

Chapter 2

Genomic Context of Metal Response Genes in *Cupriavidus metallidurans* with a Focus on Strain CH34

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Abstract *Cupriavidus metallidurans* CH34 has been studied for over 30 years, mostly because of its resistance to numerous heavy metals. Many of these metal resistance determinants were rapidly associated with native megaplasms. However, its genome sequencing and whole genome expression profiling not only revealed the complex structure of its multiple replicons and complex responses to metals, but also revealed the presence of unnoticed/unstudied metal resistance determinants on the different replicons. In this chapter, the genomic context of the metal response genes in *C. metallidurans* CH34 will be described with a focus on its mobilome including insertion sequence elements, transposons, integrative and conjugative elements and genomic islands.

Keywords *Cupriavidus metallidurans* • Megaplasms • Chromid • Mobile genetic elements • Metal resistance • Adaptation

2.1 A Genome with Multiple Replicons

All *Cupriavidus* and *Ralstonia* genomes are typified by the presence of a large replicon in addition to their chromosome and megaplasms. These large replicons with a size of around 2–3 Mb have been designated both ‘second or secondary chromosomes’ as well as ‘megaplasms’. The ambiguity in their nomenclature arises from the chimeric features of these replicons since they carry essential genes making them indispensable for cell viability (second chromosome) and they use a plasmid-type replication system rather than a chromosomal one (megaplasms). To distinguish this particular replicon, which does not completely fit the term

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chromosome or plasmid, Harrison and colleagues (2010) coined the term “chromid”. Three main criteria were put forward in defining these replicons: (i) chromids have plasmid-type maintenance and replication systems, (ii) chromids have a nucleotide composition similar to that of the chromosome and (iii) chromids carry core genes that are found on the chromosome in other species (Harrison et al. 2010). However, the presence of chromids is not limited to these genera or to the β -proteobacteria since chromids have also been observed in γ -proteobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, *Deinococcus-Thermus*, Firmicutes and Spirochaetes (Harrison et al. 2010; Van Houdt and Mergeay 2012).

Most *Cupriavidus* and *Ralstonia* strains carry one or more megaplasms with a size of 100 kb or larger (for a detailed review see Schwartz 2009). Acquiring these megaplasms was probably key to the adaptation of the strains to certain ecological niches, considering the distinct and specialized functions contained within the plasmids. For instance, pMOL28 and pMOL30 from *C. metallidurans* CH34 are both involved in heavy metal resistance, pHG1 of *C. eutrophus* H16 is involved in hydrogenotrophy and chemolithotrophy (Schwartz et al. 2003; Schwartz 2009), while pRALTA of *C. taiwanensis* LMG19424 carries nitrogen fixation and legume symbiosis functions (Amadou et al. 2008). In addition to the megaplasms, other plasmids can be present, most of which are broad host range (BHR) plasmids such as the IncP-1 β plasmid pJP4 from *C. pinatubonensis* JMP134 (*R. eutropha* JMP134) (Don and Pemberton 1981, 1985; Lykidis et al. 2010; Sato et al. 2006) and the PromA plasmids captured by *C. metallidurans* CH34 (Top et al. 1994; Van der Auwera et al. 2009; Gstalder et al. 2003; Mela et al. 2008; Tauch et al. 2002; van Elsas et al. 1998).

This chapter mainly focuses on the genomic context of *C. metallidurans* type strain CH34 in close comparison with other *C. metallidurans* strains (Table 2.1). DNA-DNA hybridization (DDH) was estimated via the Genome-to-Genome Distance Calculator (Meier-Kolthoff et al. 2013) to confirm the taxonomic position of recently isolated and sequenced *Cupriavidus* strains. This analysis indicated that *Cupriavidus* sp. HMR-1 belongs to the *C. metallidurans* species (DDH value of 72.2 %), while *Cupriavidus* sp. BIS7 (Hong et al. 2012) does not (DDH value of 30.0 %). In addition, the available genomes of other *Cupriavidus* species, *Ralstonia* species and other closely related β -proteobacterial genomes were taken into account for a comprehensive analysis. These include *C. pinatubonensis* JMP134 (Lykidis et al. 2010), *C. eutrophus* H16 (*C. necator* H16, *R. eutropha* H16) (Fricke et al. 2009), *C. necator* N-1 (Poehlein et al. 2012), *C. taiwanensis* LMG19424 (Amadou et al. 2008), *R. solanacearum* species complex (Salanoubat et al. 2002; Remenant et al. 2011), and *R. pickettii* strains 12J and 12D (Yang et al. 2010). Other β -proteobacterial genomes included are *Delftia acidovorans* SPH-1, *Comamonas testosteroni* KF-1 and *Bordetella petrii* DSM12804. The latter strains only have one chromosome of ~ 6 Mb, in contrast to the various replicons found in *Cupriavidus* strains, although totalling a comparable amount of genetic material.

Table 2.1 *C. metallidurans* strains compared in this study

Strain	Isolation site	Isolation place	References
31A	Galvanization tank, metal factory	Holzminden, Germany	Schmidt et al. (1991)
43015	Human cerebrospinal fluid	Göteborg, Sweden	CCUG ^a
45957	Pharmaceutical industry	Sweden	CCUG
AS167	Mine tailings	Likasi-Sud, Congo	Brim et al. (1999)
AS168	Mine tailings	Likasi-Sud, Congo	Diels and Mergeay (1990)
AS39	Mine tailings	Likasi-Sud, Congo	Diels and Mergeay (1990)
CH34 ^{T*}	Decantation tank, zinc factory	Liège, Belgium	Janssen et al. (2010)
CH42	Polluted sediments, zinc factory	Liège, Belgium	Brim et al. (1999)
CH79	Polluted sediments, zinc factory	Liège, Belgium	Brim et al. (1999)
H1130 [*]	Patient with nosocomial septicaemia	Québec, Canada	Monsieurs et al. (2013)
HMR-1 [*]	Wastewater treatment plant	Hong Kong, China	Li et al. (2013)
KT01	Wastewater treatment plant	Göttingen, Germany	Timotius and Schlegel (1987)
KT02	Wastewater treatment plant	Göttingen, Germany	Schmidt et al. (1991)
KT21	Wastewater treatment plant	Göttingen, Germany	Timotius and Schlegel (1987)
NA1 [*]	Water storage system	ISS ^b	Monsieurs et al. (2014)
NA2	Contingency water container	ISS	Mijnendonckx et al. (2013)
NA4 [*]	Water recovery system	ISS	Monsieurs et al. (2014)
NE12 [*]	Cleanroom Kennedy Space Center	Florida, USA	Monsieurs et al. (2014)
SV661	Non-ferrous industry	Beerse, Belgium	Diels and Mergeay (1990)

*Genome sequence available

^aCulture Collection, University of Göteborg, Sweden^bInternational Space Station

2.2 The Megaplasms pMOL28 and pMOL30

Like *C. metallidurans* CH34, most of the related bacteria isolated from metal-rich biotopes carry megaplasms conferring high level resistance to heavy metals. The size of these megaplasms ranges from 180 to 450 kb and some of them are conjugative with much higher transfer frequencies than those found in CH34, based

on zinc, cobalt or cadmium as selective marker (Diels et al. 1989, 1995). However, up to now, only pMOL28 and pMOL30 from *C. metallidurans* CH34 have been sequenced (fully closed), thoroughly annotated and studied at the transcriptomic level after various challenges by heavy metals (Janssen et al. 2010; Monchy et al. 2006a).

Plasmid pMOL28 (171 kb) confers resistance to nickel and cobalt (Liesegang et al. 1993; Mergeay et al. 1985), chromate (Nies et al. 1990) and mercury (Diels et al. 1985). It is self-transferred at low frequency (Diels et al. 1989), although it contains an almost complete conjugative gene set similar to that carried by megaplasmid pHG1 from *C. eutrophus* H16, which is self-transferrable at high frequency (Friedrich et al. 1981). The conjugative transfer of pMOL28 can be enhanced through co-transfer with the BHR IncP-1 α plasmid RP4. This boosted transfer is assisted through transposition of Tn4378 (see Sect. 2.4.2), cointegrate formation and resolution. In addition, pMOL50, a derivative of pMOL28 obtained from a survivor of growth at 37 °C, was found to be highly conjugative and was used to make a circular genetic map of the *C. metallidurans* CH34 chromosome (Sadouk and Mergeay 1993). It was not perceived at that time that this map did not include the genes now known to be carried by the chromid.

Two distinct conserved synteny blocks are shared by pMOL28, plasmids carried by other *C. metallidurans* strains such as NA1, NA4, NE12 and HMR-1, pHG1 from *C. eutrophus* H16 (Schwartz et al. 2003), pBB2 from *C. necator* N-1 (Poehlein et al. 2012), and to a lesser extent pRALTA from *C. taiwanensis* LMG19424 (Amadou et al. 2008) and pBVIE02 from *Burkholderia vietnamiensis* G4. In pMOL28, and likely also for similar plasmids from other *C. metallidurans* strains, these synteny blocks are separated by genomic islands (GIs) that group all genes involved in heavy metal resistance (Mergeay et al. 2009). This indicates that related β -proteobacteria carry plasmids with similar backbone functions (replication, maintenance and transfer) but with additional and different accessory genes contributing to the adaptation to their specific niches. The backbone similarity between pMOL28 and pHG1, pRALTA or pBB2 is quite variable but low for *parAB* and *repA* suggesting plasmid compatibility.

Plasmid pMOL30 (234 kb) confers resistance to mercury (Diels et al. 1985), zinc, cadmium and cobalt (Mergeay et al. 1985; Nies 1995), lead (Borremans et al. 2001), silver (Bersch et al. 2011), and copper (Collard et al. 1994). It is not self-transmissible but it can be mobilized at very low transfer frequency probably with the aid of conjugation genes located on other replicons. Comparable with pMOL28, its conjugative transfer can also be enhanced through co-transfer with the BHR plasmid RP4 via transposition of Tn4380 (see Sect. 2.4.2). All pMOL30 genes related to heavy metal resistance are also located on genomic islands, while the backbone is only shared with plasmids from other *C. metallidurans* strains such as NA1, NA4 and NE12 and to lesser extent with pBVIE01 of *B. vietnamiensis* G4 (Mergeay et al. 2009).

Recently, *C. metallidurans* strains from different sites, ranging from the pharmaceutical and space industry to metal mining and metal industries, waste treatment plants and even human infection, have all (except one) been shown to carry at least

one megaplasmid (Mijnendonckx et al. 2013; Van Houdt et al. 2012b). For all strains, comparative genome hybridization (CGH) showed a strong conservation of the pMOL28 and pMOL30 GIs and their accessory genes related to metal resistance, while more variation was observed for the backbones (Van Houdt et al. 2012b). For instance strain 31A, which carries the two megaplasms pTOM8 and pTOM9 (Schmidt and Schlegel 1989), showed only a positive hybridization signal with the pMOL28 backbone, indicating that one of the plasmids has a backbone related to pMOL28, while the other has a backbone unrelated to pMOL30 (Van Houdt et al. 2012b). These observations extend the biotopes where *C. metallidurans* hosts that carry typical metal resistance plasmids are found beyond just metal-contaminated sites. Therefore, these resistance determinants could be acquired earlier in evolution, which would be consistent with the hypothesis that toxic metal resistance systems pre-existed the recent anthropogenic activities and arose soon after life began, in a world already polluted by volcanic activities (Silver and Phung 1996). However, taking into account that most of the metal determinants are on the native megaplasms and the GIs thereon, it could be argued that anthropogenic activities and industrially polluted environments provided a selective pressure for the conservation of these determinants or even the acquisition of more, considering both the arsenal of determinants as well as the level of resistance.

2.3 The *C. metallidurans* CH34 Chromid: A Diversified Reservoir of Metal Response Genes

As previously mentioned, all *Cupriavidus* and *Ralstonia* genomes are typified by the presence of an additional replicon designated as the chromid. For *Cupriavidus* chromids, the gene cluster *csp repA csp parA parB xerD*, which is involved in replication and partitioning, is strongly conserved. The *repA* upstream region contains 3 putative DnaA boxes and many repetitive 17 nt-long elements with a highly conserved motif, which may be RepA binding sites (Janssen et al. 2010). The partition proteins belong to the ParAB superfamily, as is the case for most chromids, and these Par proteins tend to phylogenetically group with plasmid-encoded Par proteins rather than those encoded by primary chromosomes (Dubarry et al. 2006). Analysis of the similarity between and phylogenetic relationships of the chromid-encoded ParA and ParB proteins from 19 strains belonging to the *Cupriavidus/Ralstonia* and *Burkholderia* lineages, indicates that two distinct plasmids may have been the origin of the chromids present in the genera *Cupriavidus/Ralstonia* and *Burkholderia* (Lykidis et al. 2010).

The majority (7 out of 12) of the RND (Resistance-Nodulation- Division)-driven efflux systems of *C. metallidurans* CH34 are located on the chromid. Most of these systems still remain to be fully elucidated like *cusDCBAF*, *zniBAC* and *zneBAC*, while others are most likely inactive (*nimBAC* inactivated via insertion of *ISRme3*

in *nimA*; *hmyCBA*, insertion of IS1088 into *hmyA*; *hmvCBA*, nonsense mutation in *hmvA*; *czcC₂B₂A₂*, insertion of Tn6050 in *czcB₂*). It could be that the chromid allowed adaptation to environments with moderate heavy metal concentrations, for instance in a (volcanic) biotope colonized by an ancestor of CH34. The formation and acquisition of the megaplasmids pMOL28 and pMOL30 may well be key to surviving high metal concentrations in industrial biotopes, and could putatively have led to the inactivation of redundant genes.

Two particular RND loci on the CH34 chromid attract attention. The first locus codes, in addition to the RND system (*czcI₂C₂B₂A₂*), also for an ATPase (*zntA*). It is part of a larger conserved synteny block in (at least) CH34, H1130, NA4 and NE12, indicating that it is part of the chromid core. The RND locus is separated from its divergently transcribed cognate two-component regulatory system *czcRS* by *ubiG* coding for a 3-demethylubiquinone-9 3-methyltransferase. The latter participates in the biosynthesis of ubiquinone, a mobile redox carrier in the respiratory chain. In *Escherichia coli*, ubiquinone and demethylmenaquinone have been shown to affect the ArcB sensor kinase activity of the two-component system AcrBA (Sharma et al. 2013; Alvarez et al. 2013). Further downstream, the *czcL*, *hns* and *mmmQ* genes are present, coding for an unknown protein, an H-NS like protein and a small stress responsive protein, respectively. The CzcC₂B₂A₂ efflux pump is highly identical (60–80 %) to CzcCBA encoded by pMOL30, suggesting efflux of similar divalent cations (Cd²⁺, Zn²⁺ and Co²⁺). In CH34, however, *czcB₂* is inactivated by Tn6050 insertion and *czcI₂C₂B₂'* is separated from *czcB₂"A₂* through a large chromosomal inversion via homologous recombination with a second Tn6050 copy (Van Houdt et al. 2009). The efflux pump appears to be intact in all other strains.

The second locus holds two RND systems and the uncharacterized membrane-anchored protein ZnfP (Fig. 2.1). Again the locus is part of a larger conserved synteny block in (at least) CH34, H1130, NA4 and NE12, indicating that

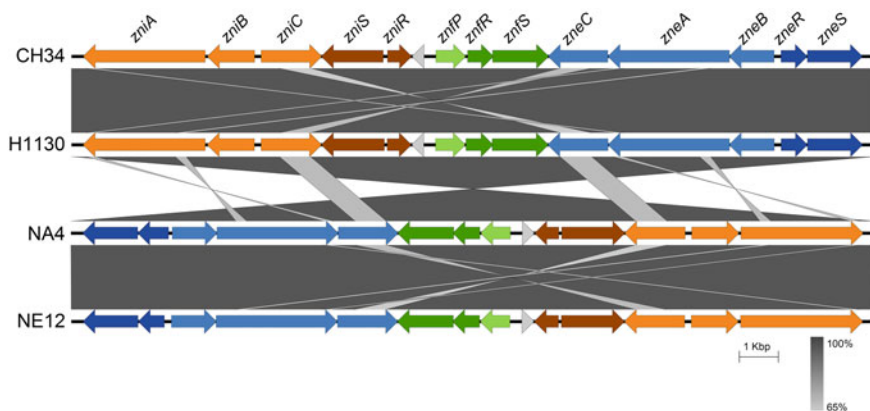


Fig. 2.1 Map showing the genomic organization of two RND systems on the chromid of *C. metallidurans* CH34, H1130, NA4 and NE12. Cognate two-component regulatory systems are coloured darker than their proposed regulon. The grey scale indicates the levels of synteny

it is part of the chromid core. The *zne* cluster is likely linked to zinc resistance (see volume II) and has a *BAC* gene order instead of the more common *CBA*. Also the *zniABCSR* cluster has an uncharacteristic structure, with both the efflux pump as well as the regulatory system coding genes being divergently transcribed (Fig. 2.1). Although *zne* and *zni* systems with similar gene order arrangements are found in many *Ralstonia* spp., they are not present in other *Cupriavidus* species. Next to the RND-driven efflux systems, the chromid also carries a cluster of genes involved in chromate resistance and one involved in copper resistance. Both have a more extensive counterpart on pMOL28 and pMOL30, respectively (see Chap. 3). The chromid-located cluster involved in chromate resistance codes for a chromate efflux pump ChrA₂ (Rmet_3865), a transcriptional repressor ChrF₂ (Rmet_3864), and a putative transcriptional activator ChrB₂ (Rmet_3866) (Juhnke et al. 2002). Both the chromid- and pMOL28-encoded efflux pump belong to the CHR2 subgroup (Diaz-Perez et al. 2007) of the CHR transporter family (first described by Nies et al. 1998). The locus is located on a highly conserved synteny block (>160 kb) shared by CH34, NA4 and H1130. Strains NA1, NA2 and HMR-1 also share this highly conserved synteny block, but not the chromate cluster. The chromid-located basic *copS₂R₂A₂B₂C₂D₂* encodes for homologs of the Cop/Pco system responsible for the handling of periplasmic copper (Bondarczuk and Piotrowska-Seget 2013).

2.4 Mobile Genetic Elements and Genomic Islands in *C. metallidurans* CH34

2.4.1 Genomic Islands

The *C. metallidurans* CH34 genome harbours at least 22 GIs, which were identified via a composite set of criteria (Van Houdt et al. 2009; Mergeay et al. 2009) and CGH (Van Houdt et al. 2012b). Thirteen GIs were identified on the chromosome, four on the chromid, three on pMOL28 and two on pMOL30 (Table 2.2). The available information on these GIs, which was detailed in previous studies (Van Houdt et al. 2009, 2012b; Mergeay et al. 2009), will be summarized and updated below.

2.4.1.1 GIs on the CH34 Chromosome

The largest island (109 kb) on the chromosome, CMGI-1, is almost identical (>99.95 % DNA sequence similarity) to PAGI-2 isolated from the pathogenic *Pseudomonas aeruginosa* strain clone C (Klockgether et al. 2007; Larbig et al. 2002). However, the integrase gene of CMGI-1 is inactivated by Tn6049. CMGI-1 and PAGI-2 do not carry any specific catabolic functions but encodes proteins for the complexation and transport of heavy metals. Both belong to the large

Table 2.2 Genomic islands identified in *C. metallidurans* CH34

Element (size kb)	Location (Rmet_)	Features
CMGI-1 (109.6)	CHR (2287-2408)	Identical to island PAGI-2C of <i>Pseudomonas aeruginosa</i> clone C, TBSSR ^a inactivated by Tn6049
CMGI-2 (101.6)	CHR (1236-1351)	Tn4371 ICE family (ICE _{Tn4371} 6054), involved in hydrogenotrophy and degradation of aromatic compounds
CMGI-3 (97.0)	CHR (1465-1560)	Tn4371 ICE family (ICE _{Tn4371} 6055), involved in hydrogenotrophy and CO ₂ fixation
CMGI-4 (56.5)	CHR (2987-3045)	Tn4371 ICE family (Δ ICE _{Tn4371} 6055), partial element
CMGI-5 (25.4)	CHR (2824-2847)	Remnant of integrated plasmid
CMGI-6 (17.6)	CHR (1997-2020)	TBSSR, no defined function for accessory genes
CMGI-7 (11.0) ^b	CHR (0316-0326)	TBSSR is part of a four-gene module (PRQ)
CMGI-8 (12.3)	CHR (2549-2561)	TBSSR, no defined function for accessory genes
CMGI-9 (20.6)	CHR (2156-2172)	TBSSR, no defined function for accessory genes
CMGI-10 (21.0)	CHR (3347-3368)	No defined function for accessory genes
CMGI-11 (10.8)	CHR (1660-1668)	Putative fimbrial operon, flanked by two IS elements (IS _{Rme7})
CMGI-12 (9.1)	CHR (2662-2670)	No defined function for accessory genes
CMGI-13 (15.9)	CHR (2723-2737)	Genes involved in polysaccharide biosynthesis
CMGI-A (87.1)	Chromid (4428-4305)	No defined function for accessory genes
CMGI-B+D (160.7)	Chromid (4475-4496) Chromid (4598-4715)	Multiple genes coding for phage-related proteins (associated to phages of <i>R. solanacearum</i>)
CMGI-C (7.1)	Chromid (4450-4556)	TBSSR fragment, gene coding for mannose-binding lectin
CMGI-E (120.2)	Chromid (5454-5568)	Tn7-related genes at one extremity, genes putatively involved in degradation of aromatic compounds
CMGI-28a (45.9)	pMOL28 (6212-6333)	heavy metal resistance genes (<i>mer</i> , <i>cnr</i> and <i>chr</i>), flanked by IS1071 elements
CMGI-28b (23.0)	pMOL28 (6252-6263)	TBSSR is part of a four-gene module (PRQ), three <i>rhs</i> -like genes
CMGI-28c (15.0)	pMOL28 (6320-6332)	TBSSR, no defined function for accessory genes

(continued)

Table 2.2 (continued)

Element (size kb)	Location (Rmet_)	Features
CMGI-30a (74.4)	pMOL30 (6002-6171)	TBSSR, heavy metal resistance genes (<i>mer</i> , <i>czc</i> and <i>pbr</i>) flanked by Tn4380 elements (one intact and one truncated copy)
CMGI-30b (88.0)	pMOL30 (6153-6067)	TBSSR, heavy metal resistance genes (<i>sil</i> , <i>ncc-nre</i> , and <i>cop</i>)

^aTBSSR: tyrosine-based site-specific recombinase

^bUpdated compared to previous studies

pKLC102/PAGI-2 family of elements that share a core gene set (Miyazaki et al. 2013; Klockgether et al. 2007) and are integrated downstream of tRNA genes (Larbig et al. 2002). Self-transfer of PAGI-2 has not been detected so far, in contrast to the *clc* element of *Pseudomonas knackmussii* B13, the best studied of this family, which enables the metabolisation of chlorocatechols (Miyazaki et al. 2013). Accordingly, most of the PAGI-2C genes are transcriptionally silent in *P. aeruginosa* clone C (Klockgether et al. 2008). CMGI-1 carries the *pbrR₂ cadA pbrC₂* cluster involved in cadmium, zinc and lead resistance (see Chap. 3) (Monchy et al. 2006b, 2007; Monsieurs et al. 2011; Legatzki et al. 2003). Through the analysis of conserved synteny blocks, the latter cluster led to the identification of transposon Tn6052 in *Burkholderia xenovorans* LB400 (recently re-evaluated as *Paraburkholderia xenovorans*; Sawana et al. 2014), which carries eight copies of this transposon harbouring the accessory genes *pbrR cdfX pbrC*, with *cdfX* coding for a putative metal-efflux system that is distantly related to cation diffusion factors (Van Houdt et al. 2009). Thus, it provides a new mobile combination of metal-processing genes. This is one of the examples for which the analysis of synteny blocks reveals the presence of a mobile genetic element (MGE), thereby uncovering the diverse variety of MGEs from environmental β -proteobacteria.

Three of the chromosomally-located *C. metallidurans* CH34 GIs (CMGI-2, CMGI-3 and CMGI-4) belong to the Tn4371 ICE family (Van Houdt et al. 2013, 2009; Toussaint et al. 2003). This family harbours MGEs with a quadripartite structure consisting of three core sets involved in integration (via a tyrosine-based site-specific recombinase (TBSSR)), maintenance/stability and conjugation, and one variable set containing accessory genes with diverse functions including xenobiotic catabolism, heavy metal resistance, antibiotic resistance and chemolithotrophic metabolism. The accessory genes are typically located between the conjugative genes *rlxS* (*virD2*) and *traR* (Van Houdt et al. 2013; Ryan et al. 2009; Toussaint et al. 2003; Merlin et al. 1999). Currently, this ICE_{Tn4371} family harbours more than 40 elements residing in β - and γ -proteobacteria with sizes ranging from 38 to 101 kb. CMGI-2 (ICE_{Tn4371}6054) harbours accessory genes involved in the degradation of aromatic compounds as well as in hydrogen oxidation. CMGI-3 (ICE_{Tn4371}6055) harbours genes involved in carbon dioxide fixation (Calvin-Benson-Bassham cycle) and hydrogen oxidation. Therefore, the ability of CH34 to degrade toluene or to

grow on hydrogen gas and carbon dioxide are enabled by CMGI-2 and CMGI-3. The third GI from this family, CMGI-4 ($\Delta\text{ICE}_{\text{Tn}437/6056}$), is only a partial element.

As mentioned in previous studies, CMGI-2 harbours two RIT (recombinase in trio) elements consisting of three adjacent and unidirectional overlapping genes encoding tyrosine-based site-specific recombinases (Van Houdt et al. 2009). The three TBSSRs in these RIT elements are associated with distinct TBSSR subfamilies and the elements were later found to be much more widespread (Van Houdt et al. 2012a; Ricker et al. 2013).

The contribution of the remaining chromosomally-located GIs to particular CH34 functions is unclear (Table 2.2). Although the cluster involved in arsenic resistance (Rmet_0327-Rmet_0334) was initially attributed to CMGI-7, this becomes less evident when compared with recent *C. metallidurans* genome sequences. The latter indicates that the arsenic resistance cluster is part of a conserved synteny block in all *C. metallidurans* strains (chromosomal backbone) irrespective of the presence of other CMGI-7 genes. Therefore, CMGI-7 probably only contains the Rmet_0316-Rmet_0326 region, which includes transposon Tn6049 (in case of CH34) and a PRQ module in addition to two conserved genes coding for unknown functions. The PRQ term was used to label a synteny module coding for three putative TBSSRs and a conserved hypothetical protein. In fact, two different PRQ modules, which share very little sequence identity, were found in strain CH34. The second one is associated with genomic island CMGI-28b in plasmid pMOL28. Both share synteny with gene blocks conserved in other bacterial genomes.

2.4.1.2 GIs on the CH34 Chromid and on Megaplasms pMOL28 and pMOL30

At least five GIs were identified on the *C. metallidurans* CH34 chromid (Table 2.2) (Van Houdt et al. 2012b). One island, CMGI-B+D, carries multiple genes coding for phage-related proteins. CMGI-E carries genes putatively involved in the degradation of aromatic compounds (vanillate O-demethylase oxygenase and phthalate 4,5-dioxygenase) (Van Houdt et al. 2012b).

As indicated in Sect. 2.2, plasmid pMOL28 and pMOL30 contain large genomic islands that harbour all plasmid-borne genes involved in the response to heavy metals. Plasmid pMOL30 carries CMGI-30a and -30b that convey resistance to cadmium, zinc, cobalt, lead and mercury, and copper and silver, respectively. Within CMGI-30a, a 25-gene cluster is highly conserved (>99 % nucleotide similarity) in all *C. metallidurans* strains (Van Houdt et al. 2012b) and in other strains like *R. pickettii* 12J (Fig. 2.2). This cluster contains next to the *czc* cluster, genes involved in membrane-related functions with *gtrM1* (Rmet_5966) and *gtrB1* (Rmet_5968) encoding for a glycosyl transferase, *gtrA1* (Rmet_5967) for a bactoprenol-linked glucose translocase, *ompP* (Rmet_5974) for a porin predicted to form a channel for the diffusion of small hydrophilic molecules, and *flgB* (Rmet_5971) for a flagellar basal body rod protein. Furthermore, this cluster is flanked by a TBSSR from a distinct family (Van Houdt et al. 2012a), which is

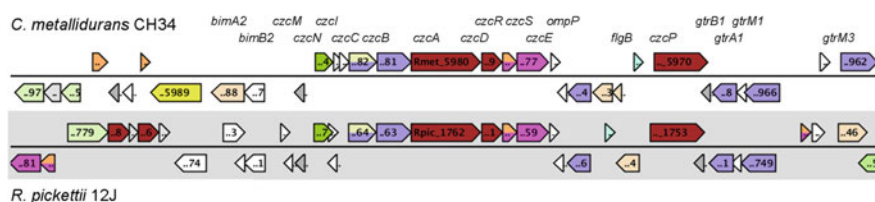


Fig. 2.2 Genomic context of the *czc* cluster on pMOL30 from *C. metallidurans* CH34 and in *R. pickettii* 12J constructed with MGcV (Overmars et al. 2013). Genes are coloured based on COG assignment (Overmars et al. 2013)

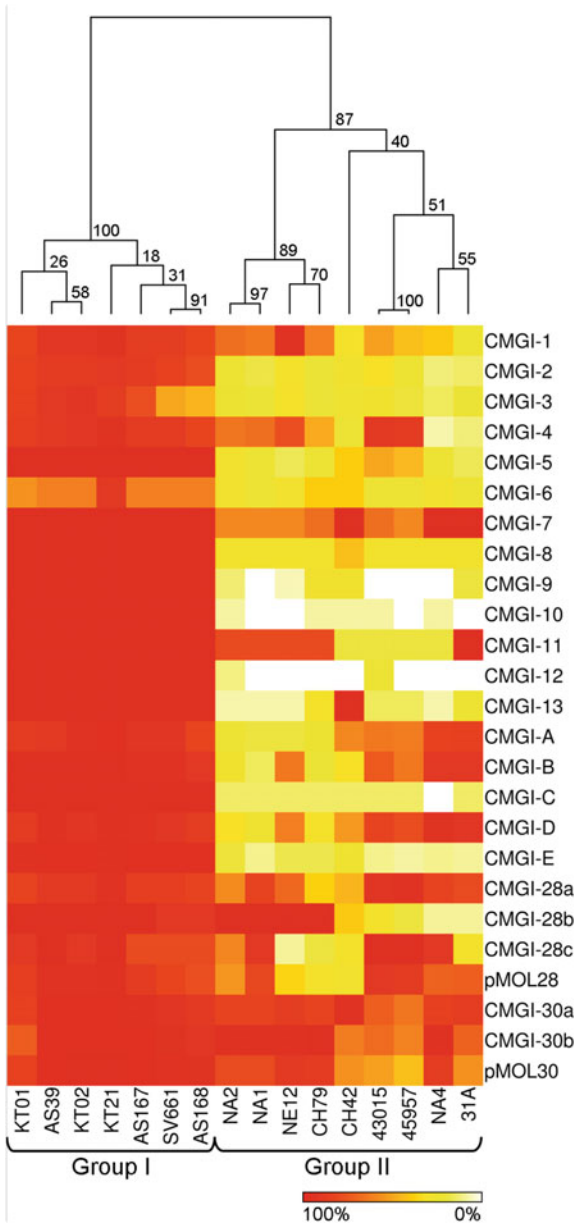
always associated with a second conserved protein of unknown function (putative transcriptional repressor) making up a bipartite module (BIM) (Van Houdt et al. 2009, 2012a). CMGI-30b contains a large cluster of 32 genes induced by copper (Monchy et al. 2006a, 2007; Monsieurs et al. 2011; Mergeay et al. 2009), which is unique with respect to its complexity compared to other copper resistance mechanisms described so far (see Chap. 3). Similar to the *czc* cluster on CMGI-30a also the *cop* cluster contains genes involved in membrane-related functions (*gtrM*₂, *gtrB*₂, *gtrA*₂ and *ompP*₂).

Three GIs were defined on plasmid pMOL28 (Table 2.2). CMGI-28a is delimited by two inactive Tn3-like insertion sequences (*IS1071* inactivated by *Tn6049* and *ISRme18*) and carries all metal resistance determinants of pMOL28, thereby providing resistance to mercury, chromate and cobalt/nickel (see Chap. 3). The functions of the accessory genes of the two other GIs are unidentified. As indicated above, CMGI-28b carries the second PRQ module (*Rmet_6252-6255*; *prqA2B2C2R2*). In addition, it contains three genes coding for RHS-repeat-containing proteins. Rhs-like family proteins have been associated with various phenotypes such as social motility, virulence, toxin production and contact-dependent growth inhibition (Youdarian and Hartzell 2007; Poole et al. 2011; Busby et al. 2013; Chen et al. 2014; Koskiniemi et al. 2013, 2014; Kung et al. 2012; Sisto et al. 2010). However, none of the three proteins carry the Rhs repeat-associated core domain (IPR022385, Hunter et al. 2012) nor the conserved motif, ending in DPXG-(18)-DPXG, that is shared by all Rhs proteins (Jackson et al. 2009).

2.4.1.3 GIs on Other *C. metallidurans* Strains

Interestingly, CGH of *C. metallidurans* strains from different sites, ranging from the pharmaceutical and space industry to metal mining and metal industries, waste treatment plants and even human infection, indicated that these strains could be divided into two main groups based on their GI content. One group carries almost all GIs identified in CH34, while these GIs were much less abundant in the second group (Van Houdt et al. 2012b) (Fig. 2.3). Although the incidence of GIs was in agreement with the functional phenotypes carried by them such as degradation of

Fig. 2.3 Hierarchically (complete-linkage) clustered heat map based on CGH results related to plasmids and GIs of 16 different *C. metallidurans* strains to a whole-genome oligonucleotide DNA microarray of type strain CH34. Bootstrap values (%) from 1000 times resampling are shown at each dendrogram node (Van Houdt et al. 2012b)



toluene or the ability to grow on hydrogen gas and carbon dioxide, the isolation site characteristics and location (geographic) showed no clear correlation with the occurrence of GIs.

2.4.2 Insertion Sequence Elements and Transposons

Insertion sequence (IS) elements are small elements (typically less than 3 kb) that are capable of independent transposition and carry one or more open reading frames generally only encoding gene products essential for their mobility. They are flanked by short inverted repeat sequences and generate short directly repeated sequences of the target DNA at the point of insertion, which are 2–14 bp in size and specific for a certain element (Mahillon and Chandler 1998; Siguier et al. 2014). The CH34 genome harbours 21 distinct IS elements totalling 57 intact IS elements (Table 2.3), which are dispersed over the four replicons with most of them located on the chromosome (the largest replicon). All IS elements were categorized into 10 families, with the IS3, IS30, and IS5 families being the most abundant. The IS3 and IS5 families are in fact the most abundant families among bacterial genomes (together with IS1 and IS481) (Touchon and Rocha 2007; Wagner et al. 2007).

IS elements and their transposition have been implicated in the evolution of their hosts as they contribute to a variety of genomic rearrangements (deletions, inversions and duplications) that can result in gene inactivation and/or affect the expression of

Table 2.3 Distribution of IS elements in *C. metallidurans* CH34

Element	Family (sub)	Length (bp)	CHR	Chromid	pMOL28	pMOL30
ISRme20	IS21	1977	1			
ISRme4	IS21	2469	2			
ISRme9	IS21	2688			1	
IS1090	IS256	1343	4			
ISRme11	IS3 (IS150)	1231		2		
ISRme12	IS3 (IS150)	1454	1			
ISRme17	IS3 (IS150)	1678	1			
IS1087B	IS3 (IS2)	1330	2			
ISRme3	IS3 (IS3)	1288	3	5		2
ISRme15	IS3 (IS51)	1325		1		1
IS1086	IS30	1106	1	1	1	
IS1088	IS30	1103	3	6		
ISRme10	IS30	1063				1
ISRme8	IS4	1455	1	1		
ISRme5	IS481	1041	3	1		
ISRme1	IS5 (IS427)	1331	2	2		
ISRme6	IS5 (IS427)	913	1			
ISRme7	IS6	840	2			
ISRme19	IS66	2227				1
IS1071	Tn3	3204	3		1*	
ISRme18	Tn3	ND			1	

CHR chromosome. ND not determined

*Copy inactivated by Tn6049

adjacent genes (Bentley et al. 2008; Bickhart et al. 2009; Hubner and Hendrickson 1997; Lin et al. 2007). Therefore, they are seen as a significant force in the adaptive and evolutionary response of their host, conferring genome plasticity that allows rapid adaptation to new environments (Mira et al. 2002; Schneider and Lenski 2004).

The involvement of IS elements in adaptation has been demonstrated for a number of the IS elements in *C. metallidurans*. IS1086 and IS*Rme1* transposed into the positive selection vectors pJV240 (Dong et al. 1992) and pGBG1 (Schneider et al. 2000). A spontaneous zinc resistant mutant of *C. metallidurans* AE126 was characterized by IS1087 transposition into *cnrY*, which codes for an anti-sigma factor. Inactivation resulted in constitutive expression of the cobalt and nickel resistance determinant *cnr* and in increased (nonspecific) zinc efflux (Collard et al. 1993; Tibazarwa et al. 2000; Grass et al. 2000). Increased heterologous expression of the *czt* (cadmium zinc resistance) operon of *P. aeruginosa* CMG103 in *C. metallidurans* AE104, which resulted in an increased zinc resistance, was mediated by insertion of IS1088 or IS1090 into the *czt* promoter region (Talat 2000). Transposition of IS1086, IS1087B and IS*Rme3* was involved in increased silver resistance (Mijnendonckx et al. 2011). Finally, at least 32 ISs are inserted inside a coding sequence, thereby inactivating the gene and concurrent gene product. Examples are the insertion of IS*Rme3* in *nimA* and IS1088 in *hmyA*, consequently affecting the efflux pumps of the Nim and Hmy heavy metal tri-component efflux systems.

Transposons differ from IS elements as they also carry accessory genes, which confer clinically or metabolically important properties on the host cell (Frost et al. 2005). The CH34 genome harbours five distinct transposon families totalling 19 intact transposons (Table 2.4), which are dispersed over the four replicons with most of them located on the chromosome (the largest replicon). The transposition modules of four transposons are related to those of mercury transposons with Tn4378, Tn4380 and Tn6050 belonging to the Tn21/Tn501 family and Tn6048 to the Tn5053 family. The Tn501 family transposons are typically delineated by 38 bp terminal inverted repeats, contain two genes involved in transposition (*tnpRA*), and generate 5 bp direct target DNA sequence repeats upon insertion (Brown and Evans 1991; Mindlin and Petrova 2013). The Tn5053 family transposons are typically delineated by 25 bp terminal inverted repeats, contain four genes involved in transposition (*tniABQR*), and generate 5 bp direct repeats upon insertion (Kholodii et al. 1995; Mindlin and Petrova 2013). The latter transposons have been designated *res* site hunters as they exhibit a striking insertional preference for the *res* regions of naturally occurring plasmids (Minakhina et al. 1999). The transposition module of Tn6049 could, at this moment, not be categorized.

Transposons Tn4378 and Tn4380 carry identical mercury resistance determinants (see Chap. 3) and are located on pMOL28 (in CMGI-28b) and pMOL30 (in CMGI-30a), respectively (Diels et al. 1985; Mergeay et al. 2009; Van Houdt et al. 2009). Transposon Tn4380 is present in most of the *C. metallidurans* strains (Van Houdt et al. 2012b) and is largely recognizable (>99 % nucleotide similarity) in plasmid BRA100 and pMAB01 from the actinobacterial strains *Mycobacterium abscessus* subsp. *bolletii* F1725 and INCQS 00594 suggesting an effective

Table 2.4 Transposons identified in *C. metallidurans* CH34

Transposon	Family	Accessory genes	Function	Length (kb)	CHR	Chromid	pMOL28	pMOL30
Tn4378	Tn21/Tn501	<i>merRTPADEurf-2</i>	Mercury resistance	8.2			1	
Tn4380	Tn21/Tn501	<i>merRTPADEurf-2</i>	Mercury resistance	8.0				1
Tn6048	Tn5053	<i>mnfABC₇DC₂RSM</i>	Unknown, induced by Zn and Pb	10.4	1	2		
Tn6049		(<i>gspA</i>)	Unknown	3.5	7	3	1	1
Tn6050	Tn21/Tn501	<i>sulP, uspA, dksA</i>	Unknown	6.8		2		

interphylum horizontal gene transfer. This is supported by the fact that both pMAB01 and BRA100 are BHR plasmids from the IncP-1 β subgroup (Leao et al. 2013). The Tn4380 *mer* genes are also observed with 100 % identity in the IncP-1 β plasmid pJP4 from *C. pinatubonensis* JMP134, mainly studied for its herbicide degradation genes (Don and Pemberton 1985; Lykidis et al. 2010), and other BHR plasmids such as the IncP-1 β plasmid pADB1 (Martinez et al. 2001) and the IncP-1 γ plasmid pQKH54 (Haines et al. 2006).

While Tn4378 and Tn4380 carry mercury resistance determinants (see Chap. 3), Tn6048 and Tn6050 carry other less common accessory genes. Tn6048 harbours the *mmfABC₁DC₂RSM* cluster encoding a two-component regulatory system (MmfRS) putatively regulating the expression of a peptidase (MmfA), a phosphatase (MmfB), two uncharacterized membrane-anchored proteins (MmfC₁ and MmfC₂) and a drug/metabolite transporter (DMT) super family permease (MmfM). Both MmfC₁ and MmfC₂ show high similarity (37.9 and 78.3 %, respectively) with ZnfP encoded by the *zni-znf-zne* cluster on the chromid (Sect. 2.4.1.2). Transposons highly similar to Tn6048 were found in *C. metallidurans* NA1 (>97 % nucleotide similarity), *Burkholderia* sp. TJI49 (>99 % nucleotide similarity) and in plasmid pBVIE02 from *B. vietnamiensis* G4 (>98 % nucleotide similarity; disrupted by IS insertions). In addition, clusters similar to the *mmf* cluster were found in *Acidovorax delafieldii* 2AN and *Dechloromonas aromatica* RCB. Although the function of this *mmf* cluster is still unknown, these genes apparently participate in the response to heavy metals as they are strongly induced in the presence of zinc and lead. Furthermore, MmfB shows 38 % sequence similarity to BcrC of *Bacillus subtilis*, which has undecaprenyl pyrophosphate phosphatase activity (Bernard et al. 2005), and the undecaprenyl pyrophosphate phosphatase activity of PbrB has been shown to be involved in lead resistance (Hynninen et al. 2009; Taghavi et al. 2009). In addition, overexpression of undecaprenyl pyrophosphate phosphatase in *Bacillus subtilis* increased resistance to bacitracin, which prevents cell wall synthesis by inhibiting the dephosphorylation of undecaprenyl pyrophosphate, and the rare earth element scandium (Bernard et al. 2005; Inaoka and Ochi 2012). Noteworthy, identical copies of Tn6048 were also identified on BHR plasmids pMOL98 (Van der Auwera et al. 2009) and pAKD16 (Sen et al. 2011). However, both plasmids probably captured Tn6048 as a result of the used exogenous isolation procedure with a CH34 derivative as recipient (Top et al. 1994; Van der Auwera et al. 2009; Sen et al. 2011). The accessory genes of Tn6050 code for a sulfate permease (SulP), a universal stress protein (UspA) and a DnaK suppressor-like protein (DksA). Their role is still unknown but a functional association could be assumed as these accessory genes (or a part) are found alone or in combination with antibiotic resistance determinants in other Tn3-related transposons such as Tn6001, Tn1403 and Tn1404* derived from clinical and environmental *Pseudomonas* strains (Tseng et al. 2007; Stokes et al. 2007), and Tn1013 from the IncP-1 α plasmid pBS228 (Haines et al. 2007). The DksA protein has been shown to be a global transcriptional regulator that acts as co-regulator along with the archetypical alarmone ppGpp for controlling the stringent response (Paul et al. 2005; Kanjee et al. 2012). Furthermore, the redox active thiols in the 4-cysteine zinc-finger motif of DksA

(which is also present in the DksA encoded by Tn6050) sense oxidative and nitrosative stress, thereby fine-tuning the expression of the translational machinery and amino acid assimilation and biosynthesis (Henard et al. 2014). Therefore, although speculative, Tn6050 could contribute to the adaptation to metabolic stress imposed by harsh environmental conditions.

These observations illustrate the modular character (of this group) of transposons, that is, different transposition modules associated with diverse mercury resistance cassettes as well as other accessory genes.

Transposon Tn6049 harbours four genes (*tnmB*, *gspA*, *tnmA*, and *tnmC*). The GspA protein sequence shows a conserved domain related to ATPases associated with type II protein secretion systems. However, the function of its putative accessory genes is still unknown. The association of Tn6049 with genomic islands is striking (at least 8 out of the 12 copies) (Van Houdt et al. 2009). Therefore, it appears to play a peculiar role in “stabilizing” genomic islands. Furthermore, inactivating transposase/integrase genes should immobilize and fix the affected genomic island in the genome.

2.5 Concluding Remarks

The genome of *C. metallidurans* CH34 harbours a high number and diversity of mobile genetic elements such as plasmids, genomic islands, integrative and conjugative elements, transposons and insertion sequence elements, but also novel types of putative elements such as the RIT elements, BIM and PRQ modules. Many of these elements contribute to its adaptation potential to certain niches. In particular for heavy metal resistance, an important role for the megaplasmids pMOL28 and pMOL30 has repeatedly been demonstrated, conferring resistance to cadmium, chromate, cobalt, copper, mercury, nickel, lead and zinc. Since the backbones of these megaplasmids are similar to those found in other strains unable to withstand high heavy metal concentrations, particular traits probably integrated into an ancestor, resulting in pMOL28 and pMOL30. Other mobile genetic elements that contribute to the heavy metal response range from genomic islands and transposons carrying specific accessory genes to insertion sequences that affect regulatory functions.

In addition to these mobile genetic elements, also the CH34 chromid carries a diversified reservoir of metal response genes. For instance, the majority of the RND-driven efflux systems are located on the chromid, although most of these systems are inactivated or have unknown functions. Inactivation of these efflux systems could be due to redundancy by the acquisition of pMOL28 and pMOL30. Alternatively, these efflux systems acquired by an ancestor of CH34 could efflux elements rarely found in high concentrations in modern industrialized environments, thereby relaxing selection pressure.

While most metal resistance determinants are shared by all *C. metallidurans* strains, irrespective of the strain’s isolation type and place (bear in mind that all

isolates come from anthropized environments), substantial differences in the diversity and size of their mobile gene pool was observed. For instance, reflected by a strain's ability to degrade toluene or to grow on hydrogen gas and carbon dioxide. The varying genomic and genetic contexts can affect the interaction patterns and phenotypic compensations between the involved proteins, therefore, comparative studies using different *C. metallidurans* strains may be helpful to map the diversity in regulatory circuits and to characterize unknown response mechanisms to stressors and heavy metals in particular. Furthermore, comparative studies will allow revealing the full adaptation potential of the *C. metallidurans* species to heavy metals.

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