Cupriavidus metallidurans CH34, a historical perspective on its discovery, characterization and metal resistance

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Abstract

Cupriavidus metallidurans, and in particular type strain CH34, became a model bacterium to study bacterial resistance to metals. Although nowadays the routine use of a wide variety of omics and molecular techniques allow refining, deepening and expanding our knowledge on adaptation and resistance to metals, these were not available at the onset of *C. metallidurans* research starting from its isolation in 1976. This minireview describes the early research and legacy tools used to study its metal resistance determinants, characteristic megaplasmids, ecological niches and environmental applications.

Introduction

This essay describes the early steps of the research focusing on *Cupriavidus metallidurans* CH34, starting from its isolation in 1976 (Houba 1976) up to its first environmental applications in the early nineties. During most of this period, *C. metallidurans* was still named *Alcaligenes eutrophus* before its transfer to the genus *Ralstonia* (Yabuuchi *et al.* 1995) and later to the genus *Cupriavidus* (Vandamme and Coenye 2004). *In vivo* genetic tools in combination with restriction

maps and DNA hybridization laid the foundation for understanding CH34, its metal resistance and future molecular and applied approaches.

Isolation of zinc- and cadmium-resistant bacteria in a region with historical pollution

More than fifty years ago, resistance to metals, e.g. cadmium, mercury and arsenic, was discovered in antibiotic-resistant pathogenic and commensal bacteria and both resistance determinants were found to be harbored by transmissible plasmids (Novick and Roth 1968; Peyru, Wexler and Novick 1969; Silver *et al.* 1981; Smith and Novick 1972; Summers and Silver 1972; Summers and Silver 1978). Although these observations were initially focused on clinically relevant settings and pathogens, other environments attracted attention to assess and compare the occurrence and diversity of metal-resistant bacteria and their plasmids in soils, polluted waters and sediments from highly polluted industrial environments.

In Liège, Belgium, pollution by heavy metals in the Meuse valley was a major concern and initiated a collaboration, led by J. Remacle, focusing on waters from a decantation basin of a zinc factory in Engis (Mergeay 2015). These waters contained 2000 mg/L Zn^{2+} and 2 mg/L Cd^{2+}, and showed a very low viable count on rich media supplemented with 2 mg/L Cd^{2+} (Houba 1976). This led to the isolation of cadmium-resistant bacteria that resisted higher Cd^{2+} concentrations than enteric and pathogenic bacteria in which cadmium-resistance was described. As expected, resistance to Zn^{2+} was confirmed for these isolates and strain CH34 was selected as a representative (Houba 1976; Mergeay, Houba and Gerits 1978). These first observations were complemented with evidence on the extrachromosomal nature of CH34's metal resistance genes via plasmid curing and conjugational transfer assays (Mergeay, Gerits and Houba 1978). As such, these studies pioneered in describing the molecular background of bacteria metal resistance and their impact on ecology.

CH34's attractive phenotypic traits and the beginning of a long taxonomic roaming

Its resistance to metals was further explored, revealing resistance to nickel, cobalt and copper, and even mercury. The cells appeared to be pseudomonad-like and utilization of a broad range of carbon sources was tested, demonstrating among others growth on alcohols (e.g. mannitol) and long chain dicarboxylic acids (e.g. azelaic acid), carbon dioxide and hydrogen gas (hydrogenotrophy), and the inability to use sugars. These characteristic observations turned out to be valuable selection and counterselection tools in heterospecific matings. After the first global phenotypic characterization, CH34 lost designation as *Pseudomonas* and subsequently was reclassified as *Hydrogenomonas palleroni*, followed by a series of confusing taxonomic changes from *Alcaligenes eutrophus* to *Ralstonia eutropha*, *Ralstonia metallidurans*, *Wautersia metallidurans* and finally *Cupriavidus metallidurans* (Mergeay, Gerits and Houba 1978; Mergeay *et al.* 1985; Vandamme and Coenye 2004; Vaneechoutte *et al.* 2004; Yabuuchi *et al.* 1995).

A chemolithautotroph carrying megaplasmids encoding for metal resistance

The presence of megaplasmids carrying a plethora of genes involved in metal resistance, with the archetypes pMOL28 (Figure 1) and pMOL30 (Figure 2) from CH34, turned out to be one of the most characteristic and well-studied features of *C. metallidurans* CH34 and the entire species (Mergeay *et al.* 1985). In fact, the first heavy metal efflux (HME) Resistance Nodulation cell-Division (RND) tri-component efflux systems were identified in pMOL30, with *czcCBA* conferring resistance to Cd^{2+}, Zn^{2+} and Co^{2+} (Nies 1995; Nies *et al.* 1989; Nies and Silver 1989), and pMOL28, with *cnrCBA* conferring resistance to Ni^{2+} and Co^{2+} (Nies, Nies and Silver 1989; Nies, Nies and Silver 1990; Siddiqui, Benthin and Schlegel 1989; Siddiqui and Schlegel 1987).

To summarize, pMOL30 (Figure 2) encodes resistance to zinc, cadmium and cobalt, lead, mercury, silver, and copper. Three different efflux mechanisms are involved in resistance to Cd^{2+}, Co^{2+} and Zn^{2+}, i.e. the cation diffusion facilitator (CDF) CzcD, the P_{IB4}-type ATPase CzcP and the HME-RND (heavy metal Resistance-Nodulation-Division)-driven system CzcCBA. Lead

resistance is mainly mediated by the P_{IB}-type ATPase PbrA and the undecaprenyl pyrophosphate phosphatase/lipoprotein signal peptidase PbrB/PbrC, and mercury by the cytoplasmic mercuric reductase enzyme MerA, the transport system MerTP and the broad-spectrum mercury transporter MerE on Tn*4380*. Silver is detoxified by the HME-RND-driven system SilDCBA, and copper by the P_{IB1}-type ATPase CopF, the HME-RND-driven efflux system SilDCBA and the periplasmic copper detoxification system CopABCD. Plasmid pMOL28 (Figure 1) encodes resistance to nickel and cobalt, chromate and mercury. Nickel and cobalt resistance are mediated by the HME-RND-driven system CnrCBA, chromate is detoxified by the chromate efflux pump $ChrA_1$, and mercury by Tn*4378* (a Tn*21*/Tn*501*-family transposon with a *mer* cluster identical to Tn*4380*). Next to metal resistance, also its chemolithoautothrophic properties attracted attention. Similar to type strain *Cupriavidus necator* H16 (at that time *Alcaligenes eutrophus* H16; see also Vandamme and Coenye (2004) and Makkar and Casida (1987)), strain CH34 also possesses two hydrogenases (Mergeay, Faelen and Schlegel 1985; Mergeay *et al.* 1985). However, the genomic location of the coding genes is different, being on chromosomally integrated Tn*4371*-family conjugative elements for strain CH34 (Merlin *et al.* 1997; Toussaint *et al.* 2003; Van Houdt *et al.* 2013) and on megaplasmid pH16 for strain H16 (Schwartz *et al.* 2003).

Novel metal-resistant bacteria carrying *czc*-like genes

These characteristics of strain CH34 initiated efforts to isolate bacterial strains from various ecological niches that are resistant high metal concentrations based on hybridization processes with specific probes related to *czc* (conferring resistance to Cd^{2+}, Zn^{2+} and Co^{2+}) (Diels *et al.* 1995a; Diels and Mergeay 1990; Diels *et al.* 1995b). Isolates were retrieved from soils and refuse surrounding non-ferrous metal industries and areas desertified by aerial zinc deposits in Belgium (Diels *et al.* 1988b), and soils contaminated with mine refuses from Congo (the copper belt of Katanga) (Diels *et al.* 1988a). Taxonomic studies showed that almost all isolates could be

classified as *Cupriavidus* (Brim *et al.* 1999; Diels and Mergeay 1990). Interestingly, CH34 is more closely related to *C. metallidurans* strains isolated from Congo than from Belgium, which supports the assumption that strain CH34 was transported into Belgium by the import of ores from Congo (Brim *et al.* 1999; Diels and Mergeay 1990; Van Houdt *et al.* 2012). In contrast, isolates from the zinc-desertified areas were classified as *Cupriavidus campinensis* or *Cupriavidus basilensis*. One particular isolate, *C. campinensis* DS185, attracted attention because of its capacity to grow on rich medium at 37°C in contrast to CH34 (see TIMM below) and the very high conjugation frequency of its plasmids harboring metal resistance genes (Diels, Sadouk and Mergeay 1989). The latter reveals genetic diversity and metabolic capabilities among these isolates, which could point to further niche adaptation.

The high prevalence of *Cupriavidus*, and specifically *C. metallidurans*, in harsh environments with nutrient scarcity and toxic compounds such as metals and recalcitrant organics indicated that it is an important member of microbial communities in these environmental niches and initiated subsequent research on the molecular aspects of metal resistance as well as their use in biotechnological applications.

First genetic tools applied to CH34

Early manipulations revealed an intriguing phenomenon of decreased survivability when grown on rich medium at an increased temperature of 37 °C compared to the normal growth temperature of 30 °C (Mergeay *et al.* 1985). The phenomenon was coined as "temperature-induced mortality and mutagenesis" (TIMM) as many surviving cells exhibited one or more aberrant phenotypes, including loss of metal resistance (e.g. Zn^{2+}, Co^{2+}), auxotrophy for amino acids, loss of autotrophy, loss of nitrate utilization, loss of ammonium utilization as nitrogen source, and loss of tyrosine as carbon source (Mergeay *et al.* 1987; Sadouk and Mergeay 1993). Although this complex and unexpected TIMM phenomenon is not yet completely deciphered, a stress response

upon septation defects is involved (Arroua *et al.* 2014). Nevertheless, many of these mutants, and specifically auxotrophic mutations, were very valuable for constructing a chromosomal linkage map of strain CH34. The latter included also mutants obtained by penicillin enrichment but was mainly performed with broad-host-range IncP plasmids carrying a non-infectious derivative of bacteriophage Mu, which enables the formation of R-prime plasmids (Faelen, Resibois and Toussaint 1979; Faelen and Toussaint 1976). The mini-Mu derivative tool developed by Faelen and Toussaint (1976) integrates in the bacterial chromosome and acts a powerful transposable element, producing a kind of "*in vivo* cloning" tool. In this way, a circular map and first description of CH34' chromosome was made before other molecular mapping tools became available and more performant (Sadouk and Mergeay 1993). Noteworthy, the large replicon in addition to the chromosome, which is typical for all *Cupriavidus* and *Ralstonia* genomes, was not revealed at that time (Janssen *et al.* 2010). These large replicons with a size of around 2–3 Mb have been designated both second or secondary chromosomes as well as megaplasmids as they carry chimeric features such as essential genes making them indispensable for cell viability as well as a plasmid-type replication system. Later, to correctly distinguish this replicon, Harrison *et al.* (2010) coined the term "chromid".

The mini-Mu system was also used in *Pseudomonas fluorescens* 6.2, isolated from the rhizosphere of legumes, for which auxotrophic mutants were successfully complemented with derivatives of pULB113 (RP4::mini-Mu) carrying CH34 fragments (Lejeune *et al.* 1983). In addition, R-prime plasmids generated in *P. fluorescens* 6.2 could also complement auxotrophic mutants of CH34 (Lejeune *et al.* 1983). Both examples highlighted the transfer and expression of genes into CH34, which could be exploited for accessing the catabolic/biosynthetic potential of environmental bacteria (Craig, Chang and Brady 2009). Broad-host-range plasmids, more specifically the IncP-1α plasmid RP4, were also used to study the megaplasmids pMOL28 and

pMOL30 in other hosts by increasing their conjugational transfer. Via the formation of a cointegrate mediated by Tn*4378* or Tn*4380* transposition, pMOL28 and pMOL30 could be co-transferred with RP4, thereby increasing transfer frequency from 10^{-8} to 5 x 10^{-5} and less than 10^{-8} to 10^{-3}, respectively (Diels *et al.* 1985; Mergeay *et al.* 2009). Next to conjugative plasmids, mobilizable IncQ plasmids carrying CH34's *czc* resistance determinant were used to isolate conjugative plasmids without having to culture the original host and to assess the plasmid mobilization potential of ecosystems (Top *et al.* 1994; Van der Auwera *et al.* 2009). The use of heterologous complementation, "*in vivo* cloning" and conjugation paved the way not only for cloning and molecular analyses of metal resistance genes but also to catalog the diversity of mobile genetic elements, including transposons and insertion sequences, from strain CH34 (Diels *et al.* 1985; Dong *et al.* 1992; Mijnendonckx *et al.* 2011; Van Houdt *et al.* 2009).

Environmental applications

The combination of different and redundant metal resistance determinants, the ability to degrade recalcitrant organics, and its genetic flexibility allowing easy exchange and expression of genetic material is not only advantageous for *C. metallidurans* CH34 to survive and persist in severe habitats (Taghavi *et al.* 1997), it also allowed the development and use in many environmental technology applications (Collard *et al.* 1994; Diels *et al.* 1999; Diels *et al.* 1987; Diels *et al.* 1991; Diels *et al.* 2009; Simon, Remacle and Mergeay 1985; Springael *et al.* 1993; Springael, Diels and Mergeay 1994; Van Roy *et al.* 1997). In this way, strain CH34 is a fine example on how fundamental knowledge on biological mechanisms can be exploited as instruments to facilitate the development of biotechnological applications (summarized by Diels *et al.* (2009)). Strain CH34's metal solubilization (via the production of siderophores) and bioprecipitation capacity were used in a BioMetal sludge reactor to treat sandy soils contaminated with heavy metals (Cd, Zn, Ni, Co, Cu, Pb), resulting in a tenfold reduction of the soil metal concentrations

(Diels 1997; Diels *et al.* 1999). Metal removal from wastewater was achieved by inoculating a sand filter (Metal Removal by Sand Filter Inoculation - MERESAFIN) or immobilizing cells, e.g. on a Zirfon ultrafiltration membrane (Bacteria Immobilized Composite Membrane Reactor) (Diels *et al.* 2009). However, as polluted environments often contain a mixture of different toxic components, the bioremediation process of one group could be impacted by other components, for instance metals could inhibit the biodegradation of organic compounds and vice versa. To augment bioremediation processes in such conditions, strain CH34 as well as related strains expressing both catabolic and metal resistance markers were constructed via conjugation (Diels *et al.* 1991; Springael *et al.* 1993; Springael, Diels and Mergeay 1994). Strain CH34 was also used to address the concerns of using genetically modified organisms, e.g. for bioremediation purposes, and their negative impacts on the environment, revealing that certain soils and environmental conditions are conducive to gene transfer (Top *et al.* 1990). In addition to these bioremediation applications, strain CH34 and related strains were also used to assess the bioavailability of metals. These BIOMET sensors are based on gene fusions between metal resistance operons and the promoterless luciferase operon *luxCDABE* of *Vibrio fischeri*, constructed for the detection of Zn and Cd, Cu, Pb, Ni, As and Cr, and applicable to soil, sediments, solid wastes, suspended solids and mineral wastes (Corbisier, Mergeay and Diels 1992; Corbisier, Thiry and Diels 1996; Corbisier *et al.* 1994; Corbisier *et al.* 1999; Tibazarwa *et al.* 2001; Van der Lelie *et al.* 2000).

Looking back

Rather than being an extensive overview, which has recently been performed (Mergeay and Van Houdt 2015, Vandenbussche, et al. 2015), the present essay tried to relate how *C. metallidurans* CH34 research originated, why this peculiar organism attracted attention in a multidisciplinary

scientific environment and the first exploratory steps from formal bacterial genetics to environmental applications.

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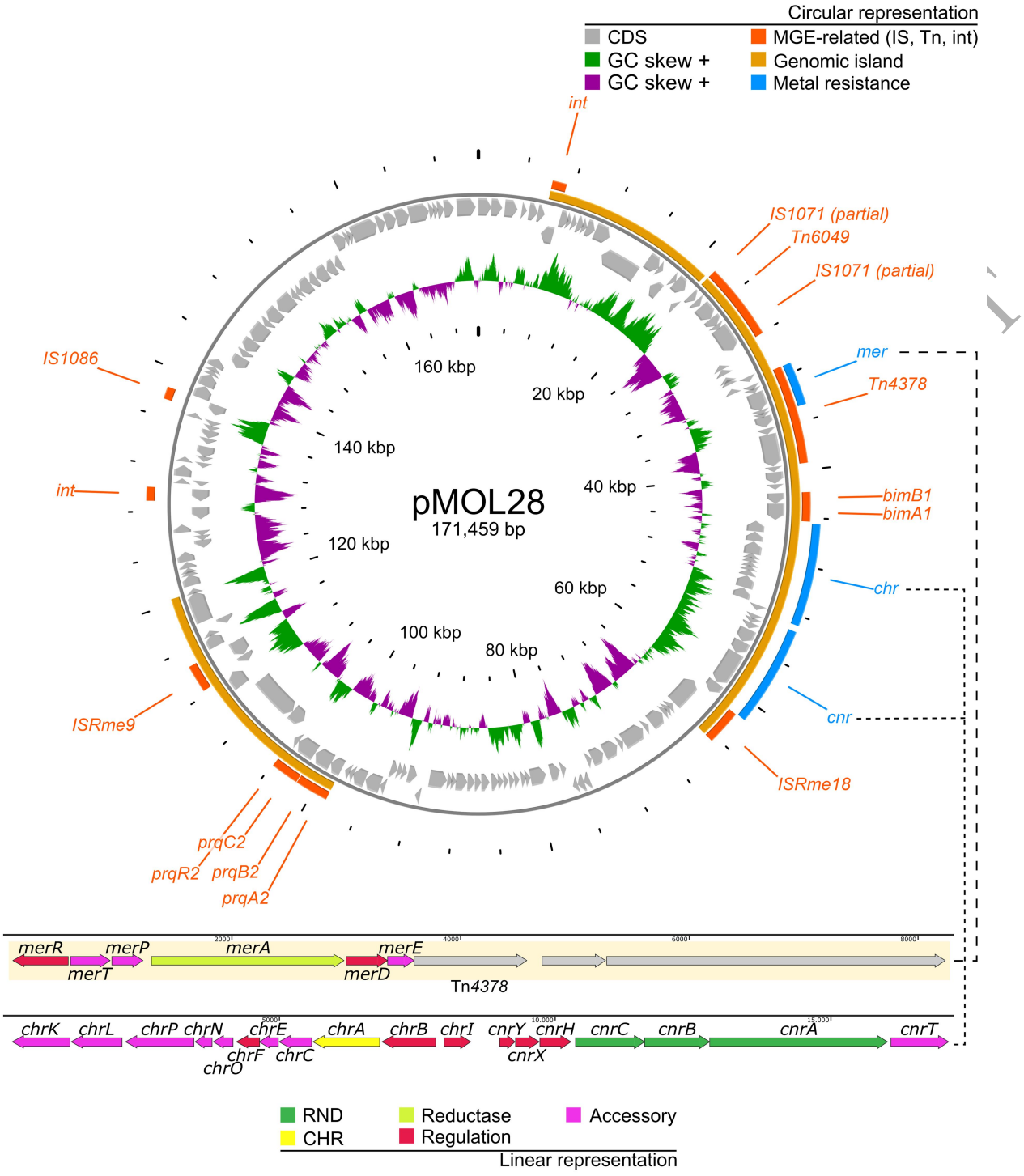

Figure 1. Representation of plasmid pMOL28 from *Cupriavidus metallidurans* CH34. In the circular representation, the rings display (from the outside): (i) position of the metal resistance determinants, (ii) mobile genetic elements and related CDS, and (iii) genomic islands, (iv)

predicted coding sequences transcribed in clockwise and (v) counterclockwise direction, and (vi) GC skew. The linear representations displays the function of the coding sequences of the main metal resistance determinants (RND: Resistance Nodulation cell-Division tri-component efflux system; CHR: chromate transporter).

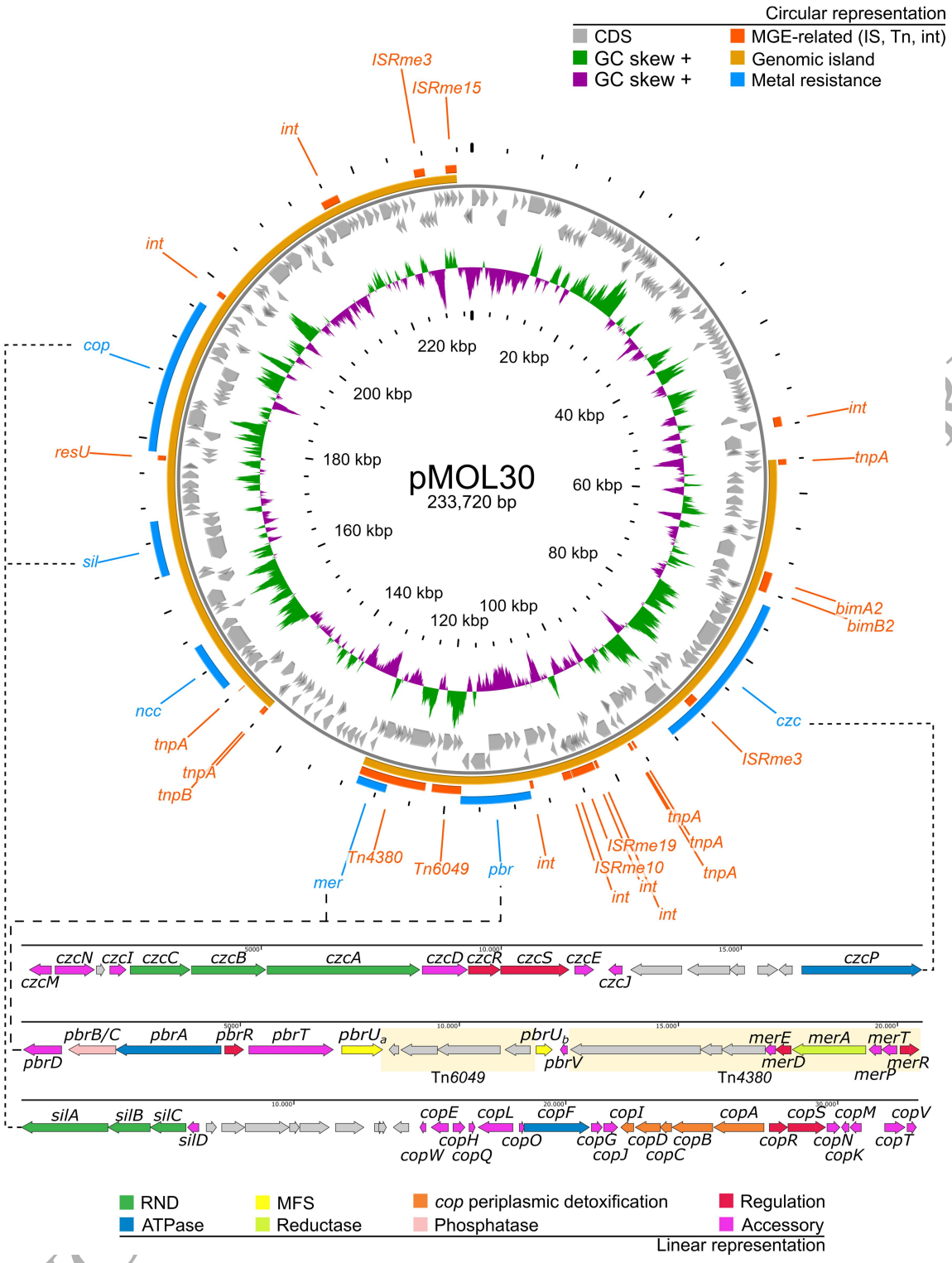

Figure 2. Representation of plasmid pMOL30 from *Cupriavidus metallidurans* CH34. In the circular representation, the rings display (from the outside): (i) position of the metal resistance

determinants, (ii) mobile genetic elements and related CDS, and (iii) genomic islands, (iv) predicted coding sequences transcribed in clockwise and (v) counterclockwise direction, and (vi) GC skew. The linear representations displays the function of the coding sequences of the main metal resistance determinants (RND: resistance nodulation cell-division tri-component efflux system; MFS: major facilitator superfamily).