2016•2017

FACULTY OF MEDICINE AND LIFE SCIENCES

Master of Biomedical Sciences

Master's thesis

Understanding the role of an ABC-type sugar transporter involved in zinc resistance in *Cupriavidus metallidurans* CH34

Supervisor :
Prof. dr. Jerome HENDRIKS

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Lawson Ngome Sone

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences

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List of abbreviations

ABC	ATP-binding cassette
ABCdb	ABC transporter database
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
bp	Base pair
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
Fw	Forward
IR	Inverted repeats
IS	Insertion sequence
Km	Kanamycin
Km^R	Kanamycin resistant
LB	Luria-Bertani medium
MGE	Mobile Genetic Element
MIC	Minimum inhibitory concentration
NBD	Nucleotide-binding domain
OD	Optical density
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
Pi	Inorganic phosphorus
Rpm	revolutions per minute
Rv	Reverse
SBP	Substrate-binding protein
TBE	Tris/Borate/EDTA buffer
Tc^R	Tetracycline resistant
tet	tetracycline
TMD	Transmembrane domain
Zn	zinc
Zn^R	zinc resistant

Acknowledgements

I express my gratitude towards Dr. Natalie Leys and my supervisor Dr. Ir. Rob Van Houdt for giving me the opportunity to undertake my senior practical internship at the Microbiology unit of SCK.CEN. I specially thank Dr. Ir. Rob Van Houdt for his guidance with the experiments I performed, and particularly for his efforts in reviewing this manuscript. I would also like to thank my institutional supervisor Prof. Dr. Jerome Hendriks, not forgetting Prof. Dr. Niels Hellings for their continuous guidance and follow-up during this period of my training. I remain grateful to Ann Provoost and Randy Vermeesen for guiding me with the necessary techniques during the early months of my training. My appreciation also goes to Ali Muntasir and Van Eesbeeck Valerie for their continuous words of encouragement and guidance. Moreover, I thank the almighty God for making it possible to travel this far for my studies and for giving me the parents who have always been a source of encouragement.

Abstract

Cupriavidus metallidurans CH34 resists high concentrations of several metal ions. A recent study reported an ABC-type sugar transporter to be putatively involved in its increased zinc resistance. This transporter has six subunits encoded by the Rmet_2229 to Rmet_2234 genes arranged in an operon and regulated by GlpR encoded by Rmet_2235. However, the role of this transporter in zinc resistance is unknown. The study of how this bacterium resists high zinc concentrations is important as it may offer opportunities in bioremediation and/or bioaugmentation, not leaving out its potential application as an advanced heavy metal whole cell biosensor. This will reduce human exposure to heavy metal-contaminated sites as contamination with heavy metals poses significant threat to human life and the entire ecosystem. Our main aim was to understand the role of this transporter in zinc resistance in *Cupriavidus metallidurans* CH34. We hypothesized that this transporter is responsible for the increased zinc resistance in a laboratory-evolved *C. metallidurans* strain. We had as objectives to describe and classify the various subunits by doing a database similarity search with ABCdb, an online database for prokaryotic ABC transporters. To confirm the involvement of this transporter in zinc resistance. To compare the level of glycerol utilization in strain CH34 and its derivatives. To test the function of the individual transporter subunits and to quantify the intracellular levels of zinc in in all the strains. A database similarity search for all the subunits was performed using ABCdb. A plasmid harboring the six genes was used to complement a CH34 derivative in which *glpR* and the transporter were deleted. Individual knockouts of the six subunits were created in the plasmid by site-directed mutagenesis and used for complementing the CH34 deletion derivative. A dose-response experiment with zinc was performed for all the strains and their growth rates with glycerol as sole carbon source evaluated. A detailed description and classification of the subunits was performed. The involvement of this transporter in zinc resistance was confirmed. The different complementation strains were created, each having a deletion of one of the transporter subunits. All strains could significantly utilize glycerol as sole carbon source. The lower resistance of the CH34 deletion derivative compared to the laboratory-evolved strains confirmed the transporter's involvement in zinc resistance. The ability of this deletion derivative to utilize glycerol as the sole carbon source suggests that this transporter is not essential for glycerol uptake in this bacterium. The function of the individual subunits could not be tested because the control (complete construct) could not be complemented. Hence, for the six other constructs we do not know if they are able to complement. Evaluation of the complementation strain showed that secondary mutations induced when creating the deletion mutant may have neutralized complementation.

1 Introduction

Bacteria have evolved several different mechanisms that enable them to adapt successfully to their environment. They have intrinsic genetic determinants that are expressed when they are challenged with different stress factors (1). Examples of such environmental stressors include antibiotics, chemicals, ionizing radiation, high salinity, extreme hydrostatic pressure, extreme temperatures and heavy metals (1, 2). When faced with single or multiple challenges, a series of transcriptional networks are upregulated to code for proteins that counteract such adverse conditions (1, 2). One environmental stress factor increasingly being studied is exposure to heavy metals, using the model bacterium *Cupriavidus metallidurans* CH34 that is known to resist high concentrations of heavy metals (3-6). The study of this bacterium's ability to resist high metal ion concentrations is important as it may offer opportunities in bioremediation or bioaugmentation, as heavy metal-contaminated sites pose significant problems to human health and the entire ecosystem (7). Several genetically-encoded players have enabled this bacterium to tolerate or resist high concentrations of metals, some of which shall be discussed in this review, particularly the putative involvement of an ATP-binding Cassette (ABC)-type sugar transporter in zinc resistance (3).

1.1 Bacterial response to heavy metal challenges

Regulating the intracellular concentration of metals is important for all bacteria since they must react fast to extreme fluctuations of metabolically essential metals, or the presence of their toxic counterparts (8). Although metals like zinc, iron, manganese and copper are essential for bacterial metabolism (9), their intracellular concentration must be carefully regulated as their imbalance is detrimental to cellular integrity. Metals like mercury, chromium, cadmium, selenium, uranium and several others, pose significant toxicity to bacteria. For example, they promote oxidative stress even in lower concentrations, leading to the production of reactive oxygen species (ROS) that damage DNA which could lead to cell death (10, 11). To protect themselves from metal intoxication, bacteria rely on several mechanisms, including the use of efflux pumps that extrude metals, proteins that oxidize metals to a less toxic state, and proteins that bind metals either within or outside of the cell and render them less toxic (12). In addition, mobile genetic elements (insertion sequences, transposons etc.) also play an important function in bacterial response to heavy metal stress. They bind and interrupt coding sequences of genes involved in metal resistance and can also deactivate their regulatory sequences leading to their derepression (13, 14).

1.2 *Cupriavidus metallidurans* CH34, a model organism to study heavy metal response in bacteria

Cupriavidus metallidurans CH34 is a gram-negative bacterium which is mostly used as a model organism for the study of heavy metal response in bacteria (3-6, 15-18). It is a member of the class β-proteobacteria and belongs to the family *Burkholderiaceae* (19). Sequencing results reported this bacterium to contain four replicons: two circular chromosomes (CHR1 and CHR2) and two megaplasmids (pMOL28 and pMOL30). CHR1 and CHR2 have a size of 3,928,089bp and 2,580,084bp respectively, while pMOL28 and pMOL30 have as size 171,459bp and 233,720bp respectively (6). Strain CH34 has the highest number of genes involved in the resistance and adaptation to high concentrations of heavy metals, of which a majority are found on its two

megaplasmids. Each of its four replicons contains a multitude of metal response gene clusters with the two megaplasmids mostly encoding resistance to five transition metals (zinc, lead, cadmium, nickel, and cobalt). Among these two megaplasmids, pMOL30 codes for resistance to zinc (4), cadmium, cobalt, lead, silver and copper (20). pMOL28 on the other hand, codes for nickel, cobalt, chromate and mercury resistance (20).

Many studies have been performed on this bacterium since it was initially isolated from a zinc sludge in the vicinity of Liege, Belgium (3). This bacterium is mostly isolated from soils contaminated with heavy metals, and also several strains are frequently recovered from industrial sites such as facilities used for spacecraft manufacturing (21), and also from hospital-related sources (22, 23). They have been recovered from patients suffering from cystic fibrosis (22), and one strain has been shown to be the cause of an invasive hospital-acquired septicemia (23, 24). The study of this bacterium generates a keen interest within the scientific community because of its ability to resist toxic concentrations of heavy metals. Hence, potential exploitation of this feature in bioremediation is engaging (25).

1.2.1 The biological availability, importance, and regulation of zinc

Natural levels of zinc typically range between 10 and 300mg/kg in the environment with an average of 70mg/kg (26). Its concentration is higher in certain places due to the natural process of erosion, as well as the influence of human activities that propagate its release in the environment. For instance, activities like mining, the use of zinc in industry, including its agricultural and medical applications (26). Living organisms are known to have evolved in the presence of natural levels of zinc and zinc plays an essential role in many biological pathways. For instance, many bacterial enzymes have zinc in their active or functional sites. An environment that either has a too low or too high concentration of zinc has detrimental effects on living systems. (27). The variant of zinc that has a toxic effect on living systems is the free ion (Zn^{2+}), and high zinc concentrations greatly disrupt biological systems (28). Conversely, added to its antimicrobial properties, zinc has long been used to boost the immune system in elderly patients (29). As zinc has both beneficial as well as undesirable effects in living organisms, its intracellular concentration needs to be carefully regulated to achieve a desired internal concentration range.

1.2.2 The different zinc resistance mechanisms in *C. metallidurans* CH34

Strain CH34 removes excess zinc using different efflux systems. The most important operon that is responsible for zinc resistance is the *czc* operon (3, 4, 30). It codes for three important metal efflux systems i.e. the Heavy Metal Efflux (HME) system of the Resistance Nodulation cell Division (RND) superfamily (CzcCBA), the CDF family of transporters and P-type ATPases. The main zinc efflux system is the CzcCBA which is made up of three components that go across the membrane i.e. CzcC (an outer membrane protein), CzcB (a membrane fusion protein) and CzcA (an inner membrane protein that binds substrates)(15, 30, 31). Twelve potential HME-RND-driven systems have been detected in CH34 which, however, are not all functional due to different mutations and effects of transposition of mobile genetic elements (6, 32). In addition to CzcCBA, there exist other zinc efflux mechanisms in strain CH34. The first of such transporters is CzcP, a member of the P-type ATPase family encoded by *czcP* which is the second cluster of the *czc*

operon (3). It belongs to the class IB P-type (P_{IB}) ATPases which is the main branch of the P-type ATPases that is responsible for transporting metal ions. The genome of CH34 encodes eleven P-type ATPases with eight of them predicted to be P_{IB}-ATPases (3, 17). This high number of P_{IB}-ATPases in strain CH34 compared to other bacteria, is perhaps an important reason why strain CH34 can survive in metal-rich environments. Two other P-type ATPases, ZntA and CadA, which are found on the chromosomes are responsible for removing zinc and cadmium respectively (33). Also, *czcD* codes for a CDF family transporter located downstream of CzcCBA (34). Several studies are ongoing to elucidate other potential players in the increased zinc resistance of *C. metallidurans* CH34. One of such studies has incriminated an ATP-binding cassette (ABC)-type sugar transporter as a potential candidate (18).

1.3 What are ABC transporters? How do they help in bacterial adaptation?

ABC transporters are a family of transporters that transport a large variety of substrates and are among the major families of transporters in all living organisms (35). They import or export a wider variety of molecules compared to other transporter families, from single ions to whole proteins across cell membranes by active transport (35). They utilize the energy that results from the hydrolysis of ATP, and the ensuing changes in their conformation to facilitate the movement of substances across the membrane (36-38). Thus, they help organisms to cope with their environment by tightly controlling the movement of substances between the cell and the extracellular milieu (39). In prokaryotes, ABC transporters are confined to the plasma membrane, and ATP hydrolysis occurs in the cytoplasm. In eukaryotic cells, however, they are also located in the membranes of organelles like the mitochondria and chloroplasts where ATP hydrolysis occurs inside those organelles and not exclusively in the cytosol (40). Generally, ATP binds and gets hydrolyzed on the side of the membrane termed the cis-side, which is found in the cytosol, while the side opposite to it is termed the trans-side. These transporters function either as importers, which take up nutrients to the inside (from the trans-side to the cis-side) or as exporters, which pump out undesirable substances to the outside (from the cis-side to the trans-side) of the lipid bilayer (35, 40). Over the years, scientists have been interested in the study of ABC transporters, not only because of their unique mechanism of transport, but also because their malfunction is the cause of many human diseases like cystic fibrosis (41). Some ABC exporters are also involved in drug resistance in bacteria and cancer cells. A well-studied multidrug ABC exporter of bacteria is sav1866 of *Staphylococcus aureus* which is structurally related to the model ABC multidrug exporter LmrA, of *Lactococcus lactis* and P-glycoprotein. P-glycoprotein is an ABC transporter involved in drug resistance in cancer cells (42). While ABC exporters are found in all kingdoms, importers are apparently only present in prokaryotes (37).

1.3.1 Structure of ABC transporters

With the application of X-ray crystallography, developments in determining the structure of ABC transporters have been achieved within the last two decades (26, 37, 40, 43-50). A typical ABC transporter has a unique architecture composed of at least four protein subunits or domains which all have a specific function. There are two hydrophobic transmembrane domains (TMDs) also known as membrane-spanning domains (MSDs) that are integrated into the lipid bilayer and two nucleotide-binding domains (NBDs) or ATP-binding cassettes (ABCs) located in the cytoplasm

that are hydrophilic (40, 45-48) (Figure 1A). The two TMDs, likewise the two NBDs can form complexes made up of two of the same protein (homodimer) or different proteins (heterodimer). NBDs and TMDs can be fused together in various ways. The majority of ABC transporters have homodimeric NBDs plus two different TMDs (51). The NBDs interact with the cytoplasmic protrusions of TMDs to supply energy for active transport after ATP hydrolysis (52). Another major component of ABC transporters is the substrate binding domain (SBD) or substrate binding protein (SBP). SBPs are soluble components of ABC transporters located on the trans-side of the membrane and binds the substrate first in ABC transporters functioning as importers. Additional proteins can bind to the TMDs and/or NBDs serving different functions such as in regulating transporter activity (45). The genes encoding the various components of ABC transporters are often arranged in a single gene cluster (operon), with a few exceptions (46).

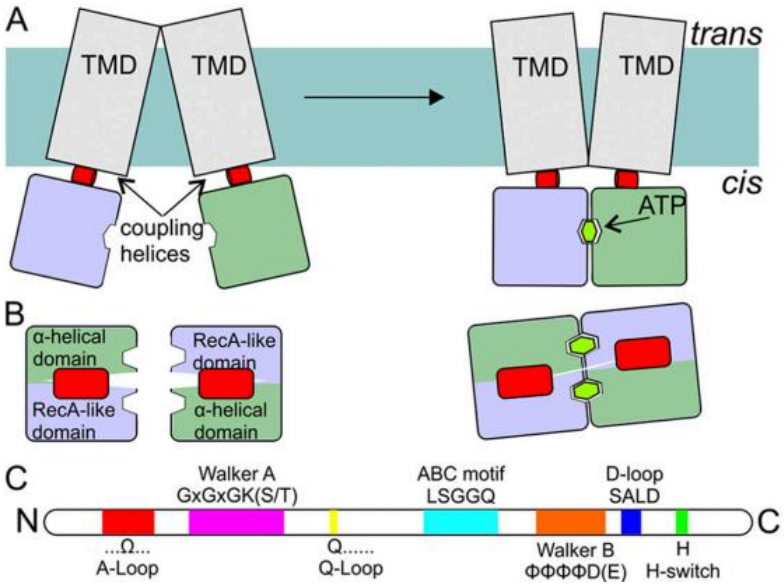

Figure 1: Schematic of an ABC transporter. A) Blue and green are the two domains of the NBD which are attached to the TMDs (gray) by short α-helices (red). B) The two subdomains of each NBD that capture the two ATPs (hexagonal green). C) The different sequence motifs and their relative positions in NBDs all represented with different colors. C and N are the amino and carboxylic acid terminals of NBDs. Adapted from ter Beek *et al*., 2014 (40).

1.3.2 Structure of the nucleotide-binding domains (NBDs)

The NBDs have many conserved sequence motifs with different functions and are highly similar to each other (Figure 1C). Hence, they serve as special features used to identify this family of transporters (36, 49). Each NBD is made up of about 200 amino acids and has two subdomains: the RecA-like domain which is larger and accommodates the P-loops (Walker-A motif) and the α-helical domain which is more diverse in structure and accommodates the LSGGQ motif that is typical of ABC transporters (37, 40). Both NBDs can be aligned differently to each other i.e. they can be packed tightly against each other (the closed conformation) or be separated slightly (the open conformation) (Figure 1B). Two ATP-binding sites are present at the meeting point between the two NBDs. The binding sites are called the ATP-binding cassettes from where this superfamily of transporters is named. The binding of ATPs to these sites orchestrate the formation of the closed conformation because each ATP interacts with motifs from the two NBDs, hence bringing them

closer to each other (40). During this process, the RecA-like and the α-helical subdomains, lying face-to-face to each other between the two NBDs, rotate towards each other when ATP is bound and move away upon its hydrolysis. ATP hydrolysis is only possible when the two NBDs are tightly packed against each other. Several other sequence motifs are found in NBDs with each having a specific function (Figure 1C) (40).

1.3.3 Structure of the transmembrane domains (TMDs)

Compared to the NBDs, the sequences and buildup of TMDs are less conserved, which reflects the ability of ABC transporters to transport a wide variety of substances (45). The TMDs make up a translocation pathway which can be reached by the substrate from either the trans-side or cis-side of the membrane. Ideal TMDs have at least twelve α-helical transmembrane (TM) segments (6 per TMD), forming the passage through which a substrate crosses the membrane. The α-helices are intertwined with each other and go across the membrane several times in a zig-zag manner (38). The translocation pathway is found at the meeting point between the two TMDs that are coiled around each other and embedded in the membrane. The TMDs ensure the specificity of the transporter because they have specific sites called substrate-binding sites where substrates bind (38). Because of their varied structure, they have been used to categorize ABC transporters.

1.3.4 Coupling helix

A coupling helix is a short α-helix on the cytoplasmic ends of the TMDs that attaches to an NBD and coordinates signals between the TMDs and NBDs. Coupling helices have been identified in ABC exporters as well as in certain importers (47).

1.3.5 Substrate-binding proteins (SBPs)

SBPs are additional components of ABC transporters found on the trans-side of the membrane that first binds the substrate in ABC importers (Figure 2B and C). The SBP could be linked to a TMD, or joined to the membrane by a lipid or be diffused freely in the periplasm as in gram-negative bacteria (40, 45). Certain transporters, however, do not require SBPs for their functioning (40). Although SBPs can differ in their size and sequence and are also specific for different substrates, they all have an identical build with two domains or lobes linked by a hinge (40). When a substrate is absent, the two lobes stay open, but upon encounter with a substrate, they close up to trap the substrate and deliver it to the TMDs (44). SBPs can be classified based on their structure into six different groups (43).

1.4 **Classification of ABC transporters.**

ABC transporters have been classified into four groups based on four different folds of TMDs as detected by X-ray crystallography. Three of these are associated with import functions while one is an exporter. The three importers are classified as Type I and Type II importers and the energy coupling factor (ECF) transporters (also called Type III importers) (40).

1.4.1 Type I ABC importers

Both TMDs of type I importers are either homodimeric or similar in structure. For example, the two TMDS of the *E. coli* maltose transporter $MalEFGK_2$ are related in structure, although they

share only a 13% sequence homology, having 5 TM helices per TMD. Some of their TMDs have up to 8 TM helices per TMD. $MalEFGK_2$ is one of the most studied Type I ABC importer (40). Type I importers mostly take up substrates required in large quantities like sugars, amino acids and ions (40).

1.4.2 Type II ABC importers

Two identical TMDs of10 TM helices each are characteristic of Type II ABC importers. They take up substrates that are required in small quantities like metal chelates e.g. haem or cobalamin (vitamin B12). Type II substrates are generally larger than the Type I substrates (40). The uptake of haem from the host has been the basis of virulence by certain pathogenic bacteria. (53). ABC transporters of transition metals import only the essential ones as it is disadvantageous for bacteria to invest energy in importing rather toxic compounds (36).

1.4.3 ECF transporters

In ECF transporters, no relationship exists between the two TMDs, either structurally or functionally. One of the TMDs is termed the T-component with 5 TM helices while the other is termed the S-component with 6 TM helices (40).

1.4.4 ABC exporters

ABC exporters are found in all completely sequenced organisms. Their substrates are to date unknown or uncertain for several bacteria or eukaryotes, but for the fact that they are involved in multidrug resistance in bacteria and cancer cells (42). Even though these transporters recognize different substrates, they all have a common build consisting of 6 TM helices per TMD. The two TMDs either share sequence identity or are similar in structure (48).

1.5 Mechanism of ABC transporter function: the "alternating access" model

For ABC transporters, an "alternating access" model for substrate translocation has been proposed which is highly similar for both importers and exporters (37, 50, 54) (Figure 2). The hallmark of this model is the presence of the substrate-binding site on the TMDs that can be accessed by the substrate either from the extracellular or the intracellular compartment of the membrane. These correspond to the state of the transporter termed the "outward-facing" and the "inward-facing" conformations, respectively (47). The Type I, and II importers rely on SBPs to capture their substrates from the trans-site and to deliver the substrates to the TMDs (40). ECF transporters and ABC exporters do not need SBPs for their functioning. ECF importers rather bind their substrates using one of the TMDs called the S-component, which has a very high affinity for its substrates (40). Like the word alternating says, the transporter can switch conformation, thereby exposing its substrate binding site to either side of the membrane. ABC transporters function by transporting substrates against a concentration gradient, and ATP hydrolysis is pivotal for this transport as it provides the required energy for this process to be successful (37, 40, 50, 52, 54).

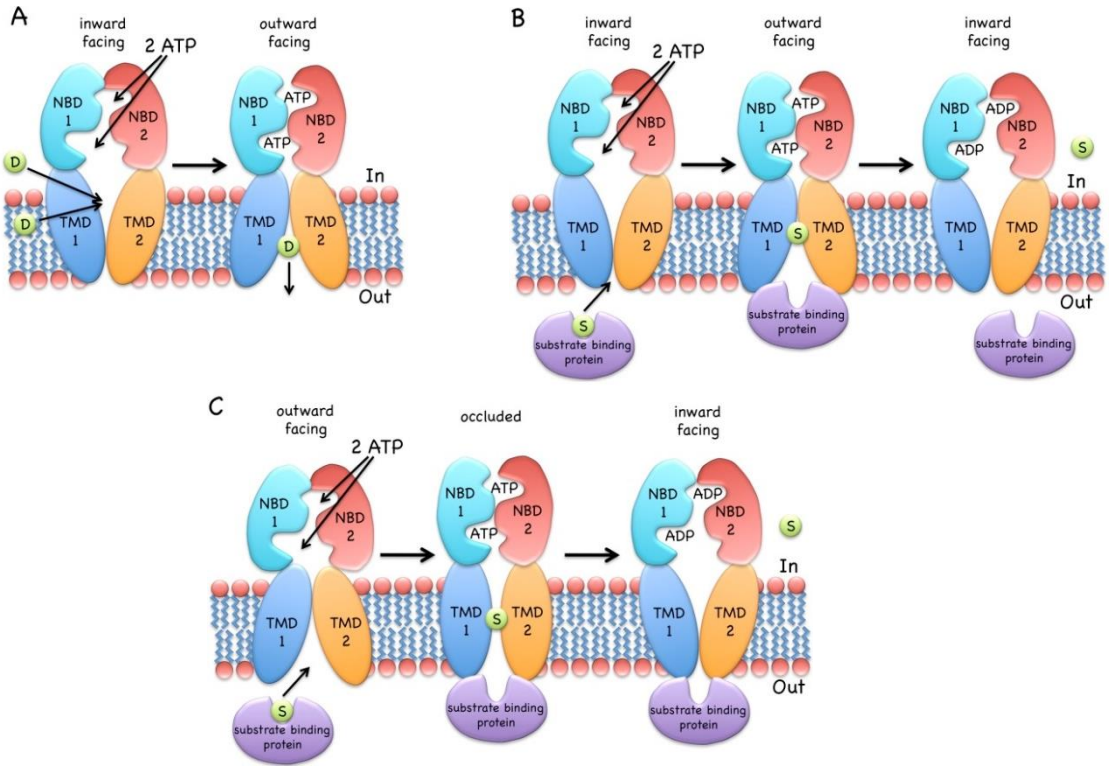

Figure 2: Cartoon showing the full structure of an ABC transporter and the ATP-driven alternating access model. A) An exporter (inward-facing), binds drug, "D" from the cytoplasm or the inner membrane. The NBDs bind two ATPs and attain a closed conformation. ATP hydrolysis causes the conformation of the TMDs to switch from the inward to the outward facing conformation, and the drug gets released to the exterior of the cell. The hydrolysis of ATP, release of ADP and Pi and the dissociation of the NBDs, reverts the transporter to its default inward-facing conformation. B) The inward-facing type I importer (e.g., $MalFGK_2$) with its SBP that first binds the substrate "S". NBDs bind 2 ATPs and dimerize resulting in the outward-facing conformation. Substrate leaves the SBP and binds to the substrate binding site on TMDs. ATP gets hydrolyzed, its products released, and NBDs dissociate reverting the transporter to its original inward-facing conformation. C) The outward-facing type II importer (e.g. BtuCD). SBP binds substrates and delivers to TMDs, NBDs gets dimerized by binding of ATPs. Substrate gets stock in a sealed cavity mid-membrane making transporter to attain an occluded conformation. ATP hydrolysis and NBD dissociation occur subsequently allowing the escape of the substrate into the cytoplasm. Transporter assumes its default conformation. Arrows indicate the movement of the substrate to their TMD and ATP binding sites and the progression of the translocation cycle. Adapted from Stephan Wilkens, 2015 (50).

With the phrase "alternating access", it would be misleading to assume an ABC transporter can serve as an importer and at the same time an exporter. ABC transporters normally transport substances unidirectionally, except for one known multidrug efflux transporter LmrA that can both import and export under certain conditions (55). The successful translocation of a substrate by ABC transporters consists of multiple steps. In the first step, the substrate is captured by SBPs and later transferred to the TMDs (in the case of importers) (Figure 2 B and C), or the substrate binds directly to the TMDs (in the case of exporters) (Figure 2A). Secondly, the binding of the substrate induces the NBDs to bind ATPs. Two ATP molecules bind to the NBDs and get hydrolyzed (Figure 2A-C). The resulting energy from ATP hydrolysis is then converted to "conformational energy" that is then transferred to the TMDs via the coupling helices (40, 47, 50, 54). This energy propagates the conformational switches in the TMDs leading to the alternating exposure of their

binding site containing the substrate to the other side of the membrane which, however, depend on the type of transporter involved (40, 54). For importers, it is assumed that the outward-facing conformation has a higher affinity for a substrate than the inward-facing conformation, and the opposite is true for exporters (54). Lastly, the release of the substrate, phosphate, and ADP from the NBDs causes the transporter to revert to its default conformation for the next cycle to begin. Although all or some of these steps must occur at some point in the transportation cycle, the order and details of these steps, however, depend on the type of transporter involved, whether importer or exporter (50).

1.6 Regulation of ABC transporter activity

In addition to the four core subunits of ABC transporters, other subunits called accessory domains can be found in different parts of an ABC transporter. They can be found inside or outside of the membrane and perform either regulatory or catalytic role within the transporter (45). These domains are classified into four different groups i.e. extracytoplasmic domains, membrane-embedded domains, cytosolic regulatory domains and cytosolic catalytic domains (45). In the case of extracytoplasmic domains, SBPs that have not bound any substrate can sometimes bind to TMDs and inhibit transport. For membrane-embedded domains, many transporters have an additional TM segment that is not part of the main TMD. Although their function is not known, they are termed accessory domains and predicted to have regulatory roles (45). Many ABC transporters are regulated at the level of gene expression as a single regulator controls the expression of the entire operon depending on the level of stress (46). A typical example is the glycine betaine uptake system of *L. lactis* encoded by *busA* which is being expressed during high osmotic stress and regulated by BusR (56). BusR is a transcriptional regulator in bacteria belonging to the GntR family (56).

1.7 The role of ABC transporters in zinc resistance in *C. metallidurans*

A recent study showed that *C. metallidurans* CH34 is able to adapt to high zinc concentrations (24 mM), probably by the involvement of an uncharacterized ATP-binding cassette (ABC)-type sugar transporter (18). In that study, a whole genome expression analysis was performed to identify determinants in the laboratory-evolved CH34 strain ($CH34^{ZnR}$). The latter was obtained by first inoculating the parental strain in liquid MM284 medium with 12mM Zn^{2+} (its minimum inhibitory concentration), and subsequently selecting them on MM284 agar plates with 24 mM Zn^{2+} (4). Results showed a constitutive expression of an ABC-type sugar transporter, putatively incriminating it as a novel player in zinc resistance in strain CH34. This ABC-type transporter is composed of six proteins transcribed from a single locus: one periplasmic protein, one small integral membrane protein, two permeases and two ATPases, which are encoded by the Rmet_2229 to Rmet_2234 genes, respectively. This operon is most probably regulated by a single regulator (GlpR), which is a DeoR-type regulator found upstream of the transporter and encoded by Rmet_2235 (38). Sequence analysis indicated that *glpR* was inactivated by IS*1088* insertion in the laboratory-evolved strain, resulting in the derepression and constitutive expression of the ABC-type transporter. Likewise, glycerol was also reported to increase zinc resistance in this bacterium as it interacts with GlpR and derepresses the ABC-type sugar transporter (18).

When the ABC gene cluster was deleted in both the parental and laboratory-evolved strain, the laboratory-evolved deletion mutant displayed a decreased zinc resistance compared to the parental deletion mutant after a dose-response experiment with zinc. This proved the hypothesis that constitutive derepression of the ABC-type transporter is responsible for the high zinc resistance phenotype of the laboratory-evolved strain, providing additional evidence of the ABC-type transporter's involvement in increased zinc resistance. The parental deletion mutant was still able to utilize glycerol as the only carbon source, demonstrating that the ABC-type transporter may not be necessary for glycerol uptake in *C. metallidurans* CH34, as is the case with its homolog in *R. leguminosarum* bv. *viciae* VF39. Other transporters in this bacterium might have been responsible for glycerol utilization. The interaction of *C. metallidurans* with zinc has already been well studied (4, 30, 31, 57), although little is known about the role of ABC transporters and metal resistance. No examples of ABC metal exporters are known in prokaryotes. One study showed that the ABC transporter AtmA of strain CH34 protects *C. metallidurans* and *E. coli* from cobalt and nickel toxicity (58). In that study, an AtmA deletion mutant of strain CH34 displayed a decreased resistance to cobalt and nickel compared to its parent. In *E. coli*, AtmA also increased resistance to these metals when its other cobalt and nickel resistance determinants were deleted.

Uptake of zinc in *C. metallidurans* is achieved by a set of broad spectrum metal importers which, unlike the ZnuABC uptake importer of *E. coli*, are not selective for zinc. In contrast, a single transporter (ZupT) imports zinc in severe shortages (59). To date, nothing is known about the role of ABC transporters in zinc resistance in *C. metallidurans*. Likewise, the role of this ABC-type sugar transporter in zinc resistance of strain CH34 remains unknown. i.e. whether it has a direct or an indirect effect in zinc resistance. No information is available whether it is an importer or an exporter, although a database similarity search and literature show that it is likely to be an importer. Moreover, how it mediates resistance to zinc remains elusive.

1.8 The research plan

The main goal of this study was to understand the role of this ABC-type sugar transporter involved in zinc resistance in *C. metallidurans* CH34. We hypothesized that the ABC-type sugar transporter is responsible for the increased zinc resistance of a laboratory-evolved *C. metallidurans* strain. To verify this hypothesis, five objectives were defined. A database similarity search was performed using ABCdb, an online database for prokaryotic ABC transporters to properly describe and classify each subunit. To confirm the involvement of this transporter in zinc resistance, a dose-response experiment with zinc was performed with the different strains of *C. metallidurans* CH34 and their growth evaluated by measuring their optical densities at day five of incubation. To compare the level of glycerol utilization among the different strains of *C. metallidurans* CH34, the strains were grown in MM284 without gluconate but supplemented with 1% (w/v) glycerol as sole carbon source and their optical densities evaluated for 14 days. To test the function of the individual subunits, a plasmid harboring the genes of the six subunits was used to complement a CH34 derivative which has both its transporter and regulator deleted. Individual genes were inactivated on this plasmid and the mutated plasmid used to make different complementation strains to be scored against the complete complementation strain in dose-response experiments with zinc. Finally, to analyse the intracellular concentration of zinc in all the strains by ICP-OES.

2 Material and methods

2.1 Strain, media, and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *C. metallidurans* strains were cultured routinely at 30˚C under aerobic conditions in Tris-buffered mineral sodium supplemented with 0.2% (w/v) sodium gluconate (MM284) as previously described (4). Liquid cultures were grown in the dark at 30˚C on a rotary shaker at 150rpm, while cultures on agar plates did not require shaking. For culturing on agar plates, 2% (w/v) agar (Thermoscientific, USA) was added. *E. coli* stains were routinely cultured at 37˚C in LB broth (Invitrogen, USA) with shaking at 200rpm, while cultures on agar plates did not require shaking. When relevant, the following substances from Sigma-Aldrich or Thermoscientific were added to the medium at final concentrations indicated with their symbols in bracket: kanamycin 50µg/mL (Km^{50}) for *E. coli* or 1500µg/mL (Km^{1500}) for *C. metallidurans* CH34, tetracycline 20µg/mL (Tc^{20}), chloramphenicol 30µg/mL (Cm^{30}), Zn^{2+} (5, 10, 15, 20 and 25mM $ZnSO_4.7H_20$). Glycerol (Merck Millipore, Germany) was added to the growth medium at a final concentration of 1% (w/v).

2.2 Phenotypic analyses

2.2.1 Dose-response experiments

The response of the different *C. metallidurans* strains to zinc was assessed by dose-response experiments. This was performed in a flat bottom 24-well cell culture plate (Greiner Bio-one). Pre-cultures were incubated at 30ºC up to stationery phase, diluted 1:100 in 2mL fresh MM284 medium supplemented with 5, 10, 15, 20 and 25mM Zn^{2+}. All tests were performed in biological triplicates and incubated at 30˚C in the dark with shaking. The optical densities were determined at 600nm in a flat-bottom 24-well cell culture plate which was placed into a Clariostar® (BMG Labtech, Germany) and measured at day 5.

2.2.2 Glycerol growth experiment

Growth experiments were performed for the laboratory-evolved strains, the mutant, the complementation strain and the parent strain in MM284 without gluconate but with 1% (w/v) glycerol. Experiments were performed by a 1:10 inoculation of stationery phase cultures in fresh MM284 supplemented with glycerol in biological triplicates. Precultures were washed twice with PBS before inoculation. Tubes were incubated at 30˚C with shaking, and 200µL of each replicate was added to a 96-well cell culture plate and the optical density measured at 600nm for fourteen days.

2.2.3 Evaluating the plasmid retention capacity of the complementation strain

A tenfold serial dilution was performed for cells cultured in MM284 and MM284 plus 10mM Zn^{2+}. 10µL drops of each dilution were spotted on LB plates and LB Km^{1500} plates and incubated for two days at 30˚C. The number of colonies were counted and reported as colony-forming units per mL (CFU/mL).

Table 1: Bacterial strains and plasmids used in this study

Strain	Genotype/relevant characteristic	Reference
C. metallidurans		
CH34	pMOL28 pMOL30 resistant up to 12mM	Mergeay *et al*., 1985
$CH34^{ZnR1}$	pMOL28 pMOL30 Zn^R	Vandecraen *et al*., 2016
$CH34^{ZnR2}$	pMOL28 pMOL30 Zn^R	Vandecraen *et al*., 2016
CH34ΔRmet_29_35*::tet*	Rmet_2229 to Rmet_2235 replaced by *tet*, Tc^R	Van Houdt, 2016
CH34ΔRmet_29_35*::tet* + pRmetABC_29-34	Rmet_2229 to Rmet_2235 replaced by *tet*, and complemented with pBBR1MCS2 + Rmet_2229_2234, Tc^R and Km^R	This study
E. coli		
DG1	*mcrA* Δ(*mrr-hsdRMS-mcrBC*, modification-, restriction-) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ*(ara-leu)7697 galU galK rpsL endA1 nupG*	Eurogentec, Belgium
DH5α	F^- φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 λ^- thi^-1 gyrA96 relA1	Laboratory collection
Plasmids		
pBBR1MCS2	lacZα KmR *ori* pBBR1 *oriT*	(Kovach *et al*., 1995)
pRmetABC_29-34	pBBR1MCS2+Rmet_2229_2234, Tc^R and Km^R	Laboratory collection
pRmetABC_30_31_32_33_34	Rmet_2229 inactivated on pRmetABC_29-34, Tc^R and Km^R	This study
pRmetABC_29_31_32_33_34	Rmet_2230 inactivated on pRmetABC_29-34, Tc^R and Km^R	This study
pRmetABC_29_30_32_33_34	Rmet_2231 inactivated on pRmetABC_29-34, Tc^R and Km^R	This study
pRmetABC_29_30_31_33_34	Rmet_2232 inactivated on pRmetABC_29-34, Tc^R and Km^R	This study
pRmetABC_29_30_31_32_34	Rmet_2233 inactivated on pRmetABC_29-34, Tc^R and Km^R	This study
pRmetABC_29_30_31_32_33	Rmet_2233 inactivated on pRmetABC_29-34, Tc^R and Km^R	This study

2.2.4 Viable cell count

Viable cell counts were performed to evaluate plasmid stability in *C. metallidurans* CH34 and during sample preparation for ICP-OES. Precultures were incubated at 30˚C right up to stationery phase and a tenfold serial dilution was performed in triplicates for each sample. 10 to 20µL of each dilution was then used to inoculate plates of LB or MM284, and incubated for 48h. The number of colonies were counted and reported as colony-forming units per milliliter (CFUs/mL).

2.3 **Plasmid DNA extraction and purification**

Plasmid DNA was extracted from *E. coli* DG1 transformants using the Wizard® Plus SV Minipreps DNA Purification System (Promega, USA). *E. coli* DG1 with the plasmid insert was grown in 20mL LB broth supplemented with Km^{50} overnight with vigorous shaking at 37˚C. The next day, another 20mL LB was added to the culture and incubated for further 4-6 hours. This was then divided into four tubes of 10mL each, and the plasmid was extracted following the manufacturer's protocol. For plasmids sent for sequencing, 3mL of LB was added to 5mL of an overnight culture and further incubated for 2hours with vigorous shaking at 37˚C. DNA was eluted with 50µL nuclease-free water, and the quality analyzed using the Nanodrop spectrophotometer. DNA quality was checked using the DropSense spectrophotometer (Trinean NV, Belgium) and concentrated using the Speed VAC concentrator (Eppendorf concentrator plus, Germany).

To remove impurities like salts and organic matter after PCR amplification, the mutated PCR product was purified using the Wizard® SV Gel and PCR clean-Up system (Promega, USA) following the centrifugation protocol. An equal volume of membrane-binding solution was added to the PCR products, the mixture transferred to a minicolumn and incubated for 1min at room temperature after which it was centrifuged at maximum speed for 1min and the flow-through discarded. 700µL of column wash solution was added, centrifuged for 1 min and the flow-through discarded. This step was later repeated with 500µL of column wash solution and centrifuged at maximum speed for 5mins. The minicolumn was transferred to a new tube, centrifuged for 2mins with the microcentrifuge tube open, and then left open at room temperature for 15mins to ensure effective evaporation of any residual ethanol. DNA was eluted with 50µL nuclease-free water, and the quality analyzed using the Nanodrop spectrophotometer.

2.3.1 Restriction enzyme digest of plasmid DNA

To ensure the correct and uniform size for all extracted plasmids, a restriction enzyme digest was performed with the following enzymes from Thermoscientific; *Bam*HI, *Xba*I, and *Bgl*II. All digestion experiments were setup on ice and for each digestion, 3µg of plasmid DNA was used in a 20µL reaction containing 1µL of the restriction enzyme and 2µL of the appropriate buffer. This was gently mixed with the tip of a sterile pipette and incubated for 1-2 hours at 37˚C. The fragments were later visualized by checking the band pattern after gel electrophoresis. The empty vector (pBBR1MCS2) was used as a control for the digestion reactions.

2.3.2 Agarose gel electrophoresis

The PCR and digestion reactions were evaluated by agarose gel electrophoresis using a Bio-Rad electrophoresis chamber (Bio-Rad, USA). The running buffer was TBE, and the gel was prepared to a final concentration of 0.8% (w/v) in TBE plus 5µL of a 10000× Gel Red (Biotium, USA). A 1/2 dilution of the sample in 6X loading dye (Thermoscientific, USA) was loaded into the wells of the electrophoresis chamber. All electrophoresis reactions were done at 100V for 85mins. The size and the amount of the PCR products were visualized using an ultraviolet spectrophotometer (Vilber, Germany) and the bands were evaluated by comparing the sample band position and intensity with that of the 1kb plus Gene Ruler (Invitrogen, USA).

2.4 **Site-directed Mutagenesis and Cloning experiments**

Individual genes of the ABC-type transporter carried on the pBBR1MCS2 vector were inactivated using the Phusion site-directed mutagenesis kit (Thermoscientific, USA). This was done following the procedures described below.

2.4.1 Primer design

Primers were designed using the SnapGene® 3.3.1 software (GSL Biotech, USA), and following the recommendations of the Thermoscientific mutagenesis protocol. The primers were designed such that a point mutation was induced at the start codon to inactivate the gene to be studied, except for Rmet_2231 where another method was used to inactivate it. The point mutations were created by designing a mismatch in the mutagenic primer to delete a nucleotide in the start codon. The desired mutation was designed to be in the middle of the forward primer with approximately 8-13 perfectly matched nucleotides on each side. For Rmet_2231, an alternative method was used to design its primers due to the challenges in inducing a point mutation at the start codon. In this case, the primers were designed such that a short in-frame fragment of the gene was created. All primers were phosphorylated at their 5'ends such that they anneal back-to-back for easy circularization of the mutated PCR products. Primers used in this study are as listed in Table 2.

2.4.2 Polymerase Chain Reaction

Plasmid DNA was amplified using the Phusion high-fidelity DNA polymerase (Thermoscientific) with the primers designed for the mutation of interest. The annealing temperatures were calculated using the Thermoscientific Tm calculator at a primer concentration of 0.5µM. The following experimental conditions were used; approximately 10pg of plasmid DNA was used as template in a total reaction volume of 50µL, containing 0.04U/µL of Phusion high-fidelity DNA polymerase, 200µM dNTPs, 0.5µM forward and reverse primer and a 1× GC buffer. Each reaction was performed for 30 cycles, each cycle consisting of an initial denaturation step at 98˚C for 1min, a denaturation step at 98˚C for 10s, annealing temperature depending on the Thermoscientific Tm calculator for 30s, extension at 72˚C for 6mins and a final extension at 72˚C for 10mins. The annealing temperature for Rmet_2230, Rmet_2231 and Rmet_2234 was 70˚C while for Rmet_2229, Rmet_2232 and Rmet_2233, annealing temperatures of 59˚C, 67˚C and 63˚C were used respectively. PCR Amplification was done using the GeneAmp PCR system (Applied

Biosystems). The empty vector (pBBR1MCS2) was used as a control for the amplification reactions.

Table 2: Primer pairs used in this study

Primer pair	**5'-3' Sequence (5' phosphorylated)**
Rmet_2234_Fw	TGGAGACTGGATCAGCTCAGCCTG
Rmet_2234_Rv	ATATTCTTCTTGTGACAGCTCAGCCACT
Rmet_2233_Fw	GAGCCTGATAGGCACGCATC
Rmet_2233_Rv	CCGACTCGGTAATCGCGG
Rmet_2232_Fw	AACTGGTCCAAGAAGCCCGTC
Rmet_2232_Rw	CCTCGTTGACGTAGAAGCAGGTATG
Rmet_2231_Fw	GCGCCGAGGAGATTCCGAAGGCTGAGCAT
Rmet_2231_Rv	CCGCCGTGCCGGCGCGTTGCATCCAGTT
Rmet_2230_Fw	GAGGCACCGTGCTGAGCTG
Rmet_2230_Rv	CCTCAAACGCGGCCCATCGCGAA
Rmet_2229_Fw	AGACACAGAGAAAGAGCGCG
Rmet_2229_Rv	CCTCATGAACCCCTTCCC
pBBR1MCS2_Fw	CGATTAAGTTGGGTAACGCC
pBBR1MCS2_Rv	ATTAGGCACCCCAGGCTTTA
Seq_Rmet_2233	ACGTTCGTGCATGTCGATAC

When appropriate, a colony PCR was performed to determine the presence of the correct plasmid DNA in *E. coli* DG1 cells. Single colonies were picked using a sterile toothpick and dissolved in 30µL of sterile water. 5µL of this mixture was used in a total reaction volume of 50µL containing 0.04U/µL of Phusion high-fidelity DNA polymerase, 200µM dNTPs, 1× GC buffer and 0.5µM forward and reverse pBBR1MCS2 which are primers specific for the vector sequence. Each reaction was performed for 30 cycles, with each of the cycles consisting of an initial denaturation step at 98˚C for 1min, a denaturation step at 98˚C for 10s, annealing temperature at 63˚C for 30s, extension for 3.5mins at 72˚C and a final extension at 72˚C for 10mins. The success of the amplification reactions was evaluated by agarose gel electrophoresis, the product purified and digested with *Dpn*I.

2.4.3 ANZA™ *Dpn*I digest of template DNA in PCR product

A restriction enzyme digest was also performed on the PCR products to get rid of the background dam^+ methylated GATC sites on the template DNA that could interfere with cloning. ANZA™ *Dpn*I (Thermoscientific) was used as the choice restriction enzyme as it cuts only DNA that has been dam methylated. The restriction digest was performed by incubating a 20µL reaction containing 17µL of a 0.2µg/µL DNA, 1µL ANZA™ *Dpn*I, and 2µL ANZA™ 10× buffer at 37˚C for 2-3 hours. The reaction was stopped by inactivating the enzyme at 80˚C for 20mins.

2.4.4 Self-circularization of linear plasmid DNA

The mutated PCR products were then ligated using the Thermoscientific self-circularization of linear DNA kit. 100ng/µL of linear DNA was mixed with 5µL of Anza™ T4 DNA Ligase Master Mix in a 20µL reaction. The mixture was then incubated overnight at room temperature to ensure effective ligation. The ligation products were used to chemically transform *E. coli* DG1 cells.

2.5 **Transformation**

2.5.1 Chemical transformation

Ligated PCR products were then transformed into *E. coli* DG1 chemically competent cells (Eurogentec, Belgium) and inoculated by spreading on LB Km^{50} plates. DG1 cells from the -80˚C were left to thaw on ice for 10mins after which, 3µL of the ligation mix was added to a tube of DG1 cells and stirred gently to mix. The mixture was then incubated on ice for 30mins and the bacteria heat-shocked by placing the vial at 42˚C for 30s without shaking, and the vial transferred immediately to ice. The cells were then transferred to a small tube containing 1mL of LB and incubated for 1 hour at 37˚C with shaking. The tube was centrifuged and approximately 750µL of the supernatant discarded. 100µL of the remaining cell mix was inoculated on LB Km^{50} plates followed by another inoculation of 10× and 100× dilution. The remaining cell mix was centrifuged, the supernatant discarded and the pellet resuspended in the remaining medium. This was used to inoculate the last set of LB Km^{50} plates. Plates were incubated at 37˚C overnight. The next day, ten distinct colonies were purified on new LB Km^{50} plates to ensure effective selection of *E. coli* DG1 transformants. A plasmid DNA miniprep from the 10 *E. coli* DG1 transformants selected on LB Km^{50} plates were further confirmed by sequencing prior to transformation of the *C. metallidurans* CH34 deletion mutant.

2.5.2 Electroporation

To construct the CH34 complementation strains, the CH34 deletion mutant was made competent and transformed with the plasmid insert by electroporation as described by Kyoung-Hee Choi *et al*., 2005 (60). 2mL of a 5mL overnight culture of the deletion mutant grown in LB plus Tc^{20} was centrifuged for 2mins at 16000xg. The cell pellet was washed twice with 300mM of room temperature sucrose, and 40µL was used to resuspend the cells. 4µL of the purified plasmid was added to the mixture and mixed gently by stirring and then transferred to a 1mm gap width electroporation cuvette (Bio-Rad, USA). A pulse of 1800v was applied to the samples using a Bio-Rad Gene pulser and 1mL of room temperature LB was added immediately to the mixture and transferred to a small glass tube for incubation at 30˚C with shaking for one hour. Cells were transferred to a microcentrifuge tube, centrifuged, and 750µL of the supernatant discarded. From the remaining cell mix, 100µLwas plated on LB Km^{1500} plates and a 10× and 100× dilution also plated on LB Km^{1500} plates. The remaining volume was then centrifuged and the pellet used to inoculate another set of LB Km^{1500} plates. Ten colonies were purified on a set of LB Km^{1500} plates and later maintained on MM284 plates with Km^{1500}.

2.6 DNA sequencing and computer-assisted sequence analysis

Plasmids extracted from *E. coli* DG1 transformants were submitted for Sanger sequencing (Eurofins Genomics, Germany) to confirm the desired mutation. Samples were prepared according to the Eurofins Genomics Mix2seq protocol. The sequencing mix was composed of 15µL purified plasmid DNA of 100ng/µL concentration and 1µL of the sequencing primer in a total reaction volume of 17µL. For Rmet_2333, a sequencing primer was designed (seq_Rmet_2233) because none of the available primers was suitable. The pre-mixed sample was then pipetted into Mix2seq tubes and sent for sequencing ensuring to note the barcodes of each tube before dispatching. The SnapGene software package was used for sequence analysis and annotation.

2.7 Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) Analysis

To analyze the intracellular zinc concentrations in the laboratory-evolved strain, the deletion mutant and the parental strain, 10mL precultures of the strains were grown up to stationery phase and later diluted 1:20 in triplicates in liquid MM284. This dilution was then incubated at 30˚C for 2hrs after which a ten-fold serial dilution performed and drops of 20µL of each dilution spotted on MM284 agar plates for a viable count to serve as a control. 20mM Zn^{2+} was then added to the triplicate samples and further incubated for an hour, after which another viable count performed. From the 20mM Zn^{2+}-stimulated cells, 6mL was fixed using 2% paraformaldehyde (Sigma-Aldrich, USA) at 4˚C for 20mins and later washed thrice with an ice-cold wash buffer (10mM Tris-Hcl, pH 6.8, 100µM EDTA). 1mL was then prepared and stored in liquid Nitrogen and the remaining 5mL processed for shipping for the intracellular zinc levels to be analyzed (ActsLab, Canada). The remaining volume of the stimulated cells was then incubated for a further 4hours after which a viable count performed and samples prepared for ICP-OES as above.

2.8 ABC transporter database similarity search

The ABCdb, an online database of ABC transporters in archaea and bacteria (38), was used for similarity search between the transporter subunits and classification of ABC transporters. The command was given as "Jump to (Strain.Gene)" and the query as "Rmet_2229 right up to Rmet_2234" and clicking the search button. After a display of the various features, the protein subfamily was selected to view the characteristics of other members of the family. The parameters of interest were gene, its description or function, status, protein subfamily and features of the subfamily.

2.9 Statistical analysis

Bacterial growth curves were modeled using the DMfit 3.5 statistical program (IFR, Norwich, UK). The GraphPad Prism software (GraphPad Software inc., USA) was used for all statistical analyses. The Bonferroni multiple comparison test was used for posttest analyses. The statistical significance of differences ($p<0.05$) was evaluated with the ANOVA and the Student's t-test.

3 Results

3.1 ABC-type transporter subunits similarity search and classification

The ABCdb database, a database of all bacterial and archaeal ABC transporters, was searched to provide a detailed description and classification of the different ABC-type transporter subunits (Table 3). Rmet_2229 encodes the periplasmic component of the ABC-type sugar transporter, and it is probably the SBP with an unconfirmed status. It belongs to the S_5ab subfamily with 1237 other members, all of which are components of ABC transporters serving as SBPs with confirmed statuses. Rmet_2230 encodes a predicted small integral membrane protein with an unknown status and subfamily. Rmet_2231 codes for a permease component of the ABC-type transporter with an unconfirmed status and is one of the TMDs involved in the translocation pathway. It belongs to the M_5ab1 subfamily with 1226 other members, which are all permease proteins of ABC transporters with confirmed statuses. Rmet_2232 encodes the second permease component and TMD of the ABC-type sugar transporter although its status remains unconfirmed. This permease component is a member of the M_5ab2 subfamily with 1260 members, most of which are permease components of ABC transporters with confirmed statuses. Rmet_2233 codes for an ATPase component or NBD of the ABC-type transporter with yet an unconfirmed status. It belongs to the N_5ab1 subfamily with 64 members, which are ATP-binding proteins of ABC transporters with the majority having confirmed statuses. Rmet_2234 encodes the second ATPase or NBD component with an unconfirmed status. Its subfamily is the N_5ab2 with 63 members, majority of which are ATP-binding proteins of ABC sugar transporters with confirmed statuses.

Table 3: ABC transporter subunits similarity search and classification

Gene	**Description (function)**	**status**	**Protein subfamily**	**Subfamily credentials**
Rmet_2229	ABC-type sugar transporter, periplasmic component, probable sugar binding precursor	unconfirmed	S_5ab with 1238 members inclusive	ABC transporters: substrate-binding proteins with confirmed statuses
Rmet_2230	predicted small integral membrane protein	nil	nil	nil
Rmet_2231	ABC-type transporter, permease component	unconfirmed	M_5ab1 with 1227 members inclusive	ABC transporters: permease proteins with confirmed statuses
Rmet_2232	ABC-type sugar transport systems, permease component	unconfirmed	M_5ab2 with 1260 members	ABC transporters: permease components with confirmed statuses
Rmet_2233	ABC-type transporter, ATPase component	unconfirmed	N_5ab1 with 64 members	ABC transporters: ATP-binding proteins with majority of confirmed statuses and a few rejected
Rmet_2234	ABC-type transporter, ATPase component	unconfirmed	N_5ab2 with 63 members	ABC sugar transporters: ATP-binding proteins with confirmed statuses

3.2 **Confirming the presence of the correct plasmid DNA**

At the beginning of the study, the *E. coli* transformants did not harbor the desired construct as initially considered. This was noticed after a series of failed digestion and PCR experiments. A new batch of *E. coli* DG1 was chemically transformed with the correct plasmid DNA (i.e pBBR1MCS2+Rmet_2229-2234) and a colony PCR performed with these transformants to confirm the presence of the correct construct. Restriction enzyme digests were also performed on this plasmid DNA using *Bam*HI, *Xba*I and *Bgl*II also to confirm the presence of the right vector sequence. Gel electrophoresis was used to separate the different fragments and later photographed. It was shown that the newly transformed DG1 cells had the correct construct which was necessary to proceed with the study. The size of the construct was approximately 11.5kb.

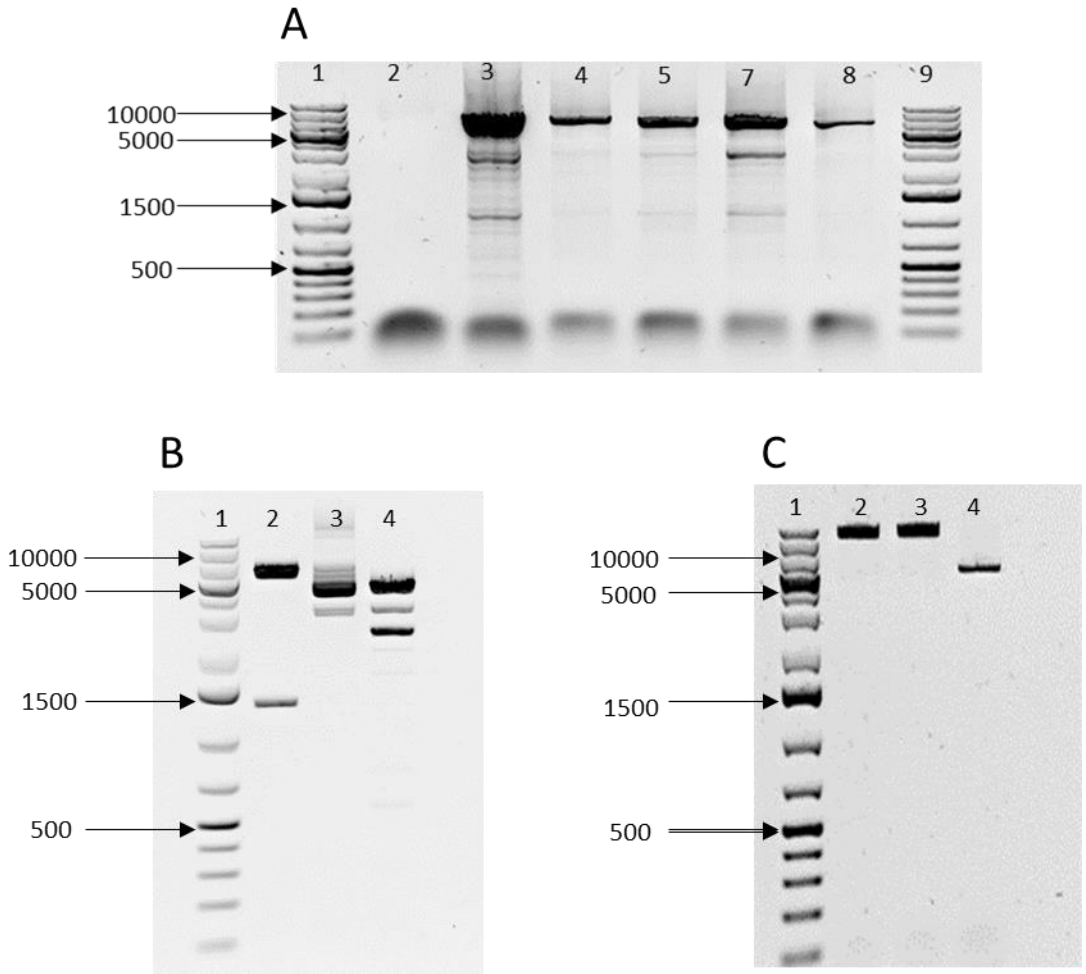

Figure 3: Checking the presence of the correct construct in newly transformed E. coli DG1 cells A) Colony PCR of E. coli DG1 transformants harboring the desired vector sequence. Water as negative control (lane 2) and selected colonies (lanes 3, 4, 5, 6, 7, 8). B) Restriction enzyme digest on the undesired construct (Lane 2, 3, 4) digested with BamHI, BglII and XbaI respectively. C) Restriction digest of the correct construct (lane 2, 3) digested with BamHI and the empty vector pBBR1MCS2 as negative control (lane 4) digested with BamHI. The numbers represent the size of the DNA in comparison with the ladder (lane 1, lane 9).

3.3 Inactivating the genes for the respective subunits on the complementation plasmid

Each of the six subunits (the periplasmic protein, the small integral membrane protein, the two permeases and the two ATPases) on the complementation plasmid were deleted by site-directed mutagenesis. This deletion was done by inducing a point mutation at the start codon of their respective genes (i.e. for Rmet_2229, Rmet_2230, Rmet_2232, Rmet_2233 and Rmet_2234). A single nucleotide base pair was deleted from the start codon (ATG) and confirmed by sequencing. For Rmet_2231, the gene was deleted by creating a short in-frame fragment. All the six subunits were successfully deleted in the complementation plasmid.

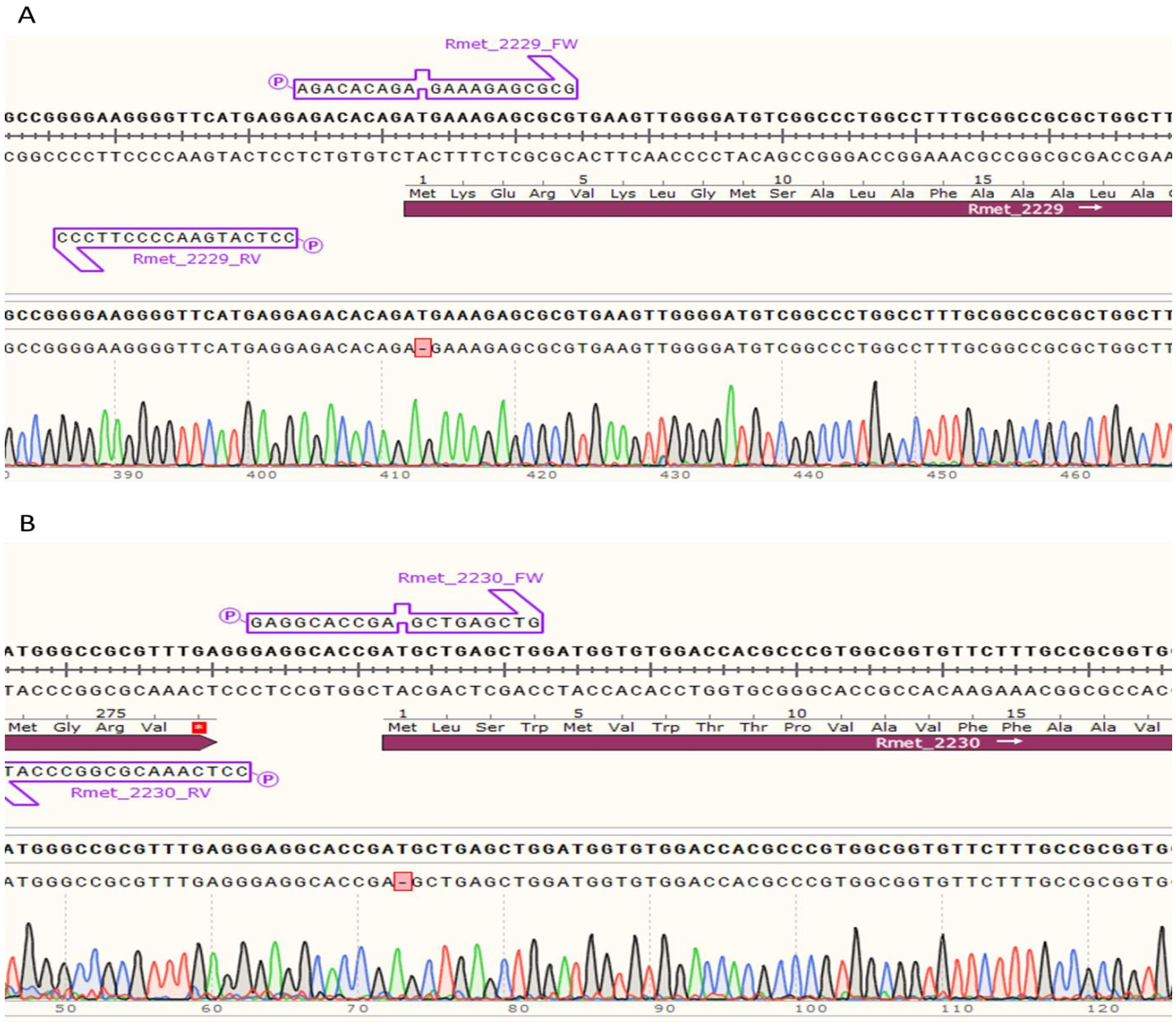

Figure 4: Sequencing results for Rmet_2229 and Rmet_2230 inactivation A) A section of Rmet_2229 showing the point mutation by the deletion of a T. The forward primer carried the intended mutation as seen with the arch which shows the missing T from the start codon (ATG). The sequences in bold are the original sequences while the plain one below is the mutated sequence showing the point mutation on the start codon with a negative sign surrounded by a pink box. Fw; forward primer, Rv; reverse primer. B) A section of Rmet_2230 showing the missing T on the start codon. The well-formed distinctive single-coloured peaks of the chromatogram and the missing base pair signifies the success of the sequencing. P; primers phosphorylated at their 5' ends. A: Adenine; T, Thymine, G: Guanine, C: Cytosine.

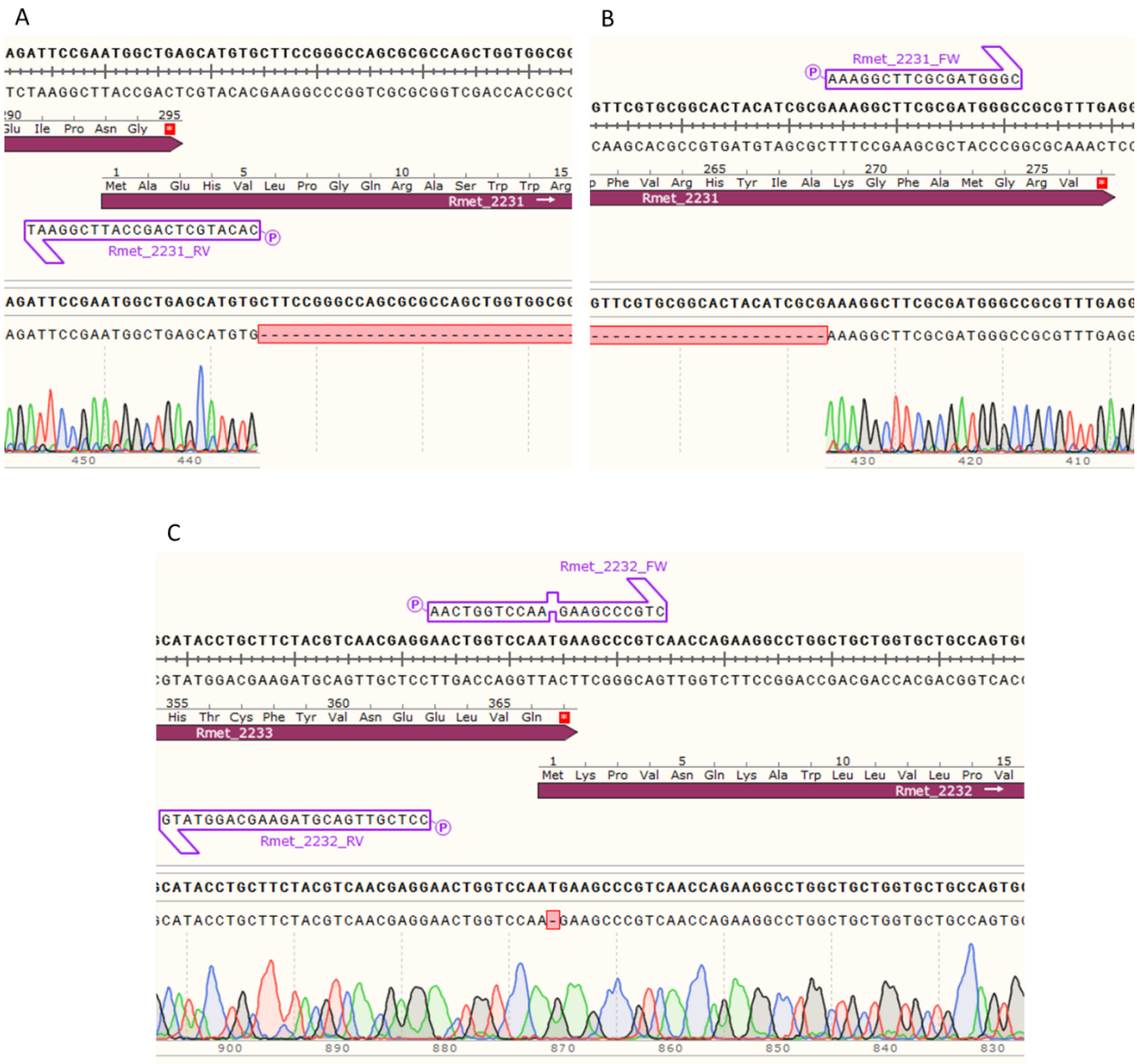

Figure 5: Sequencing results of Rmet_2231 and Rmet_2232 A) Two sections of Rmet_2231 showing the deletion by the alternative method. Only a short portion of the gene remains active, a few bps upstream and downstream. The deleted strand is shown with the many negative signs in the DNA. Fw; forward primer, Rv; reverse primer. B) A section of Rmet_2232 showing the missing T on the start codon. The well-formed distinctive single-coloured peaks of the chromatogram and the missing T signifies the success of the sequencing. P; primers phosphorylated at their 5' ends. A: Adenine; T, Thymine, G: Guanine, C: Cytosine.

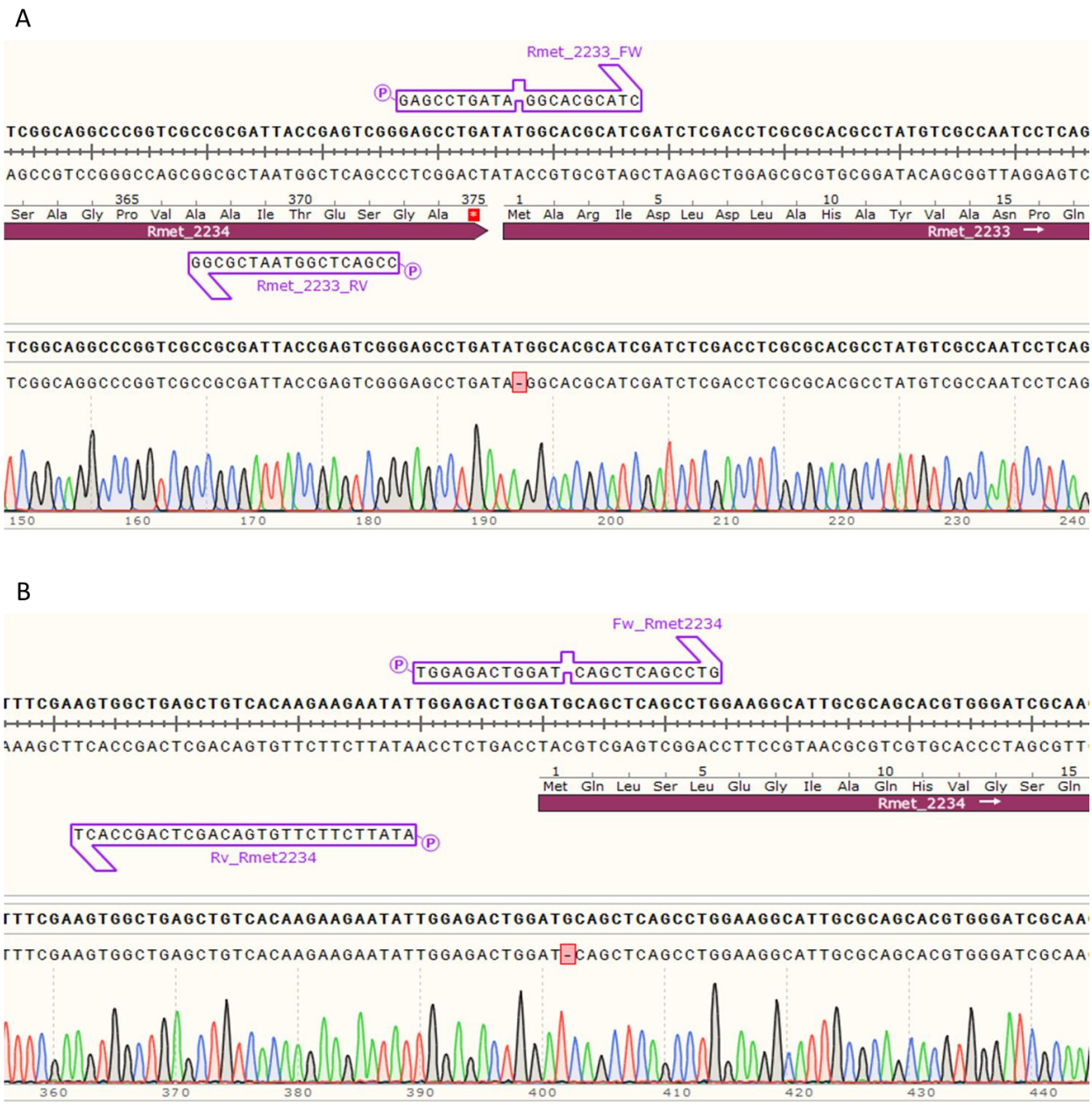

Figure 6: Sequencing results of Rmet_2233 and Rmet_2234 A) A section of Rmet_2233 showing the point mutation by the deletion of a T. The forward primer carried the intended mutation as seen with the arch which shows the missing T from the start codon (ATG). The sequences in bold are the original sequences while the plain sequence below is the mutated sequence showing the point mutation on the start codon with a negative sign surrounded by a pink box. Fw; forward primer, Rv; reverse primer. B) A section of Rmet_2234 showing the missing G on the start codon. The well-formed distinctive single-coloured peaks of the chromatogram and the missing base pair signifies the success of the sequencing. P, primers phosphorylated at their 5' ends. A: Adenine; T, Thymine, G: Guanine, C: Cytosine.

3.4 **The different complementation strains with the respective subunit inactivated**

The six deletions were successfully created on the complementation plasmid. These mutated plasmids were then used to complement the CH34 deletion mutant. A full description of the six complementation strains with the respective inactivation are outlined in Table 4 below.

Table 4: CH34 complementation strains with the six individual knockouts of the various subunits.

Complementation strains	**Genotype/characteristic**
CH34ΔRmet_29-35*::tet* + pRmetABC_30_31_32_33_34	Rmet_2229 to Rmet_2235 replaced by *tet*, and complemented with pRmetABC_30_31_32_33_34
CH34ΔRmet_29-35*::tet* + pRmetABC_29_31_32_33_34	Rmet_2229 to Rmet_2235 replaced by *tet*, and complemented with pRmetABC_29_31_32_33_34
CH34ΔRmet_29-35*::tet* + pRmetABC_29_30_32_33_34	Rmet_2229 to Rmet_2235 replaced by *tet*, and complemented with pRmetABC_29_30_32_33_34
CH34ΔRmet_29-35*::tet* + pRmetABC_29_30_31_33_34	Rmet_2229 to Rmet_2235 replaced by *tet*, and complemented with pRmetABC_29_30_31_33_34
CH34ΔRmet_29-35*::tet* + pRmetABC_29_30_31_32_34	Rmet_2229 to Rmet_2235 replaced by *tet*, and complemented with pRmetABC_29_30_31_32_34
CH34ΔRmet_29-35*::tet* + pRmetABC_29_30_31_32_33	Rmet_2229 to Rmet_2235 replaced by *tet*, and complemented with pRmetABC_29_30_31_32_33

3.5 Evaluating the zinc resistance phenotype of the laboratory-evolved strains

To confirm the higher zinc resistant phenotype of the laboratory-evolved strains, a dose-response experiment with zinc was performed with the parental strain and the two laboratory-evolved strains and incubated for 5 days after which the optical density was determined at 600nm in a 24-well cell culture plate which was placed into a Clariostar. The two laboratory-evolved strains significantly displayed a higher zinc resistant phenotype compared to the parent strain (Figure 7)

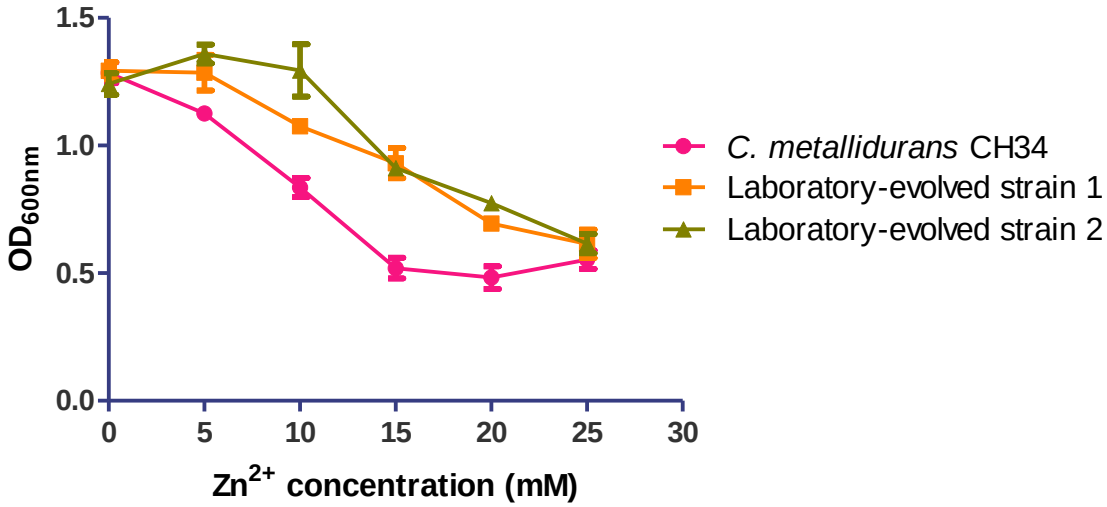

Figure 7: Increased zinc resistance phenotype of the laboratory-evolved strains compared to the parental strain. The optical densities were determined after 5 days of incubation. The X-axis represents the different zinc concentrations in millimolar (mM) while the Y-axis represents the optical densities at 600nm. Graphs are the mean value out of three biological replicates and error bars indicate standard deviation.

3.6 **Confirming the transporter's involvement in zinc resistance**

To confirm the involvement of the studied transporter in zinc resistance, the parental strain, the two laboratory-evolved strains, the deletion mutant and the complementation strain were grown in MM284 with different concentrations of zinc and incubated for five days after which the optical density was determined at 600nm in a 24-well cell culture plate using a Clariostar. The two laboratory-evolved strains significantly displayed the highest zinc resistance levels compared to the mutant and also the parental strain. This is in conformity with the findings of Vandecraen *et al*., 2016 (18), who reported that this transporter is involved in the increased zinc resistance of a laboratory-evolved CH34 strain. The complementation strain unexpectedly displayed a lower level of resistance compared to the laboratory-evolved strains.

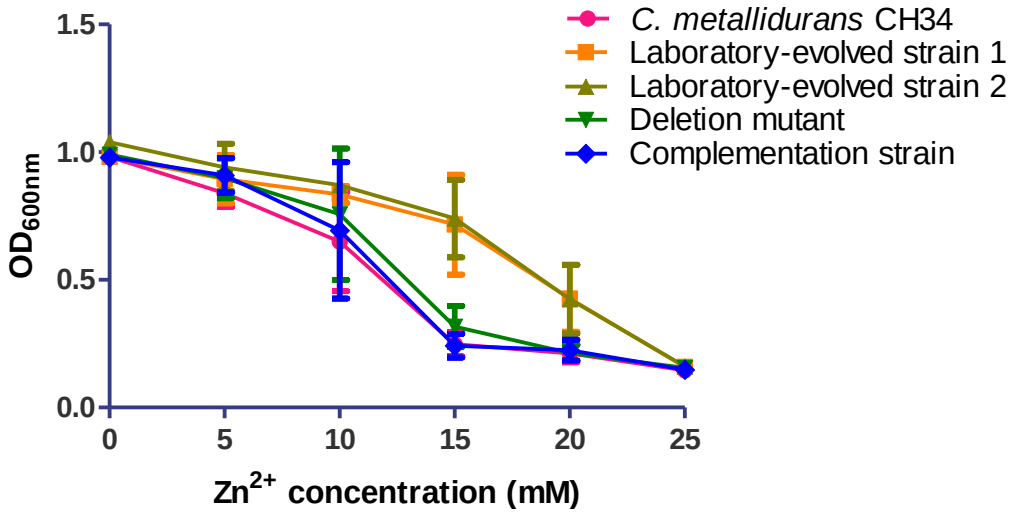

Figure 8: Confirming the transporter's involvement in zinc resistance. Dose-response experiments with zinc were performed with the laboratory-evolved strains, the deletion mutant, the parent strain and the complementation strain of Cupriavidus metallidurans CH34. The optical densities were determined after 5days of incubation. The X-axis represents the different zinc concentrations in millimolar (mM) while the Y-axis represents the optical densities at 600nm. Graphs are the mean value out of three biological replicates and error bars indicate standard deviation.

3.7 The response of *C. metallidurans* CH34 and its derivatives to high zinc exposure

As part of the sample preparation for ICP-OES, the response of one laboratory-evolved strain, the parental strain and the deletion mutant was examined in 20mM Zn^{2+}. Precultures were incubated at 30˚C until stationary phase, diluted 1:20 in triplicates in MM284 and a viable count performed after two hours of incubation. 20mM Zn^{2+} was then added, after which another viable count performed after one hour and four hours. It was observed that the number of colonies formed per ml for the mutant was significantly reduced after one hour of incubation in MM284 with 20mM Zn^{2+}. Only the laboratory-evolved strain could survive in MM284 with 20mM Zn^{2+} after four hours of incubation.

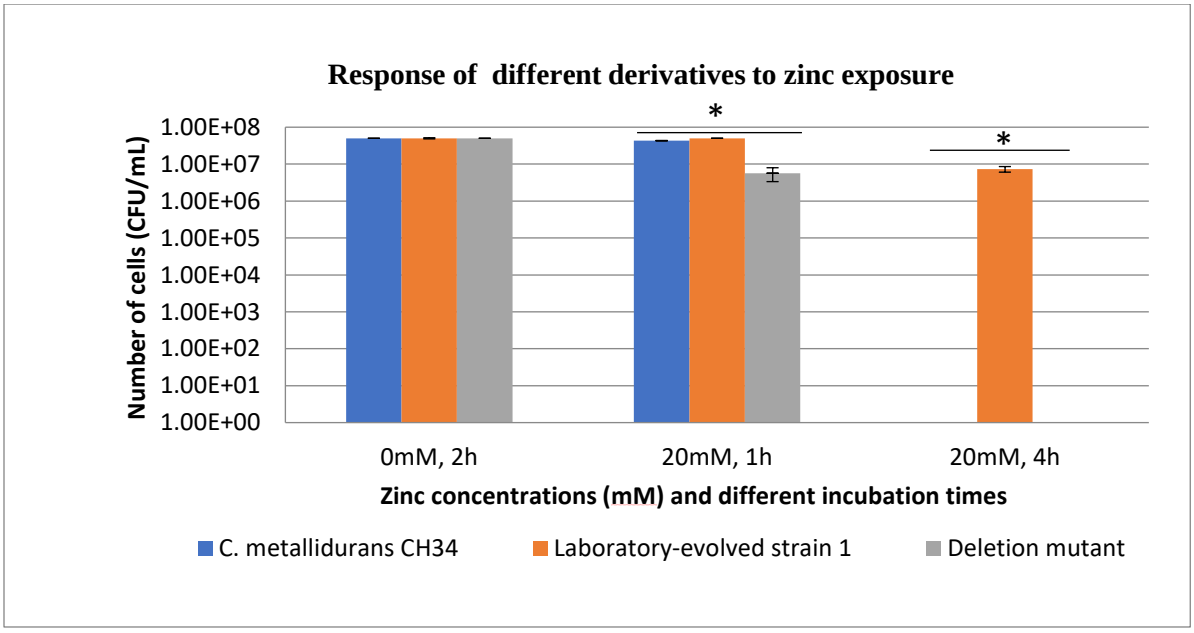

Figure 9: Bacteria exposure to 20mM Zn^{2+}. Precultures of the parental strain, the laboratory-evolved strain 1, and the mutant were exposed to 20mM Zn^{2+} and a viable count performed after the first and the fourth hour of incubation. 0mM, 2h; Bacteria culture without Zn^{2+} and incubated for 2h. 20mM, 1h; 20mM Zn^{2+} added and incubated for 1hr. 20mM, 4h; The 20mM Zn^{2+} cultures incubated for another 4hrs. Bars are the mean value out of three biological replicates and error bars indicate standard deviation. (*) indicates significant difference.

3.8 Evaluating the stability of the complementation plasmid in *C. metallidurans* CH34

The stability of the complementation plasmid in higher concentrations of zinc was evaluated after the unexpected lower resistance level of the complementation strain compared to the laboratory-evolved strains in dose-response experiments. Viable counts were performed with precultures from MM284 and MM284 plus 10mM Zn^{2+}. Precultures from MM284 plus 10mM Zn^{2+} spread on LB and LB Km^{1500}, were scored against precultures from MM284 without zinc. Precultures from MM284 with 10mM Zn^{2+} had fewer number of colonies on both LB and LB Km^{1500} compared to that without zinc. Km^{1500} served as a selective marker for the complementation strain.

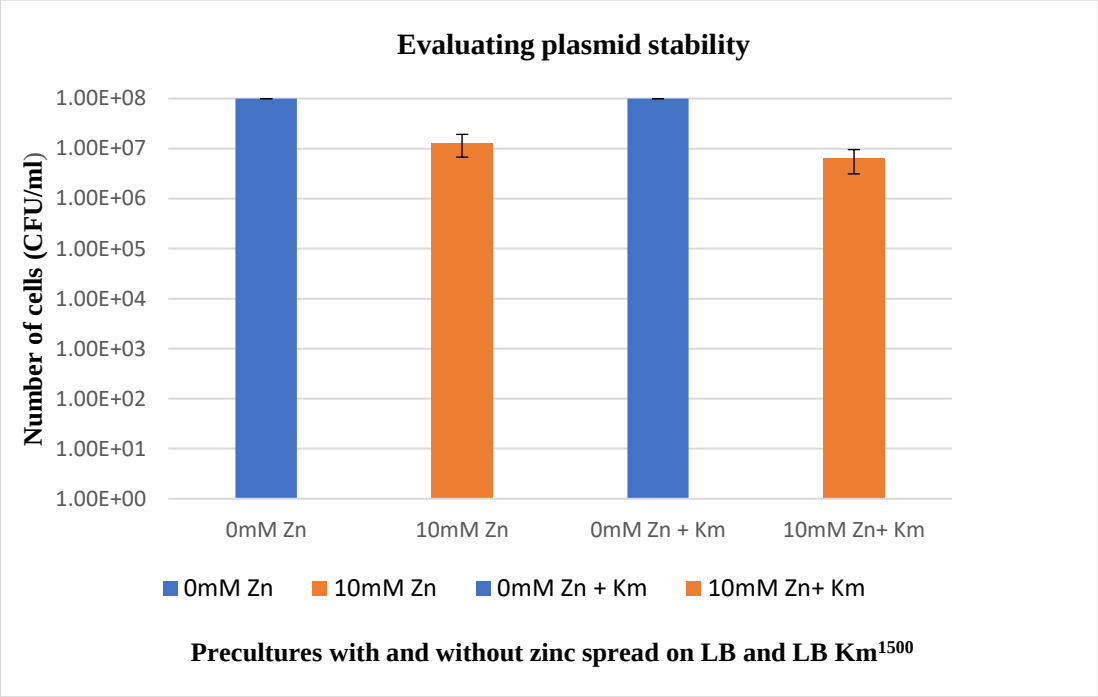

Figure 10: Plasmid stability. The X-axis represents the medium in which the precultures were grown (MM284) and the medium on which viable counts was performed (LB). Y-axis represents the number of cells per ml after a viable count. 0mM Zn; precultures without Zn^{2+} plated on LB. 0mM Zn + Km; precultures without Zn^{2+} plated on LB km^{1500}. 10mM Zn; precultures with 10mM Zn^{2+} plated on LB. 10mM Zn + Km; precultures with 10mM Zn^{2+} plated on LB km^{1500}. Bars are the mean value of three biological replicates and error bars represent standard deviation.

3.9 Comparing the level of glycerol utilization in the laboratory-evolved strains, the mutant, the complementation strain and the parental strain

To compare the level of glycerol utilization, the laboratory-evolved strains, the mutant, the parental strain and the complementation strain were grown in MM284 medium with 1% (w/v) glycerol and their growth evaluated over time by measuring optical densities for 14 days. All strains could significantly utilize glycerol as sole carbon source, although at different rates. The laboratory-evolved strains exhibited the longest lag phase with substantial growth observed only after day nine. The prolonged lag phase of *C. metallidurans* CH34 in glycerol is in conformity with the findings of Peitzch and colleagues (1998)(61), who reported that growth of *C. metallidurans* CH34 and AE104 in glycerol was slow when compared to growth in gluconate.

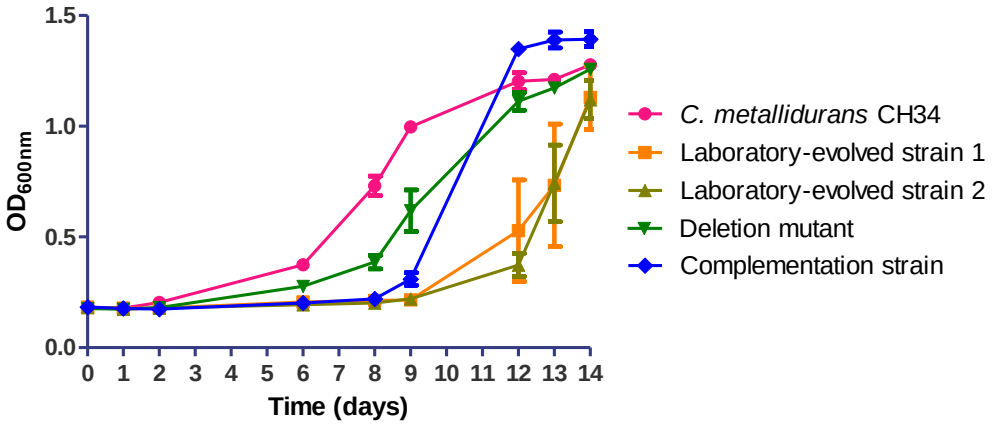

Figure 11: Glycerol growth experiment. Growth experiments were performed for five different derivatives of CH34 in MM284 without gluconate but supplemented with 1% glycerol. Graphs are the mean value out of three biological replicates and error bars indicate standard deviation.

4 Discussion

This study confirms the involvement of an ABC-type sugar transporter in zinc resistance in *Cupriavidus metallidurans* CH34. Here, a unique approach was used that merged mutagenesis, cloning, ICP-OES and phenotyping to investigate the role of an ABC-type sugar transporter in zinc resistance in a laboratory-evolved *Cupriavidus metallidurans* CH34. This strain was obtained by inoculating the parent strain on liquid MM284 with 12mM $Zn^{2+}$and then selecting them on plates of MM284 with 24mM Zn^{2+}. A derivative of *C. metallidurans* CH34 was created which has both its transporter and regulator deleted. This deletion mutant was then complemented with a plasmid harboring the genes for the six subunits. Six complementation strains were made harboring the complementation plasmid with each of the subunits deleted. A detailed description and classification of the six subunits was performed after an ABCdb database similarity search. The involvement of this ABC-type transporter in zinc resistance was confirmed. It was demonstrated all derivatives of *C. metallidurans* CH34 could utilize glycerol as sole carbon source and that this transporter is not essential for glycerol utilization in this bacterium.

4.1 Optimization experiments

The first step in gene deactivation using site-directed mutagenesis is amplification of the template DNA with primers designed for the intended mutation. The very first amplification reactions were unsuccessful as either no bands, aspecific bands, primer dimers or smears were observed upon imaging after gel electrophoresis. After a series of failed amplification reactions, it was noticed that the starting *E. coli* DG1 transformants harbored a construct different from the desired construct. This was noticed after a restriction enzyme digest with *Bam*HI, *Xba*I, *and Bgl*II was performed on the plasmid and observing incorrect band patterns different from the desired plasmid DNA. After confirming the correct construct, the plasmid was extracted as earlier mentioned and another digest performed using the above-mentioned restriction endonucleases. Samples that had the desired band with *BamH*I were then used to proceed with the cloning experiments.

As self-designed, and not standard commercial primers were used, optimization was necessary to attain the optimum annealing temperatures as the annealing temperatures generated by the Thermoscientific Tm calculator were not ideal for the reactions. After a series of unsuccessful amplification reactions, the respective annealing temperatures for the various primers were finally attained as mentioned under material and methods. At first, the recommended 15s/kb base pair for the extension step was used, but later 30s/kb was ideal which gave a total extension time of 6min for the nearly 12kb DNA. With this, and only after stepping up the concentration of the Phusion DNA polymerase to 0.04U/µL, we could achieve successful amplifications. After successful reactions with the first two primer pairs (i.e. for Rmet_2230 and Rmet_2234), the PCR products were cleaned and ligated with Thermoscientific T4 DNA ligase, transformed into *E. coli* DG1, plasmids extracted from 20 isolated colonies and sent for sequencing. Of the twenty samples sent, none of them contained the desired mutation. To optimize the efficiency, *Dpn*I was used to digest the methylated template DNA in the PCR product prior to ligation. Without this digestion, many false positive transformants will be observed because the circular template DNA is present which transforms efficiently. The ANZA ligation Master Mix was used for the ligation experiments. When the next batch of samples were processed, and sent for sequencing, there were many colonies

having the desired mutation. After successfully creating the two strains, primers for the remaining genes were ordered and the cloning experiments were conducted with successful results. After inactivating the individual genes of the six subunits in the plasmid, each of these mutated plasmids was used to complement the deletion mutant. Each of the clones was maintained on plates of MM284 + km^{1500} and stored at -4˚C until when required for experiments. The *E. coli* DG1 transformants with the different subunits inactivated were also processed for storage in liquid nitrogen and logged into SCK.CEN's databank. The series of long PCR optimization experiments, other optimizations and wait times for sequencing results and delivery of orders, were time-consuming and delayed the study progress, although these are factors which most often cannot be influenced.

4.2 Subunit similarity search and classification

A database similarity search was performed for the six subunits to identify homologs of each subunit and properly classify them. The search was necessary as it correctly classifies each unit to give an overview of its features in relation to its homologs, and served as a pointer to the function of each unit with respect to the subfamily to which it belongs. From this classification, it is likely that this transporter is an importer as its architecture correlates with that of different importers as outlined in this database and the literature review. For example, substrate-binding proteins have been reported only in importers and not exporters. In this case, Rmet_2229 codes for the periplasmic component of the ABC-type sugar transporter, and is probably the sugar binding precursor, although its status remains unconfirmed when compared to other members of its subfamily, which are all SBPs with many of confirmed statuses. The ABC-type transporter probably crosses the inner membrane by the two permeases (which are the two TMDs) and the small integral membrane protein, because this integral membrane protein has an α-helical structure which is mostly found in the cytoplasmic membrane. The periplasmic protein is probably the SBP that will bind the substrate and deliver it to the two TMDs. The two ATPases are the NBDs which will fuel the transport reaction by hydrolyzing ATP. However, current data is insufficient to decipher whether the transporter is an exporter or importer, or whether it has a direct or an indirect effect in zinc resistance.

4.3 Evaluating the phenotype of the laboratory-evolved strains as well as others

A quality control was done on the two laboratory-evolved strains, the deletion mutant, and the complementation strain before commencing the study. All the strains could grow well on MM284 and MM284 plus 1mM Zn^{2+}. As expected, only the mutant and the complementation strain could grow on plates of MM284 plus Tc^{20} while only the complementation strain could grow on MM284 plus Km^{1500}. This was done to ensure that the strains are zinc resistant and to check that the mutant and the complementation strain have the correct markers. i.e. the tetracycline cassette for the mutant and kanamycin with tetracycline cassettes for the complementation strain. The mutant and the complementation strains were always maintained on MM284 agar plates containing Tc^{20} and MM284 agar plates with Km^{1500} respectively, to ensure effective selection of both strains before precultures. Moreover, precultures of the complementation strain were always performed in liquid MM284 with Km^{1500}.

4.3.1 Maintaining the phenotype of the laboratory-evolved strains

In the early phase of this study, the laboratory-evolved strains could not display the expected level of zinc resistance when compared to the other strains suggesting that these strains could have lost their high zinc resistance phenotype with time. To maintain this high zinc resistance phenotype, the two strains were maintained on MM284 agar plates with 10mM Zn^{2+}. This was to maintain the deactivation of *glpR* by IS*1088* ensuring the continuous derepression of the ABC transporter operon downstream because excision of IS*1088* can render an intact *glpR*. When a dose-response experiment with zinc was performed on these re-stimulated laboratory-evolved strains and the parent strain, it was observed that these strains could once again display a higher zinc resistance phenotype compared to its parent. This re-stimulation was therefore pivotal in maintaining the observed higher zinc resistance of the laboratory-evolved strains (Figure 7).

4.4 Confirming the involvement of the ABC transporter in zinc resistance

Validating the involvement of the studied transporter in zinc resistance was necessary to verify if the initial results could be reproduced (18). Results have been checked a few times but MIC determinations with zinc are not always the same, as they even varied between batches of agar. To confirm the involvement of this transporter in increased zinc resistance, the laboratory-evolved strains, the parental strain, the deletion mutant and the complementation strain were grown in increasing concentrations of zinc right up to 25mM. The lower resistance level of the mutant compared to the laboratory-evolved strains confirms the involvement of this transporter in zinc resistance (Figure 8). Likewise, an optimization experiment where the parent strain was maintained on 10mM Zn^{2+}, showed that this strain could express a higher level of zinc resistance of 18mM Zn^{2+} but not 23mM Zn^{2+} as observed with the laboratory-evolved strains. The deletion mutant maintained on 10mM Zn^{2+} could not exhibit this higher resistance levels as it could not grow on 18mM zinc plates like the parental strain. This also shows that exposure to zinc is necessary for strain CH34 to gain a survival advantage in elevated levels of zinc. Inoculating the three strains on plates of MM284 plus 25mM Zn^{2+} was bactericidal as no colonies could emerge even after extended periods of incubation. The complete complementation strain unexpectedly displayed a lower resistance level compared to the laboratory-evolved strains in dose-response experiments. This shows that complementation of the mutant was not successful. Moreover, when the complementation strains with the various subunits deleted were inoculated on MM284 plus 10mM $Zn^{2+}$and Km^{1500}, only a few colonies could emerge for Rmet_2229 and 2232 knockouts after extended incubation of approximately two weeks. This suggests that this transporter could have a role in mediating resistance to elevated concentrations of zinc.

4.5 Evaluation of the complementation strain

Several factors are implicated to have been responsible for this failed complementation assay, either acting individually or concomitantly. First, it was speculated that the bacteria may not be able to retain its plasmid in elevated levels of Zn^{2+}. Second, the plasmid may not be ideal for complementation with *Cupriavidus metallidurans* CH34. Third, polar mutations may have resulted from the deletions performed on the mutant. Last, Insertion Sequence transposition, transposons

and secondary mutations resulting from the deletion procedures in the mutant can neutralize complementation.

4.5.1 Evaluating the stability of the complementation plasmid in *C. metallidurans* CH34

It may be possible that the generated plasmids did not have the correct base pair, a situation termed structural plasmid instability, or daughter cells may have proliferated without acquiring a plasmid, another situation termed segregational plasmid stability. Likewise, although many plasmids have high expression levels, it may be possible that the gene dosage necessary for expression is relatively small in which case a plasmid is said to be of a low copy number (62). To assess the plasmid stability in the complementation strain, precultures were done in triplicates in MM284 with and without 10mM Zn^{2+} from were a viable count was performed on LB plates with and without Km^{1500}. Kanamycin was used as a selective marker for the complementation strains. The lower CFUs/mL on both LB and LB Km^{1500} for precultures with 10mM Zn^{2+}compared to precultures with no zinc, suggests that the plasmid could be less stable in increasing concentrations of zinc, although the difference was not statistically significant (Figure 10). Moreover, the complementation strain could not grow on plates of MM284 containing both 10mM Zn^{2+} and Km^{1500}suggesting that the plasmid is less stable in higher zinc stress. Therefore, during bacterial cell division, the daughter cells may have proliferated without acquiring the plasmid from the parents because the rate of cell division is probably higher than that of the plasmid replication. Likewise, the new generation may have acquired plasmids with the wrong base pair. Hence, the daughter cells without the plasmid or with a faulty plasmid are more or less like the mutant which is unable to survive elevated levels of zinc. Moreover, because the vector is of a low copy number, the number of plasmids per offspring may be insufficient for the expression of the genes it harbors. Nevertheless, this difference in colony-forming units is not statistically significant to conclude that plasmid instability is the reason for the unsuccessful complementation.

4.5.2 Analyzing the competence of pBBR1MCS2 in complementation experiments with *C. metallidurans* CH34

It was speculated that pBBR1MCS2, a derivative of pBBR1MCS may not be ideal for complementation experiments with *Cupriavidus metallidurans*. However, another study by our group successfully used this same vector for complementation. In that study, complementation with this vector was used to confirm the role of the *cnrYXHCBAT* operon to be capable of exerting zinc resistance in *Cupriavidus metallidurans* AE104 (63). Strain AE104 is a derivative of strain CH34 void of a plasmid and sensitive to zinc and had been reported to be unable to evolve beyond 0.8mM Zn^{2+}. The *cnrCBAT* operon encodes the RND-driven efflux system and this locus is regulated by another operon found upstream i.e. the *cnrYXH* which encodes a membrane-bound anti-sigma factor CnrY, the sensor protein CnrX, and the ECF family sigma factor CnrH. Here, complementation of strain AE104 with the entire *cnr* locus resulted in its ability to acquire zinc resistance at a frequency of $0.96\pm0.38x10^{-6}$ after 48hour exposure to 0.8mM Zn^{2+}. Moreover, complementation with a full and modified *cnr* locus from AE126 zinc resistant derivative ($AE126^{R1}$), exerted full resistance resulting in a ten-fold increase in its MIC. However, in a complementation to check if the sigma factor (CnrH) alone could induce zinc resistance, no resistance could be acquired suggesting the *cnrCBAT* alone is necessary to induce zinc resistance

at high frequencies in strain AE104 and AE126. The successful use of pBBR1MCS2 in these complementation experiments suggest that it is an ideal vector for use with complementation assays in *C. metallidurans* CH34 and that other factors and not the plasmid might be responsible for the failed complementation.

4.5.3 Polar effects resulting from gene deletions can lead to failed complementation

It is logical to think a polar mutation might be the cause of the failed complementation. A polar mutation is one that affects the expression of downstream genes or operons, and it mostly occurs in polycistronic mRNA. Hence, it is speculated that the deletion of Rmet_2229 might affect the expression of Rmet_2228 downstream which may also be a part of the operon or have an indirect effect in mediating zinc resistance by this operon. However, chances are low that a polar mutation might be the cause of the failed complementation because there is more evidence supporting Rmet_2228 not being part of the transporter operon. For instance, it is likely that Rmet_2228 has its own promoter since it is 184 amino acids away from Rmet_2229, meaning that there are lesser chances of Rmet_2228 being affected upon deletion of Rmet_2229. This protein is also highly conserved among several bacteria where it is not part of an operon and its function in majority of these bacteria remains unknown. Moreover, the gene upstream of Rmet_2235 (which codes for the transporter's repressor) is transcribed in the reverse direction from the transporter operon adding to the conviction that a polar mutation is likely not responsible in inactivating complementation in this case.

4.5.4 Insertion sequence transposition and secondary mutations can neutralize complementation

C. metallidurans CH34 has several mobile genetic elements (transposons and insertion sequence elements) whose transposition have been reported to influence determinants that are not only involved in metal resistance, but also in adaptation to other stress factors (13, 14, 18, 20, 63). They are regarded as the simplest MGEs which are widespread in all domains of life. Such small elements that are typically below 3kb are capable of independently transposing themselves. Flanked by short terminal inverted repeats (IRs), they generally harbor either single or multiple open reading frames that code for products only necessary for their own mobility. The transposition of these elements can either cause its insertion or its excision in a DNA sequence, propagating the adaptation of an organism. By either contributing to mutations such as deletions, inversions and duplications, they promote genomic plasticity. In this way, they can either cause the inactivation of a gene or modulate the expression of neighboring genes (Figure 12). IS elements are different from transposons in that transposons encode other proteins that can alter the metabolism of the host cell. The most common effect of IS transposition is perhaps in inactivating genes. They can also inactivate regulatory sequences and modulate the expression of resistance determinants. Transposition of these elements into a non-coding region of a DNA can change the expression pattern of neighboring genes. They can also provide promoter sequences which are either entirely or partially included within the IS.

When a repressor is inactivated by IS insertion, the gene it regulates becomes derepressed subsequently leading to its increased expression. One example of a scenario where the deactivation of a repressor was beneficial to strain CH34 is the inactivation of *glpR* by IS*1088* insertion leading

to the derepression of the ABC-type transporter and subsequent increased resistance to elevated levels of Zn^{2+} (18). It could be possible that a mobile genetic element may have inserted into the vector sequence leading to its mutation although the frequency of insertion is quite low. Also, mutation of a gene that has an indirect effect on this transporter in mediating Zn^{2+} resistance can also neutralize complementation. In one study that aimed at understanding the potential of complementation in rescuing mutations in the *argG* and *hisA* gene, it was observed that complementation activity was inactivated by IS insertion and also by partial deletion of the cloned DNA in the plasmid (64). The *argG* and *hisA* are genes that code for the biosynthesis of arginine and histidine respectively. In that study, DNA derived from the bacterium *Methanococcus voltae* was digested with *Pst*I and cloned into the vector pBR322 to generate recombinant plasmids (pAW1 for *argG* and pAW2 for *hisA*). These recombinant plasmids were used to transform *E. coli* that has a mutation in their *argG* and *hisA* and this complementation rescued the effect of these mutations. However, derivatives of pAW1 harboring insertion elements Tn*5* and γδ, could no longer complement the mutation in *argG* indicating that pAW1 was inactivated by each of these insertion elements. Moreover, only a small number of pAW2 cells into which Tn*5* was inserted were still capable of complementing *hisA.*

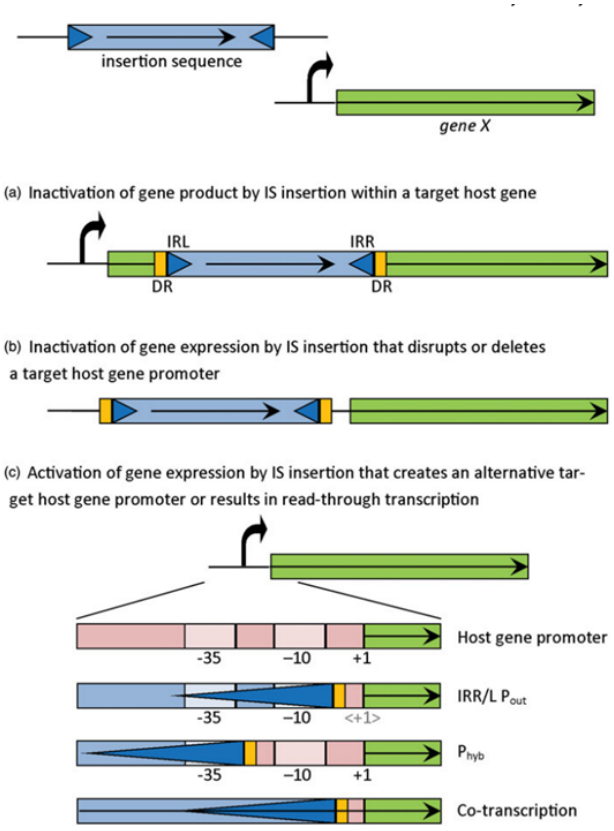

Figure 12: The effect of insertion sequence transposition on gene activation or inactivation. The IS is represented as blue rectangle surrounded by short terminal inverted repeats (IR; blue triangles) and flanked by directed repeats (DR; yellow rectangles) and the target gene as a green rectangle. The mRNA products are the straight arrows while the promoters of the target genes are the curved arrows made up of -35 to -10 components and the transcriptional start

site +1. <+> is the altered transcriptional start site of the target gene which harbors an IS carrying a complete out-ward promoter P_{out}.

It may also be possible that when deleting *glpR* and Rmet_2229-2234 from the CH34 deletion mutant, secondary deletions could have occurred thereby altering the general metabolism of the mutant which neutralised complementation. To test the effect of a secondary deletion in inactivating complementation, an *Acc*I fragment of 1.2kb in size which includes the entire *Hind*III fragment was deleted from the pAW2 plasmid. These deletion derivatives failed to complement *hisA* demonstrating that secondary deletions or mutations in the mutant can inactivate complementation. Hence, not only can secondary mutations in the complementary DNA alone inactivate complementation, but also unintended mutations that were induced when creating the deletion mutant.

This issue of failed complementation has not only been observed in prokaryotes but also in Eukaryotes. In one study which aimed at evaluating the reason for false positive and false negative complementation experiments, it was observed that secondary mutations that resulted from deleting or inactivating a gene of interest, could result in failed complementation experiments. As shown, complementation failed to rescue the effect of a mutation in the *ald* gene which lead to defects in meiosis and mitosis in *Drosophilia (65)*. It was later realized that this secondary mutation was as a result of the presence of a transposable element known as *Doc* in the gene *CG18212* which is found downstream of *ald*. This secondary mutation negatively affected the expression of the *ald* gene (Figure 13). Hence, it may also be possible that one of the many mobile genetic elements found in strain CH34 could have exerted an influence on the transporter operon or better still other neighboring genes downstream or upstream and inactivate it. Perhaps the inactivated gene could be acting synergistically with the ABC transporter operon.

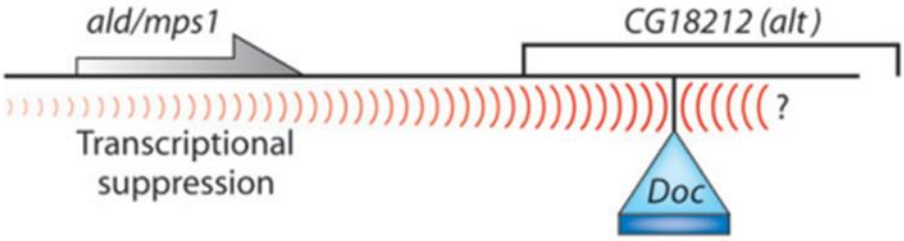

Figure 13: The influence of a transposable element (Doc) on the *ald* gene of Drosophilia leading to a failed complementation experiment aimed at rescuing mutations of the *ald* gene which lead to defects in its reproduction cycle. The transcription of *ald* was greatly suppressed. Adapted from Hawley *et al*., 2006 (65).

With the available data, it is quite challenging to decipher which is the correct argument: whether it is the insertion of a mobile genetic element that may have inactivated the complementation or the effect of secondary mutation(s) in the mutant. However, it is convincing to rule out the speculations on plasmid instability and the polar effect. Nevertheless, the effect of the mobile genetic elements and the secondary mutations have been increasingly reported in the literature and cannot be under looked. Moreover, all the above determinants might be acting synergistically in inactivating complementation.

4.6 Intracellular zinc quantification

We had earlier anticipated to send all the strains including the complementation strains to analyze their intracellular concentrations of zinc by ICP-OES. However, this was delayed due to the unsuccessful complementation. So, we resorted to send just the parental strain, one laboratory-evolved strain and the deletion mutant. Due to the limited duration of this study, samples that were prepared for analyzing their intracellular zinc concentration could not be sent for analysis as it was estimated that the time for delivery, processing and reception of results will exceed the duration of the study. Nevertheless, substantial information was gotten from the viable counts that were performed alongside the sample preparation (Figure 9).

4.7 Comparing the level of glycerol utilization in the evolved strains as well as others

Glycerol has been reported to induce high zinc resistance in strain CH34 as it represses GlpR (18). So, the phenotype of the five strains in glycerol was compared. To compare the level of glycerol utilization, the laboratory-evolved strains, the parent strain, the complementation strain and the deletion mutant were cultured in MM284 medium with 1% (w/v) glycerol and their optical densities evaluated for 14 days. The ABC-type transporter of *C. metallidurans* CH34 is similar to the operon that is responsible for glycerol utilization as the sole carbon source in *Rhizobium leguminosarum* bv. *viciae* VF39 which is carried on a plasmid (Figure 14) (66). This operon houses a gene (*glpR*) that codes for GlpR which belongs to the DeoR-family of repressors, as well as two other genes downstream which are all involved in glycerol utilization and under the control of GlpR. These family of regulators often repress the uptake and breakdown of sugars and usually control the expression of neighboring genes. Immediately after *glpR,* is *glpD* which encodes glycerol-3-phosphate dehydrogenase, and *glpK*, which encodes glycerol kinase is found at the rear of the operon. These two genes border the *glpSTPQUV* genes coding for an ABC transporter and the entire operon, all regulated by GlpR. This arrangement is different for *E. coli* where only *glpD* is located closer to *glpR* and not *glpK*. These two enzymes are responsible for the utilization of glycerol as the only carbon source after its uptake by the ABC transporter of *Rhizobium leguminosarum* bv. *viciae* VF39. This mechanism of glycerol utilization is probably very common in most bacteria as the genetic composition is highly conserved amongst several *Rhizobium*, *Sinorhizobium* and *Agrobacterium* species and other α-proteobacteria, often encoded by a plasmid. The expression of genes involved in glycerol utilization in both *E. coli* and *R. leguminosarum* bv. *viciae* VF39 are induced by glycerol itself and its phosphorylated product (glycerol-3-phosphate) as they interact with GlpR perpetrating the derepression of the entire operon (66).

In *C. metallidurans* CH34, *glpK* and *glpD* are proximal to *glpR* and Rmet_2229-2234 but are not found in the same operon with the transporter, an arrangement which is quite different from that of the glycerol utilization operon of *R. leguminosarum* bv. *viciae.* Hence, it may be possible that they have their own regulatory mechanism independent of GlpR. This ABC-type transporter of CH34 has six protein components arranged in an operon: one periplasmic protein, one small integral membrane protein, two permeases and two ATPases, which are encoded by the Rmet_2229 to Rmet_2234 genes respectively. This operon has a single regulator (GlpR) which is found upstream of the transporter and encoded by Rmet_2235. Its similarity with the glycerol ABC transporter operon of *R. leguminosarum* bv. *viciae* VF39, indicates a possibility of glycerol uptake

and probably other sugars by this uncharacterized ABC-type transporter. For instance, Rmet_2229 which encodes the periplasmic protein shares a percentage amino acid similarity of 63.4 with *glpV* of *R. leguminosarum* bv. *viciae* VF39 (66).The utilization of glycerol by strain CH34 is characterized by a prolonged lag phase which is typical for other bacteria (67). The longest lag phase observed with the laboratory-evolved strains suggests that certain factors could have negatively regulated glycerol uptake with subsequent slow relieve as an adaptive option in the prolonged presence of glycerol only. Although glycerol increases the zinc resistance phenotype of this bacterium, it could be possible that in an already higher zinc resistant strain, these factors can block the uptake of glycerol. As a survival option for this bacterium in the presence of glycerol only, such factors can be slowly deactivated or repressed by other adaptive mechanisms that may have been induced.

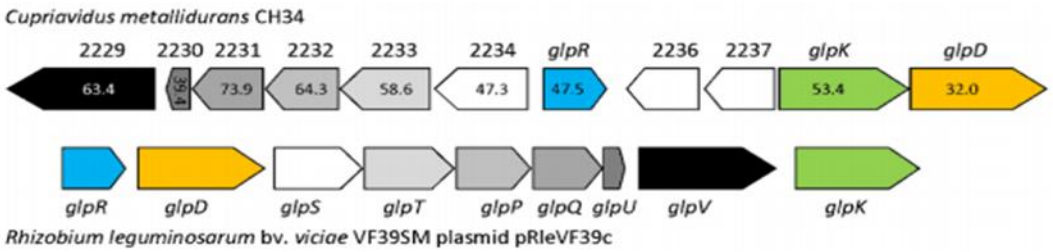

Figure 14: Similarities between the locus of the ABC-type transporter of strain CH34 and plasmid pRleVF39c in *R. leguminosarum bv viciae* VF39. The genes are presented as arrows and those of the same color represent homologs, with the number inside the arrow representing the % amino acid identity shared between both bacteria. Adapted from Ding *et al*., 2012 (66)

4.8 The ABC-type sugar transporter is not essential for glycerol uptake in strain CH34

Until now, it was not yet known if this transporter is necessary for glycerol utilization in *Cupriavidus metallidurans* or not. In the case of *R. leguminosarum*, an ABC transporter is responsible for the uptake of glycerol effectuated by ATP hydrolysis. In *E. coli*, however, an outer membrane porin channels glycerol into the periplasm of the cell and GlpF is responsible for the uptake of glycerol as it mediates transport of this sugar into the cell by facilitated diffusion (68). While in the cell, glycerol kinase immediately catalyzes the phosphorylation of this sugar to Glycerol-3-phosphate which is subsequently trapped in the cell. This type of glycerol uptake system is typical for most gram-negative bacteria and gram-positive bacteria like *Pseudomonas aeruginosa* and *Bacillus subtilis*, respectively (66, 68). As for CH34, no specific mechanism has been defined to be responsible for glycerol uptake. It could be possible that *Cupriavidus metallidurans* employs a similar mechanism to utilize glycerol as the only carbon source, since it has an aquaporin-like protein that is 30% similar in amino acid composition with GlpF of *E. coli.* Although the percentage homology is quite low, it may render the same function or even to a lesser extent. The fact that all the strains could utilize glycerol, indicates that the studied transporter is not essential for glycerol transport in this bacterium and other transporters may take up glycerol. The putative aquaporin-like glycerol uptake system could explain the ability of the CH34 deletion mutant to utilize glycerol as the only available carbon source. Hence, the ABC-type sugar transporter may not be essential for glycerol transport in this bacterium as its homolog, the *glp* operon of *R. leguminosarum* bv. *viciae* VF39.

5 Conclusion

This study aimed at understanding the role of an ABC-type sugar transporter that was reported to be putatively involved in increased zinc resistance in *Cupriavidus metallidurans* CH34. This bacterium is known for its ability to tolerate and resist high concentrations of several heavy metals. The transporter is composed of six subunits encoded by genes Rmet_2229 to Rmet_2234, with each subunit having a specific function. However, it is only the second case of an ABC transporter being reported to be involved in its metal resistance.

This study is the first of its kind to study how an ABC-type sugar transporter mediates resistance to high zinc concentrations, whether it has a direct or an indirect effect, or whether it exports or simply blocks the entrance of Zn^{2+}. To understand the function of this transporter in zinc resistance, mutagenesis, cloning, and phenotypic analyses were merged. An adaptive evolution was done on *C. metallidurans* CH34 to obtain two laboratory-evolved strains. A plasmid that harbors the genes for the six subunits was used to complement a CH34 derivative which has both its transporter and regulator deleted. The involvement of this transporter in zinc resistance was confirmed by the observed lower zinc resistance of the deletion mutant compared to the laboratory-evolved strains in dose-response experiments with zinc. The complementation strain displayed an unexpected lower resistance compared to the laboratory-evolved strains and several optimization experiments to resolve this are still ongoing. Current data is insufficient to conclude that these strains are unable to retain their plasmids in higher concentrations of zinc as there was no significant difference with the plasmid stability assay. Likewise, the speculation of polar effect and incompetency of the vector (pBBR1MCS2) in complementing *C. metallidurans* CH34 are likely not incriminated in the failed complementation. Moreover, it is convincing that this vector is capable of complementing *C. metallidurans* CH34 as observed with *C. metallidurans* AE104 and AE126 which are all zinc sensitive derivatives of strain CH34. Nevertheless, we can conclude that secondary mutation(s) may have been induced during the creation of the deletion mutant and that this greatly affected the phenotype of this strains such that complementation is neutralized by these mutations. Moreover, the effects of mobile genetic elements (insertion sequences and transposons etc.) could have been active players in deactivating the complementation although their frequencies of transposition are low.

Glycerol has been reported to induce zinc resistance in this bacterium as it represses GlpR relieving the transporter operon. The level of glycerol utilization in this bacterium was compared among the two laboratory-evolved strains, the parental strain, the deletion mutant and the complementation strain. It was observed that all the strains could significantly utilize glycerol. The fact that even the deletion mutant could utilize glycerol, suggests that this transporter is not essential for glycerol uptake as with its homolog in *R. leguminosarum* bv. *viciae* VF39. Other transporters may have mediated this glycerol uptake like the aquaporin-like protein which is similar to GlpF, the glycerol uptake system of *E. coli*.

Because complementation was not successful, the function of the respective subunits in this transporter could not be studied as earlier anticipated. Otherwise, the complete transporter complementation strain would have served as a standard in scoring the six complementation strains with the individual subunits deleted. However, we could confirm the involvement of this

transporter in zinc resistance and we could also show that the transporter is not essential for glycerol transport.

In the future, it will be ideal to continue with the ICP-OES analysis to quantify the intracellular zinc concentrations in the laboratory-evolved strains, the deletion mutant and the parental strain for a start. Second, to study the function of each subunit, future experiments should be aimed at re-evaluating the complementation assay to check the reasons for its failure. Since gene dosage determines the expression levels of a protein, experiments should be geared at detailly analyzing plasmid stability. qPCR can be used to determine the integrated plasmid copy number and attempts at increasing the expression level of the genes in the vector should also be considered. Thirdly, the presence of secondary mutations in the deletion mutant can also be checked at the level of making the deletion mutant. This can be performed by whole genome sequencing to know the actual position and type of mutation. That is, approximately 20 colonies of the deletion mutant should be selected and screened for secondary mutations and only those with the sole mutations of interest should be maintained for complementation assays. If the complementation remains unsuccessful, a change in the experimental setup should then be considered which should be focused on phenotypic analysis like viable cell counting in detail.

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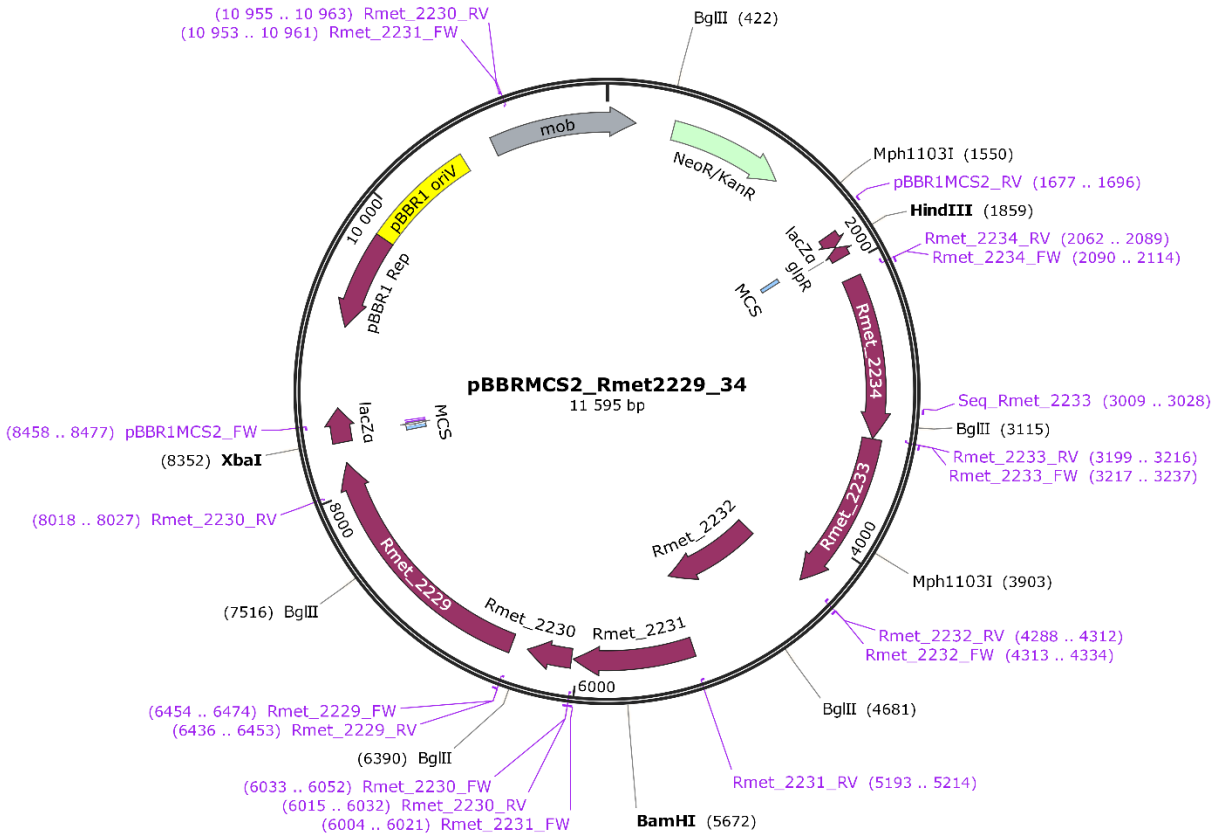
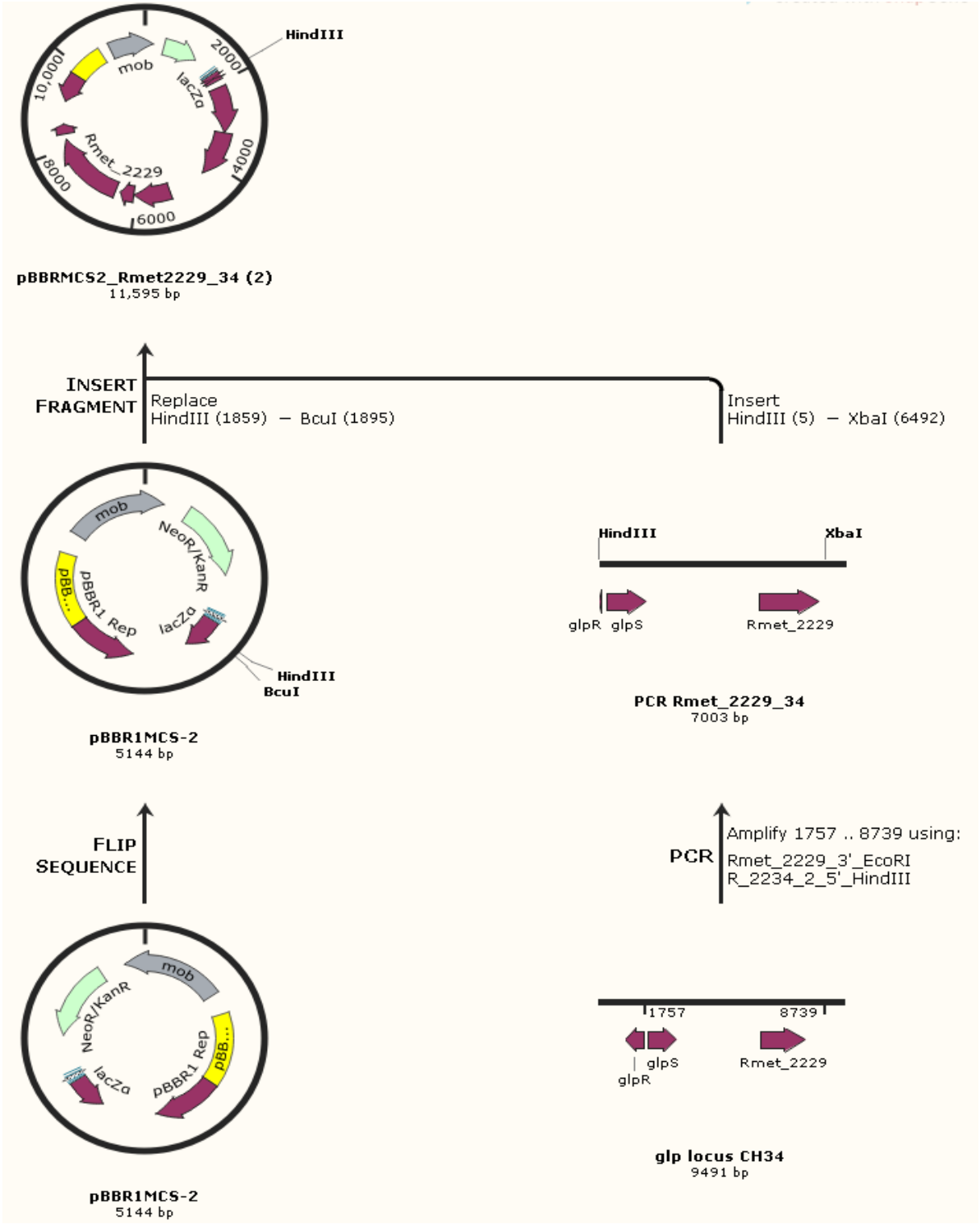
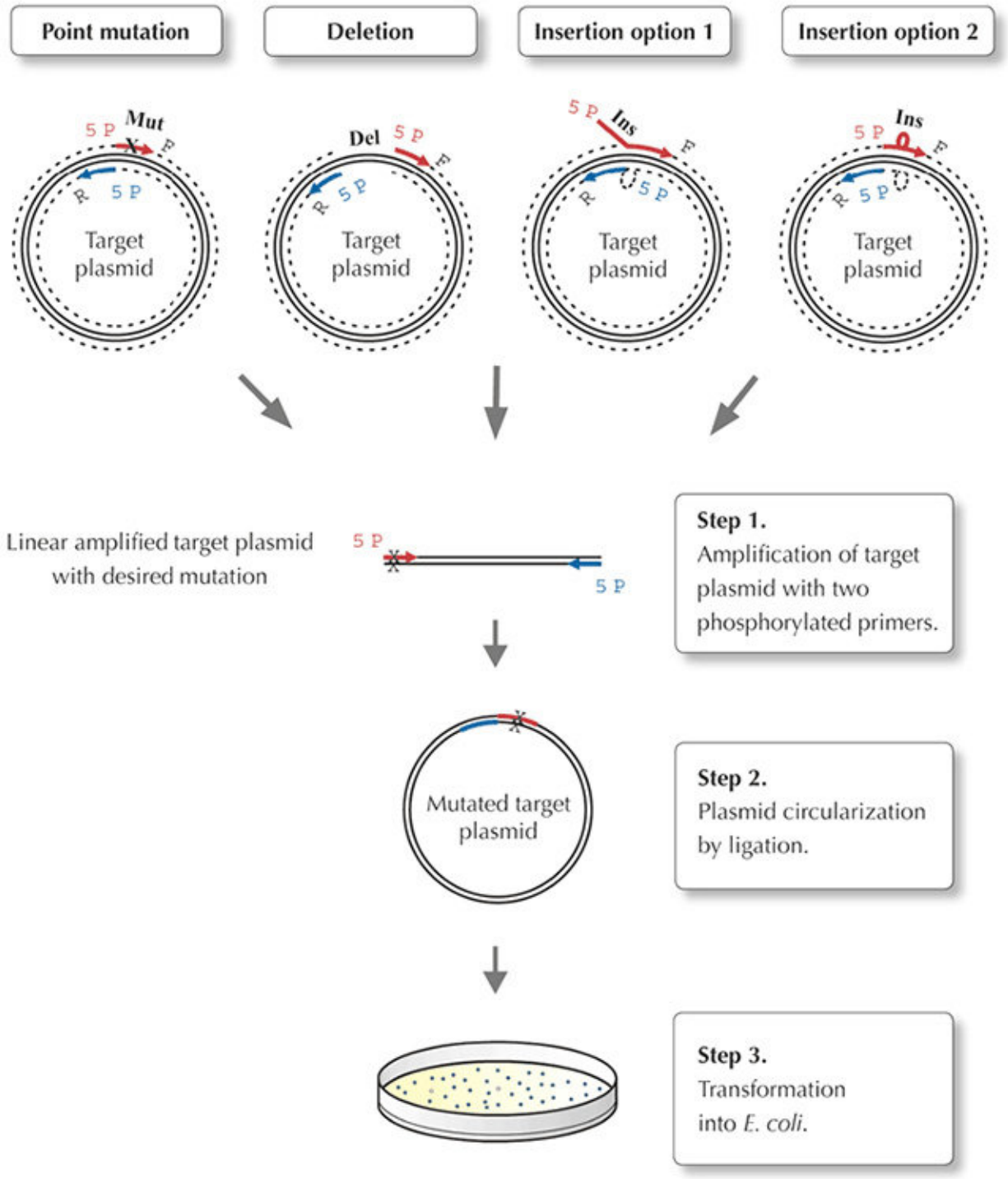
Supplementary



Supplementary Figure 1: Map of pBBR1MCS2 harboring the genes of interest, from Rmet_2229 to Rmet_2234. Also displayed are the various restriction sites and endonucleases *Bgl*II, *Xba*I, *Bam*HI, *Hind*III, Mph11031. Also featured are the genes *mob* and *rep* (which are necessary for both mobilization and replication respectively), lacZα, and the multiple cloning site (MCS). The kanamycin resistant cassette (KanR) is used as a selective marker for transformed cells. The position of each primer is indicated in purple.



Supplementary Figure 2: Steps involve in constructing the complementation plasmid beginning with the empty vector pBBR1MCS2.



Supplementary Figure 3: Site-directed mutagenesis protocol