5 μM to around 13 % at 50 μM.

At a smaller scale, an expression study using qPCR has been applied for the pMOL30 *cop* cluster (Monchy et al. 2006a), leading to results that are deviating from the gold transcriptomic data. The pMOL30 *cop* genes of *C. metallidurans* CH34 were exposed to different concentrations of Cu^{2+}, and the fold change in expression after 30 min exposure was measured using qPCR. Instead of obtaining fold changes that increase proportionally with the applied concentration a dramatic decrease in fold change was observed once the copper concentration added to the growth media reached the MIC.

Based on the two data sets described above, it is very difficult to predict what the exact effect of increasing metal concentrations, even exceeding the MIC, will have on the transcriptional process in *C. metallidurans*. Indeed, where the fold changes still increase for gold when applying a concentration more than 100 fold higher than the MIC, the opposite is observed for the pMOL30 *cop* genes with exposure to copper concentrations just below the MIC. As these conclusions are only based on a limited number of experiments, a more thorough analysis of the transcriptomic profile above the MIC is required, using a wider range of metals in a well-defined experimental setup.

3.3.3.2 Time Effect

Transcriptional regulation is a dynamic progress, and the type of genes upregulated after exposure to metals will also depend on the time point when gene expression is measured. Datasets assessing transcriptional regulation at different time points after exposure to metals in *C. metallidurans* are limited. However, data from a transcriptomics experiment measuring genome-wide transcription after exposure to gold and a qPCR experiment assessing the expression levels after exposure to different concentrations of copper allow us to derive some general trends.

Looking at the overlap of the upregulated genes at 10 and 30 min after exposure to 50 μM gold, around 50 % of the upregulated genes are the same at both time points. It is not clear based on the functional classification of these genes, which physiological effect can possibly explain this shift in gene expression, as for both time points the same functional classes are over- or underrepresented. However, when focusing on metal-specific genes (i.e. genes with a limited amount of

orthologs in related bacteria), a change in two opposite directions can be observed. First, for some metal-specific clusters, a clear increase of gene expression was observed at 30 min compared with 10 min. An illustrative example for this phenomenon is the *cop* cluster on pMOL30. Despite the fact that this region is already partially upregulated at 10 min (9 out of 19 genes; average fold change 3), a significant increase is observed at 30 min (14 out of 17 genes; average fold change 9). As a second example, Rmet_4682-Rmet_4686 is already fully activated at 10 min with an average fold change of 9, however increasing to an average fold change of 46 at 30 min. In contrast, some of the known metal-resistance clusters are upregulated immediately after exposure of *C. metallidurans* to gold, but the effect decreases with time (almost no differential expression detected after 30 min). Examples of this behaviour are the *mer*-cluster on Tn*4378* (average fold change of $\sim$3 at 10 min vs. 1.70 at 30 min), the *pbr* operon on the chromid (fold change $\sim$6 at 10 min vs. $\sim$2 at 30 min) and the *ars* operon on CMGI-1 (average of 13 at 10 min vs. 1.80 at 30 min).

This might indicate that the first reaction of the bacterium is a general stress response, thereby activating a wide range of metal resistance mechanisms, where a more specific response is seen at 30 min. This hypothesis is confirmed by qPCR data (Monchy et al. 2006a), following up the transcriptional profile over time of the pMOL30 *cop* genes after exposure to Cu^{2+}. Indeed, where after 10 min an increase in fold change can already be observed for most *cop* genes, this effect is even more pronounced after 30 min.

On the other hand, in the case of the Tn*4378 mer* operon, MerR activation of expression is balanced by MerD acting to dissociate Hg^{2+}-bound MerR from P_{merT} as a feedback control mechanism (Champier et al. 2004). Many transcription studies have concentrated on activation of gene expression in response to metals, but being able to switch off expression is as important for the organism to maintain a dynamic response to changing internal and external conditions.

3.4 Concluding Remarks

C. metallidurans, with type strain CH34 used as case study, is highly adapted to hostile environments with a high burden of multiple metals. Because of these environments, it is not unexpected that its metal resistance determinants and their transcriptional network would be similarly complicated, overlapping, robust and to contain a degree of redundancy. Human engineering solutions to critically important safety and survival systems also build in system duplication, robustness and redundancy, to avoid catastrophic failure in case components of the system fail to operate correctly. In the real world (rather than the lab) it is very unlikely that this strain would ever be exposed to single toxic metal species at 99.9 % purity in controlled growth conditions, it is more likely that exposure would be to multiple metals maybe in different forms, alongside other physical insults (pH, water activity, nutrient, temperature).

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