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Signals and responses of ColRS two-component system in *Pseudomonas putida*

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Institute of Molecular and Cell Biology, University of Tartu, Estonia

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Mumm, K., K. Ainsaar, S. Kasvandik, T. Tenson, and R. Hõrak. 2016. Responses of *Pseudomonas putida* to Zinc Excess Determined at the Proteome Level: Pathways Dependent and Independent of CoIRS. Journal of Proteome Research. 15:4349–4368.

Ainsaar, K., H. Tamman, S. Kasvandik, T. Tenson, and R. Hõrak. 2019. The $TonB_m$-PocAB System Is Required for Maintenance of Membrane Integrity and Polar Position of Flagella in *Pseudomonas putida*. Journal of Bacteriology. 201.

My contribution to the publications is following:

Ref I – constructed plasmids and strains, performed most of the experiments and contributed to the manuscript writing.

Ref II – performed proteome data verification experiments, participated in data analysis and contributed to the writing and editing of the manuscript.

Ref III – constructed plasmids and strains, performed most of the experiments and wrote the manuscript.

ABBREVIATIONS

CA domain	catalytic and ATP-binding
D	aspartate residue
DHp domain	dimerization and histidine phosphotransfer
ECF	extracytoplasmic function
HAMP domain	named from being present in histidine kinases, adenylate cyclases, methyl accepting proteins and phosphatases
H	histidine residue
HK	histidine kinase
HPt domain	histidine phosphotransfer
LPS	lipopolysaccharide
MIC	minimal inhibitory concentration
OMP	outer membrane protein
PAS domain	Per-ARNT-Sim (period and single-minded, and the vertebrate aryl hydrocarbon receptor nuclear transporter)
PDC domain	PhoQ-DcuS-CitA
REC domain	receiver domain
RNAP	RNA polymerase
RR	response regulator
TCS	two-component signal transduction system

INTRODUCTION

Bacteria live in a very wide range of environmental habitats with different and often changing conditions. To survive in the fluctuating surroundings, it is important for the bacteria to sense the environment and appropriately act if needed. One of the primary means by which bacteria can sense and respond to external, but also to intracellular conditions, is by using two-component signal transduction systems. These are systems typically consisting of two types of proteins: a sensor histidine kinase that detects the signal and a response regulator that converts the signal into an appropriate physiological change in the bacterial cell.

In the past decade the number of described histidine kinases and response regulators has grown enormously due to the rise in the availability of complete genome sequences (Gao et al., 2019). Also, the number of high-resolution crystal structures of various domains of histidine kinases and response regulators has increased immensely (Bhate et al., 2015). This has improved remarkably the knowledge about how histidine kinases recognise the signal, how the signal is passed on to the response regulator and how the response regulator generates the cellular response. Yet, despite all this recent progress, for most two-component systems, their importance and the sensed signal have remained undetermined (Zschiedrich et al., 2016).

Typically, bacteria encode dozens of different two-component systems. The number varies and depends mainly on the size of the genome and the unpredictability of the environment in the niche the bacterium inhabits (Laub, 2011). *Pseudomonads* that exhibit considerable nutritional and metabolic versatility are found in a broad variety of environmental niches ranging from soil and water to plants and animals. Due to the diversity of their habitats they tend to possess more than the average number of two-component systems which allows them to sense and respond to a wide diversity of signals (Rodrigue et al., 2000). This thesis focuses on one two-component system that is highly-conserved in *Pseudomonads* – the ColRS system. The first part of the thesis gives an overview about the two-component systems in general: the modular architecture of the histidine kinases and the response regulators, and the modes of action taking place from the signal recognition to the response generation. The experimental part describes the signal for the ColRS system of *P. putida.* It focuses on determining the role of the ColRS system by analysing the cellular effects that the absence of ColRS signalling has for *P. putida*.

REVIEW OF LITERATURE

1. Overview of two-component signal transduction systems

Bacteria as well as archaea often possess dozens or hundreds of two-component signal transduction systems (TCS). They are one of the main mechanisms by which bacteria sense and respond to environmental stimuli. TCSs have also been found in some fungi and plants but are absent in the animal kingdom (Wuichet and Zhulin, 2010). In general, TCSs are composed of two kinds of proteins: sensor histidine kinases (HK) and response regulators (RR) (Figure 1A). HKs contain a conserved histidine residue (H) and RRs have a conserved aspartate (D), both of which are essential for signal transduction. In order to generate a certain change in cell's physiology the histidine in the HK is autophosphorylated in response to an environmental stimulus and the phosphoryl group is transferred to the conserved aspartate in the RR (Zschiedrich et al., 2016). There are, of course, many variations of this two-step signal transduction pathway. Relatively common are the phosphorelays (Figure 1B) in which the phosphoryl group is not directly transferred to the RR but instead is first passed on to a receiver (REC) domain in the HK,

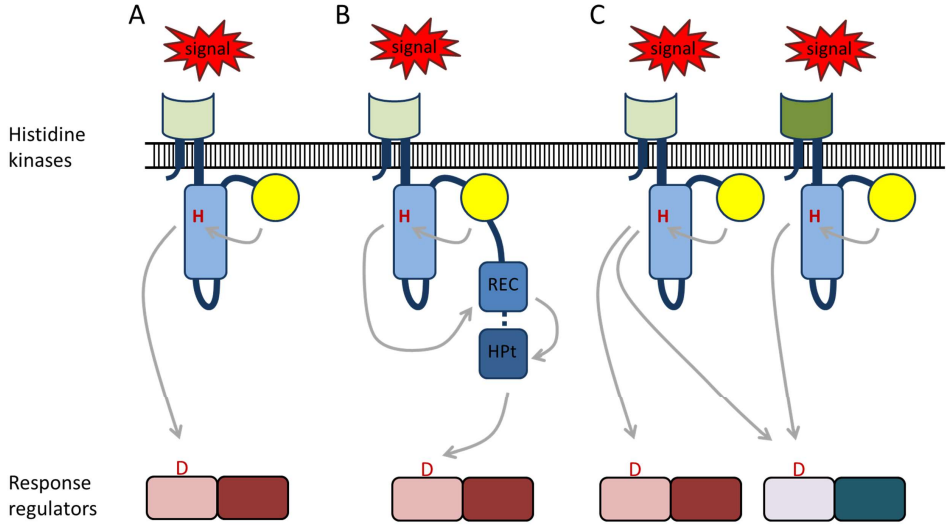

Figure 1. Schematic representation of phosphotransfer pathways. (A) A typical TCS. A conserved histidine residue, marked as H, in the HK is autophosporylated in response to the stimulus and the phosphate group is transferred from the histidine to a conserved aspartate residue, marked as D, in a RR in order for it to carry out a change in cell's physiology. (B) A phosphorelay system where the phosphate group is transferred first to a receiver (REC) and phosphotransfer (HPt) domain before RR is phosphorylated. HPt domain may locate in a separate protein. (C) An atypical HK that is able to phosphorylate several RRs and a RR that can be phosphorylated by multiple HKs.

from there to a phosphotransfer (HPt) domain, which may locate in a separate protein, and only then to the RR. There are also examples of multiple HKs phosphorylating the same RR and a single HK phosphorylating several RRs (Figure 1C) (Stock et al., 2000). Most commonly phosphorylated RRs act as transcriptional regulators, although enzymatic, RNA-, ligand- or protein-binding RRs exist as well (Galperin, 2010). When the stimulus is not present, HKs function as phosphatases and accelerate the rate of dephosphorylation of the RR (Laub, 2011).

1.1. Architecture of TCS proteins

In principle, TCSs are composed of dimeric HKs and monomeric RRs (Figure 2) but RRs often form di- or multimers in response to phosphorylation (Zschiedrich et al., 2016). Nearly all characterized HKs are homodimers. Only a few exceptions such as monomeric light sensing HK from *Erythrobacter litoralis* (Rivera-Cancel et al., 2014) and trimeric ammonium sensing HK from *Candidatus* Kuenenia stuttgartiensis have been described (Pfluger et al., 2018).

Overall, HKs and RRs can be considered as multidomain proteins. They contain a set of modules that can be arranged in different combinations and numbers to produce a wide variety of signalling proteins (Jacob-Dubuisson et al., 2018). All HKs have two highly conserved cytoplasmic domains: a dimerization and histidine phosphotransfer (DHp) domain and a catalytic and ATP-binding (CA) domain (Figure 2) (Jacob-Dubuisson et al., 2018). The DHp domain is a four-helical bundle that is composed of the helices of both HKs in the dimer and can be divided into three regions. Top third of the DHp domain contains the conserved catalytic histidine residues. The middle part of the bundle is highly symmetric and stable and the bottom third of DHp creates a binding surface for the RR. The bottom of the DHp domain also contains hairpin loops that determine whether the autophosphorylation occurs in *cis* or in *trans* (see chapter 1.3 Kinase activation in a prototypical TCS). The CA domain is attached to the DHp domain with an α-helical linker and has an ATP (or ADP) molecule bound to it (Bhate et al., 2015). In addition to DHp and CA most HKs possess a sensor domain that detects the stimulus. A typical HK is a transmembrane protein that has its sensor domain in the extracytoplasmic space but the sensor may as well locate in the cytoplasm. A typical HK also has at least two transmembrane helices which participate in signal transmission from the extracytoplasmic sensor domain to the cytoplasmic parts of the HK (Möglich et al., 2009). Some HKs have one or several linker regions such as HAMP (found in **h**istidine kinases, **a**denyl cyclases, **m**ethyl-accepting proteins and **p**hosphatases) or PAS (**P**er-**A**RNT-**S**im) domains following the transmembrane region and preceding the DHp domain (Figure 2) (Bhate et al., 2015). The PAS domain may, in addition to being a signal transmitter, also serve as a cytoplasmic sensory domain (Möglich et al., 2009; Sobran and Cotter, 2019). Depending if the HK is part of a simple TCS or a multistep phosphorelay system, the HK may end with the CA domain or have additional receiver (REC) and HPt domains attached to it (Figure 1) (Zschiedrich et al., 2016).

A RR usually has a simpler architecture. Most commonly it consists of two domains: a receiver (REC) domain and an effector domain. The REC domain contains the phosphoryl group receiving aspartate (D) and the effector domain carries out the adaptive response (Laub, 2011).

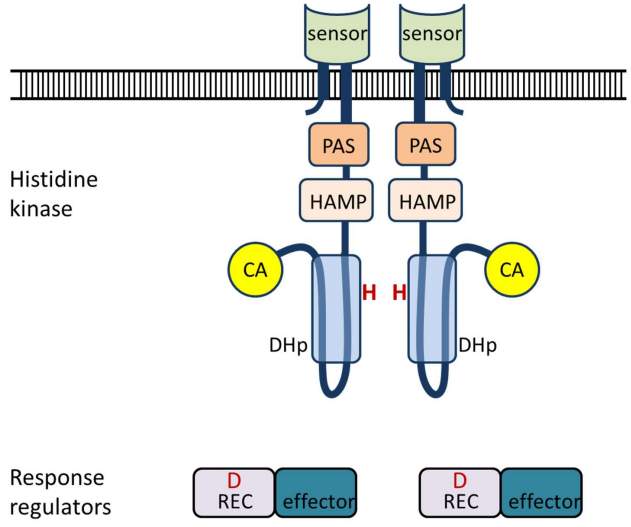

Figure 2. Schematic representation of a TCS. A dimeric membrane-anchored HK consisting of extracytoplasmic sensor domain, cytoplasmic linker domains (PAS and HAMP), dimerization and phosphotransfer domain (DHp) and a catalytic and ATP-binding domain (CA) is presented. H marks the catalytic histidine. RRs are shown as monomers composed of receiver (REC) and effector domain. D represents the phosphate receiving aspartate residue.

1.2. Signals sensed by TCSs

TCSs can either directly or indirectly detect a very wide variety of different signals. The sensed stimuli can be various small molecules, ions, toxic compounds, dissolved gases, pH, temperature, osmotic pressure, light or redox state of the cell (Jacob-Dubuisson et al., 2018). Generally, TCSs sense the signal via a sensor domain of the HK. Depending on the location of the sensor domain the detected signal can come from the extracellular or cytoplasmic environment or be a membrane condition (Figure 3) (Mascher et al., 2006).

Extracellular (or periplasmic) sensing HKs constitute the largest group. These are transmembrane HKs with an extracellular sensory domain and at least two transmembrane helices (Figure 3A). Typically they sense solutes and nutrients (Mascher et al., 2006). For example CitA, the HK of CitAB TCS that regulates the expression of citrate fermentation genes in *Klebsiella pneumoniae* (Bott et al., 1995), detects citrate, but not its close analogues (Kaspar et al., 1999). PhoQ, the sensor kinase of *Salmonella enterica*'s PhoPQ system, detects the presence of certain antimicrobial peptides in the environment and alters the expression of

proteins that modify lipopolysaccharides and inactivate antimicrobial peptides (Bader et al., 2005). PhoQ also responds to low pH which activates the system (Bearson et al., 1998) and to high concentrations of bivalent cations like Mg^{2+} and Ca^{2+} which repress its activity (Montagne et al., 2001). Other systems, such as the PmrAB of *Salmonella enterica* and CusSR of *E. coli*, respond to toxic conditions. PmrB detects high levels of extracytoplasmic Fe^{3+} by directly binding ferric ions (Wösten et al., 2000). CusSR that upregulates the expression of copper-removing RND family efflux pump (Gudipaty et al., 2012) is activated by elevated concentrations of copper (Munson et al., 2000) and silver (Franke et al., 2001).

The second largest group contains HKs that sense cytoplasmic stimuli. They are either membrane-anchored or soluble cytoplasmic proteins (Figure 3B) that detect intracellular solutes and mainly report the metabolic or developmental state of the cell (Mascher et al., 2006). Some of the best-studied TCSs of this group are KdpDE, FixJL and ArcBA, which have membrane-integrated HKs, and NtrBC that is entirely cytoplasmic. KdpDE senses K^+ concentration and osmotic upshift in *E. coli* and controls genes that are important for the fast adaptive change in turgor (Heermann and Jung, 2010). Interestingly, KdpD is a bifunctional receptor that is able to sense both extra- and intracellular K^+ levels (Laermann et al., 2013; Schramke et al., 2016). High extracellular K^+ is sensed through the periplasmic loop locating between transmembrane domains (Schramke et al., 2016), while intracellular K^+ levels are detected by the cytoplasmic sensor domain (Laermann et al., 2013). FixJL detects O_2 concentration in *Rhizobium meliloti* and regulates the expression of nitrogen fixation related genes (Wright et al., 2018). ArcBA of *E. coli* responds indirectly to O_2 levels and is responsible for regulating facultative anaerobic metabolism (Bekker et al., 2010). The stimulus for ammonia assimilation regulating NtrBC is nitrogen supply which is sensed indirectly through binding of deuridylylated GlnB protein (van Heeswijk et al., 2013).

Least abundant are HKs that recognize membrane-associated stimuli (Figure 3C). They have usually 2 to 20 transmembrane regions, which are connected by very short intra- and extracellular linkers. The stimulus is sensed from within the membrane or is derived from membrane-bound components and their sensing mechanism is associated with the transmembrane segments (Mascher et al., 2006). For example, DesK of *Bacillus subtilis* has five transmembrane helices and it responds to low temperatures by sensing membrane fluidity (Cybulski et al., 2010). Its cognate RR DesR regulates the expression of fatty acid desaturase Des, which controls the saturation level of phospholipids and allows membrane to stay fluid in colder temperatures (Aguilar et al., 1999). Another TCS of this group is RegBA, a global regulatory system in *Rhodobacter capsulatus* that controls energy-generating and utilizing processes (Swem et al., 2001). RegB has six transmembrane helices that are necessary for interacting with cytochrome oxidase and sensing the redox state of aerobic electron transport chain (Potter et al., 2002; Swem et al., 2003).

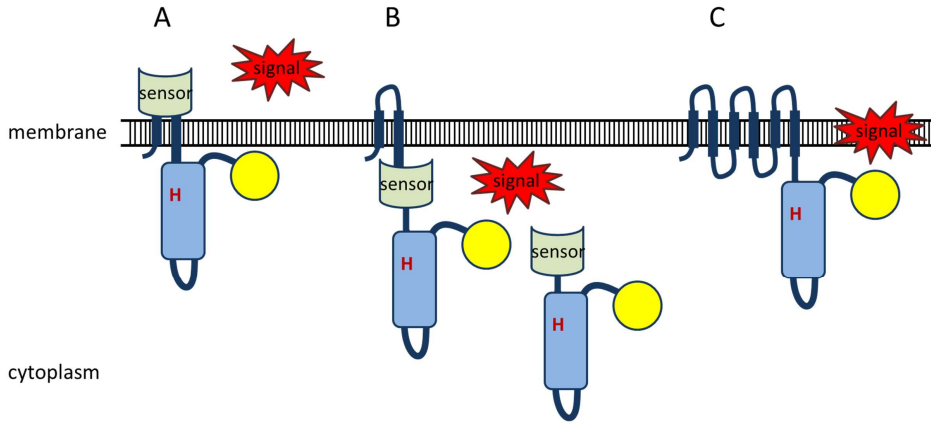

Figure 3. Architecture of HKs depending on the origin of the signal they sense. (A) Extracellular (or periplasmic) stimuli sensing HK. The HK is membrane-anchored and has a sensor domain outside of the cytoplasm. (B) Cytoplasmic stimuli sensing HKs can be membrane-anchored or soluble proteins that have their sensor domain in the cytoplasm. (C) A membrane condition sensing HK that has several transmembrane regions and no distinct sensor domain.

1.3. Kinase activation in a prototypical TCS

The general mechanism of kinase activation is that signal detection induces conformational changes in the sensor domain that lead to a series of conformational rearrangements along the following parts of the HK dimer. This includes moving the CA domain to close proximity with the conserved histidine residue which causes the autophosphorylation of the histidine and activation of the kinase (Bhate et al., 2015).

Binding of the stimulus molecule or a molecule affected by the stimulus is usually the first trigger for the rearrangements (Mascher et al., 2006). Sensor domains are extremely variable in their amino acid sequences but have been found to contain conserved structural motifs. The most commonly found motifs in extracellular sensor domains are PDC (PhoQ-DcuS-CitA) motifs composing of mixed α-β folds, and all-α-helical folds which often directly participate in stimulus recognition (Möglich et al., 2009). For example, the sensor domain of PhoQ in the PhoPQ system relies on a PDC domain for sensing antimicrobial peptides. The PDC domain of PhoQ contains acidic amino acids and creates a flat and highly negatively charged surface (Cho et al., 2006). This surface is in close proximity to the lipid surface of the cytoplasmic membrane which also has a negative charge. In between there are at least three Mg^{2+} or Ca^{2+} ions that tether the surfaces together and in this state the PhoPQ system is inactive. It is proposed that antimicrobial peptides displace the cations and by disrupting the interactions between the PhoQ and the membrane they lift the PDC domain off the membrane which causes the activation of the PhoPQ system (Cho et al., 2006).

In a typical transmembrane HK the message of signal detection has to be passed on through the cytoplasmic membrane. This step involves the transmembrane helices. Since the mobility of the transmembrane helices is restricted, there are only a limited number of motions that can occur. The possible motions are moving of the helices toward or away from each other, moving perpendicular to the membrane plane, pivoting and rotation of the helices (Gushchin and Gordeliy, 2018). These movements are not mutually exclusive and are found to co-occur in different HKs (Gushchin et al., 2017; Yang and Spudich, 2001). In some HKs the transmembrane helices are followed by one or many HAMP domains (Figure 2) while others are directly linked to the DHp domain. The HAMP domains exist in two conformational states and there are several models of how the domain passes on the signal but the common feature is that transition from one conformational state to the other is crucial for kinase activation (Zschiedrich et al., 2016).

In an inactive state the CA and DHp domains of the HK are in a symmetric conformation (as shown in Figure 2). Several studies have suggested that only one of the HKs in the dimer is autophosphorylated during the activation of the TCS (Casino et al., 2014; Jiang et al., 2000). The proposed mechanism of activation is that the conformational changes in the transmembrane helices or linker regions induce an asymmetrical bend in the top third region of the DHp stem. The asymmetrical motion causes one of the CA domains to develop strong interactions with the DHp domain and retains it in an inactive state, while the other

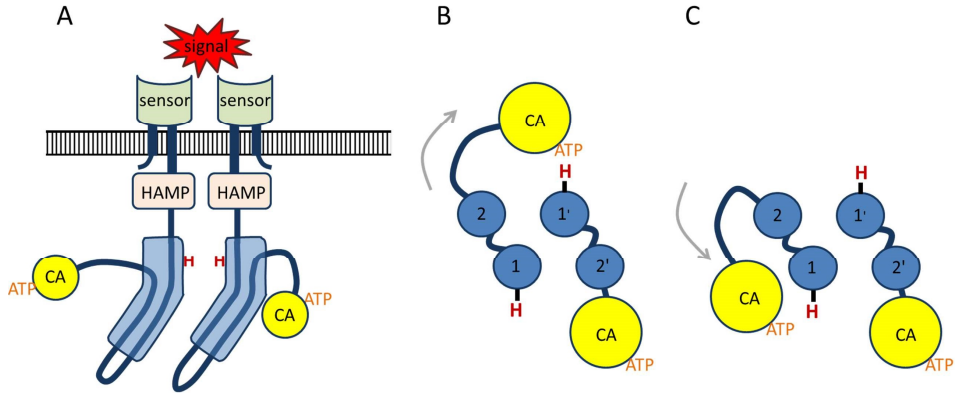

Figure 4. Activation of a HK. (A) Detecting signal induces an asymmetrical bend in the stem of a DHp domain in the HK dimer. This causes one of the CA domains to develop strong interactions with the DHp domain and stay inactive, while the other CA domain is released and is free to interact with one of the catalytic histidines. (B) *Trans* autophosphorylation. Due to the conformational rearrangements in the HK dimer a CA domain moves closer to the catalytic histidine (H) of the other HK monomer (the helixes of which are presented with numbers 1' and 2') and a terminal phosphate from the ATP bound to the CA is transferred to the histidine of the other monomer. (C) *Cis* autophosphorylation. A CA domain moves closer to the catalytic histidine (H) of its own monomer and the histidine of the same HK monomer is phosphorylated.

CA domain is released and is able to interact with the catalytic histidine (Figure 4A) (Mechaly et al., 2014). As a result of that a terminal phosphate from the ATP bound to the CA domain is transferred to the N atom of the catalytic histidine and a high-energy N-P bound is formed (Bhate et al., 2015). The hairpin loop at the bottom of the DHp domain determines if the autophosphorylation takes place in *cis* or in *trans*. If, in response to the signal, the hairpin loop turns right, the CA domain of one monomer moves closer to the catalytic histidine of the other monomer and the HK autophosphorylates in *trans* (Figure 4B). In other HKs where the hairpin loop turns left, the CA domain gets closer to its own histidine and the autophosphorylation takes place in *cis* (Figure 4C) (Ashenberg et al., 2013).

1.4. Responses to TCS's activation

For kinase activation to have an effect on cell's physiology, the phosphoryl group has to be passed on to the RR. For this a RR docks itself onto the DHp domain of the HK by using its REC domain and catalyses the phosphoryl group transfer from the histidine residue to its conserved aspartate (Galperin, 2010; Stock et al., 2000). Although activation of each individual RR is different in detail, phosphorylation of the aspartate usually causes conformational changes in the REC domain which evokes changes in the effector domain. Many RRs also undergo di- or multimerization after phosphorylation in order to achieve their active state. This is often mediated by a region called α4β5α5 face in the REC domain (Bourret, 2010; Gao and Stock, 2010).

Phosphorylated RRs can trigger very different responses in the cell. Some affect development while others influence cell division, metabolism, pathogenicity, resistance or taxis (Jacob-Dubuisson et al., 2018). The mode of RR's action is determined by its effector domain. Most RRs (about 65% of the described RRs) have a DNA-binding motif in its effector domain and act as transcriptional regulators (Gao et al., 2019). An example of this kind of RR is PhoB, a RR of the PhoBR system in *E. coli* which is also the prototype for the largest subfamily of DNA binding RRs. PhoB recognizes a 22 bp long DNA sequence with two 11 bp repeats. To bind onto this sequence, two PhoB monomers form a dimer in a head-to-tail arrangement (Bachhawat et al., 2005). In unphosphorylated form, the REC domain of PhoB hinders dimerization by sterically blocking the binding of PhoB monomers (Blanco et al., 2002). The detection of limitation of inorganic phosphate in the external environment by PhoR leads to its activation, which in turn results in phosphorylation and dimerization of PhoB monomers. PhoB dimer then binds to its cognate DNA sequence and recruits σ^{70} subunit of the RNA polymerase. This triggers the initiation of transcription from genes that control phosphate uptake and the usage of alternative phosphorus sources (Santos-Beneit, 2015). In addition, PhoB binding may also alter DNA bending which could contribute to transcription activation (Blanco et al., 2002).

Another relatively large group of RRs (8%) consists of RRs with enzymatic effector domain (Gao et al., 2019). A large subgroup of these are regulators of cyclic di-GMP, an important messenger in bacteria which has shown to regulate biofilm formation, motility, virulence, cell cycle and differentiation (Römling et al., 2013). One such RRs is WspR in *Pseudomonas* species which acts as a di-guanylate cyclase (Huangyutitham et al., 2013). The activation of WspR involves Wsp complex where a membrane-bound sensor protein WspA senses a signal that is associated with growth on a surface. The signal is communicated to a HK WspE that ultimately leads to phosphorylation of RR WspR. Phosphorylated WspR produces cyclic di-GMP, which in turn stimulates biofilm formation by inducing exopolysaccharide and adhesin production (Huangyutitham et al., 2013).

About 1% of the RRs possess a protein-binding domain and function through interacting with other proteins (Gao et al., 2019). An interesting example of this class is PhyR which is considered a general stress response regulator in *Methylobacterium extorquens* (Gourion et al., 2008). PhyR has an effector domain that is very similar to the σ^E subunit of RNA polymerase (Francez-Charlot et al., 2009). σ^E is an extracytoplasmic function (ECF) sigma factor that plays a key role in envelope stress response in Gram-negative bacteria (Amar et al., 2018). However, PhyR does not act as a sigma factor but instead controls gene expression indirectly. It is proposed that under normal conditions an ECF sigma factor σ^{EcfG1} is sequestered by its anti-sigma factor NepR. In response to stress, PhyR is phosphorylated and interacts with NepR, thus releasing σ^{EcfG1}, which can then associate with RNA polymerase and transcribe stress genes (Francez-Charlot et al., 2009).

A few RRs have been found to contain a RNA-binding motif in their effector domain. These RRs primarily regulate gene expression at translation initiation level by forming an antiterminator structure instead of a terminator in the 5′ leader region of a RNA and allow translation to occur (Ramesh et al., 2012). One such RR is EutV that regulates expression of ethanolamine utilization genes in *Enterococcus faecalis. EutV is phosphorylated by* EutW in response to detecting ethanolamine (Del Papa and Perego, 2008) and undergoes dimerization (Ramesh et al., 2012). The EutV dimer binds specific 13-bp long sequences in the mRNA transcribed from *eut* operon and disrupts the multiple hairpin and terminator structures found in there (Fox et al., 2009).

1.5. Dephosphorylation of RRs

RRs will stay in their active conformation until they are dephosphorylated. Dephosphorylation of the RR is a highly important step in signal transduction pathway, because it terminates the current response and enables the system to respond again to a new signal. For the dephosphorylation reaction to take place, a water molecule needs to be lined with the acyl phosphate bond that is to be broken. Generally, most of the amino acid residues involved in the coordination of these molecules locate in the REC domain of the RR. Thus, most RRs are capable of autodephosphorylating in the absence of any partners (Gao et al.,

2019). For some RRs, the autodephosphorylation reaction takes seconds, while others would stay in their active form for days (Thomas et al., 2008). The reaction rate is enhanced by the phosphatase activity of the bifunctional HKs or by some auxiliary phosphatases that help to position the necessary molecules. In contrast to the kinase state, in the phosphatase-competent state, both HK monomers display symmetrical arrangements of their DHp and CA domains and their DHp helices are straight (as shown in Figure 2). Onto this complex bind symmetrically two response regulators, one to each DHp bundle, and catalyse the dephosphorylation of their aspartate residues which leaves them in an inactive state (Jacob-Dubuisson et al., 2018).

1.6. Specificity of TCSs

On average, bacteria possess dozens of different TCSs. However, the number varies from zero to almost 140 and strongly correlates with the size of the genome and the environmental niche that the species inhabits (Capra and Laub, 2012). To maintain the bacterium's ability to adequately respond to different stimuli, it is important that the HKs specifically phosphorylate only their cognate RRs. This is achieved predominantly by three mechanisms (Podgornaia and Laub, 2013).

The primary mechanism is molecular recognition, i.e. the HK's ability to recognize its cognate RRs. It is driven by the amino acid residues participating in HK-RR binding, some of which locate in the DHp domain of the HK and others that are in the REC domain of the RR. If the specificity-determining residues in the HK do not correspond with the specificity-determining residues in RR, then the RR is not able to dock onto the HK in a way needed for phosphotransfer (Gao et al., 2019). It has been shown that as few as three residues can be sufficient to ensure HK-RR pair's specificity. For example, normally the osmolarity sensing HK EnvZ of *E. coli* phosphorylates its cognate RR OmpR but by replacing three specificity-determining residues, it can be rewired to start phosphorylating RstA, the RR of RstAB system (Skerker et al., 2008).

The second specificity-ensuring mechanism is the phosphatase activity of the HKs. If a RR is inappropriately phosphorylated, it is soon dephosphorylated with the help of its cognate HK complex that is in the phosphatase-competent state in the absence of a signal (Podgornaia and Laub, 2013).

Thirdly, specificity is further enhanced by the relatively higher abundance of RRs than HKs. The synthesis rate of RRs is often much higher than that of HKs (Li et al., 2014). It has been found that the ratio of EnvZ and OmpR is around 1:35 in *E. coli* (Cai and Inouye, 2002) and several other TCSs have likely have similar HK:RR ratios (Miyashiro and Goulian, 2008). This means that there is a competition between the RRs for binding to an active HK complex and cognate RRs efficiently outcompete the nonspecific ones resulting in HKs predominantly interacting with their specific partners (Podgornaia and Laub, 2013).

2. The ColRS two-component system

ColRS is a two-component system that is conserved in *Pseudomonas* species but is also found in some other Gram-negative bacteria such as the *Xanthomonas*. It consists of a HK ColS and a RR ColR. ColS is a membrane-anchored protein with two transmembrane regions and a sensor domain in the periplasmic space. ColR is a DNA-binding RR that belongs to the OmpR/PhoB subfamily of RRs (Winsor et al., 2016).

The system was first described in *Pseudomonas fluorescens* where *colS* deficiency was reported to impair the bacterium's ability to colonize the roots of different plants (Dekkers et al., 1998). Since then, the system has been extensively studied in *Pseudomonas putida* and over the years it has been found to be involved in a variety of processes, such as the regulation of transposon Tn*4652* transposition (Hõrak et al., 2004), phenol tolerance (Kivistik et al., 2006), heavy metal resistance (Hu and Zhao, 2007) and membrane integrity maintenance (Putrinš et al., 2011; Putrinš et al., 2008). In *P. putida* strain PaW85 *colR* and *colS* deficiency causes the translocation frequency of transposon Tn*4652* to be decreased (Hõrak et al., 2004). Later, it was discovered that the *colR*- and *colS*-deficient strains are sensitive to phenol (Kivistik et al., 2006) and as the Tn*4652*'s transposition assay involved growing the bacteria on phenol-containing medium it was concluded that the role of the ColRS system in Tn*4652*'s transposition is most likely related to the reduced phenol tolerance of the mutant. ColRS system is also shown to be needed for the maintenance of membrane integrity when *P. putida* uses glucose as the sole carbon source (Putrinš et al., 2008). This phenotype result from the inability of the *colR* and *colS* mutants to tolerate the rise in the abundance of sugar channel protein OprB1 in outer membrane (Putrinš et al., 2011). That finding strongly indicated that the role of the ColRS system is related to maintaining membrane integrity in *P. putida*. Interestingly, a study conducted with a cadmium-resistant *P. putida* strain CD2 associated the ColRS system with heavy metal tolerance, as the *colR*- and *colS*-deficient strains of the *P. putida* CD2 were found to be considerably less tolerant to Mn^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+} and Pb^{2+} excess than the parent strain (Hu and Zhao, 2007).

Besides *P. putida* and *P. fluorescens*, the ColRS system has been studied in *P. aeruginosa* and some *Xanthomonas* species. In *P. aeruginosa* ColRS contributes to polymyxin resistance (Gutu et al., 2013) and virulence against *C. elegans* (Garvis et al., 2009). In plant pathogenic *Xanthomonas* species it is mainly found to be required for virulence but the system also contributes to various other cellular processes (Subramoni et al., 2012; Zhang et al., 2008; Yan and Wang, 2011). For instance, *colS* deficiency causes *X. oryzae* to produce siderophores even in iron-replete conditions (Subramoni et al., 2012). *X. citri* cells with inactivated *colRS* are impaired in growth, biofilm formation, catalase activity, LPS production and in resistance to several stresses (Yan and Wang, 2011). In *X. campestris*, ColRS system is required for the tolerance of several antibiotics, NaCl, $CdSO_4$ and phenol (Zhang et al., 2008). It is tempting to speculate that some of these phenotypes could be related to membrane issues.

The RR of the ColRS system, the ColR, is a DNA-binding protein that can up- or downregulate the expression of its target genes (Kivistik et al., 2009). In several species some of the ColR-regulated genes have been described. For example, ColR positively regulates the expression of virulence genes in *X. campestris* and *X. citri* (Zhang et al., 2008; Yan and Wang, 2011) and the LPS synthesis gene *rfbC* and catalase gene *katE* in *X. citri* (Yan and Wang, 2011). In *P. fluorescens* it controls the expression of an operon coding for a probable methyltransferase and lipopolysaccharide kinase WapQ that locates just downstream the *colRS* operon (de Weert et al., 2006). The homologues of these two genes, PP_0903 and PP_0904, are also upregulated by ColR in *P. putida* and were used to first describe the ColR-binding sequence (Kivistik et al., 2009). The ColR-binding motif consists of 8 fully conserved nucleotides in a 16-bp-long core binding box ((T/C)(T/C)NA(C/G)NN(T/C)TTTTT(C/G)AC) and it is found in the promoter region of all known ColR-regulated genes in *P. putida*. Altogether eight loci have been described to be directly regulated by ColR in *P. putida* strain PaW85 (Kivistik et al., 2009). ColR negatively regulates the expression of an outer membrane protein coding *oprQ* and LPS modification related PP_0737 genes and induces the transcription of PP_0035-33 and PP_0903-905 operons, PP_0036, PP_0900, PP_1636 and PP_3766 (Kivistik et al., 2009). Most of these genes code for either membrane proteins or lipopolysaccharide synthesis related proteins pointing cell membrane as the most probable target of the ColRS system. However, the signal that the ColRS system responds to and the mechanism of how membrane homeostasis is potentially regulated, has remained undetermined.

THE AIM OF THE THESIS

Pseudomonas putida is a ubiquitous soil bacterium that is known for its ability to exploit toxic organics and degrade aromatic compounds (Timmis, 2002). Due to the versatility of the conditions it may encounter in soil, *P. putida* possesses a relatively high number of TCSs which contribute to its survival in such a fluctuating environment (dos Santos et al., 2004). One two-component system that is highly-conserved in *Pseudomonads* is the ColRS system.

The ColRS two-component system has mainly been associated with maintaining membrane integrity but there have also been cues suggesting that it might be involved in metal tolerance. For instance, the ColRS system is necessary for a cadmium-resistant *P. putida* strain CD2 for the tolerance of high concentrations of Mn^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+} and Pb^{2+} (Hu and Zhao, 2007). In *X. citri* it provides tolerance to Cu^{2+} (Yan and Wang, 2011) and in *X. campestris* to Cd^{2+} excess (Zhang et al., 2008).

P. putida encodes more than 60 genes that are proposed to be involved in the regulation, uptake, extrusion and chelation of metals and it seems to have more efflux systems than many other bacteria (Canovas et al., 2003). Several of these genes are controlled by TCSs such as CopRS (Canovas et al., 2003), CadAR (Lee et al., 2001) and CzcRS (Liu et al., 2015) that are known to detect excess of metal ions in the extracellular environment. Based on the hints that ColRS system might be related to metal tolerance, the aims of this thesis were to

- evaluate if the ColRS system of *P. putida* PaW85 is involved in metal tolerance
- test whether metal excess is the signal sensed by the ColS histidine kinase
- determine the cellular response to metal excess in a *colR*-deficient strain

RESULTS AND DISCUSSION

3. ColRS system is essential in *P. putida* for tolerating excess amounts of zinc, iron, manganese and cadmium (Ref I)

The involvement of ColRS system in metal tolerance was first shown in a *Pseudomonas putida* strain CD2 where *colRS* deficiency was reported to significantly reduce the tolerance to Mn^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+} and Pb^{2+} (Hu and Zhao, 2007). Given that CD2 strain was isolated from the sewage sludge of Wuhan Steel Factory as a cadmium resistant strain of *P. putida* (Hu and Zhao, 2007), it may display several differences from other *P. putida* strains and these results may not be applicable to *P. putida* in general.

To investigate whether the ColRS system is required for tolerating metal excess in *P. putida*, we tested the metal tolerance of *P. putida* strain PaW85 and its *colR*- and *colS*-deficient derivatives. The results revealed that the mutants were clearly more sensitive to Zn^{2+} and Fe^{2+} and slightly more sensitive to Mn^{2+} excess (Ref I, Table 1 and Fig. 1). In liquid, but not on solid medium, the *colR*- and *colS*-deficient strains also demonstrated a slightly increased sensitivity to Cd^{2+} (Ref I, Table 1 and Fig. 1). Complementation of *colR* and *colS* mutants with a *colR* and a *colS* gene, respectively, restored the Zn^{2+}, Fe^{2+} and Mn^{2+} tolerance (Ref I, Fig. 1). This confirmed that the ColRS system indeed increases the tolerance of Zn^{2+}, Fe^{2+} and Mn^{2+} in *P. putida* and possibly also contributes to Cd^{2+} tolerance.

In order to analyse whether the signal transduction between the ColS and ColR is necessary for the metal tolerance, we also tested a *colR*-deficient strain complemented with a phosphorylation-deficient variant of ColR designated as $ColR_{D51A}$. Since the expression of $ColR_{D51A}$ did not alleviate the Zn^{2+}, Fe^{2+} or Mn^{2+}sensitivity of the *colR* mutant (Ref I, Fig. 1), it indicated that not only is the presence of ColR and ColS needed but also there has to be signal transduction from ColS to ColR to maintain the bacterium's tolerance to the excess of these metals.

Unlike the *colR*- and *colS*-deficient strains of *P. putida* CD2, the *colR* and *colS* mutants of *P. putida* PaW85 were not more sensitive to Co^{2+}, Cu^{2+} and Ni^{2+} excess than the wild-type (Ref I, Fig. 1). There could be several reasons for this difference. The CD2 strain was isolated as a cadmium resistant strain but seems to be overall more tolerant to the excess of different metals than PaW85. For instance, the CD2 strain can grow in the presence of 7.3 mM Cd^{2+}, 12.8 mM Zn^{2+}, 8.5 mM Ni^{2+} and 11.6 mM Co^{2+} (Hu and Zhao, 2007), i.e. at the metal concentrations that are by far intolerable to *P. putida* PaW85 (Ref I, Table I). This suggests that *P. putida* CD2 has acquired some additional mechanisms to cope with the high concentration of metal ions that it encounters in its environment. It could be that these additional mechanisms have some overlap with the ColRS-regulated response in *P. putida* CD2. Since this additional layer of metal protection is absent in the *P. putida* PaW85, there is no change in the Co^{2+}, Cu^{2+} and Ni^{2+} tolerance of the *colR*- and *colS*-deficient derivatives of the PaW85 either. The other explanation

would be that the activation of ColRS system is in general beneficial in metal excess conditions but the metals that activate the ColRS are somewhat different in these two strains. This, however, is not very probable, as the ColRS systems are highly similar in the two strains – the identity between the ColR proteins of the CD2 and PaW85 strains is 100% and between the ColS proteins 97% (Hu and Zhao, 2007). Also, it is possible, although rather unlikely, that the promoter regions of the genes that provide tolerance to the Co^{2+}, Cu^{2+} and Ni^{2+} excess are different in the two strains and contain a ColR-binding sequence in the CD2 but not in the PaW85 strain.

4. The signal for the ColRS system

Many metals are essential for bacteria in certain amounts as they are co-factors for enzymes that participate in processes such as respiration, photosynthesis and nitrogen fixation or they act as structural stabilizers of proteins. On the other hand, all metals become toxic in excess. Some, such as Cu^{2+} and Zn^{2+}, are toxic because in overabundance they are capable of substituting native metals from the metalloproteins and thereby disrupt the functionality of the protein. Others, iron in particular, are harmful because they generate reactive oxygen species. Because of the essentiality and toxicity of metals, bacteria need to closely monitor the availability of metal ions in the environment and according to the circumstances modulate their transport into or out of the cell. This can be achieved by using the TCSs (Chandrangsu et al., 2017). Since the ColRS system was confirmed to be necessary for the tolerance of several metals and also because the ColRS-mediated tolerance was dependent on the signal transduction between the ColS and ColR, we hypothesised that metal ions could be the signal sensed by the ColS.

4.1. ColRS system is activated by Zn^{2+}, Fe^{3+}, Mn^{2+} and Cd^{2+} ions (Ref I)

To test the possibility that the ColRS system responds to metal excess, the promoter regions of eight ColR-regulated genes were cloned in front of a *lacZ* gene and the expression levels of these transcriptional fusions were determined. It was concluded that all the tested ColR-regulated promoters responded to the addition of Zn^{2+} in wild-type *P. putida* (Ref I, Fig. 2). With the exception of PP_0035-PP_0033 operon's promoter, the promoters did not respond to Zn^{2+} in *colR*- and *colS*-deficient strains (Ref I, Fig. 2) indicating that zinc activates the ColRS system. The PP_0035-PP_0033 promoter displayed partial ColR-independent zinc-promoted activation, possibly by some other regulatory mechanism. Two promoters, PP_0903 and PP_0268, were selected to test other metals and both of these promoters responded to Fe^{2+}, Mn^{2+} and Cd^{2+} in a *colS*-dependent manner (Ref I, Fig. 3), whereas Cu^{2+}, Co^{2+} and Ni^{2+} had no effect. This showed that in *P. putida* ColRS system is activated by the presence of excess amounts of

zinc, iron, manganese and cadmium. In line with our results, the ColRS system was later also shown to respond to zinc in *P. aeruginosa* (Nowicki et al., 2015).

Several other TCSs are known to detect the excess of iron and all of them seem to discriminate between the ferrous Fe^{2+} and ferric Fe^{3+} ions. A PmrAB system of *Salmonella enterica* detects only Fe^{3+} (Wösten et al., 2000), whereas BqsRS of *P. aeruginosa* and FirRS of *H. influenza* sense Fe^{2+} (Kreamer et al., 2012; Steele et al., 2012). As we used the $FeSO_4$ solution to generate the excess of iron for bacteria we presumed that the ColRS system responds to Fe^{2+}. However, Fe^{2+} is easily oxidized to Fe^{3+} in the presence of oxygen. Therefore, to determine which form of iron activates the ColRS system, we tested transcription from the PP_0903's promoter in the presence of ferrous and ferric ions. Sodium ascorbate, which reduces Fe^{3+} to Fe^{2+} (Wyckoff et al., 2006), was used to keep iron in its Fe^{2+} state. The results showed that in the presence of sodium ascorbate the PP_0903's promoter did not respond to iron (Ref I, Fig. 7), indicating that the ColRS system is activated only by Fe^{3+}. The control experiment with zinc and sodium ascorbate confirmed that the reducing agent itself has no effect on the metal-responsiveness of the PP_0903 promoter (data not shown). Given that *P. putida* is a soil bacterium that typically inhabits aerobic environments in which iron would most often be found in the ferric form, it is reasonable for it to predominantly sense the extracellular Fe^{3+} concentrations.

Iron, zinc and manganese are physiologically important metals for bacteria. Iron is a cofactor for many essential enzymes such as cytochrome c which participates in the respiratory chain (Thöny-Meyer, 1997) and ribonucleotide reductase, a key enzyme that mediates the synthesis of DNA precursors (Torrents, 2014). Zinc is known to be required for the activity of more than 300 enzymes but also has a role in maintaining protein structure in zinc-finger motifs (McCall et al., 2000). These motifs, although more common in eukaryotic cells, have been found in the DNA-binding domains of a few bacterial transcriptional regulators as well (Malgieri et al., 2015). The usage of manganese is more species specific. In many bacteria it is considered a trace element (Chandrangsu et al., 2017) and is often found in enzymes that have the same function as an iron-containing enzyme but are used under conditions of oxidative stress or iron starvation (Kaur et al., 2014). For example, in *P. aeruginosa* the Fe-cofactored superoxide dismutase SodB is the main enzyme used to neutralize superoxide radicals in iron-replete conditions, while Mn-cofactored SodA is expressed in iron limitation (Hassett et al., 1995). Cadmium, on the other hand, has no biological role in the cells and is highly toxic, mainly because of its tendency to replace Zn^{2+} from proteins (Khan et al., 2016).

The metal tolerance assay with *colR*- and *colS*-deficient strains showed that the ColRS system is highly relevant for the tolerance of zinc and iron but its impact on manganese and cadmium tolerance is modest (Ref I, Table 1 and Fig. 1). Manganese and iron are interchangeable in the metal-binding sites of many proteins because the ionic radiuses of these cations are similar (Jakubovics and Jenkinson, 2001). An analogous scenario is true for cadmium and zinc ions. The chemical properties of cadmium are quite similar to the ones of zinc, making it possible for cadmium ions to replace zinc ions from its native proteins (Malgieri

et al., 2015). Because the effect of the ColRS system on manganese and cadmium tolerance was small, we propose that the ColRS system is able to recognize manganese and cadmium due to their similarity to iron and zinc but the main biological role for the system is to detect the excess of zinc and iron.

4.2. Conserved glutamic acids of an ExxE motif in ColS are involved in signal recognition (Ref I)

The periplasmic region of the ColS protein, which is most probably the sensor domain for this HK, is 96 amino acids long (Ref I, Fig. 5A and 5B) and contains 13 amino acids that are conserved in all the 639 ColS proteins from different *Pseudomonas* species that had chromosome sequences available in March 2020 (Figure 5). In 2014, when the periplasmic sequence analysis of ColS proteins was conducted with 47 sequences, 14 amino acids were found to be fully conserved (Ref I, Fig. 5C). The finding that the huge amount of new ColS sequences has not significantly changed the sequence conservation logo (compare Figure 5 and Ref I, Fig. 5C) illustrates how similar all the periplasmic sequences of the ColS proteins are. Assuming that the function of the ColRS system is also relatively conserved in *Pseudomonads*, we anticipated to find the amino acids participating in metal sensing among the most conserved residues. Zinc is usually coordinated by cysteine and histidine residues in proteins (Malgieri et al., 2015), whereas glutamic and aspartic acids are mainly used to bind iron (Ma et al., 2009). There are four highly conserved glutamic acids in the ColS sequences in positions 38, 96, 126 and 129 and one aspartic acid in position 57 (Figure 5). Also, three histidine residues at positions 35, 95 and 105 are relatively conserved (Figure 5).

To investigate whether the conserved glutamic acids, aspartic acid or histidine residues participate in signal recognition, we constructed ColS derivatives carrying the replacements of these amino acids and analyzed the metal-promoted activation of the PP_0903 promoter. The results revealed that only the substitution of the glutamic acid E126 or E129 of the ColS protein abolished the metal responsiveness of the PP_0903 promoter (Ref I, Fig. 6). Surprisingly, both iron- and zinc-promoted activation was lost, implying that these amino acids are required for sensing both iron and zinc. The arrangement of the E126 and E129 in the ColS sequence resembles the ExxE motif that is found to be important for Fe^{3+} sensing in the PmrA HK of *Salmonella enterica* (Wösten et al., 2000). The positioning also has some resemblance to a RExxE motif that has been shown to bind iron in several eukaryotic proteins (Stearman et al., 1996; Wedderhoff et al., 2013). Given that in PmrA the glutamic acids of two ExxE motifs directly bind ferric ions (Wösten et al., 2000), we propose that the E126 and E129 of the ColS may also recognize iron excess by directly binding Fe^{3+} ions. To our knowledge the ExxE motif has not been shown to bind Zn^{2+} but considering that there are examples of proteins where zinc is coordinated by glutamic acids (Laitaoja et al., 2013), it is not impossible.

Contrary to the E126 and E129 substitutions, mutating H35, E38, D57 or H105 in the periplasmic region of the ColS resulted in partial activation of the PP_0903 promoter already in the absence of metal excess and increased the promoter's responsiveness to zinc and iron (Ref I, Fig. 6). It shows that these amino acids do not participate directly in metal sensing but still have an important role in the sensor domain of the ColS, presumably in regulating the metal sensing sensitivity of the ColS.

Both iron and zinc ions are usually coordinated by four amino acids (Laitaoja et al., 2013; Wedderhoff et al., 2013) but ColS seems to possess only two amino acids that are involved in the signal recognition. However, the majority of described HKs exist as homodimers (Zschiedrich et al., 2016) and since nothing has suggested that the ColS is an atypical HK, it most likely functions as a homodimer. Therefore, the signal sensing of the ColS is probably a collaboration of two ColS proteins where each monomer contributes with one ExxE motif and altogether four glutamic acids are used to bind the metal ions (Ref I, Fig. 8).

Figure 5. The conservation of the periplasmic region of ColS proteins. 639 sequences from 570 different *Pseudomonas* strains were used to create the logo with WebLogo server tool (Crooks et al., 2004). The degree of sequence conservation at each position is indicated by the total height of a stack of letters and is measured in arbitrary "bit" units, with a theoretical maximum of 4.3 bits at each position. Asterisks (*) indicate the highly conserved glutamic and aspartic acids and "+" indicates the well-conserved histidine residues.

4.3. ColRS system may respond to membrane damage (Ref III)

Previous work with the *colR*- and *colS*-deficient strains of *P. putida* has indicated that the membrane integrity of these mutants is decreased (Putrinš et al., 2011; Putrinš et al., 2008) and in search of the reason for this, several other proteins that are required for membrane homeostasis have been identified. Among these are the $TonB_m$, PocA and PocB proteins that together constitute an inner membrane localized complex.

In the absence of the $TonB_m$-PocAB complex cells have increased membrane permeability (Ref III, Fig. 2) and they share several phenotypes with the *colRS* deficient bacteria. For example, both $TonB_m$-PocAB and ColRS deficient strains are susceptible to Mn^{2+} and Zn^{2+} (Ref I, Fig. 1, Ref III, Fig. 3B and data not shown) and experience glucose-specific cell lysis caused by the rise in the levels of OprB1 sugar channel (Putrinš et al., 2011) (Ref III, Fig. 2). Still, there are also several differences between the $TonB_m$-PocAB and ColRS deficient strains. The

$TonB_m$-PocAB complex has been shown to be needed for the polar localization of flagella and type IV pili in *P. aeruginosa* (Cowles et al., 2013) and we showed that it has a similar role in *P. putida*. The Δ*tonB*$_m$, Δ*pocA* and Δ*pocB* deletions strains have randomly localized flagella (Ref III, Fig. 1B) because the placement of FlhF, a protein that determines the place where new flagella are synthesized (Pandza et al., 2000), is random in these strains (Ref III, Fig. 1D). The Δ*tonB*$_m$ strain is also susceptible to a greater variety of chemicals than the *colRS* deficient strain (Ref III, Fig. 3A and 3B), its maximal growth rate is decreased (Ref III, Fig. 3D) and the magnitude of these effects seems to be growth phase dependent (Ref III, Fig. 3). It all suggests that despite some similarities, the $TonB_m$-PocAB and ColRS systems function independent of each other in providing membrane homeostasis.

In order to get some insight of what is happening in bacteria when they lack the $TonB_m$-PocAB complex, a proteome analysis of exponentially growing and stationary phase Δ*tonB*$_m$ as well as wild-type was carried out. To our surprise, the proteome analysis indicated that the abundance of ColR regulon proteins PP_0903, PP_5151 and OprQ was changed in the exponentially growing Δ*tonB*$_m$ in a way consistent with the activation of ColRS signaling (Ref III, Fig. 7), although no metal excess was present. To control the possibility that the ColRS signaling is active in the Δ*tonB*$_m$ strain, the activities of four ColR-regulated promoters were determined. All the promoters responded in the Δ*tonB*$_m$ strain but did not in the Δ*tonB*$_m$ *colR* double mutant (Ref III, Fig. 8), confirming that the ColRS system must be activated when the $TonB_m$-PocAB system is deficient.

The $TonB_m$-PocAB deficient *P. putida* has two main phenotypes: its membrane permeability is increased (Ref III, Fig. 2) and its flagella arrangement is random (Ref III, Fig 1B). The flagella arrangement is random due to the random placement of FlhF, which determines the place where new flagella are synthesized (Cowles et al., 2013). In the absence of FlhF bacteria have slightly less flagella but nevertheless the random distribution of flagella is similar to the Δ*tonB*$_m$, Δ*pocA* and Δ*pocB* deletion strains (Ref III, Fig. 1B and 1C). On the other hand, the Δ*flhF* mutant does not have any indicators of membrane stress that are characteristic to the $TonB_m$-PocAB deficient cells (Ref III, Fig. 1E and 2), which made us conclude that the mislocalization of flagella is not the cause for membrane defects in the Δ*tonB*$_m$ strain. Contrary to that we proposed that the impaired membrane homeostasis may be the main issue that causes the inability of FlhF to locate on to its correct place in the cell pole.

Membrane state is a condition that can be sensed by TCSs. Besides the TCSs that specifically detect only membrane conditions, some TCSs with extra- or intracellular sensor domain can also respond to membrane state. For example, the PhoPQ system that responds to the presence of antimicrobial peptides (Bader et al., 2005), low pH (Bearson et al., 1998) and high concentration of Mg^{2+} and Ca^{2+} ions (Montagne et al., 2001) can detect osmotic upshift in the extracellular environment through the physical properties of the membrane (Yuan et al., 2017). Taking into consideration that the ColRS system is also partially activated when bacteria grow on glucose minimal medium or in the presence of phenol (Kivistik

et al., 2009) which cause membrane stress (Putrinš et al., 2011), we propose that the activation of the ColRS system in the Δ*tonB*$_m$ strain originates from the membrane damage.

In general, the activation of a HK that possesses an extracytoplasmic sensor domain occurs when a signal molecule induces a conformational rearrangement in the sensor domain. Membrane conditions, however, are usually sensed by using the transmembrane regions (Mascher et al., 2006). The activation of the PhoPQ system in response to osmotic upshift was also shown not to require the sensor domain of the PhoQ, which is important for detecting all other signals it responds to, but depended on the transmembrane region. Apparently it is the height of the transmembrane domain, which changes when the membrane thickness increases due to the osmotic upshift, that is the primary signal for the PhoQ in the changed osmotic conditions (Yuan et al., 2017). Therefore, we do not think that the activation of the ColRS system in TonB$_m$-PocAB deficient strain occurs due to the sensor domain of the ColS detecting a distinct signal. Rather, the ColRS responds in the Δ*tonB*$_m$ strain because the membrane damage of this strain alters the positioning or physical properties of the transmembrane regions of the ColS proteins, which causes the HK to shift into its active form.

Taken together, the ColRS system of *P. putida* responds to Fe^{3+}, Zn^{2+}, Cd^{2+} and Mn^{2+} excess and possibly also to distinctive membrane damage. The detection of metal excess involves two glutamic acids, E126 and E129, in the periplasmic region of the ColS that are arranged in an ExxE motif. It is likely that the metal excess signal is sensed by a ColS dimer where each monomer contributes with one ExxE motif.

5. The role of the ColRS system

Many bacteria live in fluctuating environmental conditions and a metal-starved bacterium may encounter a metal-rich environment relatively sudden. Since metal ions cannot be degraded, the cells' primary response to metal excess is to modulate the transport of metal ions out of the cell. To give the cell time to respond, cytosolic buffering mechanisms are used to limit the damage of metal excess until the metal uptake systems of the cell are inhibited, metal efflux is triggered and sequestration mechanisms that store the excess metal ions into storage proteins are activated (Chandrangsu et al., 2017). In addition, alternative methods such as modulation of the membrane properties may as well be used to avoid the toxicity of the metal (Nishino et al., 2006).

Most of the known ColR-regulated genes encode membrane proteins or proteins involved in membrane biogenesis (Kivistik et al., 2009; Kivistik et al., 2006) which together with the phenotypic effects of the *colR* mutant has led to the assumption that the role of the ColRS system is to regulate membrane functionality. This suggests that the responsibility of the ColRS system might be to prevent the excess metal ions from entering the cell.

5.1. The effect of known ColR-regulated genes on metal tolerance is small (Ref I)

As the *colRS*-deficient strains were sensitive to several metals and the metals modulated the expression of the ColR-regulon, we reasoned that the ColR-regulated genes should be important for metal tolerance. Previous studies had identified eight loci that are ColR-regulated in *P. putida* (Kivistik et al., 2009; Kivistik et al., 2006). In order to test whether these loci affect metal tolerance, knockouts of these genes were constructed and the minimal inhibitory concentrations (MIC) of zinc and iron for the mutant strains were determined. It was presumed that inactivation of the ColR-activated genes in wild-type background will decrease the metal tolerance and disruption of ColR-repressed genes in *colR*-deficient strain will increase the tolerance. However, the results revealed that none of the strains defective of a single ColR regulon gene or operon had significant effect on iron or zinc tolerance (Ref I, Table 2). Only deleting PP_0035-33 operon slightly decreases the MIC value for Zn^{2+} (Ref I, Table 2).

To investigate if the ColR-regulon genes might be functionally redundant and therefore the effect of any individual gene on metal tolerance is masked, double, triple and quadruple mutants of the loci were constructed. While deleting as much as three loci had no further effect to the metal tolerance, the quadruple mutant of PP_0035-33, PP_2579, PP_0903-905 and PP_0900 was noticeably less tolerant to iron than the wild-type and slightly less tolerant to zinc than the PP_0035-33 deletion strain (Ref I, Table 2). This indicates that the ColR-regulated genes are almost equally slightly important and no gene contributes significantly to the metal tolerance. At the same time, as deleting any of the known ColR-regulated loci alone or in sets was not enough to decrease the cell's metal tolerance to the level of the *colRS*-deficient strain's, it suggests that the ColR-regulon identified so far is yet incomplete.

The role of the ColR-regulated PP_0035-33 operon is most likely to modify lipopolysaccharides (LPS) as the genes it encodes are homologues to the 4-amino-4-deoxy-L-arabinose transferase proteins described in *E. coli* and *S. enterica* (Trent et al., 2001). Adding 4-amino-4-deoxy-L-arabinose to the lipid A part of the LPS decreases the negative charge of the LPS and in *S. enterica* this increases the bacterium's resistance to cationic antimicrobial peptides, Fe^{3+} and Al^{3+} (Nishino et al., 2006). The genes of the 4-amino-4-deoxy-L-arabinose transferases in *S. enterica* are under the control of the PmrAB system (Gunn et al., 1998; Tamayo et al., 2005) which responds to extracellular Fe^{3+} (Wösten et al., 2000), so the modification occurs in the excess of iron. It is reasonable to think that if the PP_0035-33 operon encodes 4-amino-4-deoxy-L-arabinose transferase proteins in *P. putida*, it is important for zinc and iron tolerance for a similar reason. However, as deleting PP_0035-33 operon had only a minor effect to metal tolerance (Ref I, Table 2), the impact of this LPS modification is small in *P. putida*.

The role of the ColRS system in modifying LPS is further supported by the finding that in *P. aeruginosa* ColRS system regulates the expression of phospho-

ethanolamine transferase $EptA_{Pa}$ in response to zinc excess (Nowicki et al., 2015). Phosphoethanolamine is a cationic lipid and likewise the 4-amino-4-deoxy-L-arabinose, it can be added to the lipid A to modify the properties of the LPS. Nevertheless, as the *eptA*$_{Pa}$ mutant of *P. aeruginosa* does not have any growth defect in media with Zn^{2+} (Nowicki et al., 2015), this indicates also that adding a particular modification to the LPS cannot be the sole role of the ColRS system.

5.2. The importance of ColRS system determined at the proteome level (Ref II)

Since the zinc and iron tolerance analysis of the strains defective in known ColR-regulated genes did not reveal the reason why ColRS system is important for metal tolerance, we decided to analyse the proteomes of the *colR*-deficient strain and wild-type *P. putida* in the presence and absence of zinc excess. We expected that the proteomic approach would provide some cues about the mechanism behind the ColRS-mediated metal tolerance. In order for the results of the wild-type and *colR*-deficient strain to be comparable, we aimed to use a zinc concentration that would be low enough not to affect the growth of the zinc sensitive *colR*-deficient strain. At the same time, it was reasonable to use as high zinc concentration as possible to see the effects that the metal's excess has.

To determine the effect of zinc excess on the growth, the growth curves of *colR*-deficient and wild-type strain in the presence of various concentrations of $ZnSO_4$ were established. Adding 0.6 mM $ZnSO_4$ to the medium caused a similar slight growth inhibition for the wild-type and the *colR* knockout (Ref II, Fig. 1A), while at higher zinc concentrations the growth of the *colR* mutant was suppressed more than that of the wild-type (data not shown). To find out how long it takes for the ColR regulon to respond to the 0.6 mM $ZnSO_4$ concentration, the activity of the PP_0903 promoter was analysed in the presence of 0.6 mM $ZnSO_4$. The transcription from PP_0903 promoter was induced in wild-type cells in a *colR*-dependent mode already after 30 minutes of exposure to zinc but the full activity was achieved after an hour (Ref II, Fig. 1B). The analysis of outer membrane protein (OMP) patterns of the *colR* mutant and wild-type after exposure to 0.6 mM zinc showed also that slight differences from the wild-type are noticeable in the OMP profile of the *colR*-deficient strain after 30 minutes of zinc treatment but the differences were most pronounced after 3 h of zinc exposure (Ref II, Fig. 1C). Therefore, a 3-hour long treatment with 0.6 mM zinc concentration was chosen to determine the responses of *colR*-deficient strain and wild-type cells to zinc excess at the proteome level.

5.2.1. Zinc excess induces upregulation of efflux pumps and membrane damage avoidance proteins in both wild-type and colR-deficient cells (Ref II)

In stress-free conditions, i.e. when bacteria grow in rich LB medium, the *colRS*-deficient strain has no phenotypic differences from the wild-type. The proteome analysis of LB-grown wild-type and *colR*-deficient strains showed that none of the 2705 proteins identified were statistically differentially expressed in the two strains. This confirmed that in normal growth conditions, where the signalling through ColRS system is not active, the lack of ColRS has no effect on *P. putida*.

To determine the response induced by zinc excess, the proteome data of wild-type and the *colR*-deficient strain was compared to the data of non-induced condition. This analysis indicated that in wild-type the abundance of 60 proteins changed due to the addition of zinc (Ref II, Fig. 2A and 2B), whereas in *colR*-deficient strain 237 proteins responded to zinc excess (Ref II, Fig. 2A and 2C). There were 51 proteins that responded in the same manner in both strains (Ref II, Table 3 and 4), although some of these (17 proteins) were noticeably more affected in the *colR*-deficient strain (Ref II, Table 4).

Not surprisingly, some efflux pump proteins and porins responded in both strains (13 proteins altogether; Ref II, Table 3 and 4) as the export systems provide means to transport the excess metal out of the cells and the porins to fine tune the permeability of the cell membrane to the ions. Also, proteins related to amino acid metabolism, more specifically glutamate homeostasis (5 proteins; Ref II, Table 3), were upregulated in the wild-type as well as in the *colR*-deficient strain. This is in line with previous reports indicating that glutamate homeostasis is altered in response to Cd^{2+} excess in *P. brassicacearum* (Pages et al., 2007) and to Ni^{2+} in *P. putida* (Ray et al., 2013). Zinc excess also induced several proteins associated with stress response and cell envelope homeostasis (8 proteins; Ref II, Table 3 and 4).

The most drastic change in both strains was the upregulation of CzcB1 and CzcC1 (Ref II, Table 3), which are components of the CzcCBA1 efflux pump that provides resistance to Cd^{2+}, Zn^{2+}, Co^{2+} and possibly also to Pb^{2+} (Leedjärv et al., 2008). In addition, three other metal efflux transporters, CzcCBA2, CadA1 and CadA2, were upregulated in both strains (Ref II, Table 3 and 4), although in a smaller scale, which is in line with the finding that CzcCBA1 is the main contributor to Zn^{2+} resistance in *P. putida* (Leedjärv et al., 2008). In *P. aeruginosa* the expression of CzcCBA pump is under the control of a CzcRS TCS that responds to zinc and copper (Caille et al., 2007; Wang et al., 2017). *P. putida* possesses two *czcRS* operons, *czcRS1* and *czcRS2*, and a solitary *czcR3* gene (Canovas et al., 2003). All three regulators, CzcR1, CzcR2 and CzcR3, were upregulated by zinc excess according to our analysis (Ref II, Table 3), suggesting that they all contribute to the response of zinc excess and possibly regulate the expression of the CzcCBA efflux pumps in *P. putida*.

Zinc is known to perturb the cytoplasmic membrane of bacteria (Poole et al., 2019) and in fact a number of TCSs that respond to envelope stress such as the

AmgRS of *P. aeruginosa* (Poole et al., 2019), the BasSR of *E. coli* (Froelich et al., 2006; Ogasawara et al., 2012) and the BaeSR of *E. coli* (Lee et al., 2005) are also activated by Zn^{2+}. The proteome analysis indicated that several proteins that responded to zinc in both strains were more affected in the *colR* mutant (Ref II, Table 4). For example, the Blc and VacJ family lipoproteins PP_5037 and PP_1737 responded to zinc in the *colR*-deficient strain about two-times more than in the wild-type. These lipoproteins are proposed to be involved in membrane biogenesis and repair (Bishop, 2000; Xie et al., 2016) indicating that the 0.6 mM zinc we used caused membrane damage to the cells. Their higher abundancy in the *colR*-deficient strain is likely an indicator suggesting that the 0.6 mM zinc caused higher stress for the *colR* mutant than for wild-type, despite the absence of differences in the growth of two strains.

5.2.2. Four new putative ColR-regulon proteins were identified by the proteome analysis (Ref II)

In order to get some insight into the purpose of the ColRS system for the cells, the proteome data of wild-type grown in the presence of zinc was compared to that of the *colR*-deficient strain. We expected to find ColR-regulon proteins among the proteins that responded to zinc excess in the wild-type and not in the *colR*-deficient strain.

There were only nine proteins that responded to zinc excess in the wild-type but had no significant change in the *colR* strain (Ref II, Table 5). Five of them, OprQ, PagL, PP_0903, PP_0904 and PP_5152, were proteins previously known to belong to ColR-regulon and the abundance of all of them was changed in a direction consistent with ColRS system's activation in the wild-type. The other four, PP_0032, PP_2011, PP_2863 and PP_3954, were hypothetical proteins with unknown functions. To determine if the genes coding for these four proteins could be ColR-regulated, the binding sequence of ColR was searched in front of the genes. However, the computer search failed to identify the ColR binding site in the promoter regions of any of them (data not shown), suggesting that the direct involvement of ColR in the regulation of these genes is questionable. Several other known ColR-regulon proteins remained undetected in our proteome screen. This illustrates that there is a set of scarce or poorly ionizable proteins that were missed by this proteomic analysis, most likely including the yet unidentified ColR-regulon proteins.

5.2.3. In the absence of ColRS system maintenance of membrane integrity is compromised (Ref II and III)

Compared to the modest number of ColR-dependent changes in the wild-type, zinc caused a vast rearrangement of gene expression in the *colR*-deficient strain. The abundance of 186 proteins was changed due to zinc excess only in the *colR*-

deficient mutant (Ref II, Table 6), out of which 122 were up- and 64 down-regulated. When the proteins were categorized based on their function and the proteins with unknown function (73 proteins) were excluded, the majority of them classified into one of four categories (Ref II, Table 6) – transport and secretion (23 proteins), stress and defence response (24 proteins), metabolism (29 proteins) or chemotaxis and motility (22 proteins). All the proteins related to chemotaxis and motility as well as 14 proteins (61%) from the transport and secretion group were downregulated, whereas all other categories contained predominantly up-regulated proteins. Interestingly, also 11 proteins involved in signaling or transcription regulation were altered in the *colR* mutant in response to zinc stress.

The most noticeable change in the *colR* strain was the drastic upregulation of alginate biosynthesis proteins (Ref II, Table 6). Alginate biosynthesis genes are clustered into a large 12-gene operon, *algD-8-44-KEGXLIJFA*, and all the proteins encoded by this operon, except for Alg8, were highly induced by zinc excess. Also three alginate synthesis regulators, AmrZ, AlgB, and AlgP, were upregulated. Alginate is an exopolysaccharide that in *P. putida* plays an important role in the tolerance of water limitation as it creates a well hydrated environment around the bacterium (Chang et al., 2007). In *P. aeruginosa* alginate contributes to biofilm formation (Hentzer et al., 2001) and protects bacteria from antibiotics and the immune system of the host (Leid et al., 2005; Ramsey and Wozniak, 2005). The regulation of alginate synthesis is a complex process and involves several regulators. The most upstream regulator of the cascade is the envelope stress response sigma factor AlgU (Wood et al., 2006), which in normal growth conditions is bound to the anti-sigma factor MucA. MucA anchors the AlgU to the cytoplasmic membrane and prohibits it from regulating gene expression (Qiu et al., 2007). However, when the cells encounter envelope stress, induced either by cell wall-acting antibiotics or other compounds that disrupt bacterial membranes (Wood et al., 2006; Wood and Ohman, 2009), MucA is degraded and AlgU is released into the cytoplasm where it regulates the expression of large numbers of genes (Wood and Ohman, 2009).

When we compared the zinc induced response in the *colR*-deficient strain with the AlgU regulon (Wood and Ohman, 2009), a remarkable overlap was observed: out of 186 proteins 58 (30%) were orthologs of the *P. aeruginosa* AlgU regulon proteins. Furthermore, 55 out of 58 proteins responded in a similar manner as AlgU-regulated genes in *P. aeruginosa*, i.e., they were either activated (47 proteins) or repressed (8 proteins) in response to stress. This strongly indicated that the zinc excess caused the activation of AlgU sigma factor in the *colR*-deficient cells, which in turn refers that the membrane integrity of these cells was perturbed. As the AlgU activation was not detected in zinc-treated wild-type, it shows that without the functional ColRS system, zinc is able to cause an extensive damage to the cell envelope.

Interestingly, the AlgU regulon activation was also determined by proteome analysis in the exponentially growing $\Delta tonB_m$ strain (Ref III, Fig. 7 and Table S4). This prompted us to compare the proteomic response of $\Delta tonB_m$ strain to that of zinc-treated *colR*-deficient strain. When the differently than wild-type expressed

proteins of exponentially growing *ΔtonB$_m$* cells were compared to the proteins that responded only in zinc-treated *colR*-deficient strain, 61 overlapping proteins were found, all of which were altered in both strains in the same direction (Ref III, Fig. 7 and Table S4). This also included 37 proteins that were not part of the AlgU regulon. Since the common characteristic of the *ΔtonB$_m$* and zinc-treated *colR*-deficient strain is their membrane damage, it is likely that these 61 proteins are responding to membrane distress. In this case, it furthermore illustrates that a large proportion of the differently expressed proteins in the zinc-treated *colR*-deficient strain are indicators of membrane damage.

Noticeably, a considerable amount of siderophore biosynthesis related proteins were upregulated in both *ΔtonB$_m$* and zinc-treated *colR*-deficient strain (7 proteins) (Ref III, Table S4). The increased pyoverdine production of the *colR* mutant in response to zinc was confirmed by evaluating the supernatants of the wild-type and the *colR*-deficient bacteria grown in the presence of 0.6 mM zinc (Ref II, Fig. S1). Usually, siderophores are synthesised in response to iron limitation as their main role is to acquire iron from the environment (Cornelis, 2010). However, it has been shown that zinc, copper and manganese can also stimulate siderophore production (Braud et al., 2009b) and this could decrease the diffusion of these metals into the cell (Braud et al., 2010). The reason for it is that siderophores are able to chelate metals other than iron but the siderophore uptake pathways are highly selective for iron-bound siderophores (Braud et al., 2010; Braud et al., 2009a). This could mean that the production of siderophores is beneficial for the *colR*-deficient strain in the zinc excess and that it is part of the stress response.

5.2.4. Upregulation of alginate regulator AmrZ but not alginate synthesis proteins contributes to zinc tolerance (Ref II)

Alginate biosynthesis proteins coded by the large *algD-8-44-KEGXLIJFA* operon were the most altered proteins by zinc excess in the *colR*-deficient strain (Ref II, Table 6). The *algD* operon has four major regulators: AlgU, AmrZ, AlgB and AlgR (Ohman, 2009). Our proteome analysis indicated that the expression of two of these regulators, AmrZ and AlgB, was induced in the zinc-treated *colR*-deficient strain (Ref II, Table 6). The expression of AmrZ, which is considered a global regulator that besides alginate genes (Baynham et al., 1999), regulates the expression of the motility regulator FleQ and signalling molecule cyclic-di-GMP production (Martinez-Granero et al., 2014; Tart et al., 2006), was induced most significantly, about 10-times. This considerable upregulation led us to hypothesize that ColR might be a repressor for the *amrZ*.

In order to test the role of ColR in *amrZ* expression, the *amrZ-lacZ* transcriptional fusion was constructed and the promoter activity of the construct was determined in the wild-type and *colR*-deficient strain. In line with the proteome data, the activity of the *amrZ* promoter was highly induced by zinc in the *colR*-deficient strain, while no induction was observed in the wild-type (Ref II, Fig. 3A).

In silico analysis of the *amrZ* gene revealed a highly similar sequence to the ColR-binding consensus in the promoter region of the *amrZ*. To analyze whether ColR directly regulates *amrZ* expression, ColR binding to the promoter region was tested with DNA mobility shift assay and DNase I footprinting. Surprisingly, neither method showed any binding of ColR to the promoter region of *amrZ* (data not shown). This indicates that either the binding of the ColR to the *amrZ* promoter is complicated and needs some cellular factor(s) that are absent in the *in vitro* reactions or that the ColR influences *amrZ* expression indirectly. The latter possibility is more likely as it took several hours for the *amrZ* promoter to respond zinc excess in the *colR*-deficient strain (Ref II, Fig. 3A), while the full activity of the PP_0903 in the same conditions was achieved with 30 minutes (Ref II, Fig. 1B). The most likely explanation is that *amrZ* promoter responds to the activation of the AlgU.

In order to test if AmrZ is responsible for the up-regulation of the alginate biosynthesis proteins in the zinc-treated *colR*-deficient strain, the *algD-lacZ* transcriptional fusion was constructed. The promoter activity of this construct was determined in the wild-type, *colR*-deficient strain and in the *amrZ* deletion derivatives of these strains. The results showed that *algD* expression was triggered in the *colR* mutant exposed to zinc excess and the induction was abolished in the *colRamrZ* double mutant (Ref II, Fig. 3B). This indicates that similarly to other *Pseudomonads* (Baynham et al., 1999), AmrZ positively regulates *algD-8-44-KEGXLIJFA* expression in *P. putida*.

To determine if the upregulation of the alginate synthesis proteins contributes to zinc tolerance, the *amrZ, algD, amrZcolR* and *algDcolR* knockout strains were constructed and the zinc tolerance of the strains was analysed. The *algD* and *amrZ* deficiency had no effect on the zinc tolerance in the wild-type background (Ref II, Fig. 5). For the *colR* mutant, the lack of AlgD slightly increased the zinc tolerance, whereas the lack of AmrZ decreased it (Ref II, Fig. 5). This indicates that the increased expression of alginate genes seems to be a disadvantage for the *colR* mutant under zinc excess and contrary, the *amrZ* expression contributes to zinc tolerance. Notably, we did not observe a mucoid phenotype of the *colR* mutant growing in the presence of zinc, indicating that, even though the proteome data showed drastic up-regulation of alginate biosynthesis proteins, the actual alginate synthesis is low. As alginate biosynthesis and export is regulated at multiple levels, including post-translationally (Hay et al., 2009; Merighi et al., 2007), it is not entirely surprising that the upregulation of the alginate operon did not lead to increased alginate production.

Taking the proteome data together, the analysis of the *P. putida* wild-type and *colR*-deficient strain indicated that zinc causes membrane damage. In the wild-type cells, where zinc excess activates the ColRS system, the damage seems to be modest. In the *colR*-deficient strain, on the other hand, the activation of the AlgU sigma factor indicates that the lack of the ColR-dependent response leads to a massive zinc-caused envelope perturbation (Figure 6). We propose that the extensive rearrangement of gene expression recorded in the *colR*-deficient strain

in response to zinc is at least partially, if not mainly, the result of AlgU regulating the expression of large number of stress response genes. The activation of AlgU would also be the most likely explanation for the upregulation of AmrZ and alginate biosynthesis proteins in the *colR* mutant.

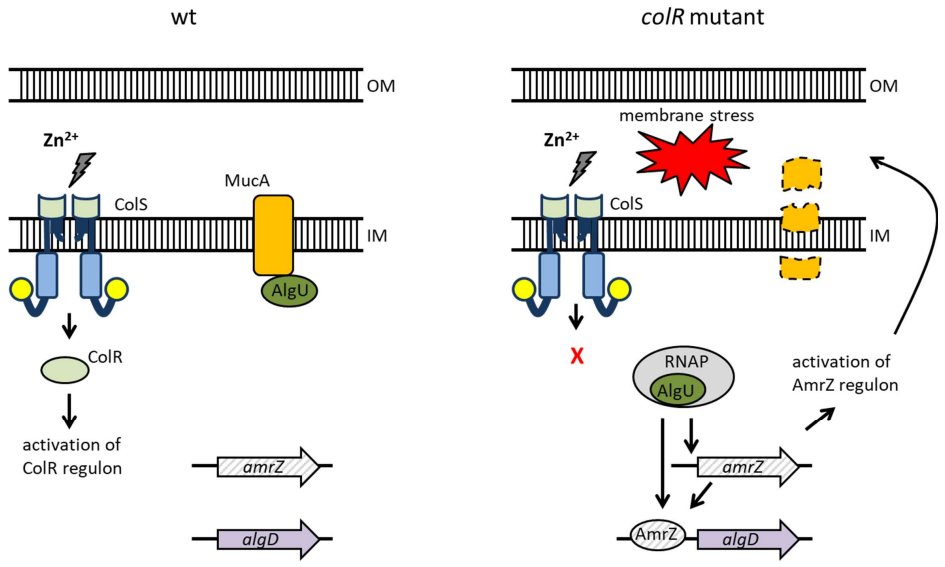

Figure 6. Representative model of the activation of AlgU-regulated pathway in zinc-treated *colR*-deficient *P. putida*. In wild-type *P. putida* (wt) zinc excess is sensed by ColRS two-component system and the expression of ColR-regulated genes contributes to zinc tolerance. In the *colR*-deficient strain (*colR*) the lack of activation of the ColR regulon in zinc excess results in membrane stress. Due to the membrane distress the anti-sigma factor MucA is degraded and the sigma factor AlgU is released. AlgU binds to RNA polymerase (RNAP) and facilitates the transcription of alginate synthesis regulator AmrZ and together with AmrZ the expression of alginate synthesis operon (*algD*). Besides activating the expression of alginate synthesis operon, AmrZ regulates the expression of other genes that contribute to zinc tolerance of *colR*-deficient *P. putida*.

CONCLUSIONS

The ColRS system is a two-component signal transduction system that is conserved among the *Pseudomonas* species. Previous studies with the ColRS system of *P. putida* strain PaW85 have suggested the involvement of this system in the maintenance of membrane integrity (Putrinš et al., 2011). Also, several ColR-regulon genes have been identified (Kivistik et al., 2009) that have pointed to the cell membrane as the most likely target of the ColRS system. Yet, the sensed signal of this system had remained unknown.

The results of this work can be summarized as follows:

- The ColRS system of *P. putida* detects the presence of excess amounts of Zn^{2+}, Fe^{3+}, Mn^{2+} and Cd^{2+}.
- The ColRS system may also respond to membrane damage.
- Two glutamic acids, E126 and E129, of a conserved ExxE motif in the periplasmic region of the ColS are involved in the metal ion detection. ColS likely senses the signal as a homodimer and altogether four glutamic acids of two ExxE motifs are used for signal recognition.
- The ColRS system is required for *P. putida* for the tolerance of zinc and iron but it slightly contributes to the tolerance of manganese and cadmium as well. Therefore, the biological role of the system is most likely to respond to the excess of Zn^{2+} and Fe^{3+}.
- Without the activation of the ColRS system, zinc causes an extensive damage to the *P. putida*'s cell envelope. The membrane damage leads to the activation of AlgU sigma factor, which induces a stress response in the *colRS* deficient cells.
- The importance of the ColRS system seems to be in maintaining membrane integrity. This is in good agreement with the previously indicated role of ColR-regulon genes and the phenotypic effects of the *colRS* deficient strains.

These results show that the ColRS system is one of the few two-component signal transduction systems described in *Pseudomonads* to detect and respond to extracellular metal excess. The importance of the ColRS system for *P. putida* lies in sensing Zn^{2+} and Fe^{3+} excess and providing tolerance against these metals. It is most likely that by strengthening the membrane the ColRS system prevents the metal ions from entering the cell. Furthermore, current study also indicates that besides metals the ColRS system can be activated by the membrane damage, making this two-component system even more general sentinel of membrane homeostasis.

SUMMARY IN ESTONIAN

ColRS süsteemi aktiveeriv signaal ning selle süsteemi roll *Pseudomonas putida* stressitaluvuses

Bakterid elavad väga erinevates ning tihti muutlikes keskkonnatingimustes, kus ellujäämiseks on oluline tunnetada ümbritseva keskkonna olusid ja vajadusel neile vastavalt reageerida. Üheks mehhanismiks, mida bakterid kasutavad nii välis- kui sisekeskkonna tunnetamiseks, on kahekomponentsed signaaliülekanderajad. Need on kahest valgust koosnevad süsteemid, milles üks komponent tunneb ära teatud keskkonnasignaali ja vastusena sellele aktiveeritakse teine valk. Teine valk on enamasti transkriptsiooniregulaator, mis mõjutab geenieksрessiooni ja põhjustab sellega signaalile vastava füsioloogilise muutuse bakteris (Zschiedrich et al., 2016).

Ühel bakteril võib olla kümneid erinevaid kahekomponentseid signaaliülekanderadu (Laub, 2011). Tüüpiliselt koosneb üks kahekomponentne signaalirada dimeersest sensorvalgust ja tsütoplasmas asuvast regulaatorvalgust. Sensorvalk on enamasti membraaniseoseline ja tunnetab väliskeskkonnas leiduvat signaali või membraani olukorda, kuid on ka tsütoplasmaatilisi sisekeskkonna seisundit tunnetavaid sensorvalke (Mascher et al., 2006). Reeglina toimub signaali äratundmine sensorvalgu sensordomääni abil, mille seondumine signaalmolekuliga kutsub esile konformatsioonilise muutuse esmalt sensordomäänis ja sellele järgnevalt ka ülejäänud sensorvalgu osades. Oluline roll kahekomponentsete signaaliradade töös on sensorvalgus paikneval konserveerunud histidiinijäägil ja regulaatorvalgus paikneval asparagiinhappejäägil. Nimelt autofosforüleeritakse sensorvalgus signaali äratundmise tulemusena konserveerunud histidiinijääk, kasutades sensorvalguga seondunud ATP fosfaatrühma. Fosforüleeritud histidiinilt kantakse fosfaatrühm edasi regulaatorvalgu asparagiinhappejäägile ning selle tulemusena aktiveeritakse regulaatorvalk (Zschiedrich et al., 2016). Enamik kahekomponentsete signaaliradade regulaatorvalkudest on transkriptsiooniregulaatorid, mille aktivatsioon muudab geenieksрessiooni, aga leidub ka ensüüme või valke, mis seonduvad RNA või teiste valkudega (Galperin, 2010). Kui signaalmolekuli enam keskkonnas ei leidu, katalüüsivad sensorvalgud regulaatorvalkude defosforüleerimist (Laub, 2011).

Üheks *Pseudomonas* perekonna liikides konserveerunud kahekomponentseks signaalirajaks on ColRS süsteem. ColRS süsteemi kirjeldati esmakordselt *Pseudomonas fluorescens*'is, kes vajab seda signaalirada taimejuurte edukaks koloniseerimiseks (Dekkers et al., 1998). Hiljem on ColRS süsteemi detailsemalt uuritud *P. aeruginosa*'s ja *P. putida*'s. *P. aeruginosa*'s on näidatud, et ColRS süsteem on vajalik nematoodi *C. elegans* (Garvis et al., 2009) vastaseks virulentsuseks ja antibiootikumi polümüksiin talumiseks (Gutu et al., 2013). *P. putida*'s on aga leitud, et ColRS süsteem on tõenäoliselt vajalik membraani terviklikkuse säilitamiseks. Sellele viitab *colRS*-defektsete rakkude lüüsumine glükoosi sööt-mel, kuna nad ei talu välismembraanis OprB1 suhkrukanalite hulga tõusu (Putrinš

et al., 2011). Lisaks toetavad ColRS süsteemi seotust *P. putida*'s membraani funktsioonidega ka teadaolevad kümmekond ColR-reguleeritavat geeni, millest enamus kodeerib kas membraanivalke või lipopolüsahhariidide sünteesiga seotud valke (Kivistik et al., 2009). Kaadmiumiresistentses *P. putida* tüves CD2 on aga leitud, et ColRS süsteem on oluline mitmete metallide (Mn^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+} ja Pb^{2+}) liia talumiseks (Hu and Zhao, 2007).

Kuigi ColRS signaaliraja osalust oli kirjeldatud erinevates bakteriliikides väga erinevates fenotüüpides, ei olnud seni teada, mis on ColRS süsteemi aktiveerivaks signaaliks ning mis on selle signaaliraja täpne funktsioon. Antud töö eesmärgiks oli analüüsida, kas ColRS süsteem on *P. putida* PaW85 tüves vajalik metalliliia talumiseks, ning teha kindlaks, kas ColRS süsteem aktiveerub vastusena keskkonnas leiduvatele metallidele.

Käesoleva töö peamised tulemused on:

- *P. putida* ColRS süsteem tunnetab Zn^{2+}, Fe^{3+}, Mn^{2+} ja Cd^{2+} liiga.
- ColRS süsteem võib aktiveeruda ka vastusena membraani kahjustusele.
- ColS valgu periplasmaatilises osas ExxE motiivis paiknevad konserveerunud glutamiinhapped E126 ja E129 osalevad signaali äratundmises. Arvatavasti tunnetab ColS signaali homodimeerse valguna ning neli glutamiinhapet kahest ExxE motiivist on vajalikud metalliiooni sidumisel.
- ColRS süsteem on *P. putida*'le vajalik tsingi- ja raualiia talumiseks. Kuigi see signaalirada panustab vähesel määral ka mangaani- ning kaadmiumiliia talumisse, on ColRS signaaliraja bioloogiliseks rolliks tõenäoliselt reageerida Zn^{2+} ja Fe^{3+} liiale.
- ColRS süsteemi puudumisel kahjustab tsink ulatuslikult *P. putida* membraani. Membraani kahjustuse tagajärjel indutseeritakse *colRS*-defektsetes rakkudes AlgU sigma faktori kontrollitav stressivastus, mis aitab leevendada ColRS süsteemi puudumisest tingitud membraanistressi.
- Antud töö tulemused näitavad, et ColRS süsteemi roll on membraani terviklikkuse säilitamine. See on kooskõlas varasemate teadmistega ColRS süsteemi kohta.

Käesoleva töö tulemused näitavad, et ColRS süsteem on üks vähestest *Pseudomonas*'e liikides teadaolevatest kahekomponentsetest süsteemidest, mis tunnetab ja reageerib väliskeskkonnas olevale metalliliiale. ColRS süsteemi roll *P. putida*-s on tunnetada Zn^{2+} ja Fe^{3+} liiga ning vastusena sellel tagada antud metallide liia taluvus. Tõenäoliselt toimub metallitaluvuse suurendamine läbi membraani läbilaskvuse vähendamise. Käesoleva töö tulemused viitavad, et lisaks metalliliia tunnetamisele aktiveerub ColRS signaalirada ka vastusena membraani kahjustusele, mis teeb sellest kahekomponentsest süsteemist veelgi tähtsama membraani homöostaasi regulaatori.

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PUBLICATIONS

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List of publications:

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2. Tamman, H., A. Ainelo, K. Ainsaar, and R. Hõrak. 2014. A moderate toxin, GraT, modulates growth rate and stress tolerance of *Pseudomonas putida*. Journal of Bacteriology. 196(1):157–69.
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4. Ainsaar, K., H. Tamman, S. Kasvandik, T. Tenson, and R. Hõrak. 2019. The $TonB_m$-PocAB System Is Required for Maintenance of Membrane Integrity and Polar Position of Flagella in *Pseudomonas putida*. Journal of Bacteriology. 201.

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