An Exploration of the Relationship Between the Small Protein MntS and the MntP Exporter Protein Regarding Manganese Homeostasis in *Escherichia coli*

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ABSTRACT

When controlled, the transition metal manganese is crucial for cellular life. However, when present in unusually high levels, manganese can restrict the ability for cells to grow. Thus, specialized export proteins, such as MntP, help combat metal toxicity induced by manganese. Recent studies have identified another new, small protein called MntS that also plays a role in maintaining homeostasis. It's mechanism of action, however, remains unknown. This research focuses on the importance that MntP holds, as well as the potential function of MntS.

First, wildtype strains of *E. coli* were modified to delete the *mntP* gene and to replace it with a gene encoding a similarly functioning export protein, known as MneA. Two different assays were used to test the importance of MntP; the first, a metal sensitivity assay, examined whether MntP was required for the cell to survive metal toxicity. The second, a two-hybrid assay, was used to determine whether MntP was required for the interaction between MntS with itself. The first set of experiments concluded that MntP is, in fact, required by the cell to survive metal toxicity induced by manganese. In contrast, the second set of experiments alluded that MntS can bind to itself even in the absence of MntP. This works provides an insight on the relationship between MntP and MntS, in the hopes of clarifying the unique function of MntS.

BACKGROUND

Manganese Homeostasis in *E. coli*
- When intracellular manganese levels are scarce, the MntH protein imports manganese
- When intracellular manganese levels are too high, the MntP protein exports excess manganese out of the cell

MntR Transcription Factor
- MntR serves as a transcription factor that regulate the synthesis of genes by sensing and directly binding to the manganese ion
- When bound to manganese ($MntR:Mn_2$), MntR induces expression of MntP
- $MntR:Mn_2$ also represses transcription of MntH as well as MntS

MntP Export Protein
- MntP acts as the *E. coli's* primary export mechanism when intracellular manganese levels are too high
- In other bacteria, it has been shown that in the absence of MntP, the cell becomes sensitive to manganese, and virulence significantly decreases

Small Protein MntS
- MntS plays a role in manganese homeostasis, although its mechanism of action is unknown
- Transcription of MntS is repressed when MntR is bound to manganese, suggesting that it functions when intracellular manganese levels are low
- It is also speculated that MntS acts by inhibiting the export ability of MntP, as an overexpression of MntS yield similar phenotypes as an absence of MntP

MneA Export Protein
- MneA is an export protein native to *Vibrio cholerae* that functions like MntP
- In this research, MneA provides a source for manganese export and allows the cellular processes of *E. coli* to be observed in the absence of MntP

RESEARCH QUESTIONS

- Overall, what is the role/function of the small protein MntS in *E. coli*?
- Does the cell require MntP in the presence of excess MntS in order to save the cell from manganese toxicity?
- Is MntP necessary for MntS to interact with itself inside the cell?

REFERENCES

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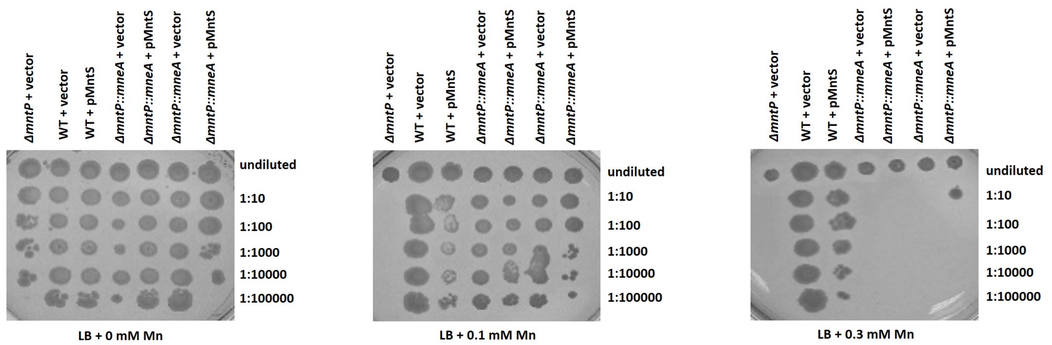
2. Waters LS: Bacterial manganese sensing and homeostasis. *Current Opinion in Chemical Biology* 2020, 55: 96-102.
3. Waters LS, Sandoval M, Storz G: The *Escherichia coli* MntR Miniregulon Includes Genes Encoding a Small Protein and an Efflux Pump Required for Manganese Homeostasis. *Journal of Biology* 2011, 193(21): 5887-5897.

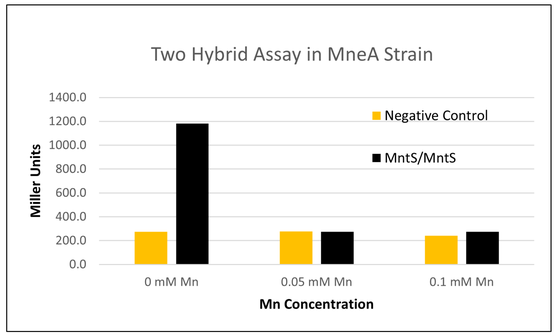
RESULTS

Figure 1. Manganese Sensitivity Assay Showing MneA Can Partially Substitute for MntP and MntS Can Still Confer Sensitivity in the Absence of MntP



Manganese sensitivity assays were conducted under conditions containing various amounts of manganese (0 mM, 0.1 mM, and 0.3 mM). This was completed to test whether the cell requires MntP to survive toxic levels of manganese.
- Under conditions containing 0 mM of manganese, all strains of bacteria grew sufficiently, suggesting that MneA serves as an alternate export protein
- In the presence of 0.1 mM of manganese, all bacterial strains grew sufficiently except Δ*mntP* + pBAD24, the strain without MntP. This suggests that an export mechanism is required in order to grow in the presence of excess manganese.
- In the presence of 0.3 mM of manganese, only the wildtype strain and the wildtype with an overexpression of MntS grew sufficiently, suggesting that MntP is required by the cell to survive manganese toxicity

Figure 2. Two-Hybrid Assay Showing the Ability of MntS to Interact With Itself in the Absence of MntP



Two-hybrid assays were completed using the MneA bacterial strain in order to test whether MntP is required by the cell to allow the interaction of MntS with itself.
- "Miller Units" represents the amount of protein-protein interaction
- Under conditions containing 0 mM of manganese, sufficient interaction occurred between MntS with itself
- Under conditions containing doses of manganese higher than 0 mM, the ability of MntS to bind with itself decreased drastically
- MntS was observed to interact only as well as the strains containing MntS bound to only one part of the protein under conditions containing manganese
- Error bars present in the results of this experiment suggest that the data is not entirely conclusive, and that techniques should be revised to generate more reliable data

Figures 3a and 3b. 96-Well Two-Hybrid Assay Showing the Ability of MntS to Interact with Itself in the Absence of MntP

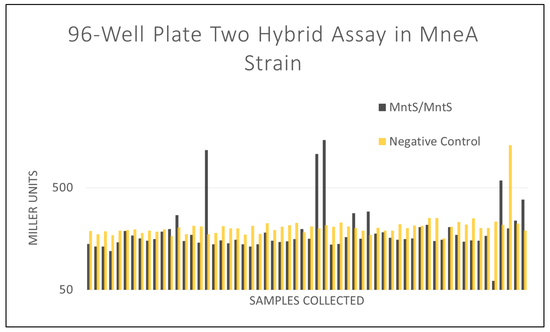


Figure 3a. Histogram Showing Quantitative Results of 96-Well Two Hybrid Assay
- 60 samples were collected from the plates for both the negative control and the samples containing MntS
- Most of the samples fell in the range of background, suggesting that in most replicates no interaction between MntS with itself had occurred

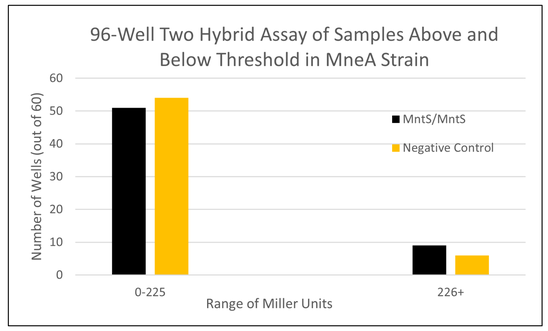
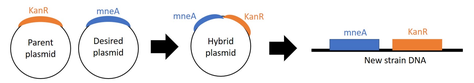


Figure 3b. 96-Well Two Hybrid Assay Showing Threshold of Interaction
- Threshold was determined based on the average miller unit and the standard deviation
- Samples were arranged according to whether they reached or fell short of the threshold
- Samples exceeding threshold were concluded to have significant MntS interaction

METHODS & PROCEDURES

Strain Construction

Figure 4. Construction of New MneA Strains of E. coli



- Antibiotic resistant gene (KanR) was isolated from the parent plasmid and inserted into the plasmid containing MneA to create a hybrid plasmid
- Hybrid plasmid was modified using a restriction enzyme to create a linear piece of DNA
- Linearized DNA was inserted into competent *E. coli* cells and recombined into the chromosome to replace the native *mntP* gene with *mneA* marked with KanR

Metal Sensitivity Assays
- Wildtype (MG1655), Δ*mntP*, and MneA (LSW298 and LSW299) strains were used to test whether the cell requires MntP to survive metal toxicity induced by manganese.
- Modified wildtype and MneA strains were also included to contain an overexpression of MntS
- Cultures were grown overnight in LB and ampicillin before performing 5 ten-fold serial dilutions from each of the undiluted cultures
- All dilutions were spotted on LB plates containing ampicillin (to select for MneA strains), arabinose (to induce MntS expression), and various concentrations of manganese

Two-Hybrid Assays
- MneA strains were transformed with plasmids expressing MntS attached to one of the two domains of adenylate cyclase
- Multiple replicates of cultures were grown overnight in LB + Amp + Kan + IPTG
- Cells were transferred to microcentrifuge tube along with Z-buffer + BME, chloroform, and SDS and left to permeabilize
- Z-buffer + ONPG was added to each tube (ONPG yields a yellow color when cleaved)
- After leaving to incubate, Na_2CO_3 stop solution was added and both OD_{600} and OD_{420} values were measured to observe the cell concentration and the amount of MntS interaction, respectively

96-Well Two-Hybrid Assay
- Colonies were grown overnight in 96-well plates containing LB + Kan + Amp + IPTG
- Cells were resuspended, diluted into a new plate, and OD600 values were measured
- Plate was left to permeabilize after adding Z-buffer + BME, Pop Culture reagent, and lysozyme
- After permeabilization, Z-buffer + ONPG was added to each well and the OD_{420} values were measured every minute for 1 hour

CONCLUSIONS

- MneA, an export protein with similar function to MntP, was successful in saving the cells from metal toxicity. This proves that MntP is required for MntS to confer manganese sensitivity
- In the absence of manganese, MntS successfully binds to itself in the MneA strain, suggesting that MntP is not required for this interaction to occur.
- In the presence of manganese, MntS does not interact with itself sufficiently, suggesting that MneA does not support this interaction as well as MntP.
- The error bars present in the two-hybrid assay bar graph are large, suggesting that the data obtained from these experiments need to be optimized.

FUTURE DIRECTIONS

- Continue with 96-well two hybrid assay experiments to optimize data and obtain qualitative results
- Develop affinity chromatography experiments designed to test whether MntS is inhibiting MntP by binding to it directly
- Continue with Western Blotting experiments to test the possibility that MntS inhibits the function of MntP by causing its degradation (see poster by Flor Martinez, Xiong, and Waters)

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