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# Study of OxcT function in Caulobacter crescentus copper tolerance

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# Study of OxcT function in *Caulobacter crescentus* copper tolerance

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# Study of OxcT function in *Caulobacter crescentus* copper tolerance

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Résumé

Malgré qu'il soit essentiel à faible dose, l'excès de cuivre (Cu) est toxique pour toutes les formes de vie. Chez le modèle alpha-protéobactérien *Caulobacter crescentus*, une réponse bimodale au Cu a été précédemment identifiée par notre équipe. Le gène *oxcT* (*CCNA_00027*), un nouvel acteur de la tolérance au Cu chez *C. crescentus* a été identifié avec un crible génétique recherchant des mutants sensibles au Cu. De plus, une expérience de RNA-seq montre une induction d'*oxcT* liée au Cu. En accord avec ces observations, la délétion d'*oxcT* (Δ*oxcT*) conduit à une sensibilité accrue au Cu dans les milieux riches et pauvres. Aucune sensibilité accrue aux autres métaux lourds n'a été observée. De façon surprenante, aucune augmentation significative de contenu cellulaire en Cu n'a pu être observée chez le mutant Δ*oxcT*. Les mesures de la concentration en Fe intracellulaire ainsi que les courbes de croissance dans des milieux à faible et forte concentration en Fe sous stress Cu ont démontré un lien entre l'homéostasie du Fe et le rôle d'OxcT dans la tolérance au Cu. Ces observations sont cohérentes avec le contrôle de l'expression d'OxcT par le ferric uptake regulator (Fur). Enfin, la purification d'OxcT suivi d'une spectrométrie de masse a permis de mettre en évidence une liste de partenaires potentiels d'OxcT. L'interaction entre OxcT et un candidat sélectionné sera étudiée dans un avenir proche.

Mémoire de master 120 en biochimie et biologie moléculaire et cellulaire

Janvier 2022

**Promoteur:** J.-Y. Matroule

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# Study of OxcT function in *Caulobacter crescentus* copper tolerance

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Summary

Despite its essentially at low dose, the excess of copper (Cu) is toxic to all forms of life. In the model alpha-proteobacterium *Caulobacter crescentus*, a bimodal response to Cu has been previously identified by our lab. The *oxcT* gene (*CCNA_00027*), a new actor of Cu tolerance in *C. crescentus*, was identified in a genetic screen seeking for Cu-sensitive mutants. In addition, a RNAseq experiment shows a Cu-dependent upregulation of *oxcT* expression. Consistent with these observations, *oxcT* deletion (Δ*oxcT*) leads to an increased Cu sensitivity in both rich and poor media. No increased sensitivity to other heavy-metals was observed. Surprinsingly, no significant increase in cellular Cu content could be observed in the Δ*oxcT* mutant. Measurements of the intracellular Fe content as well as of growth curves in low and high Fe concentration under Cu stress demonstrated a link between Fe homeostasis and the role of OxcT in Cu tolerance. These observations are consistent with the control of *oxcT* expression by the ferric uptake regulator (Fur). Finally, an OxcT affinity purification assay followed by mass spectrometry highlighted a list of potential OxcT partners. Interaction between OxcT and a selected candidate will be studied in the near future.

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# Acknowledgements

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# Introduction

## 1) Cu duality in Bacteria

### a) Cu essentiality

Three billion years ago, the photosynthetic activity of cyanobacteria triggered an accumulation of $O_2$ in the oceans. Six hundred million years later, the level of $O_2$ in the ocean saturated and $O_2$ was released into the atmosphere. This phenomenon gradually increased until the atmosphere became oxidizing. Anaerobic microorganisms use iron (Fe) as a cofactor for metalloenzymes, in heme form or not. Fe, as a cofactor, can also mediate reduction of ribonucleotide precursors of DNA. In its reduced form, $Fe^{2+}$ is soluble and bioavailable as a microelement whereas, in presence of dioxygen ($O_2$), it mainly precipitates as insoluble $Fe^{3+}$ and is no more bioavailable. The increasing concentrations of $O_2$ in the atmosphere lead to oxidation of the insoluble cuprous ions ($Cu^{+}$) into the soluble and bioavailable cupric ions ($Cu^{2+}$). Bioavailability of $Cu^{2+}$ increased and allowed the expansion of its biological role in prokaryotes and lateron in eukaryotes. It led to the evolution of cuproenzymes as well as copper-responsive regulators of genes involved in Cu homeostasis. Bacteria as well as anaerobic microorganisms requiring $Fe^{2+}$ evolved strategies to scavenge $Fe^{3+}$ with high Fe affinity siderophores and to reduce it into bioavailable $Fe^{2+}$ intracellularly (Solioz, M., 2018).

Cu is ubiquitous and widely distributed in nature. It has the ability to cycle between $Cu^{+}$ and $Cu^{2+}$. Thanks to its bioavailability and its high redox potential, Cu was then incorporated into energy capturing systems and became essential to reaction such as oxidative phosphorylation with cytochrome oxidase, Fe homeostasis with ferroxidase such as ceruloplasmin, pigmentation with tyrosinase and laccase, superoxide dismutation with superoxide dismutase and connective tissue formation with lysyl oxidases (Ladomersky, E., & Petris, M. J., 2015). Cu-dependent metalloenzymes are important for normal cell growth, differentiation, and survival in aerobic bacteria, mammals and plants. Most cuproenzymes are found in eukaryotes rather than in archea and bacteria which reflects the fact that eukaryotic cells evolved in the oxic world where Cu was more bioavailable. In both eukaryotes and prokaryotes, the most frequent metalloenzymes are zinc-binding proteins or non-heme Fe proteins and the occurrence of cupro-enzymes is quite scarce. The size of the copper proteome is generally less than 1% of the total proteome of an organism which contributes to low levels of Cu cells (Festa, R. A., & Thiele, D. J., 2012). Cu is mostly accumulated in the periplasm where cupro-enzymes are found and is usually associated with a biological ligand (Barwinska-Sendra, A., & Waldron, K. J., 2017).

### b) Cu toxicity

Despite its essentiality, Cu is toxic at high concentrations for all living organisms. Cu toxicity results from Fe displacement from iron-sulfur (Fe-S) clusters, formation of non-native disulfide bonds and oxidative stress (Dupont, C. L., *et al.*, 2011).

#### I. Degradation of Fe-S clusters

Upon Cu stress, it is thought that Cu accumulates in the periplasm and the cytoplasm. In the periplasm, $Cu^{2+}$ is the prevalent form as it is an oxidizing compartment whereas in the cytoplasm which is reducing, it is mostly found as $Cu^{+}$. Intracellular reductants like respiratory chain complexes, enterobactin, and cysteine also contribute to the formation of $Cu^{+}$. $Cu^{+}$ is also enzymatically generated for incorporation into copper-dependent enzymes (Giachino, A., &

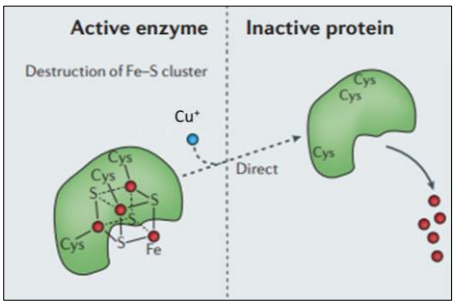


**Figure 1: Degradatation of Fe-S clusters due to Cu toxicity.** Cu displaces Fe from Fe-S cluster and inactivates proteins (Modified from Lemire, J. A., *et al.*, 2013).

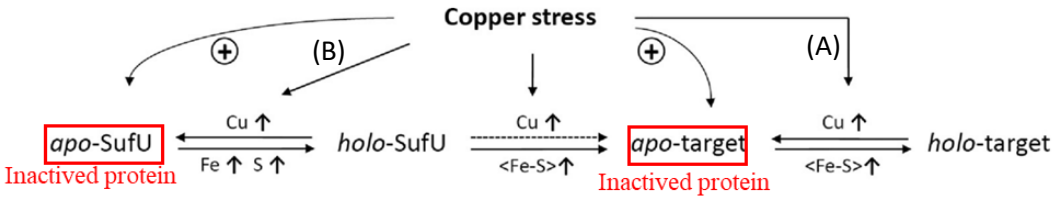


**Figure 2: Impact of Cu stress on Fe-S clusters assembly.** (A) Cu degrade Fe-S cluster from mature proteins that form holo-enzymes. (B) Cu also degrades the Fe-S cluster of SufU that is involved in assembly and delivering of Fe-S clusters to enzymes. SufU is itself an Fe-S cluster protein and is inactivated because of Cu-mediated Fe-S cluster degradation. That also contributes to protein inactivation (Chillappagari, S., *et al.*, 2010).

Waldron, K. J., 2020). $Cu^{+}$ has a higher affinity for sulfur compounds than $Fe^{2+}$. As a consequence, $Cu^{+}$ is able to displace Fe from solvent-exposed Fe-S clusters in important proteins. This results in the degradation of the Fe-S cluster and the inactivation of Fe-S cluster enzymes (Fig. 1) (Macomber, L., & Imlay, J. A., 2009; Solioz, M., 2018).

Copper targets proteins with solvent-exposed, where Cu displaces Fe from Fe-S clusters leading to degradation of the cluster and formation of an inactive apo-enzyme (Macomber, L., & Imlay, J. A., 2009). In Gram- bacteria, the cytoplasmic dihydroxy-acid dehydratase of branched-chain amino acid synthesis, the isopropylmalate isomerase (IPMI) involved in leucine biosynthesis, and the fumarase A are the most impacted Fe-S clusters-containing enzymes (Solioz, M., 2018). In *Neisseria gonorrhoeae*, it has also been found that Cu induces inhibition of heme biosynthesis through Cu-dependent inactivation of HemN. HemN is a Fe-S cluster protein that is involved in heme biosynthesis. Its inactivation results in the decrease of heme-dependent catalase activity (Barwinska-Sendra, A., & Waldron, K. J., 2017).

In Gram + bacteria, it was also shown that Cu degrades the Fe-S cluster of SufU, a scaffold protein involved in Fe-S cluster assembly by transfering the Fe-S clusters to target proteins (Fig. 2) (Solioz, M., 2018). If the Fe-S cluster is buried inside the 3D structure of the protein, Cu is not able to disrupt it. Upon disruption of Fe-S cluster, the released $Fe^{2+}$ may catalyze a Fenton and Haber-Weiss reactions leading to ROS formation and causing an oxidative stress (Barwinska-Sendra, A., & Waldron, K. J., 2017). However, this remains a hypothesis and could not be proved.

## II. Non-native disulfide bonds formation

Besides the disruption of Fe-S clusters, Cu-mediated oxidation could also lead to the formation of non-native disulfide bonds leading to misfolding and inactivation of proteins functionally dependent on free cysteines such as the periplasmic proteins RNase (Hiniker, A., *et al.*, 2005). In enterobacteria, in normal conditions, two oxidoreductases, DsbA and DsbB mediate native folding of periplasmic polypeptides. When a Cu stress occurs and non-native bonds are formed, DsbC and DsbD in *E. coli* and ScsC and ScsD in *S. enterica*, respectively, rearrange the misfolded peptides. These proteins are activated during the envelope stress response and are essential to Cu tolerance (Giachino, A., & Waldron, K. J., 2020).

## III. Mismetalation

Cu displaces essential cofactor in other proteins and inactivate them. This happens with molybdenum (Mo) and Fe as they have lower affinity for thiols than Cu. Mismetalation can not only happen on mature proteins but also during the protein maturation if Cu mismetalates the metallochaperone delivering the metal cofactor to maturating polypeptides (Giachino, A., & Waldron, K. J., 2020). For instance, in *E. coli* Cu inserts non-specifically in molybdopterin (MPT), inhibiting the insertion of Mo in MPT and thus, inhibiting the biosynthesis of the Molybdenum cofactor (Moco). This cofactor is an essential component of a group of redox enzymes involved in diverse functions such as nitrogen, sulfur and carbon compounds metabolism (Iobbi-Nivol, C., & Leimkühler, S., 2013).

## IV. Unspecific binding

Cu has a high affinity for sulfur compounds and oxidizes thiol groups. In *E. coli*, during the lipoprotein maturation, polypeptide chains are present in the inner membrane (IN) and are first sequentially acylated. The signal peptide is then removed and the chain is translocated to the outer membrane (OM). Upon Cu excess, Cu binds to the acyl-accepting N-terminal cysteine residue of maturating lipoprotein and prevent its access by enzymes. This leads to envelope stress response as toxic lipoprotein precursors are accumulated in the IM. The envelope stress response is coordinated by the two-component regulator CpxAR.

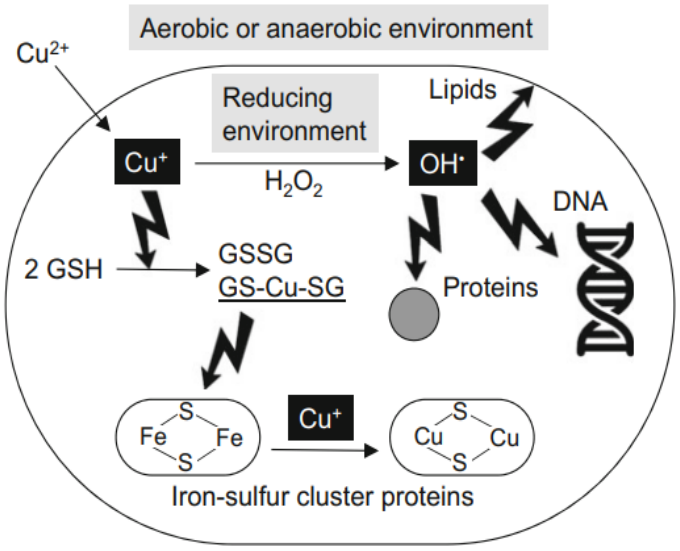


**Figure 3: Putative Cu toxicity mechanisms in bacteria.** Putative toxic effects of Cu are summarized: depletion of GSH by thiol reduction, targeting of Fe-S clusters by the GS-Cu-SG complex, and finally, oxidative stress (Solioz, M., 2018).

The *cpx* regulon represses biosynthesis of copper-sensitive lipoproteins, and induces production of maturation enzymes to remove damage (Giachino, A., & Waldron, K. J., 2020).

## V. Oxidative stress

Free $Cu^+$ ions are the most toxic because they are able to undergo Fenton-like (1) and Haber-Weiss reactions (2) in the cell leading to Reactive Oxygen Species (ROS) formation such as hydroxyl radicals (Dupont, C. L., *et al.,* 2011).

$$Cu^+ + H_2O_2 \rightarrow HO\cdot + HO^- + Cu^{2+} \quad (1)$$

$$O_2^{\cdot -} + Cu^{2+} \rightarrow Cu^+ + O^2 \quad (2)$$

ROS are highly deleterious to the cell because they can inhibit respiration, trigger lipid peroxidation, or oxidative damage of proteins (Fig. 3) (Solioz, M., 2018). However, in the Gram- bacteria *E. coli*, the presence of DNA-damage could not be demonstrated. $Cu^{2+}$ would be chelated and reduced by glutathione (GSH) and cysteine and would not be able to go through Fenton-like chemistry (Macomber, L., *et al.,* 2007).

## VI. Antioxidant depletion

GSH are the most important small-molecular weight thiols against heavy metal toxicity in prokaryotic and eukaryotic cells. In bacteria, Cu can be scavenged by other molecules such as bacillithiol, or amino acids like cysteine or histidine, or other abundant metabolites such as polyphosphates or lipoamide (Barwinska-Sendra, A., & Waldron, K. J., 2017). Other proteins with thiol groups have an antioxidant activity and enzymes such as catalase, peroxidase, superoxide dismutase and peroxiredoxin enable ROS detoxification (Rhee, S. G., *et al.,* 2001).

Free Cu ions lead to antioxidant depletion by oxidizing thiol groups in GSH. During this process, Cu cycles between the cupric and cuprous form at the expense of GSH and $O_2$. Oxidation of GSH leads to the production of hydrogen peroxide that, in turn, amplifies toxic hydroxyl radical production through cycling between the reactions (3) and (4) (Fig. 3).

$$2Cu^{2+} + 2RSH \rightarrow 2Cu^+ + RSSR + 2H^+ \quad (3)$$

$$2Cu^+ + 2H^+ + O_2 \rightarrow 2Cu^{2+} + H_2O_2 \quad (4)$$

However, antioxidant depletion might not be the main Cu toxicity mechanism as there might be too less free Cu to catalyze such reaction. In anaerobic conditions, it is thought that Cu toxicity could be exacerbated. Cu would form $Cu^+$-thiolate bonds and the GS-Cu-SG complex would enhance targeting of exposed Fe-S clusters in enzymes (Fig. 3) (Solioz, M., 2018).

Overall, it has been shown that Cu toxicity was higher in anaerobic conditions which means that oxidative stress directly caused by Cu is not the main toxicity pathway in Gram-negative bacteria (Solioz, M., 2018). Toxicity pathways occurring in anaerobic conditions such as the destruction of Fe-S clusters have the most impact (Macomber, L., & Imlay, J. A., 2009).

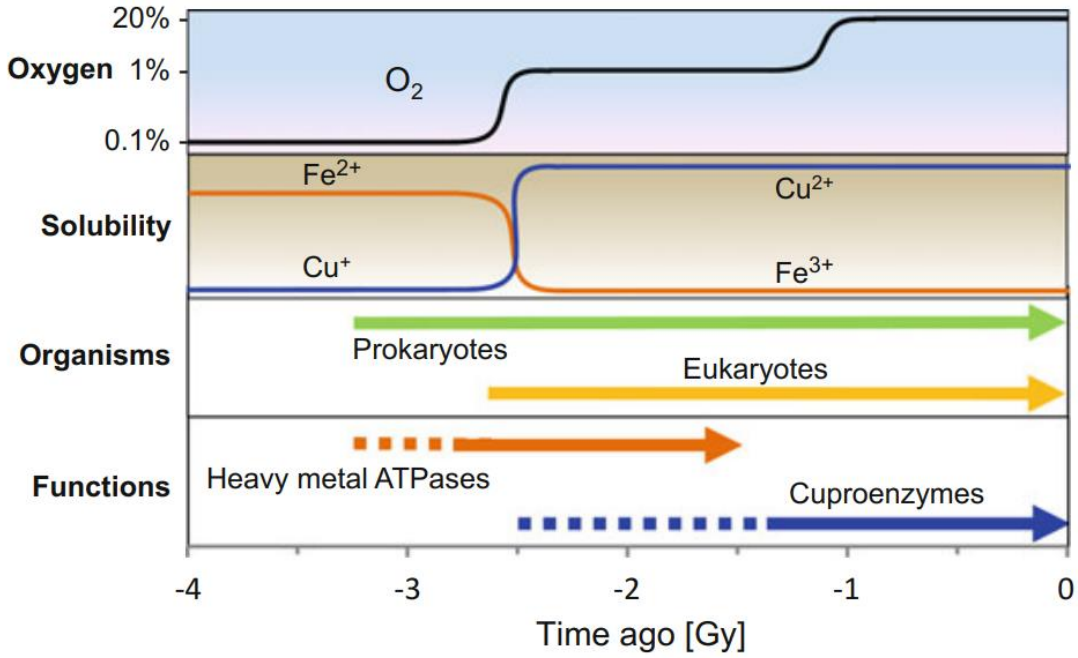


**Figure 4: Geochemical changes and evolution over time.** Heavy metal ATPases appeared earlier than cuproenzymes and independently of Cu utilization (Solioz, M., 2018).

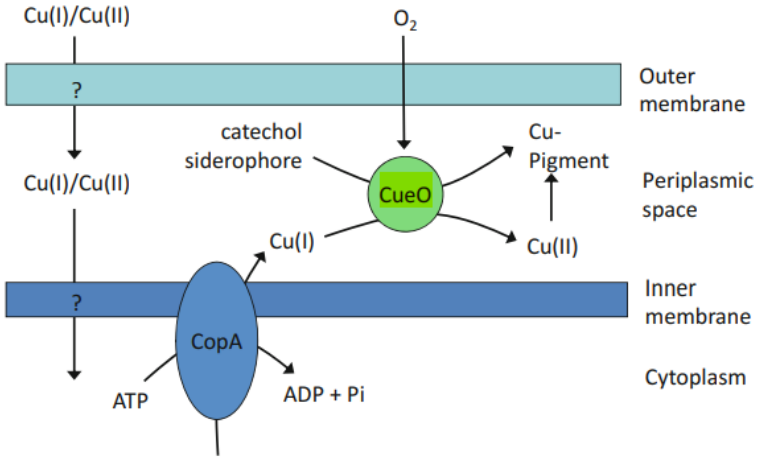


**Figure 5: Representation of the Cue system.** CopA exports Cu from the cytoplasm to the periplasm where CueO oxidizes it. CueO can also oxidize catechols and siderophores. The way Cu enters the cell is still unknown (Solioz, M., 2018).

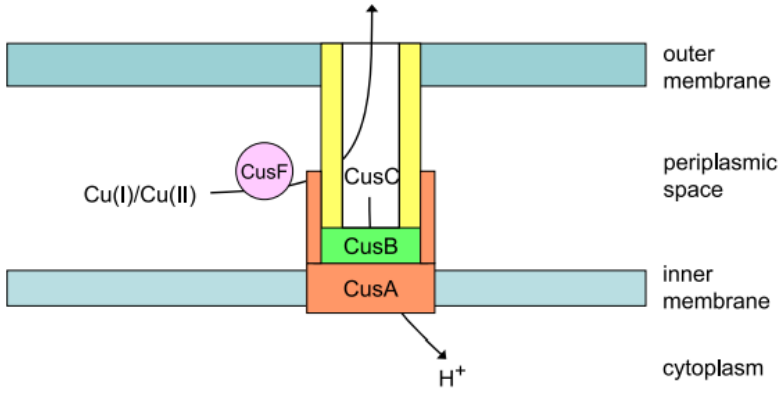


**Figure 6: Representation of the CusCBA proton-cation antiporter system.** CusA transports Cu thanks to a proton-substrate antiport. CusB is the adaptor membrane fusion protein (MFP) that links CusA in the inner membrane and CusC in the outer membrane. CusF is a chaperone that delivers Cu to the pump (Solioz, M., and David, M., 2007).

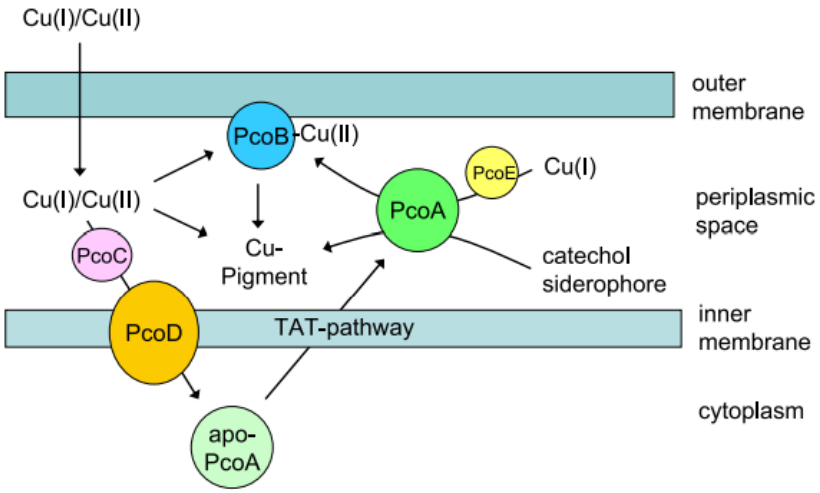


**Figure 7: Representation of the Pco system.** The PcoA multicopper oxidase is exported to the periplasm by the TAT-pathway and oxidizes Cu. PcoB exports Cu into the extracellular milieu. PcoC chaperone delivers Cu to PcoD that import Cu (Solioz, M., and David, M., 2007).

# 2) Bacterial Cu tolerance mechanisms

As described in the previous section, Cu is ubiquitous and exposure to Cu at toxic levels is deleterious. Even if eukaryotes are the ones that use the most Cu, every living organism encodes at least one Cu export ATPase from its genome. That suggests that Cu detoxification mechanisms evolved independently and earlier than redox-active cuproenzymes or oxygen-binding cuproprotein (Fig. 4) (Solioz, M., 2018).

Different Cu homeostasis systems encoded by chromosomal or plasmidic genes actively expel, detoxify or chelate Cu and maintain Cu concentration in a narrow range. As part of this project, three systems characterized in *E. coli* and *Pseudomonas syringae* are described below (Ladomersky, E., & Petris, M. J., 2015) (Fig. 5, 6 and 7).

## CueO and CopA

The Cue efflux system consists of *copA* and *cueO* (Fig. 5). Their expression is regulated in a Cu-responsive manner by CueR that is a metalloregulatory protein from the MerR-family transcriptional regulators. CopA is a P-type ATPase that exports $Cu^{+}$ from the cytoplasm to the periplasm *via* ATP hydrolysis (Solioz, M., 2018). CopZ is the cytoplasmic cupro-chaperone that delivers $Cu^{+}$ to CopA. On the other hand, CueO is a periplasmic multicopper oxidase (Giachino, A., & Waldron, K. J., 2020). In aerobic conditions, CueO converts periplasmic $Cu^{+}$ to the less toxic form, $Cu^{2+}$, thereby reducing $O_2$ into $H_2O$. Multi-copper oxidases significantly contribute to protect against Cu toxicity in the periplasm of bacteria. They have a diversity of substrates like Fe, phenols, diamines, catecholates and ascorbate. CueO is able to oxidize the catecholate groups of 2,3 dihydrobenzoic acid which is the precursor of the Fe scavenging siderophore, enterobactin. As enterobactin is known for reducing $Cu^{2+}$ into toxic $Cu^{+}$, its oxidation by CueO, at the expense of Fe scavenging might be an additional way to cope with Cu toxicity during host-pathogen competition. That is particularly useful to pathogenic bacteria such as *E. coli* as Cu excess in macrophages might be used by the host to inhibit bacterial growth (Ladomersky, E., & Petris, M. J., 2015). The core *cue* regulon is functionally conserved in all Proteobacteria. In pathogenic strains such as *Salmonella enterica*, most serotypes encode a duplicate of the entire *cue* system, the *gol* regulon, with partial redundancy. It enables the bacterium to survive within macrophages in elevated Cu concentrations (Osman, D., et al., 2010; Giachino, A., & Waldron, K. J., 2020).

## CusABC

The Cus efflux system present in *E. coli* allows the maintenance of micromolar levels of Cu in the periplasm. The copper-responsive two-component sensory system CusSR regulates the expression of CusABC and CusF. CusSR is part of the phosphate receiver response regulator family and is a two-component response regulator. CusR is the cytoplasmic response regulator (RR) and CusS is a sensor histidine kinase (HK). CusCBA is a resistance, nodulation and division (RND) + antiporter that is involved in the export of metal ions, xenobiotics, and drugs (Fig. 6). It is found in the majority of gamma proteobacteria (Ladomersky, E., & Petris, M. J., 2015). In contrast to CueO/PcoA multicopper oxidase, it is able to work in both anaerobic and aerobic conditions. Multicopper oxidase need $O_2$ to oxidize $Cu^{+}$. CusF is thought to be a periplasmic cuprochaperone (Solioz, M., and David, M., 2007). Cu sequestration by cysteine-rich metallothioneins as a mechanism to tolerate Cu is common in eukaryotes and rare in bacteria. Yet, CusF might sequestrate Cu in addition to its role in Cu delivery to the CusBC complex for export across the outer membrane (Fig. 6). The best characterized metallothionein in bacteria is the mycobacterial metallothionein (MymT) from *Mycobacterium tuberculosis* (Ladomersky, E., & Petris, M. J., 2015). In addition to its role in Cu tolerance, MymT would also protect against ROS toxicity by scavenging free radicals (Ruttkay-Nedecky, B., *et al.*, 2013).

The efflux system itself consists of CusA which is the inner membrane pump, CusB, the periplasmic protein, and CusC, the trimeric outer membrane protein which forms a channel bridging the periplasmic space (Fig. 6). CusA transports $Cu^{+}$/$Cu^{2+}$ by secondary transport thanks to a proton-substrate antiport. CusB is an adaptor membrane fusion protein (MFP) that links CusA in the inner membrane and CusC in the outer membrane (Giachino, A., & Waldron, K. J., 2020).

## Plasmid-borne Cu resistance

While the 2 previous described systems are chromosome-encoded, there are plasmid-borne systems such as the *pcoABCDRSE* and the *copABCDRS* operons in *E. coli*, and in *Pseudomonas syringae*, respectively. These Cu resistance systems are encoded on the pPT23D and on the pRJ1004 plasmids in *E. coli* and *Pseudomonas syringae*, respectively. There is a two-component regulatory system, PcoRS, that induces transcription of the *pcoABCDE* operon under Cu stress. PcoR binds DNA and activate transcription of the *pco* operon whereas PcoS is a periplasmic histidine kinase sensing Cu. The *pcoE* gene is transcribed from another promoter that is also regulated by PcoRS. Moreover, in the absence of *pcoRS*, both promoters can be induced by Cu due to the presence of a second, related two-component regulatory system that is chromosomally encoded, CusRS, and that was described earlier (Ladomersky, E., & Petris, M. J., 2015). The first component of this system is the multicopper oxidase PcoA. In analogy to CueO and CopA, this protein oxidizes $Cu^{+}$ to the less toxic $Cu^{2+}$ in the periplasm where it is exported thanks to the TAT-pathway (Solioz, M., and David, M., 2007). PcoB/CopB is the outer membrane protein and is thought to export Cu into the extracellular milieu. PcoC/CopC is another periplasmic protein simultaneously binding $Cu^{+}$ and $Cu^{2+}$ and serving as a Cu chaperone that would deliver Cu to PcoD/CopD. PcoD/CopD is a cytoplasmic membrane protein that would import Cu (Fig. 7). Finally, PcoE is periplasmic Cu chaperone (Giachino, A., & Waldron, K. J., 2020; Ladomersky, E., & Petris, M. J., 2015).

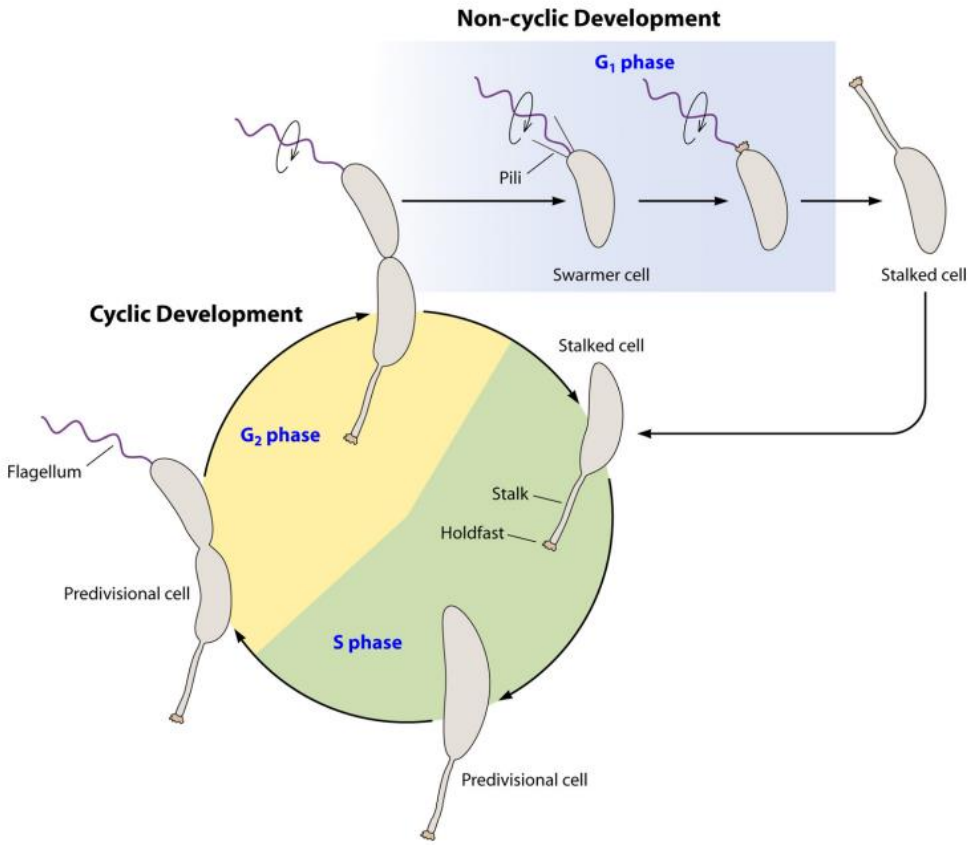


**Figure 8: Asymetrical *C. crescentus* cell cycle.** ST undergo division and forms 2 daughter cells: the ST cell and the SW. SW cannot replicate their DNA but are able to disperse into the environment before differentiation into a sessile ST cell (Curtis, P. D., & Brun, Y. V., 2010).

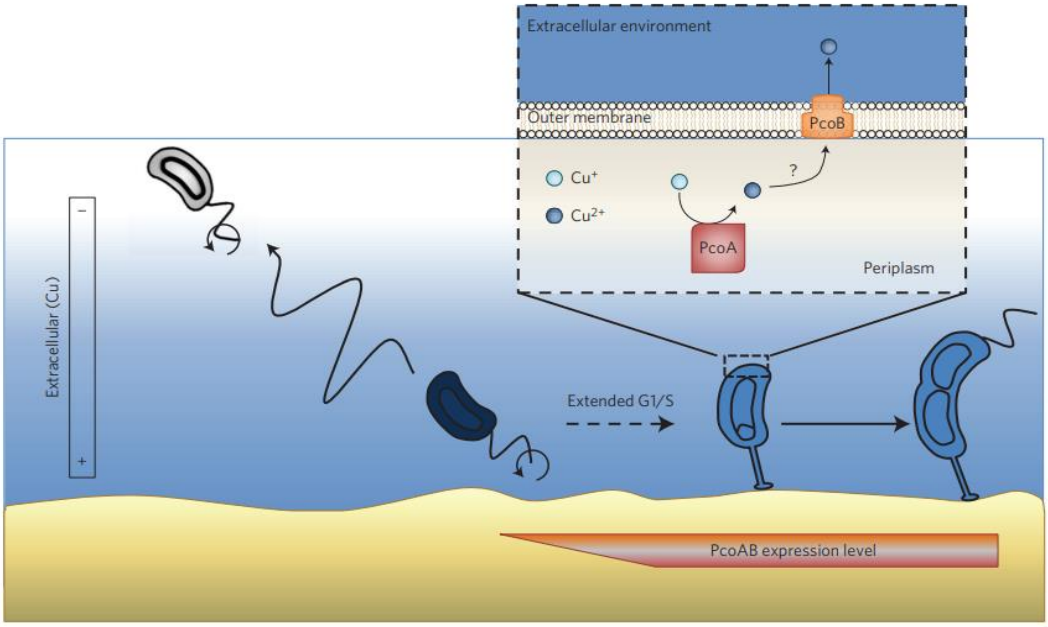


**Figure 9: Mechanisms of Cu tolerance in *C. crescentus*.** In highly Cu concentrated milieu, SW accumulates Cu which triggers negative chemotaxis. Under Cu stress, the differenciation of the SW to the ST takes longer than in non-stressed conditions. ST express the PcoAB system that allows to oxidize Cu and to export it in the extracellular milieu thanks to PcoA and PcoB, respectively (Lawarée, E., *et al.,* 2016).

## 3) *Caulobacter crescentus* as a model for Cu tolerance

Extensive studies on the well-established model *E. coli* allowed the understanding of important parts of the mechanisms at play during the Cu stress response in bacteria. However, the diversity of the bacterial world allows the development of additional models that complement the classical rod-shaped and symmetrically dividing *E. coli*. For instance, the alpha-proteobacteria *Caulobacter crescentus* has a unique strategy to cope with Cu. It exhibits a bimodal response to Cu stress; SW use negative chemotaxis whereas ST predominantly use the PcoAB system (Lawarée, E., *et al.*, 2016). That bimodal response is allowed by *C. crescentus* dimorphism. Stalked (ST) cells are sessile and undergo a division cycle, leading to the formation of two daughter cells: the ST cell and the swarmer (SW) cell. The latter cannot replicate its DNA but is able to disperse into the environment before differentiation into a ST cell thanks to the flagellum and pili (Curtis, P. D., & Brun, Y. V., 2010) (Fig. 8). ST cells are attached to their substrate thanks to a holdfast complex at the bottom of the stalk. It consists in polysaccharide anchored to the cell by the outer membrane proteins HfaA, HfaB and HfaD (Hardy, G. G., *et al.*, 2010). This sessile lifestyle forces them to use Cu detoxifying mechanisms. Among Cu homeostasis systems described previously, Cue, Cus and Pco systems are conserved in *C. crescentus.* In the Pco system, PcoA oxidizes $Cu^{+}$ into the less toxic $Cu^{2+}$ while PcoB pumps Cu outside of the cell in order to maintain low Cu concentrations (Fig. 9). In ST cells, only PcoAB could be demonstrated as essential for complete Cu tolerance so far as the deletion of *cue* and *cus* homologues studied did not impact Cu tolerance (Lawarée, E., *et al.*, 2016).

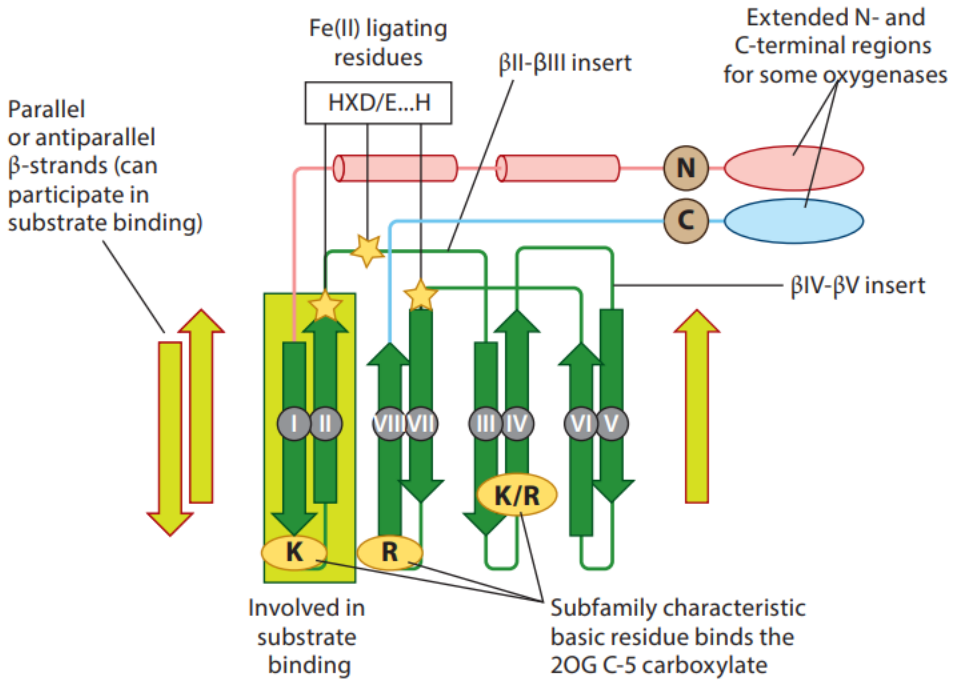


**Figure 10: Composite 2OGX topology diagram.** The 8 β-strands constituting the DSBH fold are represented in dark green. Those involved in substrate binding are annotated. The 2OG C-5 carboxylate binds residues (Lys and Arg) in the active site pocket of the 2OGX and complex Fe that binds to the HxD/Ex$_n$H conserved motif. Extented loops between βII and βIII and βIV and βV are represented as well as additional domains at the C and N-terminals (Islam, M. S., *et al.,* 2018).

Unlike *E. coli*, the PcoAB system is chromosome-encoded in *C. crescentus* (Solioz, M., and David, M., 2007). In SW, *pcoAB* is lowly expressed compared as in ST. Consequently, Cu accumulates more in SW than in ST and that activates negative chemotaxis. Negative chemotaxis is triggered by activation of the cytoplasmic chemoreceptor, McpR containing a PAS domain in its structure. Usually, PAS domains are involved in $O_2$ and redox sensing and it is suggested that oxidative stress participates to activation of negative chemotaxis (Nierman, W. C., *et al.*, 2001; Louis, G., unpublished data).

For the past 60 years, *C. crescentus* ability to adhere to surfaces as well as its dimorphic lifecycle have categorized it as an aquatic oligotroph (Nierman, W. C., *et al.*, 2001). However, recent studies shown that it would also occur in soils and would participate in the decomposition of organic matter such as plants. It would depend on water to disperse (Wilhem *et al.*, 2018). In fact, *Caulobacter spp*. have been isolated from highly diverse environments, including heavy-metal contaminated sites such as gold mines (Maertens, L., *et al.*, 2021; Inagaki, F., *et al.*, 2003). Besides its role as a bacterial intracellular organization, cellular development, and cell-cycle regulation study model, there is a huge interest in the ability of *C. crescentus* to tolerate high concentrations of heavy metals such as Cu. It could be an excellent bioremediation tool to detoxify heavy-metal contaminated sites that are a harmful to the environment (Xu, Z., Lei, Y., & Patel, J., 2010). As it will be detailed in the objectives section, this project took place from the discovery of an operon that would be involved in Cu tolerance. This operon is composed of three genes, one of them encodes a 2-oxoglutarate/$Fe^{2+}$-dependent oxygenase (2OGXs). In the next chapter, an overview of 2OGXs is presented.

# 4) 2-oxoglutarate/$Fe^{2+}$-dependent oxygenase

## a) Overview on the structure and mechanistic of 2-oxoglutarate/$Fe^{2+}$-dependent oxygenase

2OGXs are a superfamily of metallo-enzymes that use a single atom of $Fe^{2+}$ as a cofactor which distinguishes them from heme-containing dioxygenases. On the other side, 2-oxoglutarate is used as a co-substrate (Islam, M. S., *et al.*, 2018).

Regarding the structure, it has been shown that all 2OGXs contain a double-stranded β-helix (DSBH) core fold (also known as jelly-roll or Jumonji (JmjC) fold) that consists of 8 β-strands (Fig.10). The major and minor β-sheets form a squashed barrel. Alpha-helices are located at the N and C termini and allow fold stability and substrate recognition, respectively, and can also allow dimerization. It has been shown that 2OGXs could form oligomeric forms such as tetramers and hexamers (Wolf, A., *et al.*, 2013). Residues variations within loops as well as extension of their secondary structure (e.g., between strand βII and βIII and between strand βIV and βV) allow the formation of distinct subfamilies with different substrate binding and product selectivity (Fig.10). These subfamilies have different structural and catalytic characteristics. Additional domains such as α-helices and β-strands can be found at the N-terminus and C-terminus of the DSBH (Aik, W., *et al.*, 2012). They extend the major, and sometimes the minor β-sheets which also impacts structure and catalysis (Islam, M. S., *et al.*, 2018). The 2OG C-5 carboxylate group interacts with residues in the active site pocket of the oxygenase by electrostatic and hydrogen bonding (Aik, W., *et al.*, 2012). These residues involve at least two polar residues, one with a basic (Lys or Arg) and one with a polar neutral (Ser or Tyr) side chain. Fe is complexed by 2OG via its C-1 carboxylate and ketone oxygens in a bidentate manner and binds the conserved-motif $HxD/Ex_nH$ (Fig. 10). This allows the formation of a ternary oxygenase–2OG–substrate complex to which $O_2$ binds (Aik, W., *et al.*, 2012). Mechanisms underlying $Fe^{2+}$ delivery and maintenance in 2OGX in cells remain unknown (Islam, M. S., *et al.*, 2018).

Variations in $Fe^{2+}$/2OGs binding modes can confer binding and kinetics characteristics and distinguish subfamilies of 2OGXs. Still, the diversity of substrates recognized by 2OGXs is mostly due to different substrate binding modes (Islam, M. S., *et al.*, 2018).

Despite functional divergence since early on, pioneering studies on animal cells shown that 2OGXs mainly catalyze reaction of hydroxylation, and demethylation after hydroxylation. A consensus in the residues that are hydroxylated has been highlighted and reveals that there are two main subfamilies of hydroxylases; the lysyl and the prolyl hydroxylases which are involved in collagen and other animal glycoproteins biosynthesis (Aik, W., *et al.*, 2012). 2OGXs can catalyze both oligomeric substrates (proteins, nucleic acids, lipids) and small molecules when they are isolated and attached to peptides (Islam, M. S., *et al.*, 2018).

During hydroxylation, the substrate (R) is oxidized using $O_2$ which is partially incorporated to the substrate by decarboxylating 2OG into succinate and $CO_2$ (1) (Jia, B., et al., 2017):

$$R + 2OG + O_2 \rightarrow R–OH + succinate + CO_2 \quad (1)$$

The decarboxylation of 2OG oxidizes $Fe^{2+}$ into a $Fe^{4+}$oxo intermediate or other activate oxygen species. The $Fe^{4+}$oxo intermediate hydroxylates the substrate into an alcohol (R-OH) as seen on the reaction (1) (Aik, W., *et al.*, 2012).

## b) Overview of 2OGXs functions

In animals, 2OGXs are involved in primary and secondary metabolism. Primary metabolic roles include lipid metabolism, nucleic acid repair, chromatin/transcription factor modification and RNA splicing (Aik, W., *et al.*, 2012). Plants and bacteria display a wider range of function as they are also involved in the secondary metabolism of small molecules. 2OGXs in plant and bacteria are able to synthesis secondary metabolites. A few examples of those function will be given below as well as the issues raised by their study.

### Primary metabolism

The utilization of $O_2$ as well as 2OG confers a metabolic and redox states sensor role to 2OGXs within the cells. $O_2$ is a limiting factor of the reaction and enzymes such as Hypoxia-Inducible Factor Hydroxylases and related Enzymes are directly affected by variations in the level of $O_2$ (e.g., hypoxia-inducible factor (HIF) prolyl hydroxylases (PHD2)) (McDonough, M. A., *et al.*, 2010). 2OG and the succinate co-product are both part of the tricarboxylic acid (TCA) cycle and also have an important role in other aspects of the metabolism (Islam, M. S., *et al.*, 2018).

In humans, processes like aging and tumor cell metabolism are directly impacted by variations in 2OG concentrations and would be directly linked to 2OGXs activity. Studying 2OGXs involved in regulating transcription is particularly promising as the activity of 2OGXs can cause anemia and cancer. Understanding the role of the JmjC fold in substrate and 2OG binding could allow the development of inhibitors and drugs (Islam, M. S., *et al.*, 2018).

In bacteria, 2OGXs can catalyze hydroxylation of the Lipid A in the cell-wall as well as hydroxylation of amino acid (Islam, M. S., *et al.*, 2018; Smirnov, S. V., *et al.*, 2012).

## Secondary metabolism

Secondary functions include reactions of desaturation, oxidative rearrangements, epimerization and cyclisation of molecules that allow synthesis of secondary metabolites such as antibiotics as well as signalling molecules (Islam, M. S., *et al.,* 2018; Aik, W., *et al.,* 2012). Differences in substrate binding mode enable 2OGXs to have both hydroxylase and ring-forming/desaturase activities. For instance, in bacteria, cephalosporin synthesis, deacetoxycephalosporin C synthase (DAOCS) and deacetylcephalosporin C synthase (DACS) can trigger either ring expansion or methyl group hydroxylation or both depending on the amino acid residues in their side chains. Changes in these residues allow substrate and product diversity. DAOCS selectivity is currently being studied to enable the specific production of cephalosporins with hydrophobic side chains. They are easier to purify. This illustrates how bacterial 2OGXs can be used in an engineered pathway to produce a synthetic molecule that is hard to obtain otherwise (Islam, M. S., *et al.,* 2018).

In plants, 2OGXs have an important role in the synthesis of signalling molecules such as flavonoids, gibberellins, and alkaloids that allow host pathogen communication. These molecules have antioxidants, antimalarial, and possible anticancer activities that renders 2OGXs really interesting for drug development (Hausinger, R. P., 2004). 2OGXs are also important agrochemical targets for inhibition as they catalyze herbicide degradation (Islam, M. S., *et al.,* 2018).

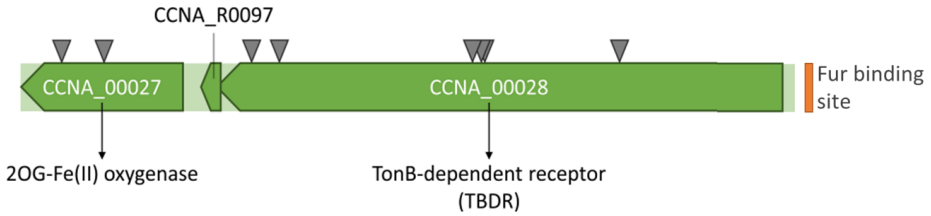


**Figure 11: Schematic representation of the studied operon, *CCNA_00027-28*.** The operon encodes the TBDR, R97 and the 2OGX, OxcT. The gray arrows represent the different mini-Tn5 insertion sites independently obtained in the genetic screen. The operon is controlled by Fur that binds upstream the transcription starting site.

# Objectives

With the aim to identify new mechanisms of Cu tolerance in *C. crescentus*, a genetic screen using mini-Tn5 transposon insertion as well as an RNA-seq were performed in the lab. One top candidate for Cu tolerance was the *CCNA_00027-28* operon (unpublished data; Maertens, L., *et al.*, 2021). This operon consists in three genes, coding for a 2-oxoglutarate $Fe^{2+}$-dependent oxygenase (*CCNA_00027*, named OxcT in this project), a small regulatory RNA (*CCNA_R0097*, R97), and a TonB-dependent receptor (*CCNA_00028*, TBDR), respectively. The *CCNA_00027-28* operon is regulated by a ferric uptake regulator (Fur) that represses the expression of the operon in Fe-replete conditions (da Silva Neto, J.F., *et al.*, 2013). The TBDR is hypothesized to be expel Cu during Cu stress and to uptake Fe upon Fe-starvation (Pauline Cherry's unpublished data). As R97 has not shown to be essential for Cu tolerance in the conditions previously tested, this project focuses on the study of OxcT (Fig. 11).

A conserved genomic and functional interaction between OxcT and the TBDR could be established. *In silico* analysis demonstrated that the organization in an operon is highly conserved among homologues of *oxcT* and *TBDR* and that the order of the genes was conserved. Then, it has been shown that upon deletion of *oxcT*, *TBDR* and the whole operon, similar growth phenotypes were observed. As there was no accumulative effect upon deletion of the operon, we could suggest that OxcT and the TBDR are involved in the same mechanism of Cu tolerance and would display different functions therein.

So far, none of the *TBDR* homologues have been described as involved in a Cu tolerance mechanism and the reason why OxcT would be associated with the TBRD in such mechanism of Cu tolerance is totally mysterious. The precise function, the substrate of OxcT as well as the mechanism in which it participates in Cu tolerance are still unknown. Therefore, the goal of this master thesis is to characterize the 2OGX OxcT. *In vivo* functional studies need to be led in order to unravel the induced Cu-tolerance. A previous master student, Hala Kasmo, already demonstrated the increased Cu sensitivity of *oxcT* knock-out strain (Δ*oxcT*) in both rich and poor media. Comparing growth profile in both media was relevant as it was demonstrated that *oxcT* did not have the same expression profile in rich and poor media (Maertens, L., *et al.*, 2021). Growth was also assessed in the presence of other heavy-metal to see if OxcT was involved in the resistance of another metal. Indeed, these heavy-metal were selected as they share toxicity pathways with Cu. Then, as *oxcT* seems to be essential for Cu tolerance, and as *TBDR* knock-out strain (Δ*TBDR*) has shown an accumulation of the intracellular Cu concentration upon Cu stress, Cu intracellular concentration needs to be measured in the Δ*oxcT* strain in both rich and poor medium using ICP-OES, after Cu exposure in both rich and poor media. As *oxcT* might also be involved in Fe homeostasis, and as it is functionally linked to an Fe importer, the impact of OxcT was measured on the Fe content in the cells using ICP-OES as well. After that, the link between Cu and Fe homeostasis should be further investigated by measuring the impact of Fe homeostasis on OxcT function in the Cu tolerance mechanism. Δ*oxcT* strain growth profile is going to be assessed in low Fe as well as high Fe conditions supplemented with Cu in controlled medium. Finally, as we suspect that the substrate of OxcT might be a protein, partners of OxcT will be highlighted by a pull-down assay followed by mass spectrometry analysis. The identification of OxcT partners, together with the investigation of the link between Fe homeostasis and OxcT function in Cu tolerance are the biggest aim of this project. It would enable to get a better idea of OxcT function and how it is involved in mechanisms at play in the cells upon Cu stress.

# Material and methods

## Strains, plasmids and growth conditions

*Caulobacter crescentus* was grown in PYE rich medium (2 g/l bacto-peptone, 1 g/l yeast extract, 0.8 mM $MgSO_4$, 0.5 mM $CaCl_2$) or M2G poor medium (0.87 g/l $Na_2HPO_4$, 0.54 g/l $KH_2PO_4$, 0.50 g/l $NH_4Cl$, 0.2 % (w/v) glucose, 0.5 mM $MgSO_4$, 0.5 mM $CaCl_2$, 0.01 mM $FeSO_4$ complexed with 0.01 mM EDTA), supplemented with 5 µg/ml kanamycin when needed. Cultures in exponential phase were used for all experiments and $CuSO_4.5H_2O$ was added when appropriate.

All the strains and plasmids used along this project are listed in *Table 1* and *Table 2*, respectively.

*Table 1: Strains used for this study*

| Strain | Genotype | Description |
|---|---|---|
| *Caulobacter crescentus* | | |
| WT | NA1000 | Synchronizable variant strain of CB15 |
| Δ*oxcT* | NA1000 Δ*oxcT* | Knock-out strain for *oxcT* gene |
| Δ*oxcT*+ | NA1000 Δ*oxcT* pMR10*oxcT* | Knock-out strain for *oxcT* gene carrying a copy of *oxcT* on the pMR10 under the control of the *lac* promoter; *KanR* |
| Δ*oxcT*+TS | NA1000 Δ*oxcT* pMR10 N-TwinStrep-*oxcT* | Knock-out strain for *oxcT* gene carrying a copy of N-terminal TwinStrep-tag fused *oxcT* on the pMR10 under control of the *lac* promoter; *KanR* |
| *Escherichia coli* | | |
| | DH10B | *E. coli* DH10B strain used for transformation and conjugation |

*Table 2: Plasmids used in this study*

| Plasmid | Description |
|---|---|
| pNPTs138 | mobRP4+ ori-R6K *sacB*; integrative vector in *C. crescentus* for in-frame deletions and insertions; $Kan^R$ |
| pMR10 | Low copy number replicative cloning vector in *E. coli* and *C. crescentus*; $Kan^R$ |

## Growth curve measurements

Cultures in exponential phase were diluted in PYE/M2G at a final $O.D._{660nm}$ of 0.05 and inoculated in a 96-wells plate. Afterwards, they were grown for 24 h at 30°C under continuous shaking in a BioTek Epoch 2 microplate spectrophotometer where $O.D._{660nm}$ was measured every 10 min. When required, bacteria were exposed to different concentrations of $CuSO_4$, $CdSO_4$, $MnSO_4$, $ZnSO_4$, $NiSO_4$ and $FeCl_3$. $FeSO_4$ concentration was also controlled in M2G to enable growth curve measurements in Fe starvation and Fe excess.

## Measure of total intracellular metals concentration

Thirty milliliters of cultures in exponential phase were used for this experiment. Half of the cultures were stressed with the appropriate Cu concentration for 5 min. Afterwards, bacteria are fixed for 20 min at 4 °C using 2% PFA (paraformaldehyde) and washed 3 times with an ice-cold wash buffer (10 mM Tris-HCl, pH 6.8; 100 µM EDTA). Pelleted bacteria are lysed under 2.48 kbar in $H_2O$ using a cell disrupter (Cell Disruption system, one-shot model, Constant). Cell debris are pelleted and 1% $HNO_3$ is added to the cellular extract and diluted into milliQ $H_2O$ for a final volume of 5 ml. The intracellular content of Cu and Fe is measured in the Chemistry department using ICP-OES.

## Affinity purification-Mass spectrometry

### Preparation of cell lysates

The strains were cultivated in 400 ml M2G and incubated at 30° C until they reach an $O.D._{660nm}$ of 0.6. Half of the cultures were stressed with 15 µM Cu for 5 min. Cells were then pelleted and washed in PBS buffer (137 mM NaCI, 2.7 mM KCI, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$). Pellets were resuspended in NP40 Lysis Buffer* and rotated for 30 min at room temperature on a wheel.

*NP40 Lysis Buffer

| | |
|---|---|
| Cellytic B 10X | 400 µl |
| 1M MgCl2 | 40 µl |
| Ready-Lyse lysozyme | 2 µl |
| DNAse I | 100 µl |
| NP40 Cell Lytic Buffer | 800 µl |
| Complete EDTA free (1 tablet/ml) | 80 µl |

The supernatant was collected and 1000 µg/ml of Avidin was added and samples were rotated for 30 min at 4° C. Thereafter, cells were lysed using sonication (5 cycles; 10 seconds - 1 min on ice). After lysis, the supernatants were kept for proceeding the affinity purification.

## Affinity purification using Strep-Tactin®XT columns

The *Strep-Tactin®XT* column was first equilibrated using a washing buffer (100 mM Tris/HCl, pH 8; 150 mM NaCl; 1 mM EDTA). Clear cell extract was applied to the column. The samples flow through and the column is then washed 5 times with the washing buffer. The elution samples were collected using an elution buffer (100 mM Tris/HCl, pH 8; 150 mM NaCl; 1 mM EDTA; 50 mM biotin). The column was then regenerated using 10 mM NaOH that was removed after. The washing buffer was used to equilibrate the column. The column is stored at 4° C (IBA and Gmbh, 2017).

## LC-MS/MS

### 1) Protein digestion

The samples were treated using the optimized Filter-aided sample preparation (FASP) protocol. Briefly, the samples were loaded onto Millipore Microcon 30 MRCFOR030 Ultracel PL-30 filters that have been rinsed and washed beforehand with, 1 % Formic Acid (FA) and 8 M urea buffer (8 M urea in 0.1 M Tris buffer at pH 8.5), respectively. The proteins on the filter were then exposed to a reducing agent (dithiothreitol (DTT)) and then alkylated with iodoacetamide (IAA). The proteins were then finally digested overnight with trypsin. The final step of the digestion is to transfer proteins in 20 µl of 2% ACN (acetonitrile) and 0.1% FA, in an injection vial for inverted phase chromatography.

### Liquid chromatography and mass spectrometry

The digested samples were analyzed by MS by MaSUN platform (Université de Namur). To search first for Oxct-TS, the data obtained are compared with *C. crescentus* NA1000_190306 database from UniRef 100. Data analysis was performed using Scaffold _4.11.1 with ion mobility module and Q module for label-free quantification (Bioinformatics Solutions Inc., Waterloo, ON). Protein identifications were conducted using the Scaffold search engine with 15 ppm as parent mass error tolerance and 0.05 Da as fragment mass error tolerance. Carbamidomethylation was allowed as fixed modification, oxidation of methionine, and acetylation (N-term) as variable modification. Enzyme specificity was set to trypsin, and the maximum number of missed cleavages per peptide was set at 3. The peak lists were searched against the Caulobacter crescentus NA1000_190306 database from UniRef 100. Peptide spectrum matches and protein identifications were normalized to less than 1.0% false discovery rate.

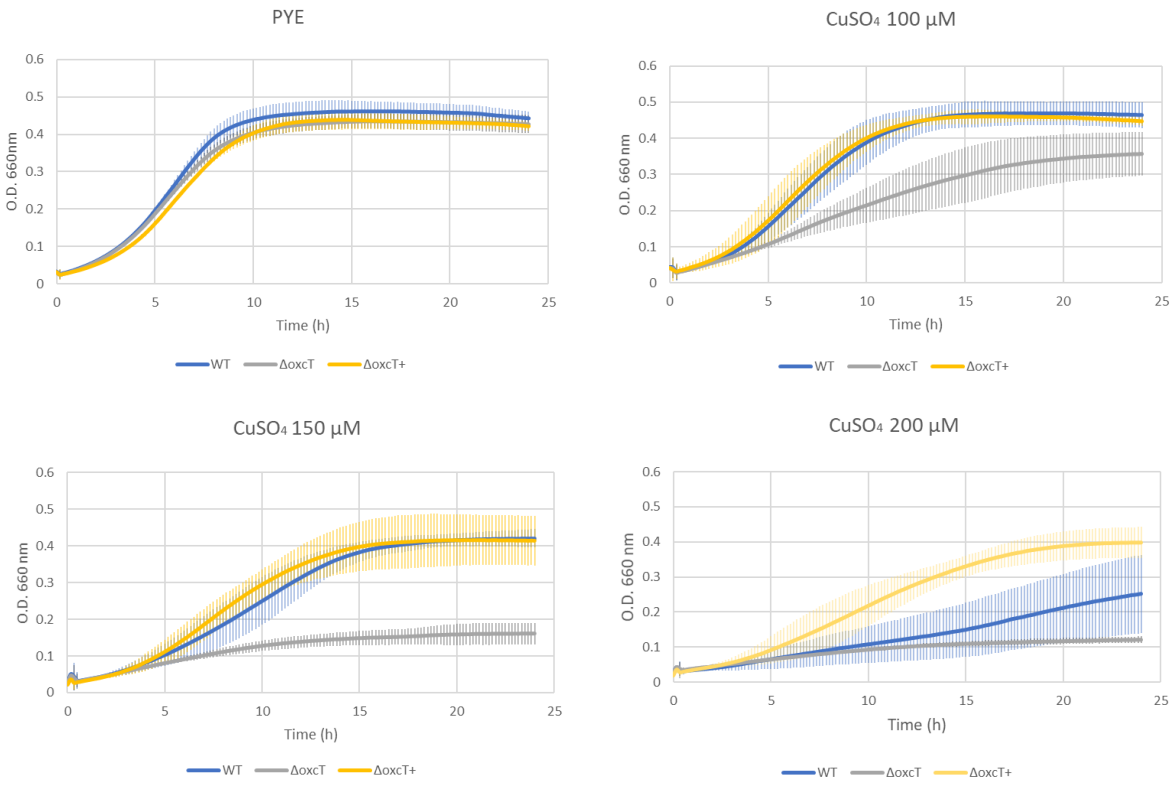


**Figure 12: Growth profile of Δ*oxcT* mutant in absence and in presence of $CuSO_4$ in rich medium.** Growth profiles of WT, Δ*oxcT,* Δ*oxcT*+ strains in PYE and in the presence of increasing concentrations of Cu (100, 150, 200 µM) were monitored (n=3). Δ*oxcT* is more impacted by Cu than the WT and Δ*oxcT*+ at all concentrations tested. The growth defect gets higher when the Cu concentration increases. WT and Δ*oxcT*+ are also impacted but to a lesser extent. Finally, Δ*oxcT*+ complements the WT phenotype perfectly and even grows better at the most toxic Cu level tested. Error bars show standard deviation (SD).

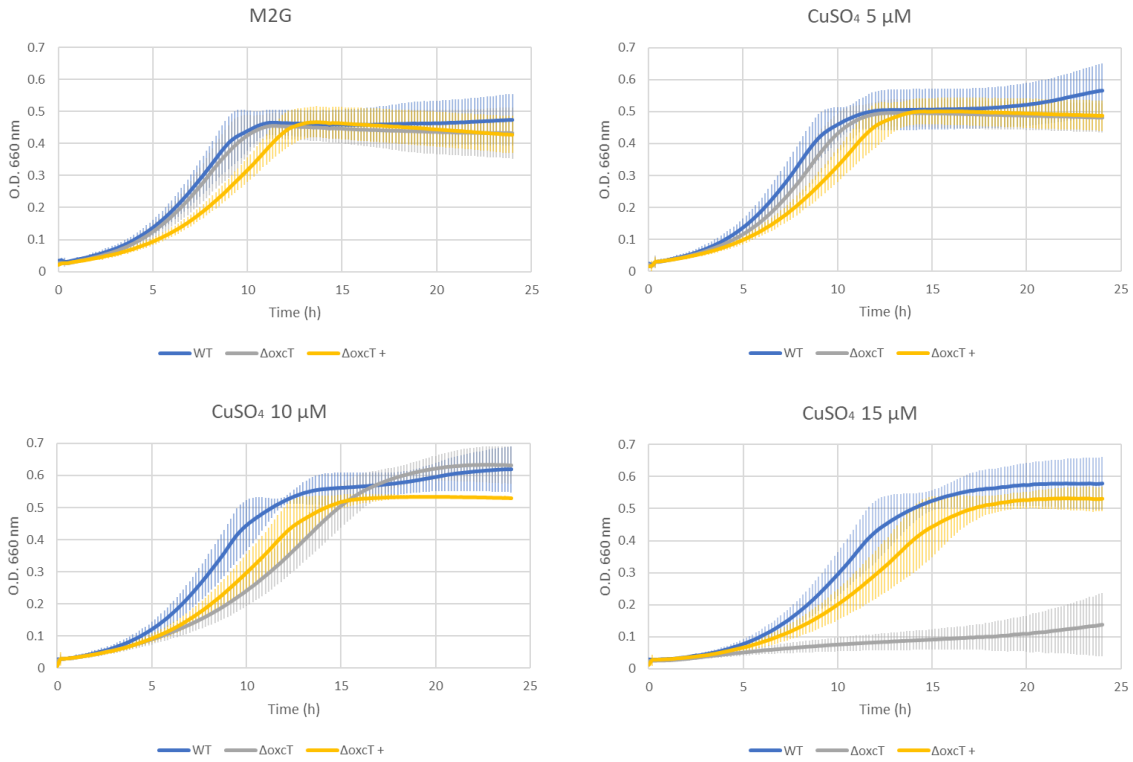


**Figure 13: Growth profile of the Δ*oxcT* mutant in absence and in presence of $CuSO_4$ in poor medium.** Growth profiles of WT, Δ*oxcT,* Δ*oxcT*+ strains in M2G and in the presence of increasing concentrations of Cu (5, 10, 15 µM) were monitored (n=3). Δ*oxcT* is more impacted by Cu than the WT and Δ*oxcT*+ at all concentrations tested. Its growth defect gets higher when the Cu concentration increases. The WT and Δ*oxcT*+ are also impacted but to a lesser extent. Δ*oxcT*+ encounters a higher growth defect than the WT but still grows better than Δ*oxcT* in Cu 15 µM. Error bars show standard deviation (SD).

# Results and discussion

## Part 1: *In vivo* characterization of OxcT

### 1) Study of OxcT specificity

#### The Δ*oxcT* mutant is sensitive to Cu in rich and poor media

OxcT encoded by the *CCNA_00027* gene on the *CCNA_00027-28* operon was highlighted during a genetic screen performed in the lab and seeking for Cu sensitive mutants. To confirm OxcT essentiality in Cu tolerance, growth profiles for the wild-type (WT), the knock-out strain for *oxcT* (Δ*oxcT*), and Δ*oxcT* transformed with a pMR10 plasmid expressing *oxcT*, under the control of the strong and constitutive lac promoter (Δ*oxcT*+), were monitored in PYE rich medium in the presence of 100, 150 and 200 µM $CuSO_4$, corresponding to low, medium and high level of Cu toxicity, respectively (Fig. 12).

The rich medium used is the peptone yeast extract (PYE). In the control condition, the WT strain as well as Δ*oxcT* and Δ*oxcT*+ strains display similar growth profiles, suggesting that OxcT is not essential for growth under normal conditions (Fig. 12). At 100 µM $CuSO_4$, corresponding to a low level of Cu stress, the Δ*oxcT* strain displays a higher Cu sensitivity relative to the WT and the Δ*oxcT*+ strains.

As the Cu concentration increases at 150 and 200 µM, Δ*oxcT* growth defect is exacerbated. On the other hand, in the Δ*oxcT*+ strain, the exogenous expression of *oxcT* complements Δ*oxcT* phenotype and even displays a better Cu tolerance than the WT strain in PYE+ Cu 200 µM (Fig 12). It could be because *oxcT* expression level would be higher in the Δ*oxcT*+ strain than in the WT and it would enhance the bacteria resistance to Cu toxicity.

It has been demonstrated that the transcription level of *CCNA_00027-28* in the WT during the Cu stress response were different in the PYE rich medium and in the controlled M2G medium. In PYE, RNA-seq data showed an induced expression of the whole operon (*CCNA_00027-28*) upon Cu stress which was not the case in M2G (Maertens, L. unpublished data). *oxcT* would be constitutively expressed in M2G compared as in PYE. Transcriptomic data also revealed that *oxcT* expression level in M2G was more than 1.4-fold higher compared to PYE (Hottes, A. K., *et al.,* 2004). The environment could considerably impact Cu tolerance mechanisms in *C. crescentus* (Maertens, L., *et al.*, 2021).

In order to determine whether this discrepancy between the poor and rich media could be reflected in the growth profiles, bacteria were grown in M2G in the presence of 5, 10, 15 µM Cu, corresponding to low, medium and high Cu stress, respectively (Fig. 13).

In M2G compared to PYE, similar levels of Cu toxicity are reach at lower Cu doses. In those two media, Cu ions are chelated in different ways and proportions. In PYE which is composed of yeast extract, $Cu^{2+}$ is mainly chelated by amino acids and GSH. In M2G, the only chelator significantly present is EDTA. However, it is hypothesized that EDTA would not be able to chelate Cu as it is chelating $Fe^{2+}$. Thus, phosphates would be the most likely ligands (Maertens, L., *et al.,* 2021). In M2G control condition, Δ*oxcT* and the WT strain have the same growth profile. The Δ*oxcT* + strain exhibits a lag of growth compared to the Δ*oxcT* and WT strains. Despite this difference, stationary phase is at a similar $O.D._{660\ nm}$ level for all the strains tested. The difference in the growth profiles could come from a toxicity induced by the high level of proteins in Δ*oxcT*+ compared as in the WT strain. Indeed, in Δ*oxcT*+ strain, *oxcT* is under the strong lac promoter whereas in the WT, *oxcT* is derepressed by Fur. The level of OxcT in Δ*oxcT*+ might be toxic in M2G and not in PYE because of medium composition as well as transcriptomic differences. As Cu concentration goes from a low (Cu 5 µM) to a medium level of toxicity (Cu 10 µM), Δ*oxcT* strain growth is considerably affected compared to the WT and Δ*oxcT*+ strains. At the highest level of toxicity tested (Cu 15 µM), the Δ*oxcT* strain does not grow anymore whereas the WT and Δ*oxcT*+ strains are impacted to a lesser extent. The growth defect of the Δ*oxcT*+ strain compared to the WT is unchanged meaning that it exhibits a similar Cu sensitivity as the WT (Fig. 13). These results demonstrate that *oxcT* is also involved in Cu tolerance in M2G. Finally, at a similar level of Cu toxicity observed on the WT in PYE and M2G (Cu 150 µM and Cu 15 µM, respectively), we can observe that the Δ*oxcT* strain is similarly affected by Cu stress relative to the WT. OxcT is essential for Cu tolerance in both media despite transcriptomic differences.

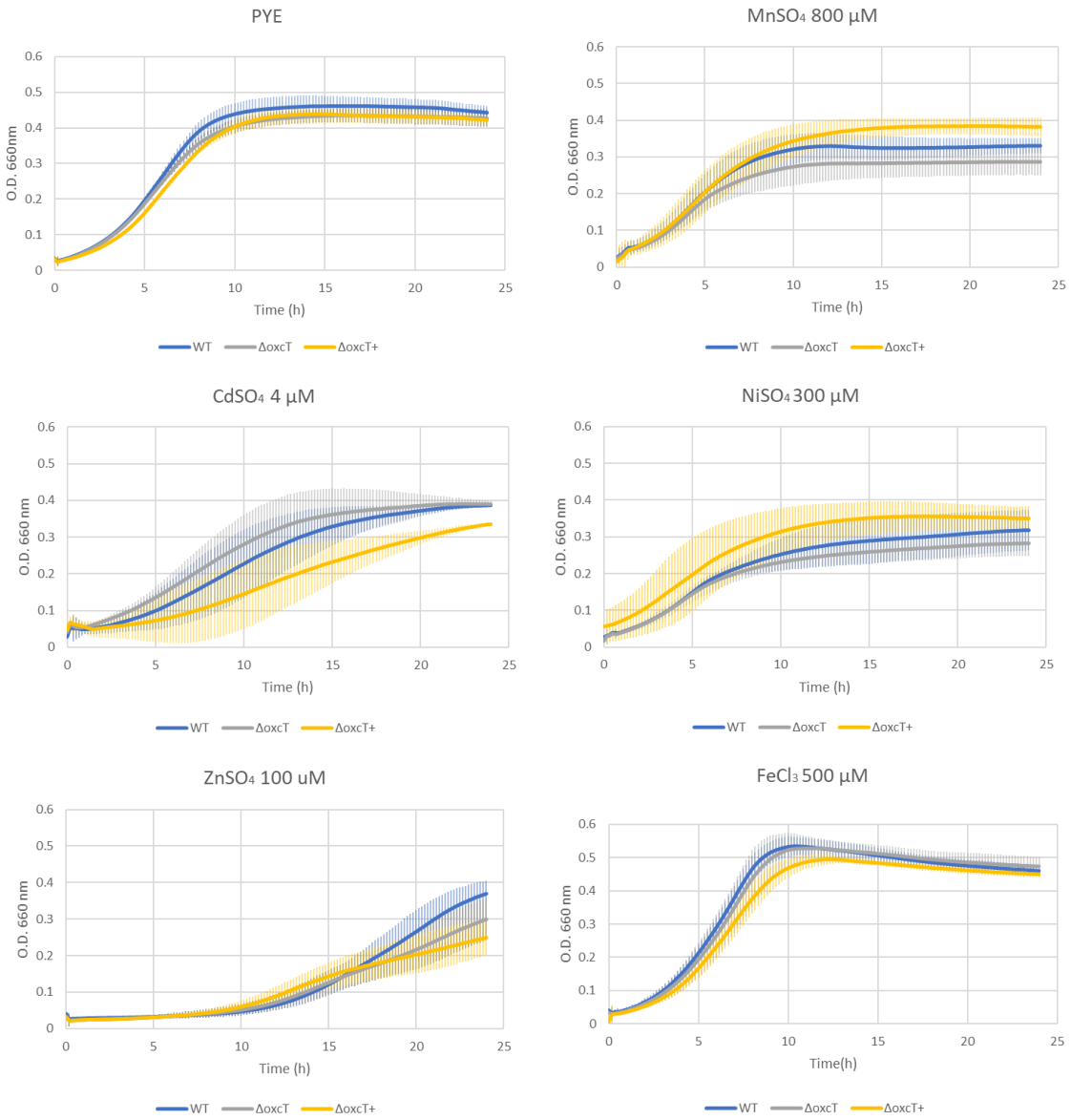


**Figure 14: Growth profiles were observed in PYE and in presence of toxic heavy-metals concentrations.** Growth profiles of WT, Δ*oxcT* and Δ*oxcT*+ were measured in PYE supplemented with 800 µM $MnSO_4$, 4 µM $CdSO_4$, 300 µM $NiSO_4$, 100 µM $ZnSO_4$ and 500 µM $FeCl_3$(n=3). The deletion and the complementation of *oxcT* do not positively or negatively impact the growth in presence of the tested heavy metals. Error bars show standard deviation (SD).

## The sensitivity to other heavy metals is not exacerbated in the Δ*oxcT* mutant

Some heavy metals such as cadmium (Cd), zinc (Zn), Fe, nickel (Ni) or manganese (Mn) share similar toxicity mechanisms to Cu. Therefore, one may expect that bacteria use the same detoxification and tolerance mechanisms against them (Xu, F. F., & Imlay, J. A., 2012). For instance, Zn, Cd, Mn and Cu all lead to degradation of Fe-S clusters and Ni, Fe and Cu all lead to ROS production (Lemire, J. A., *et al.*, 2013). In return, bacteria might use the same detoxification and tolerance mechanisms against these metals (Xu, F. F., & Imlay, J. A., 2012). As an example, mechanisms of heavy-metals tolerance such as efflux pump (e.g. CzCBA in the export of Cd, Cu, Zn) or ROS detoxification by superoxide dismutase (SOD) are shared between Cu tolerance and other heavy-metal tolerance systems such as Cd and Ni (Caille, O., *et al.*, 2007). OxcT might then not only induce tolerance to Cu but also to other heavy metals. To test this hypothesis, the growth for the WT, Δ*oxcT* and Δ*oxcT*+ strains was monitored in the presence of $MnSO_4$, $CdSO_4$, $NiSO_4$, $ZnSO_4$ and $FeCl_3$ toxic concentrations (Fig. 14).

While all the strains are perfectly growing in the PYE control condition, the addition of each heavy metal negatively impacts the growth. Despite the observed growth defect and toxicity, there is no observable difference between the 3 studied strains, suggesting that OxcT is not involved in the tolerance to other heavy metals than Cu (Fig. 14).

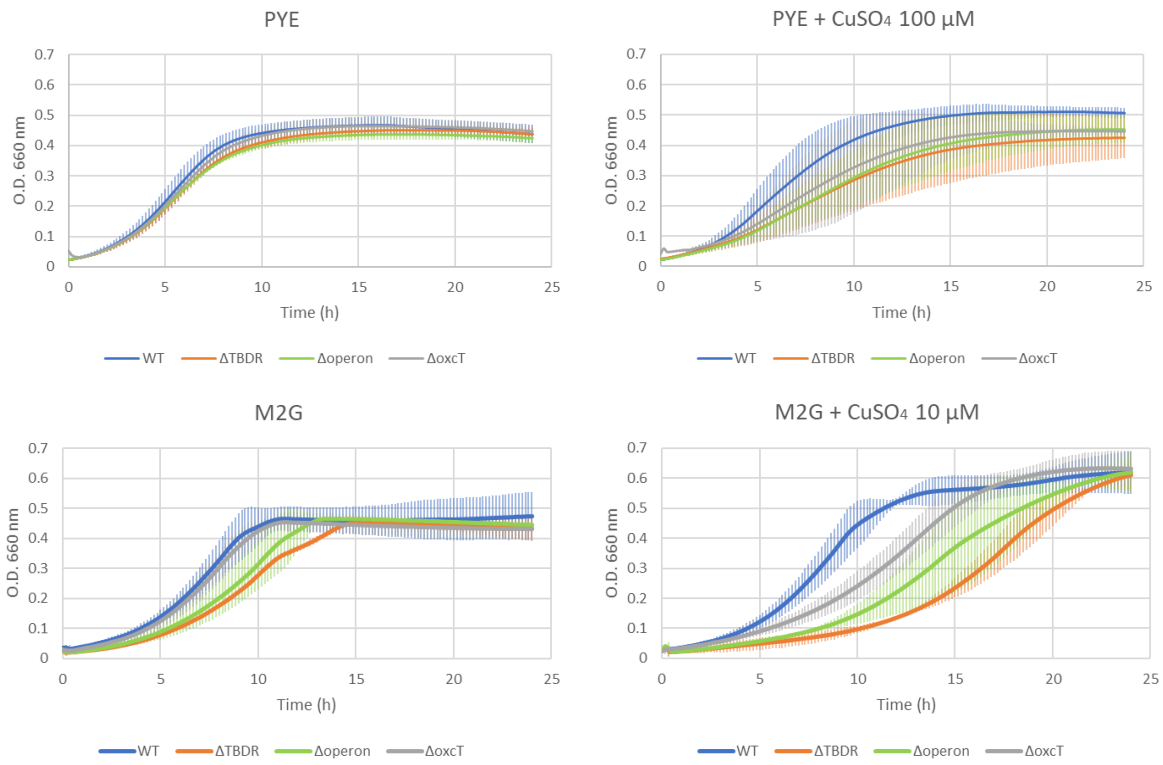


**Figure 15: Growth profile of *CCNA_00027-28* mutant in the absence and in the presence of $CuSO_4$ in rich (PYE) and poor (M2G) medium.** Growth profiles of WT, Δ*oxcT,* Δ*TBDR* and Δ*operon* mutant strains were monitored in PYE and M2G in a medium Cu stress level ($CuSO_4$ 100 µM and 10 µM, respectively) (n=4). The three knock-out strains exhibit the same phenotype as there is no accumulative effect of the deletion of both *oxcT* and *TBDR* as seen on the phenotype of Δ*operon.* Error bars show standard deviation (SD) (Pauline Cherry's data).

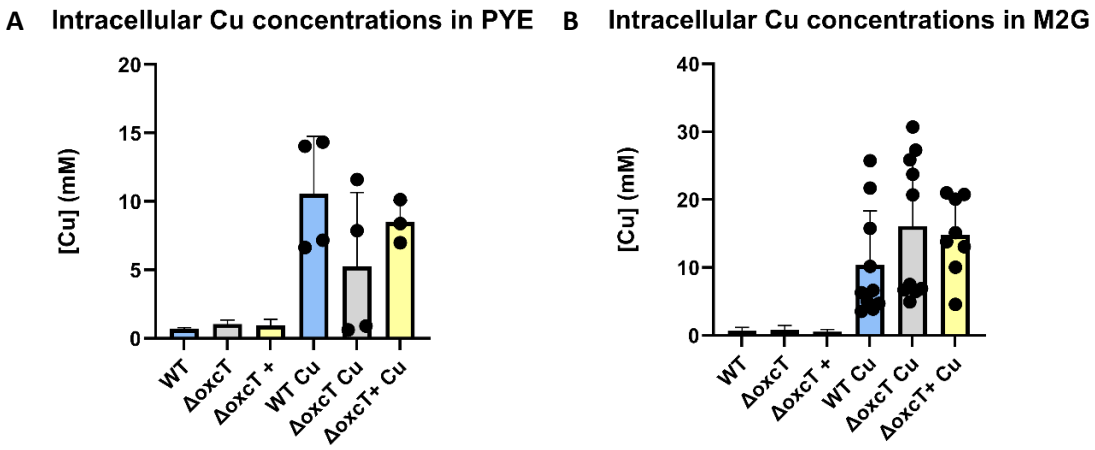


**Figure 16: Measurements of the intracellular Cu content of the total cell fraction of Δ*oxcT*.** Intracellular Cu contents were measured in PYE (A) and M2G (B) after Cu stress (175 µM and 15 µM, respectively) (n=4 and n=10, respectively). (A) While intracellular Cu concentrations are low in the control condition, an increase can be observed upon Cu stress. The Cu accumulation seems to be higher in the WT and in Δ*oxcT*+. The latter almost recover Cu content of the WT in the same condition. (B) Cu intracellular concentrations are low in the control condition and increase in Δ*oxcT* and Δ*oxcT*+ upon Cu stress. Dots represent individual biological replicates. Error bars depict standard deviation (SD).

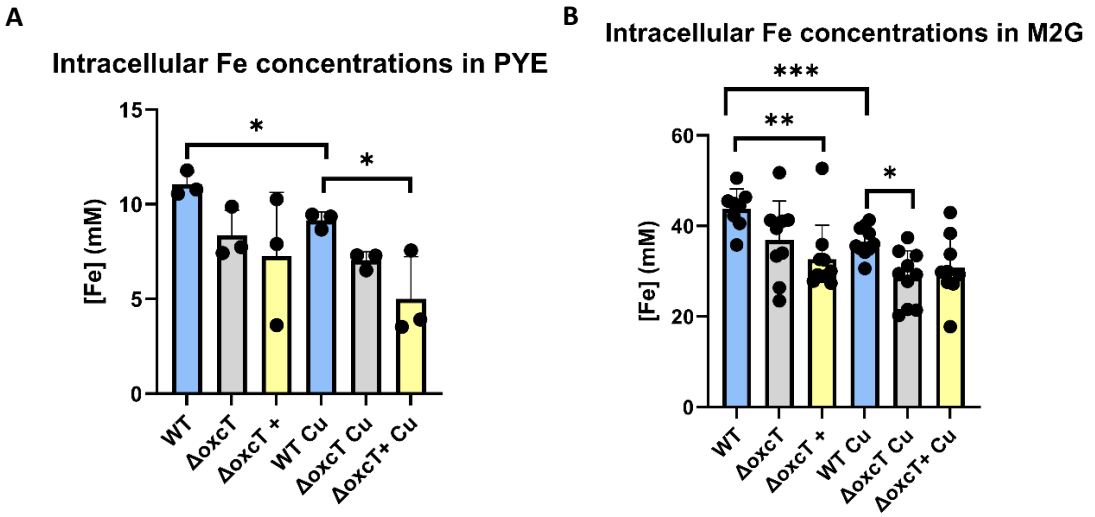


**Figure 17: Measurements of the intracellular Fe content in the total cell fraction of Δ*oxcT*.** Intracellular Fe content was measured in PYE (A) and M2G (B) after Cu stress (175 µM and 15 µM, respectively) (n=3 and n=10, respectively). (A) In control conditions, there are no significant differences. Upon Cu stress, Fe concentrations significantly decrease in Δ*oxcT*+ relative to the WT stressed with Cu (p value=0.0159 ≤0.05 *). A t-test analysis showed that there was a significant decrease in the WT upon Cu stress (p value=0.0144 ≤0.05 *). (B) In control conditions, Fe intracellular concentration only significantly decreases in Δ*oxcT*+ relative to the WT (p value=0.0085≤0.01 **). Upon Cu stress, Fe concentrations decrease significantly in Δ*oxcT* compared to the WT (p value=0.0106≤0.05 *). A t-test demonstrates a significant difference in the WT between the control and the Cu stress (p value= 0.0010 ≤0.01 ***). Dots represent individual biological replicates. Error bars depict standard deviation (SD).

## 2) Investigation of the link between Fe homeostasis and OxcT function in Cu homeostasis

OxcT and TBDR functionally interact

The fact that *CCNA_00027* encoding OxcT and *CCNA_00028* encoding a TonB-dependent receptor (TBDR) belong to the same operon suggests that their functions could be linked. As a matter of fact, the Δ*oxcT*, Δ*TBDR* and Δ*operon* mutants display similar Cu sensitivity compared to the WT in PYE and M2G (Fig. 15) (Pauline Cherry's data). It is thus likely that OxcT and the TBDR are involved in the same Cu tolerance mechanism but would play different roles therein.

### Cu content increases upon Cu stress

It seems like the Δ*TBDR* strain would accumulate more Cu than the WT under high Cu stress (Pauline Cherry's data). As OxcT and the TBDR are genetically and probably functionally linked, it is interesting to investigate whether OxcT might impact Cu content as well and also compare Cu accumulation and growth profiles.

The Δ*oxcT* strain Cu sensitivity could be explained by an increased accumulation of Cu ions in the cells inducing higher toxicity and leading to growth defect. To assess this hypothesis, total Cu content in the cell was measured using ICP-OES in PYE and M2G under normal and high Cu stress conditions. The highest stress level was tested to enhance observed phenotypes. Cu concentrations in the total fraction were normalized regarding the cell density.

In PYE control condition, Cu concentrations are really low and do not exceed 1mM Cu per cell (Fig. 16 A). This is coherent with the fact that they are only few cupro-enzymes in bacterial cells (Dupont, C. L., *et al.*, 2011). Upon a high level of Cu stress (175 µM), Cu accumulates in all the genetic backgrounds tested (Fig. 16 A). As a matter of fact, it is thought that Cu accumulates in the cells by passive diffusion along its chemical gradient (Giachino, A., & Waldron, K. J., 2020). However, no significant difference for Cu accumulation could be observed between Δ*oxcT* and WT strains. Biological variability is high and distinct populations can be observed within the different genetic backgrounds tested (Fig. 16 A). In Δ*oxcT* strain, especially, it seems like some bacteria were not even stressed with Cu, however, they were. PYE components such as cysteine, histidine and GSH could chelate Cu and impact Cu accumulation in the bacteria (Maertens, L., *et al.*, 2021). Variations in chelators concentrations within PYE might result in variable accumulation phenotypes. We could also imagine that such variation would be due to variable activation of the Cu stress response in the WT and Δ*oxcT* compared as in Δ*oxcT*+ strain where *oxcT* is constitutively expressed and where variability is a bit lower (Fig. 16 A). In M2G, Cu intracellular concentrations in the control conditions are as low as in PYE probably for the same reason. That would suggest that a similar amount of Cu is needed to load cupro-enzymes in the cells. Upon Cu stress, despite the different Cu concentrations used to stress bacteria, the accumulation of Cu in the WT strain is similar to the one observed in PYE (Fig. 16 A and B). That is probably because bacteria, whether they are in PYE and M2G, use the same Cu tolerance system to keep Cu concentrations in a certain range which is similar to both media. Relative to the WT, there is no significant accumulation of Cu in Δ*oxcT* or Δ*oxcT*+ strains which suggests that *oxcT* would not involve in Cu export (Fig. 16 B). Biological variability is once again encountered and seems higher than in PYE. That could be explained by the higher responsiveness of bacteria to Cu stress in M2G compared as in PYE. It was demonstrated that the induction of Cu responsive genes would be higher in M2G compared as in PYE. Thus, small variations in the activation timing of these genes would result in bigger variations in the Cu accumulation phenotype (Maertens, L., *et al.*, 2021).

## Fe content decreases upon Cu stress in the Δ*oxcT* mutant

*CCNA_00027-28* is negatively regulated by the ferric uptake repressor (Fur). When Fe is sufficient in the medium, it binds Fur and Fur-Fe complex represses the expression of the operon. When there is an Fe-starvation, Fur derepresses the transcription of the operon and its transcription is activated (da Silva Neto, J.F., *et al.*, 2013). *CCNA_00027-28* and thus, *oxct*, might have an impact on Fe homeostasis. Moreover, as the *TBDR* is thought to import Fe and as it functionally interacts with OxcT, we might think that OxcT would impact Fe content in the cells too. To verify this, intracellular Fe concentrations in the total cell fraction were measured by ICP-OES.

In PYE control condition, no significant difference in Fe content compared to the WT is observed (Fig. 17 A). Under Cu stress, there is a decrease in the Fe content between the WT and Δ*oxcT*+ strains (One way ANOVA p value=0.0159≤0.05 *). This decrease might explain the lag of Δ*oxcT*+ compared to the WT strain. There is also a significant decline of the Fe content within the WT genetic background and upon Cu stress (t-test p value=0,0144≤0.05 *). Fe content does not seem impacted by *oxcT* deletion in PYE.

Biological variability is encountered once again and is especially high in the Δ*oxcT* + strain (Fig. 17 A). That might be due to the toxicity of *oxcT* overexpression and the fact that OxcT uses $Fe^{2+}$ as a cofactor. That might directly reveal the link between OxcT and Fe.

In the M2G control condition, the only significant difference highlighted is the drop in Fe content in the Δ*oxcT* + strain (p value=0.0085≤0.01 **) (Fig. 17 B). Upon Cu stress, Fe content decreases significantly in Δ*oxcT* compared to the WT. In these conditions, the decrease observed in Δ*oxcT* compared to the WT is significant (p value=0.0106≤0.05 *). However, there is no significant decrease in Δ*oxcT*+ relative to the WT. With a t-test statistical analysis, we could see that within WT background, there is a significant decrease of Fe concentration in the cell related to the Cu stress (p value=0.0106≤0.001 ***). It is not known how that decrease might impact cellular physiology and if it would induce an Fe-starvation response. Inter-biological replicate variability is still high especially in Δ*oxcT* in both the control and Cu stress whereas variability in the Δ*oxcT* + strain, is encountered under Cu stress specifically (Fig. 17 B). It it seems like the deletion of *oxcT* or the overexpression of *oxcT* triggers a lot of variability in the Fe accumulation profile in M2G. That might reflect the impact of OxcT on Fe homeostasis and the fact that the cells do not tolerate variations in *oxcT* expression level.

In M2G, intracellular Fe concentrations are higher than those observed in PYE. It is possible that transcriptomic differences between PYE and M2G lead to the use of different metallochaperones. In PYE, there might be a lot more metals bioavailable in the yeast extract than in M2G where there is only $FeSO_4$. The latter is going to be more extensively used than in PYE.

To conclude, OxcT seems to have a more significant impact on Fe homeostasis in M2G than in PYE. And as the Fe content decreases even more upon Cu stress, it seems like OxcT, Cu and Fe homeostasis would be linked.

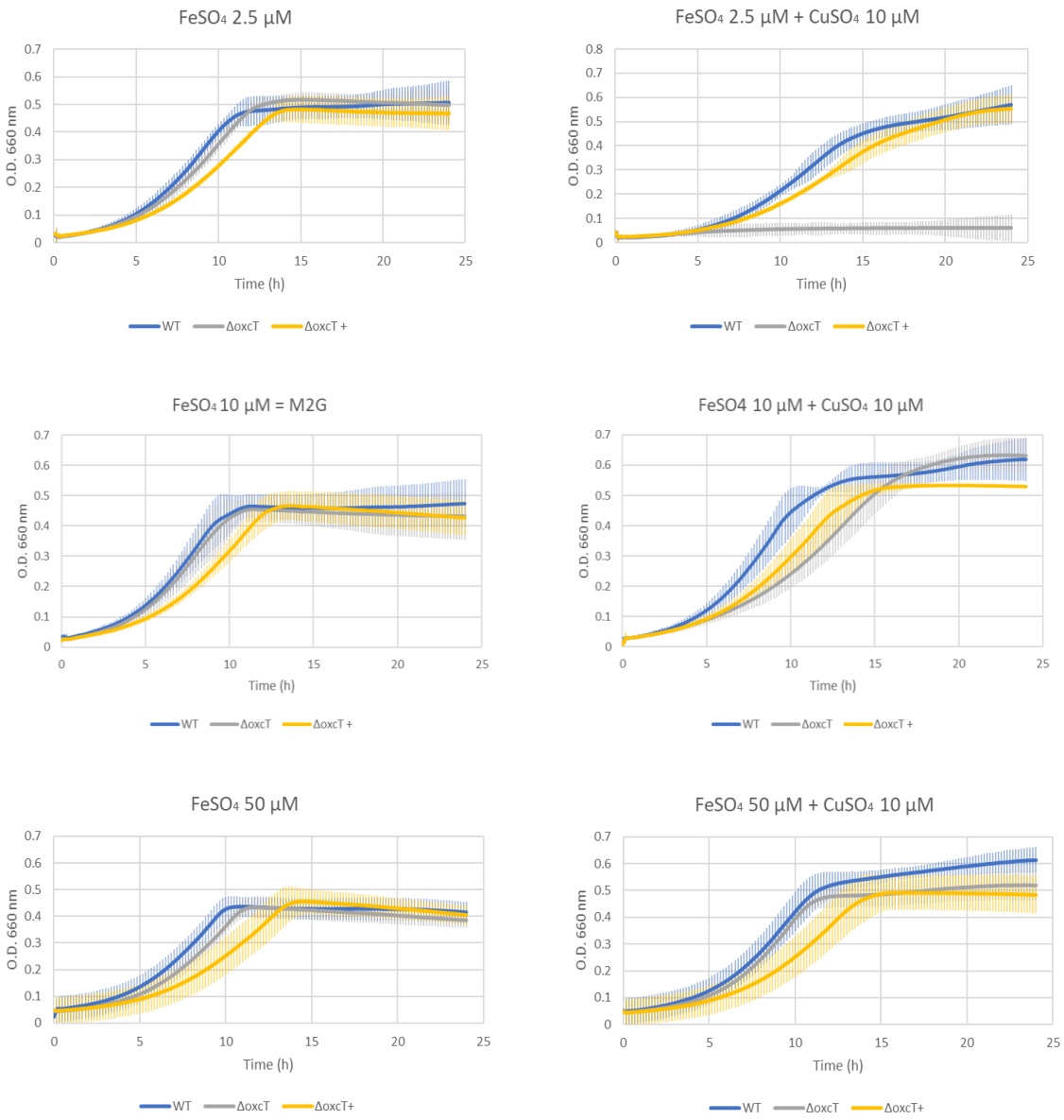


**Figure 18: Growth profiles of Δ*oxcT* in low and high Fe concentrated M2G.** Growth profiles of WT, Δ*oxcT*, Δ*oxcT*+ were monitored in low Fe concentrated M2G, Fe-sufficient M2G and excess of Fe ($FeSO_4$ 2.5, 10, 50 µM, respectively) in absence or presence of a medium level of Cu stress ($CuSO_4$10 µM) (n=4). Δ*oxcT* does not seem sensitive to low Fe concentration itself but growth is more impacted upon Cu stress and in low Fe relative to growth in the same conditions in Fe-sufficient M2G. Fe excess seems to have a protective effect as Δ*oxcT* is not sensitive to Cu stress. Error bars show standard deviation (SD).

## Cu sensitivity of Δ*oxcT* is exacerbated in low Fe conditions

As the *CCNA_00027-28* operon expression is induced in response to Fe starvation, it might be possible that, besides its function in Cu tolerance, OxcT could also play role in the Fe-starvation response and that these two mechanisms would overlap (da Silva Neto, J.F., *et al.,* 2013). To test the impact of low Fe concentration on OxcT function, bacteria were grown in M2G with different Fe concentrations and Cu stress was then added. As a minimal medium, Fe concentration in M2G can be easily controlled without the use of a chelator.

Growth curves were measured in 0, 0.5, 1 and 2.5 µM of Fe for the WT, Δ*oxcT* and Δ*oxcT*+. It appears that, from 2.5 µM of Fe, all the strains are able to grow as well as in Fe-replete M2G ($FeSO_4$ 10 µM) (Supplementary Fig. 1). A concentration of Fe 2.5 µM was chosen to measure the impact of low Fe concentration on OxcT function in Cu tolerance. As *oxcT* is not essential to tolerate an Fe concentration of 2.5 µM, by using this concentration, only effects on Cu sensitivity will be observed. A medium level Cu stress ($CuSO_4$ 10 µM) was chosen to be able to see subtle differences in the Cu sensitivity rather than using the highest Cu stress level in which Δ*oxcT* does not grow anymore.

Thus, growth profiles of WT, Δ*oxcT* and Δ*oxcT*+ were measured in lowly Fe-concentrated M2G ($FeSO_4$ 2.5 µM) in absence or presence of Cu stress (Fig. 18). During Cu stress, Δ*oxcT* does not grow anymore in lowly Fe-concentrated M2G while in M2G, growth only displays a defect (Fig. 18). The WT and Δ*oxcT*+ strains are also more impacted by Cu in Fe starved M2G relative to classic M2G but to a lesser extent (Fig. 18). Cu toxicity seems exacerbated in lowly Fe-concentrated M2G.

Then, a concentration of Fe 50 µM was used to study the impact of an Fe excess on OxcT function in Cu tolerance in M2G. Indeed, growth curves measured for the WT and Δ*oxcT* in Fe 50 µM M2G are actually similar to those measured in Fe 10 µM M2G except for Δ*oxcT*+. It exhibits a larger growth defect compared to the WT in the exponential phase in the control (Fig. 18). Upon Cu stress, Δ*oxcT* does not display any growth defect anymore relative to the WT and compared as in M2G supplemented with Cu 10 µM (Fig. 18). The excess of Fe might hide toxic effect of Cu to the point that OxcT is not essential anymore to tolerate Cu stress relative to classical M2G + Cu or Fe 2.5 µM+ Cu (Fig. 18).

Similar growth profiles in low, sufficient and high Fe with the addition of a Cu stress show that OxcT function is particularly essential to tolerate Cu upon Fe low concentration. Its role would be at the interconnection between Cu excess and Fe homeostasis as an accumulative effect has only been observed in these conditions.

As an interpretation for these results, OxcT might be involved in the response to Fe-S cluster degradation that is a direct consequence of Cu toxicity. Upon Cu stress, it has been shown in *E. coli* that Cu would inhibit the Isc system and would recquire the more resistant Suf system to cope with Cu toxicity. Isc and Suf systems are both involved in assembly of Fe-S clusters but Suf would be more resitant as it builds and transfers clusters in a more cloistered environment (Macomber, L., & Imlay, J. A., 2009). The response could be triggered to counteract Fe-S clusters degradation and to trigger activation of clusters repair systems. The growth defect in M2G Cu stress in Δ*oxcT* would be explained by the inactivation of essential Fe-S clusters enzymes for growth. On top of that, when Fe ions are displaced from the cluster and are released, there is an increase of the labile Fe pool. We might hypothesis that the free Fe from the labile pool would be able to go through Fenton reaction leading to the production of ROS and causing oxidative stress (Barwinska-Sendra, A., & Waldron, K. J., 2017). OxcT might be involved in repairing the Fe-S clusters and in triggering an uptake of Fe for the clusters assembly.

The reason why Cu is more toxic in low Fe concentration remains elusive as we cannot confirm that there is a physiological Fe-starvation in Δ*oxcT* strain. Still, in *C. crescentus*, it was demonstrated that Fe-deficiency induces oxidative stress and the SOS response, meaning that DNA-damage occurred. The SOS response is controlled by RecA and LexA. LexA is a repressor and is going to be cleaved by RecA when DNA-damage occur (Leaden, L., *et al.,* 2018). RNA-seq data also showed that genes regulated by Fur and thus, predicted as part of the Fe-starvation response could be involved in ROS detoxification and in the response to oxidative stress (Maertens, L., *et al.,* 2021). This would be coherent with the observation made in low Fe concentration and Cu stress as bacteria and especially, the Δ*oxcT* strain, were more sensitive to Cu. We could suggest that OxcT would be involved in protecting the cells against the oxidative stress as well as in the response to other Cu related toxic effects such as Fe-S cluster degradation. In PYE, growth curves need to be measured in Fe-starvation and Fe excess in Cu stress to see if we can draw the same hypothesis. Chelators such as 2,2-dipyridyl (DP) could be used but as the exact concentration in Fe in PYE is not known and might be variable, Fe concentration in DP treated bacteria could vary. It is easier to progressively add $FeSO_4$ to the minimal M2G medium in order to obtain the expected Fe concentration.

# Part 2: Identification of OxcT partners

## 1) Purification of OxcT partners

The identification of OxcT partners could allow a better understanding of its function in the Cu stress response. To this purpose, OxcT was tagged with a TwinStrep tag (TS) and the partners are copurified via the high affinity of TS to Strep-Tactin columns. The partners can then be identified by LC-MS. During her master thesis, Hala Kasmo constructed a strain encoding *oxcT* fused with the TS at the N-terminal of OxcT from a pMR10 plasmid (Δ*oxcT*+TS). Preliminary data were obtained from cells cultured in the rich PYE medium (Kasmo, H., unpublished data).

In the present project, N-terminal TS tagged OxcT (TS-OxcT) partners were sought in 4 conditions: 2 genetic backgrounds (Δ*oxcT* and Δ*oxcT*+TS); both strains were either stressed with Cu 15 µM or not for 5 min. This allows the detection of partners in both control and in specific high Cu stress conditions. Two experiments were performed and triplicates were achieved for each. The difference between the two experiment is that the purified samples were not digested at the same time. New columns were used for each experiment. Δ*oxcT* serves as a negative control as proteins pull-downed in this condition are not interacting with OxcT as it is not present.

The MS analysis depicts the number of peptides counted for each protein in each analyzed sample. A t-test statistical analysis applied between Δ*oxcT*+TS and Δ*oxcT* conditions, without distinction of the control and the Cu stress condition, revealed enriched proteins in the Δ*oxcT*+TS condition. They are potential candidates for interaction with OxcT. Only proteins with a significantly higher peptide counts in the Δ*oxcT*+TS condition were highlighted (p value$\leq$0.05, Fold Change (FC) $\geq$ 2). The two experiments were lead using the exact same protocol. For the first 3 replicates, only 12 proteins were significantly enriched in Δ*oxcT*+TS than in Δ*oxcT* with a FC higher than 2. For the last 3 replicates, 28 proteins stood out from the t-test. This increase of identified proteins comes from a better purification during the second experiment as more OxcT peptides were counted. That might also be linked to technical variations such as the resin or the volume of eluted protein. Some of the volumes eluted from the column might vary if some of this volume is lost while exchanging tubes during the pull-down assay. Some proteins might also be degraded during the digestion prior MS analysis. All proteins were not selected as some were contaminants or wrong annotated genes that do not belong to *C. crescentus*. Only the 9 most relevant candidates are presented in this table in order to be able to thoroughly discuss them (Table 1). Among the candidates selected, some were totally absent from the negative control (FC=∞). They are represented in *Table 1*, on top of the list. This reinforces the idea that if they are present in the Δ*oxcT*+TS sample, it is because they interact with OxcT. Candidates that were found in the negative control but that were still significantly more present in the Δ*oxcT*+TS conditions are also annotated in *Table 1*, after the candidates with a FC=∞. Interactions specific to Cu stress could be highlighted by comparing the MS results with the RNA seq data as well as the genetic screen in Cu stress (unpublished data). The genetic screen was performed by inserting mini-Tn5 transposons in the genome. The randomly generated mutants were grown in PYE + Cu and those that were not growing were sequenced. Another way to highlight Cu specific interactions is by comparing the Δ*oxcT*+TS control and Cu stress conditions by t-test statistical analysis. However, no significant difference was observed between control and Cu stress conditions.

**Table 3: MS candidates for interaction with OxcT.** Proteins present in Δ*oxcT*+TS in absence or presence of Cu stress (15 µM) were purified by affinity purification assay. Candidates on top of the list are those with the highest FC. The t-test p value shows the reliability of the FC number. FC and p values of the t-test are annotated. Some proteins were found only in the 1st (1) or the 2nd (2) experiment or in both (1 and 2). FC and p values for the t-test are annotated for both experiments if the candidate was present in both. RNA-seq data highlight upregulated genes in Cu (upregulation= ✓ , down or insignificant upregulation= "-"). Candidates highlighted in the genetic screen are also represented (Cu sensitive= ✓ and Cu unsensitive= "-"). Interactions specific to Cu stress (Cu) were highlighted with a t-test between Δ*oxcT*+TS control and Δ*oxcT*+TS Cu conditions.

| Protein annotation | Alternate ID | RNA-seq | Genetic screen | Cu | Experiment (n° 1 and/or 2) | FC | p value |
|---|---|---|---|---|---|---|---|
| Glutathione S transferase | CCNA_03068 | - | - | - | 1 | ∞ | 0.0042 |
| Glyoxalase family protein | CCNA_02610 | - | - | - | 2 | ∞ | 0.018 |
| TIR-like nucleotide binding protein | CCNA_02785 | - | - | - | 1 and 2 | 2.7 and ∞ | 0.048 and 0.012 |
| Myo-inositol-hexaphosphate 3-phosphohydrolase | CCNA_01353 | | - | - | 2 | 30 | <0.00010 |
| Two-component response regulator | CCNA_02783 | - | - | - | 1 and 2 | 7.2 and 11 | <0.00010 |
| Rrf2 family protein | CCNA_00131 | ✓ | - | - | 1 | 4.8 | 0.017 |
| Small heat shock protein | CCNA_03706 | ✓ | - | - | 1 | 2.8 | 0.036 |
| UvrABC system protein A | CCNA_02673 | ✓ | - | - | 2 | 2.6 | 0.0012 |
| Protein RecA | CCNA_01141 | ✓ | - | - | 1 and 2 | 2.2 and 2.3 | 0.00023 and 0.00025 |

1) Glutathione S transferase (*CCNA_03068*)

Glutathione S transferase (GST) is part of a ubiquitous superfamily of GSH-dependent enzymes. In bacteria, GST catalyze the addition of GSH to endogenous or xenobiotic, often toxic electrophilic chemicals (Vuilleumier, S., & Pagni, M., 2002). It is involved in protection against oxidative stress as well as correct folding, synthesis, regulation, and degradation of enzymes and multienzyme complexes in bacteria (Vuilleumier, S., 1997). RNA-seq data in M2G did not show any significant upregulation of *CCNA_03068* upon Cu stress and the genetic screen did not highlight CCNA_03068 as essential for Cu tolerance either (Maertens., L., unpublished data). As this GST was strongly enriched in Δ*oxcT*+TS compared to Δ*oxcT* where there was none, we can still consider it as a good OxcT partner candidate. We could suggest that OxcT interacted at some point with the GST to enable its activation. It is known that 2OGX's are able to hydroxylate proteic substrates and induce post-translational modifications (PTM). That could lead for example to conformational rearrangement and activation of the protein, mainly hydroxylation on lysine or proline residues. GST would then catalyze conjugation of GSH with $Cu^{1+}$ to oxidize it into $Cu^{2+}$. That would inhibit ROS production due to the participation of $Cu^{1+}$ in Fenton-like chemistry (Table 3).

2) Glyoxalase family protein (*CCNA_02610*)

The glyoxalase family protein is involved in secondary metabolites biosynthesis, transport and catabolism as well as in amino acid and carbohydrates transport and metabolism. *In silico* analysis reveals that C*CNA_02610* has a vicinal oxygen chelate (VOC) domain that enables binding of a divalent metal. The metal is then coordinated to a substrate, an intermediate or a vicinal $O_2$ atom. This family include the bleomycin resistance protein, the dihydroxybiphenyl dioxygenase and the glyoxalase I (He, P., & Moran, G. R., 2011). Glyoxalases are involved in the detoxification of methylglyoxal and other aldehydes produced naturally in all living organisms during glycolysis, or during enzymatic conversion of intermediates during fatty acid and amino acid metabolism. Glyoxal is a reactive α-oxoaldehyde and it could also be produced after repairing of methylated nucleic acids by AlkB (Shangari, N., & O'Brien, P. J., 2004; Tudek, B., *et al.*, 2017). In *C. crescentus*, a gene encoding an SOS-regulated glyoxalase family protein, *mmcA*, was found to be involved in DNA-damage repair. It would act independently of known DNA repair pathways and would apparently work in couple with UvrA to repair DNA-damage induce by Mytomycin C (MMC) (Lopes-Kulishev, C. O., *et al.*, 2015). Glyoxal may actually contribute to DNA-damage induction which would activate the expression of glyoxalase family proteins such as the glyoxalase I (Ozyamak, E., *et al.*, 2013). We could imagine that the reason why this glyoxalase family protein (*CCNA_02610*) was copurified is because Cu induced DNA-damage in the cells and that OxcT could interact with the glyoxalase family protein to activate or regulate DNA-damage repair (Table 3).

3) TIR-like nucleotide binding protein (*CCNA_02785*)

TIR-like nucleotide binding protein have a domain that ressembles the mammalian Toll/interleukin-1-receptor (TIR-like domain) that allows signal transduction (). *CCNA_02785* has a cyclic nucleotide binding domain that allows it to bind cAMP or cGMP which are signalling molecules. It has a jelly roll-like fold, similarly as what is found in 2OGXs. TIR-like nucleotide binding proteins are more commonly found in plants or in pathogenic bacteria where the TIR-like domain enables interference with the innate immune system of the host (Low, L. Y., *et al.*, 2007). we could still imagine that a signal would be triggered in reponse to Cu stress. OxcT would be involved in the signalling pathway by interacting with such proteins (Table 3).

4) Myo-inositol-hexaphosphate 3-phosphohydrolase (*CCNA_01353*)

Myo-inositol is an isomer of inositol and an important source of carbo-hydrate for *C. crescentus*. It can serve as an antioxidant, as a cell membrane component, as an osmolyte and as a carbon storage unit. When phosphorylated, it is a source of phosphorus (Herrou, J., & Crosson, S., 2013). Myo-inositol-hexaphosphate 3-phosphohydrolase is a phytase that catalyzes dephosphorylation of the third phosphate group from the myo-inositol hexaphosphate ring, also called phytic acid (Balwani, I., *et al.,* 2017) The interaction between the phytase and OxcT could be Cu stress specific as *CCNA_01353* was shown to be upregulated in Cu stress in M2G with RNA-seq data (Maertens, L., unpublished data). Phytic acid is not produced by bacteria and should not be present in the medium at the lab. However, the interaction of the myo-inositol-hexaphosphate 3-phosphohydrolase with OxcT specifically in Cu stress conditions might reveal the need of phosphorus. We could imagine that phosphate groups would chelate $Cu^{2+}$ and participate in Cu detoxification and that OxcT would be needed for the phytase to be functional (Mukhametzyanova, A. D., *et al.,* 2012). We could imagine that OxcT would post-translationally modify the protein by hydroxylation (Table 3).

5) Two-component response regulator (*CCNA_02783*)

A two-component response regulator was highlighted during the t-test statistical analysis and was significantly more present in the Δ*oxcT*+TS conditions but not in Cu specific conditions. Two-component system regulators usually regulates the expression of genes involved in cell cycle progression such as stalk biogenesis (Biondi, E. G., *et al.,* 2006). *In silico* analysis support that *CCNA_02783* has a guanylate cyclase domain. Guanylate cyclase domain allow the formation of messenger molecules such as cyclic di-guanosine monophosphate (GMPc) (Paul, R., *et al.,* 2004). It is also known that two-component response regulator (RR) work in pairs with histidine kinase sensor (HK) to activate signaling cascade regulating the expression of genes involved in the cell response to a stimulus. However, no characterized HK was found in the vicinity of the gene. We could imagine that, as CusSR that activates Cus efflux system, *CCNA_02783* would interact with OxcT to regulate the Cu tolerance response. OxcT might modify the RR by hydroxylation for example. However, there are many of those proteins in the cells (62 HK and 44 RR) and they could be involved in almost any type of cellular response (Biondi, E. G., *et al.,* 2006). As the TIR-like nucleotide binding protein (*CCNA_02785*) was also copurified with OxcT and is encoded near *CCNA_02783,* we could could imagine that GMPc produced by the two-component response regulator guanylate cyclase activity would bind the cyclic nucleotide binding domain. That would activate a transduction signal cascade and regulate the expression of genes involved in the Cu stress response. OxcT could mediate this regulation (Table 3).

6) Rrf2 family protein (*CCNA_00131*)

Rrf2 family is a widespread family of Fe-dependent transcriptional regulators. That includes Fe-S cluster redox sensor for Fe-S cluster status like IscR but also nitric oxide sensor (NsrR) as well as Fe metabolism sensor (RirA). Proteins from this family do not always contain an Fe-S cluster (e.g. cysteine metabolism repressor (CymR)) (Loi, V. V., *et al.,* 2018). IscR is a cytosolic Fe-S cluster redox sensor from the Rrf2 family and can regulate Fe-S synthesis and utilization pathways. In *C. crescentus*, *iscRS* and *sufBCDS* are negatively regulated by IscR (*CCNA_1942*) (da Silva Neto, J.F., *et al.,* 2013). *In silico* analysis showed that *CCNA_00131* contains a similar winged helix HrcA DNA-binding domain as IscR which suggest that it would also be a negative regulator. The apo-form of IscR binds degraded Fe-S clusters and activate the operon encoding Fe-S cluster assembly proteins (Howell,N L. W., 2010). *CCNA_00131* is upregulated in Cu stress in M2G (Maertens, L., unpublished data). The presence of such protein specifically in Cu

stress would be coherent as it would sense and activate the response to counteract the inactivation of the Fe-S cluster enzymes (Solioz, M., 2018). According to the hypothesis that OxcT would be involved in repairing Fe-S clusters, the interaction with a Rrf2 protein such as IscR would be coherent as it would enable to activate assembly and repair of Fe-S clusters degraded by Cu (Table 3).

7) Small heat shock protein (*CCNA_03706*)

Small heat shock proteins (Hsp) are among the most abundant cytosolic proteins found in eukaryotes, archaea and bacteria (Hartl, F. U., & Hayer-Hartl, M., 2002). They are generally either molecular chaperones that act to maintain the proper protein folding or protease (Reisenauer, A., et al., 1996). *In silico* analysis reveal that *CCNA_03706* has an alpha-crystallin/HSP20-like domain found at the C-terminal of ArsA proteins. The small heat shock protein copurified would be related to Hsp20 proteins. Hsp20 are chaperones that prevent other proteins to denaturate and that form large heterooligomeric aggregates upon high temperature or other environmental stresses (Hartl, F. U., & Hayer-Hartl, M., 2002). ArsA is an ATPase that exports arsenite (As) and is involved in As homeostasis (Bruins, M. R., *et al.*, 2000). *CCNA_03706* is upregulated in M2G upon Cu stress (Maertens, L., unpublished data). The presence of heat shock protein is unspecific as they are involved in any type of stress response (Hartl, F. U., & Hayer-Hartl, M., 2002). Thus, it does not really help to understand OxcT function (Table 3).

1) UvrABC system protein A (*CCNA_02673*)

The UvrABC system was discovered in the 1960's when excision of UV-induced DNA lesions from DNA in bacteria was observed. It was found that proteins encoded by *uvrA*, *uvrB,* and *uvrC* genes were responsible for this nucleotide excision repair (NER) mechanism. In response to DNA damaging agents, the SOS response is activated and upregulates *uvrA* in *C. crescentus*. Recognition and cleavage of damaged DNA is conducted by UvrABC proteins in a multistep ATP dependent reaction (Lopes-Kulishev, C. O., *et al.*, 2015; Truglio, J. J., Croteau, *et al.*, 2006). UvrA is upregulated in M2G upon Cu stress but was not found to be significantly more present in the Δ*oxcT*+TS Cu stress samples than the control ones (Δ*oxcT*+TS) (Maertens, L., unpublished data). UvrA could possibly interact with OxcT to repair DNA-damage (Table 3).

2) RecA (*CCNA_01141*)

RecA is conserved among bacteria and has an essential role in homologous recombination, DNA strand exchange, DNA repair as well as induction of the DNA damage-induced SOS response (Karlin, S., & Brocchieri, L., 1996). RecA usually binds to single stranded DNA and forms the functional form of RecA, a nucleoprotein filament. It participates in repairing DNA double strand break (DSB) by homologous recombination with the helicase/nuclease complex RecBCD. RecBCD produces a single-stranded DNA and loads RecA onto it. Rec A also has a coprotease activity: it catalyzes the auto-cleavage of the LexA repressor, resulting in the derepression of genes involved in DNA repair, including *recA* itself (Galletto, R., & Kowalczykowski, S. C., 2007). In *E. coli*, it was demonstrated that the UvrABC system and RecA would work together to repair a specific type of DNA-damage induced by Mytomycin C, interstrand crosslinks (ICLs). The gap (NER) made by UvrA would be repaired by RecA (Lopes-Kulishev, C. O., *et al.*, 2015). The enrichment of both UvrA and RecA protein in the purification could suggest that some DNA damage could occur in the Δ*oxcT*+TS and would be due to Cu excess. OxcT would interact with them to repair DNA-damages or to regulate the SOS response by interacting with RecA (Table 3).

# Conclusion and perspectives

OxcT involvement in Cu tolerance was previously confirmed in PYE as well as M2G. By comparing growth profiles, it was demonstrated that despite transcriptomic differences between the 2 media, OxcT was required for a complete Cu tolerance in both. It was also confirmed that OxcT function is specific towards Cu tolerance. Besides that, OxcT and TBDR are closely associated, both at the genomic and functional level. Indeed, growth profiles are similar in the conditions tested. Because of the link with the TBDR pump, it was hypothesized that OxcT might as well impact the intracellular Cu and Fe content. To test that, ICP-OES was undertaken to determine intracellular concentrations in Cu and Fe in Δ*oxcT*. It was concluded that OxcT does not seem to impact the Cu content but, in contrast, impacts Fe homeostasis, in M2G particularly. Indeed, we showed in M2G that intracellular Fe concentration decreased significantly upon Cu stress and deletion of *oxcT*. Intracellular Fe and Cu concentrations could also be determined by ICP-OES under Fe-poor and Fe-rich conditions. Indeed, that would allow to check if the Cu or Fe homoestasis is differently impacted than in Fe-replete M2G. Measuring Cu and Fe concentration in the Δ*CCNA_00027-28* strain would also be an additional way to verify if the deletion of both *oxcT* and *TBDR* has an accumulative effect or not and to investigate the functional interaction of the 2 proteins. To optimize the ICP-OES experiment and try to understand the apparent biological variability, a growth control along side ICP-OES measurements would allow to check if the Cu stress actually led to growth defect in Δ*oxcT*.

To further investigate on the link between Fe and Cu homeostasis systems, the impact of low and high Fe concentrations was tested on OxcT function in Cu stress. As the increased Δ*oxcT* Cu sensitivity demonstrates, OxcT is particularly essential to tolerate Cu in low Fe concentrated medium. In contrast, it seems like Fe has a protective effect on Cu toxicity as Δ*oxcT* Cu sensitivity was cleared in highly Fe concentrated medium. The reason of this phenotype remains elusive. As for the phenotype observed in low Fe concentration and Cu stress, one might think that OxcT would be involved in the regulation of the Fe-S clusters repair. That could be an attempt to explain the link between Cu stress, OxcT function and Fe homeostasis. Finally, the pull-down assay performed in M2G followed by MS highlighted interesting potential OxcT partners. The presence of an Rrf2 family protein in the purified sample reinforced the idea that OxcT could be linked to the Fe-S cluster assembly. The presence of RecA, UvrA, and the glutathione S transferase in copurified samples also suggests that DNA damage could happen in the Cu stressed Δ*oxcT*+TS strain and that the cause could be oxidative stress. As for future perspectives, it would be insightful to measure oxidative stress in Δ*oxcT* using CellRox. This method allows the detection of oxidative stress by fluorescence.

One OxcT partner candidate should be selected based on hypotheses addressed and further experiment such as coIP or bacterial Two-Hybrid system to confirm the interaction with OxcT. It is also known that 2OGXs can induce post-translational modifications (PTM) on proteic substrates and based on MS data, the presence of PTM on the candidates is currently being investigated. That might help in the selection and future study of the OxcT-partner interaction.

# Supplementary data

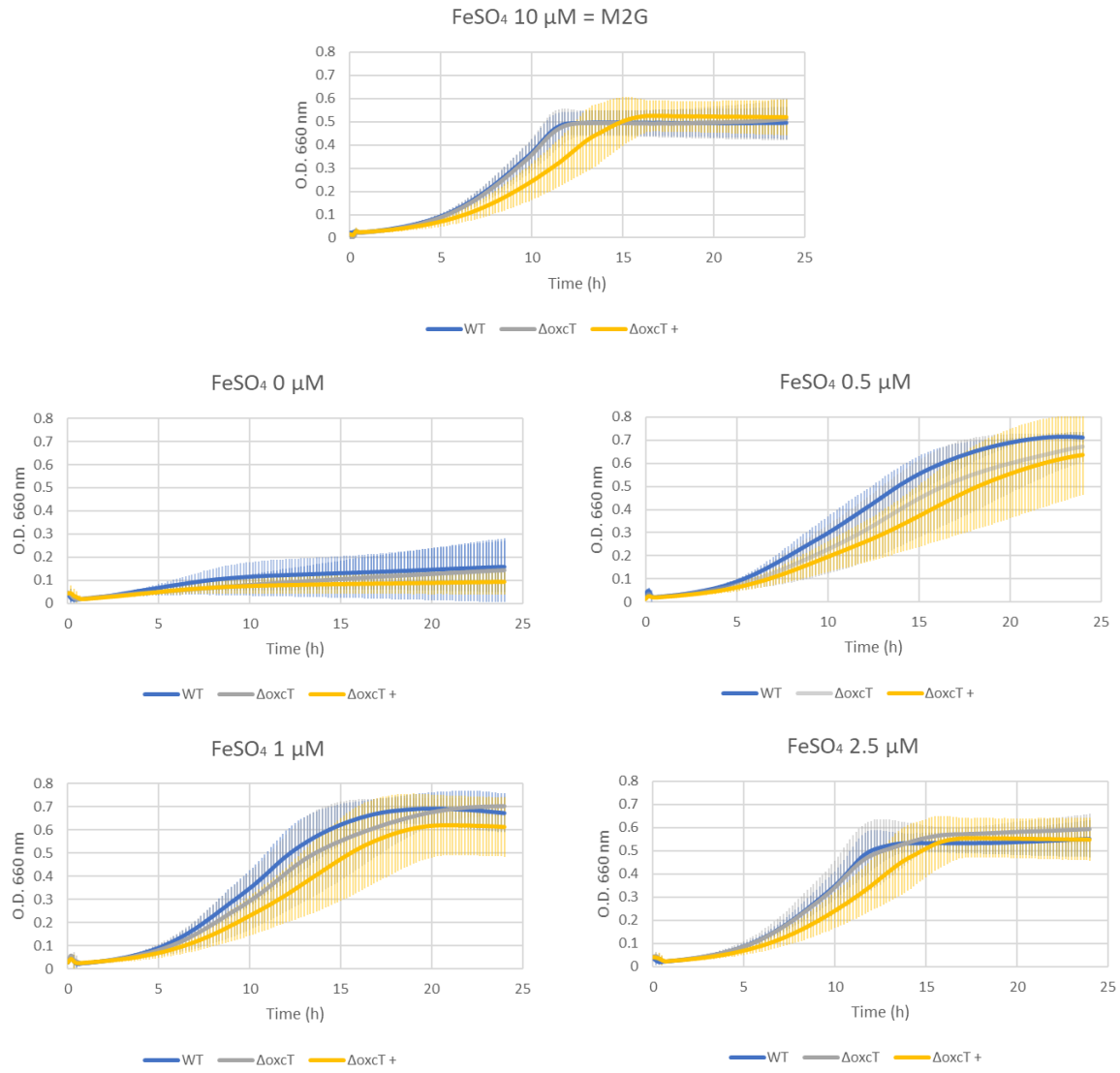


**Figure 1: Growth profiles of Δ*oxcT in* Fe starvation.** Growth profiles of WT, Δ*oxcT,* Δ*oxcT*+ were measured in M2G medium in different Fe starvation conditions ($FeSO_4$ 0 µM, 0.5 µM, 2.5 µM). At 0 µM, none of the strain can grow and at 0.5 and 1 µM, there is still a growth defect from the Fe-starvation but it looks like growth exhibit a boost. At 2.5 µM, all the strains grow as in classic M2G.

# References


1. Almeida, C. E. B., Galhardo, R. S., Felício, D. L., Cabral-Neto, J. B., & Leitão, A. C. (2000). Copper ions mediate the lethality induced by hydrogen peroxide in low iron conditions in Escherichia coli. *Mutation Research/DNA Repair*, *460*(1), 61-67.

2. Andreini, C., Banci, L., Bertini, I., & Rosato, A. (2008). Occurrence of Cu proteins through the three domains of life: a bioinformatic approach. *The Journal of Proteome Research*, *7*(01), 209-216.

3. Balwani, I., Chakravarty, K., & Gaur, S. (2017). Role of phytase producing microorganisms towards agricultural sustainability. *Biocatalysis and Agricultural Biotechnology*, *12*, 23-29

4. Bankapalli, K., Saladi, S., Awadia, S. S., Goswami, A. V., Samaddar, M., & D'Silva, P. (2015). Robust glyoxalase activity of Hsp31, a ThiJ/DJ-1/PfpI family member protein, is critical for oxidative stress resistance in Saccharomyces cerevisiae. *Journal of Biological Chemistry*, *290*(44), 26491-26507.

5. Barwinska-Sendra, A., & Waldron, K. J. (2017). The role of intermetal competition and mis-metalation in metal toxicity. *Advances in microbial physiology*, *70*, 315-379.

6. Biondi, E. G., Skerker, J. M., Arif, M., Prasol, M. S., Perchuk, B. S., & Laub, M. T. (2006). A phosphorelay system controls stalk biogenesis during cell cycle progression in Caulobacter crescentus. *Molecular microbiology*, *59*(2), 386-401.

7. Bondarczuk, K., & Piotrowska-Seget, Z. (2013). Molecular basis of active copper resistance mechanisms in Gram-negative bacteria. *Cell biology and toxicology*, *29*(6), 397-405.

8. Bruins, M. R., Kapil, S., & Oehme, F. W. (2000). Microbial resistance to metals in the environment. *Ecotoxicology and environmental safety*, *45*(3), 198-207.

9. Curtis, P. D., & Brun, Y. V. (2010). Getting in the loop: regulation of development in Caulobacter crescentus. *Microbiology and Molecular Biology Reviews*, *74*(1), 13-41.

10. Caille, O., Rossier, C., & Perron, K. (2007). A copper-activated two-component system interacts with zinc and imipenem resistance in Pseudomonas aeruginosa. *Journal of bacteriology*, *189*(13), 4561-4568.

11. Chillappagari, S., Seubert, A., Trip, H., Kuipers, O. P., Marahiel, M. A., & Miethke, M. (2010). Copper stress affects iron homeostasis by destabilizing iron-sulfur cluster formation in Bacillus subtilis. *Journal of bacteriology*, *192*(10), 2512-2524.

12. da Silva Neto, J. F., Lourenço, R. F., & Marques, M. V. (2013). Global transcriptional response of Caulobacter crescentus to iron availability. *BMC genomics*, *14*(1), 1-16.

13. de Dios, R., Rivas-Marin, E., Santero, E., & Reyes-Ramírez, F. (2020). Two paralogous EcfG σ factors hierarchically orchestrate the activation of the General Stress Response in Sphingopyxis granuli TFA. *Scientific reports*, *10*(1), 1-14.

14. Dupont, C. L., Grass, G., & Rensing, C. (2011). Copper toxicity and the origin of bacterial resistance—new insights and applications. *Metallomics*, *3*(11), 1109-1118.

15. Festa, R. A., & Thiele, D. J. (2012). Cu at the front line of the host-pathogen battle.

16. Galletto, R., & Kowalczykowski, S. C. (2007). RecA. *Current Biology*, *17*(11), R395-R397.

17. Giachino, A., & Waldron, K. J. (2020). Copper tolerance in bacteria requires the activation of multiple accessory pathways. *Molecular microbiology*, *114*(3), 377-390.

18. Hardy, G. G., Allen, R. C., Toh, E., Long, M., Brown, P. J., Cole-Tobian, J. L., & Brun, Y. V. (2010). A localized multimeric anchor attaches the Caulobacter holdfast to the cell pole. *Molecular microbiology*, *76*(2), 409-427.

19. Harris, E. D. 2001. Copper and iron: a landmark connection of two essential metals. J. Trace Elem. Exp. Med. 14:207–210.

20. Hartl, F. U., & Hayer-Hartl, M. (2002). Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science*, *295*(5561), 1852-1858.

21. Hausinger, R. P. (2004). Fe (II)/α-ketoglutarate-dependent hydroxylases and related enzymes. *Critical reviews in biochemistry and molecular biology*, *39*(1), 21-68.

22. Herrou, J., & Crosson, S. (2013). myo-inositol and D-ribose ligand discrimination in an ABC periplasmic binding protein. *Journal of bacteriology*, *195*(10), 2379-2388.

23. Hiniker, A., Collet, J. F., & Bardwell, J. C. (2005). Cu stress causes an in vivo requirement for the Escherichia coli disulfide isomerase DsbC. *Journal of Biological Chemistry*, *280*(40), 33785-33791.

24. Hottes, A. K., Meewan, M., Yang, D., Arana, N., Romero, P., McAdams, H. H., & Stephens, C. (2004). Transcriptional profiling of Caulobacter crescentus during growth on complex and minimal media. *Journal of Bacteriology*, *186*(5), 1448.

25. Howell, L. W. (2010). *The genetics of metal uptake in Caulobacter crescentus: Characterization of regulators and identification of receptors* (Doctoral dissertation, Clemson University).

26. He, P., & Moran, G. R. (2011). Structural and mechanistic comparisons of the metal-binding members of the vicinal oxygen chelate (VOC) superfamily. *Journal of inorganic biochemistry*, *105*(10), 1259-1272.

27. Hu, P., Brodie, E. L., Suzuki, Y., McAdams, H. H., & Andersen, G. L. (2005). Whole-genome transcriptional analysis of heavy metal stresses in Caulobacter crescentus. *Journal of bacteriology*, *187*(24), 8437-8449.

28. IBA and Gmbh, "Expression and purification of proteins using Strep-tag®II or TwinStrep-tag®" Version PR02-0026, January, pp. 1–33, 2017

29. Inagaki, F., Takai, K., Hirayama, H., Yamato, Y., Nealson, K. H., & Horikoshi, K. (2003). Distribution and phylogenetic diversity of the subsurface microbial community in a Japanese epithermal gold mine. *Extremophiles*, *7*(4), 307-317.

30. Iobbi-Nivol, C., & Leimkühler, S. (2013). Molybdenum enzymes, their maturation and molybdenum cofactor biosynthesis in Escherichia coli. *Biochimica Et Biophysica Acta (BBA)-Bioenergetics*, *1827*(8-9), 1086-1101.

31. Islam, M. S., Leissing, T. M., Chowdhury, R., Hopkinson, R. J., & Schofield, C. J. (2018). 2-Oxoglutarate-dependent oxygenases. *Annual review of biochemistry*, *87*, 585-620.

32. Jia, B., Jia, X., Kim, K. H., & Jeon, C. O. (2017). Integrative view of 2-oxoglutarate/Fe (II)-dependent oxygenase diversity and functions in bacteria. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1861*(2), 323-334.

33. Karlin, S., & Brocchieri, L. (1996). Evolutionary conservation of RecA genes in relation to protein structure and function. *Journal of bacteriology*, *178*(7), 1881-1894.

34. Ladomersky, E., & Petris, M. J. (2015). Cu tolerance and virulence in bacteria. *Metallomics*, *7*(6), 957-964.

35. Lawarée, E., Gillet, S., Louis, G., Tilquin, F., Le Blastier, S., Cambier, P., & Matroule, J. Y. (2016). Caulobacter crescentus intrinsic dimorphism provides a prompt bimodal response to Cu stress. *Nature microbiology*, *1*(9), 1-7.

36. Leaden, L., Silva, L. G., Ribeiro, R. A., Dos Santos, N. M., Lorenzetti, A. P., Alegria, T. G., ... & Marques, M. V. (2018). Iron deficiency generates oxidative stress and activation of the SOS response in Caulobacter crescentus. *Frontiers in microbiology*, *9*, 2014.

37. Lemire, J. A., Harrison, J. J., & Turner, R. J. (2013). Antimicrobial activity of metals: mechanisms, molecular targets and applications. *Nature Reviews Microbiology*, *11*(6), 371-384.

38. Loi, V. V., Busche, T., Tedin, K., Bernhardt, J., Wollenhaupt, J., Huyen, N. T. T., ... & Antelmann, H. (2018). Redox-sensing under hypochlorite stress and infection conditions by the Rrf2-family repressor HypR in Staphylococcus aureus. *Antioxidants & redox signaling*, *29*(7), 615-636.

39. Lopes-Kulishev, C. O., Alves, I. R., Valencia, E. Y., Pidhirnyj, M. I., Fernandez-Silva, F. S., Rodrigues, T. R., ... & Galhardo, R. S. (2015). Functional characterization of two SOS-regulated genes involved in mitomycin C resistance in Caulobacter crescentus. *DNA repair*, *33*, 78-89.

40. Low, L. Y., Mukasa, T., Reed, J. C., & Pascual, J. (2007). Characterization of a TIR-like protein from Paracoccus denitrificans. *Biochemical and biophysical research communications*, *356*(2), 481-486.

41. Macomber, L., Rensing, C., & Imlay, J. A. (2007). Intracellular copper does not catalyze the formation of oxidative DNA damage in Escherichia coli. *Journal of bacteriology*, *189*(5), 1616-1626.

42. Macomber, L., & Imlay, J. A. (2009). The Fe-sulfur clusters of dehydratases are primary intracellular targets of Cu toxicity. *Proceedings of the National Academy of Sciences*, *106*(20), 8344-8349.

43. Maertens, L., Cherry, P., Tilquin, F., Van Houdt, R., & Matroule, J. Y. (2021). Environmental Conditions Modulate the Transcriptomic Response of Both Caulobacter crescentus Morphotypes to Cu Stress. *Microorganisms*, *9*(6), 1116.

44. Magnani, D., & Solioz, M. (2007). How bacteria handle copper. In *Molecular microbiology of heavy metals* (pp. 259-285). Springer, Berlin, Heidelberg.

45. Mander, L., & Liu, H. W. (2010). *Comprehensive natural products II: chemistry and biology* (Vol. 1). Elsevier.

46. McDonough, M. A., Loenarz, C., Chowdhury, R., Clifton, I. J., & Schofield, C. J. (2010). Structural studies on human 2-oxoglutarate dependent oxygenases. *Current opinion in structural biology*, *20*(6), 659-672.

47. Mettert, E. L., & Kiley, P. J. (2014). Coordinate regulation of the Suf and Isc Fe-S cluster biogenesis pathways by IscR is essential for viability of Escherichia coli. *Journal of bacteriology*, *196*(24), 4315-4323.

48. Mukhametzyanova, A. D., Akhmetova, A. I., & Sharipova, M. R. (2012). Microorganisms as phytase producers. *Microbiology*, *81*(3), 267-275.

49. Nierman, W. C., Feldblyum, T. V., Laub, M. T., Paulsen, I. T., Nelson, K. E., Eisen, J., ... & Fraser, C. M. (2001). Complete genome sequence of Caulobacter crescentus. *Proceedings of the National Academy of Sciences*, *98*(7), 4136-4141.

50. Olea-Ozuna, R. J., Poggio, S., Quiroz-Rocha, E., García-Soriano, D. A., Sahonero-Canavesi, D. X., Padilla-Gómez, J., ... & Geiger, O. (2021). Five structural genes required for ceramide synthesis in Caulobacter and for bacterial survival. *Environmental Microbiology*, *23*(1), 143-159.

51. Olivera, A., & Spiegel, S. (2001). Sphingosine kinase: a mediator of vital cellular functions. *Prostaglandins & other lipid mediators*, *64*(1-4), 123-134.

52. Osman, D., Waldron, K. J., Denton, H., Taylor, C. M., Grant, A. J., Mastroeni, P., ... & Cavet, J. S. (2010). Copper homeostasis in Salmonella is atypical and copper-CueP is a major periplasmic metal complex. *Journal of Biological Chemistry*, *285*(33), 25259-25268.

53. Ozyamak, E., de Almeida, C., de Moura, A. P., Miller, S., & Booth, I. R. (2013). Integrated stress response of E scherichia coli to methylglyoxal: transcriptional readthrough from the nemRA operon enhances protection through increased expression of glyoxalase I. *Molecular microbiology*, *88*(5), 936-950.

54. Paul, R., Weiser, S., Amiot, N. C., Chan, C., Schirmer, T., Giese, B., & Jenal, U. (2004). Cell cycle-dependent dynamic localization of a bacterial response regulator with a novel di-guanylate cyclase output domain. *Genes & development*, *18*(6), 715-727.

55. Pelley, J. W. (2007). *Elsevier's Integrated Biochemistry*. Mosby.

56. Reisenauer, A., Mohr, C. D., & Shapiro, L. (1996). Regulation of a heat shock sigma32 homolog in Caulobacter crescentus. *Journal of Bacteriology*, *178*(7), 1919-1927.

57. Rhee, S. G., Kang, S. W., Chang, T. S., Jeong, W., & Kim, K. (2001). Peroxiredoxin, a novel family of peroxidases. *IUBMB life*, *52*(1), 35-41.

58. Ruttkay-Nedecky, B., Nejdl, L., Gumulec, J., Zitka, O., Masarik, M., Eckschlager, T., ... & Kizek, R. (2013). The role of metallothionein in oxidative stress. *International journal of molecular sciences*, *14*(3), 6044-6066.

59. Santos, R. E. R. D. S., Batista, B. B., & da Silva Neto, J. F. (2020). Ferric uptake regulator Fur coordinates siderophore production and defense against iron toxicity and oxidative stress and contributes to virulence in Chromobacterium violaceum. *Applied and environmental microbiology*, *86*(21), e01620-20.

60. Shangari, N., & O'Brien, P. J. (2004). The cytotoxic mechanism of glyoxal involves oxidative stress. *Biochemical pharmacology*, *68*(7), 1433-1442.

61. Smirnov, S. V., Sokolov, P. M., Kodera, T., Sugiyama, M., Hibi, M., Shimizu, S., ... & Ogawa, J. (2012). A novel family of bacterial dioxygenases that catalyse the hydroxylation of free L-amino acids. *FEMS microbiology letters*, *331*(2), 97-104.

62. Solioz, M. (2018). Copper—A Modern Bioelement. In *Copper and Bacteria* (pp. 1-9). Springer, Cham.

63. Truglio, J. J., Croteau, D. L., Van Houten, B., & Kisker, C. (2006). Prokaryotic nucleotide excision repair: the UvrABC system. *Chemical reviews*, *106*(2), 233-252.

64. Tudek, B., Zdżalik-Bielecka, D., Tudek, A., Kosicki, K., Fabisiewicz, A., & Speina, E. (2017). Lipid peroxidation in face of DNA damage, DNA repair and other cellular processes. *Free Radical Biology and Medicine*, *107*, 77-89.

65. Vuilleumier, S. (1997). Bacterial glutathione S-transferases: what are they good for?. *Journal of bacteriology*, *179*(5), 1431-1441.

66. Vuilleumier, S., & Pagni, M. (2002). The elusive roles of bacterial glutathione S-transferases: new lessons from genomes. *Applied microbiology and biotechnology*, *58*(2), 138-146.

67. Wolf, A., Mantri, M., Heim, A., Mueller, U., Fichter, E., Mackeen, M. M., ... & Boettger, A. (2013). The polyserine domain of the lysyl-5 hydroxylase Jmjd6 mediates subnuclear localization. *Biochemical Journal*, *453*(3), 357-370.

68. Xu, Z., Lei, Y., & Patel, J. (2010). Bioremediation of soluble heavy metals with recombinant Caulobacter crescentus. *Bioengineered bugs*, *1*(3), 207-212.