Cell Wall Endopeptidases in Pseudomonas Aeruginosa

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CELL WALL ENDOPEPTIDASES IN *PSEUDOMONAS AERUGINOSA*

by

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Area of Concentration:	Microbiology
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Prior Degrees:	B. Sc. (Biological Sciences)

I have reviewed this thesis. It represents work done by the author under my guidance/supervision.

Contents

Abstract

The bacterial cell wall is essential for viability, and thus represents an attractive target for antibiotic therapy. Although beta-lactams and certain other antibiotics target enzymes involved in cell wall synthesis, pathogens are rapidly becoming resistant. One poorly understood class of enzymes that may represent promising new therapeutic targets are endopeptidases, which cleave the cell wall. They are thought to be essential for insertion of new peptidoglycan into the wall and growth. In *Escherichia coli*, the three peptidoglycan endopeptidases, MepS, MepM, and MepH, are functionally redundant, with at least one of them being required for growth and viability. Although peptidoglycan hydrolysis is important for cell growth, uncontrolled hydrolysis can compromise the integrity of the cell wall and lead to lysis. MepS activity is controlled through the protease Prc, underscoring the importance of regulating these potentially lethal enzymes.

Pseudomonas aeruginosa is a common opportunistic pathogen that infects over 51,000 people in the United States annually. Over 6,000 cases per year are due to multidrug-resistant strains, leading the WHO to categorize it as one of the top three critical pathogens for which antibiotics are urgently needed. A better understanding of essential cellular processes, such as cell wall biogenesis, could help address the urgent need for new antibiotics to target this pathogen. The goals of this project were to identify cell wall endopeptidases in *P. aeruginosa* and investigate their regulation, which could have implications for future antibiotic development.

Bioinformatic analysis was employed to identify potential endopeptidases in *P. aeruginosa*. Based on this analysis, three genes were identified. In-frame deletion mutants of each gene were made either singly or in combination. A strain with deletion of two genes was inviable for growth as assessed through spot dilutions, suggesting that they together compromise an essential set of endopeptidases in *P. aeruginosa*.

Suppressor mutants that grow despite lacking these essential endopeptidases were sequenced. Loss-of-function mutations in one gene, encoding a protease, suppressed the growth defect and allowed the cells to grow. This was verified through inducing its expression, through a plasmid, in the loss-of-function suppressor mutants. This restricted growth in these suppressor mutants. This suggests that there may be additional redundant endopeptidase(s) proteolyzed by this protease. When the protease was deleted, these additional redundant endopeptidases can accumulate and compensate for deletion of the two other endopeptidases. In summary, *P. aeruginosa* was confirmed to have an essential set of endopeptidases analogous to *E. coli*. In addition, endopeptidases may be regulated by a protease.

Introduction

Pseudomonas aeruginosa is a common opportunistic pathogen that infects over 51,000 people in the United States annually.[1] Over 6,000 of these cases per year are due to strains that are resistant to multiple drugs, leading the Centers for Disease Control and Prevention to classify *P. aeruginosa* as a serious threat[1] and the World Health Organization to categorize it as one of the top three critical pathogens for which antibiotics are urgently needed.[2] It is also one of the ESKAPE pathogens, a group comprised of six pathogens that commonly give rise to multidrug resistant strains.[3]

P. aeruginosa can infect a wide variety of organs and susceptible patients. It commonly infects cystic fibrosis patients, who have thickened airway mucous enabling the bacteria to attach and resist clearance.[4] However, it can also cause ventilator-associated pneumonia, endocarditis, skin and soft tissue infections particularly in burn patients, catheter-associated urinary tract infections, and sepsis.

A better understanding of essential cellular processes, such as cell wall biogenesis, could help address the urgent need for new antibiotics to target this pathogen. *P. aeruginosa* is a gram-negative bacterial species. As such, it has a cell wall comprised of an inner membrane, thin cell wall, and an outer membrane with lipopolysaccharide.[5] The bacterial cell wall is comprised of glycan strands formed from repeating units of N-acetylmuramic acid and N-acetylglucosamine, which are then cross-linked through peptide bridges to form peptidoglycan.[6] Since peptidoglycan is essential for viability, this layer must be expanded and remodeled during the cell cycle to ensure it is maintained during growth and division.

peptidoglycan is synthesized in three stages.[7] First, in the cytoplasm, UDP-N-acetylmuramic acid and UDP-N-acetylglucosamine are synthesized and pentapeptides are added. Second, they are attached to a transport lipid, which is flipped across the inner membrane. Third, penicillin-binding proteins (PBPs) carry out their functions as transglycosylases and/or transpeptidases to, respectively, form peptidoglycan strands and crosslink them. Beta-lactam antibiotics work by inhibiting these PBPs;[8] however, bacteria are rapidly becoming resistant to these and other antibiotics.

Endopeptidases are a class of enzymes that cleave the peptide crosslinks between adjacent glycan strands in the mature cell wall.[6] This activity is thought to be necessary to incorporate newly synthesized peptidoglycan that is required to ultimately grow the cell. In *Escherichia coli*, three peptidoglycan endopeptidases, MepS, MepM, and MepH, are functionally redundant with at least one of them being required for growth and viability.[9] These are DD-endopeptidases, which cleave peptide links between D-alanine and meso-diaminopimelic acid of adjacent strands. Deletion of MepS alone impaired growth on nutrient agar at high temperature, and overexpression of MepM or MepH could reverse the growth defect. This suggests that MepS is the main hydrolase but MepM and MepH can compensate

under certain conditions. Deletion of both MepS and MepM impaired growth on LB but this strain could grow on minimal media. Deletion of all three, MepS, MepM, and MepH, impaired growth on both LB and minimal media, on both solid media and in liquid culture. Deletion of all three prevented incorporation of new peptidoglycan into the cell wall. More crosslinking was observed, but cells formed blobs and lysed over time after stopping induction of MepS.

Although peptidoglycan hydrolysis is important for cell growth, uncontrolled hydrolysis could theoretically compromise the integrity of the cell wall and lead to lysis. MepS activity has been shown to be controlled through a novel proteolytic system, underscoring the importance of regulating these potentially lethal enzymes.[10] The protease Prc, in combination with the adaptor NlpI, degrades MepS in a growth-phase-dependent manner. During exponential phase, MepS levels are much higher than stationary phase. Thus, Prc is potentially important for coordinating levels of peptidoglycan synthesis and cleavage for regulating bacterial growth.

However, endopeptidases have not been studied in detail in *P. aeruginosa* and past investigations have primarily focused on characterizing the enzymatic activity of the endopeptidase PBP4.[8,11] The goal of this project was to identify peptidoglycan endopeptidases in *P. aeruginosa* and how they are regulated. By characterizing essential peptidoglycan endopeptidases and regulatory factors without which peptidoglycan hydrolysis proceeds uncontrollably, the study also provides novel potential targets for antibiotic development.

Bioinformatic analysis was employed to identify orthologues of *E. coli* MepS and MepM in *P. aeruginosa*. Based on this analysis, *P. aeruginosa* PA0667 and two paralogous proteins, PA1198 and PA1199, were identified, respectively. I created strains with these genes deleted or under an inducible promoter. I characterized these strains, and used them to carry out unbiased genetic screens including suppressor mutant sequencing and transposon-sequencing. From the results of these screens, I identified additional proteins involved in endopeptidase regulation and function and characterized them.

Methods

N. Greene, a postdoctoral fellow in the Bernhardt lab, employed bioinformatic methods to identify orthologs of *E. coli* MepS and MepM in *P. aeruginosa*. Based on this analysis, *P. aeruginosa* PA0667 (MepM) and two paralogous proteins, MepS1 (MepS1) and MepS2 (MepS2), were identified. He constructed in-frame deletion mutants of each gene either singly or in combination. I assessed these deletion mutants under phase-contrast microscopy compared to the wild-type.

N. Greene and I performed spot dilutions of the various deletion mutants on LB, LB no salt, LB 1% salt, and VBMM at 30°C, 37°C, and 42°C. I identified a unique phenotype in which ΔMepM appears to grow initially on LB no salt at 42°C, but then lyses by 48 hours. By using a sharp needle and restreaking twice, I isolated suppressor mutants that are resistant to this phenotype despite lacking ΔMepM.

To delineate whether deletion of just two putative endopeptidases is sufficient to inhibit growth, I deleted MepS1 from the deletion mutant of MepM. This was done by amplifying approximately 700 bp upstream and 700 bp downstream of MepS1, and fusing them together using overlap extension PCR. This fragment was inserted into the plasmid pEXG2 using restriction enzymes and DNA ligase, and cloned in DH5a(lpir). The plasmid was then transferred to the mating strain Sm10(lpir). This mating strain containing the deletion plasmid pEXG2::MepS1 was then mated with ΔMepM. Colonies were restreaked on VBMM + Gm30 to select for bacteria containing the plasmid, then streaked onto LB + 5% sucrose to select for loss of the plasmid. Colonies were patched onto both LB + Gm30 and LB plates, and ones which grew on LB but not LB + Gm30 had colony PCR performed to assess for fragment size. Two colonies which had fragment size corresponding to deletion of MepS1 were then Sanger sequenced to verify deletion. I assessed viability of the resultant double mutant compared to control strains deleted for each gene singly and the wildtype (WT) via spot dilutions.

I constructed strains with inducible expression of MepM, MepS1, MepS2, or MepS1-S2 in the ΔMepM ΔMepS1-S2 (TKO) background. To do so, each gene was placed under the control of an IPTG-inducible promoter at the chromosomal attTn7 locus, thus enabling controlled depletion of each gene by the presence or absence of the small molecule inducer IPTG in the growth media.[12] First, through PCR and restriction-enzyme-mediated cloning or isothermal assembly,[13] the gene was placed under the control of an IPTG-inducible promoter, and then inserted into a mini-Tn7 vector.[12] Transformation of the mini-Tn7 vector, along with a helper plasmid, allows insertion of the inducible gene into the chromosomal attTn7 locus, and thus permits its expression only when the inducer is present.[12]

With N. Greene, I performed synthetic lethality screens using Tn-seq[14] on ΔMepM and ΔMepS1-S2 to identify any redundant endopeptidases, which have not yet been identified with bioinformatics, as well as any other proteins required for their activity. I constructed mutant

strains in which genes of interest identified by Tn-seq were deleted. Spot dilutions were used to test synthetic lethality suggested by the Tn-seq results.

I also isolated suppressor mutants which grew despite lacking MepM and MepS1. This suggests that they acquired additional mutation(s) that allowed them to grow in the absence of this essential set of endopeptidases. I then performed whole-genome sequencing on them to identify what mutations suppress the deletion of MepM and MepS1. I identified a candidate gene, CtpA, in which loss-of-function mutations seemed to suppress MepM and MepS1 deletion. I created plasmids in which the catalytically active or inactive version of CtpA was placed under the control of an IPTG-inducible promoter, transformed the plasmid into two suppressor mutants with a mutation in this gene, and performed spot dilutions on media with or without IPTG. Additionally, the plasmid was transformed into a wild-type background. I attempted to transform the plasmid into the suppressor strains with mutations in genes other than CtpA. I also transformed the plasmid into background ΔMepM, ΔMepS1, and ΔMepS1-S2 and assessed growth with CtpA overexpression.

Plasmids with overexpression of catalytically active and inactive MepM and MepS1 were constructed and transformed into wild-type PAO1, ΔMepM, and ΔMepS1. These strains were used to assess for effects of endopeptidase overexpression, whether overexpression of one endopeptidase could compensate for deletion of another, and dominant negative effects.

This study only involves bacteria and does not require Institutional Review Board approval.

Results

The triple knockout mutant, TKO, but not ΔMepM or ΔMepS1-S2, is severely attenuated for growth (Figure 1), indicating that MepM and either MepS1 or MepS2 (or both) may provide essential endopeptidase activity for cell growth.

ΔMepM, ΔMepS1, and ΔMepS1-S2 grew similarly to the wild-type control and under phase-contrast microscopy appeared similar. However, there was a wider shape and loss of rod structure in TKO (Figure 2). This suggests that deletion of all three endopeptidases in combination results in a strain defective for cell wall synthesis, which is likely the mechanism for the synthetic lethality.

Tn-seq was performed in ΔMepM background and ΔMepS1-S2 background to identify other possible endopeptidases or proteins required for their activity. The results indicated that PA4404, a putative endopeptidase, and *CarP*, a protein involved in calcium regulation,[15] might be synthetically lethal with MepM. PA3472, another putative endopeptidase, may be synthetically lethal with MepS1-S2. However, deleting these genes in the endopeptidase-deletion backgrounds did not affect growth, suggesting that they are not synthetically lethal with the endopeptidases (Figure 3).

Additionally, deletion of MepM and MepS1 was sufficient to result in impaired growth, suggesting that they constitute an essential set of endopeptidases and MepS2 alone is not adequate to compensate for their loss (Figure 4). There was differential growth among three ΔMepM ΔMepS1 strains, suggesting that perhaps different suppressor mutations had occurred to enable ΔMepM ΔMepS1 to grow despite lacking these two endopeptidases.

Depletion strains were constructed in the background of ΔMepM ΔMepS1-S2 using multiple combinations of inducible genes, promoters, and ribosomal binding sites. However, these strains grew normally in the presence or absence of the IPTG inducer (Table 1.1). This suggested that either there was too much expression even in the absence of the inducer or that there was insufficient expression even in the presence of the inducer leading to suppressor mutations. Multiple depletion strains were then constructed with inducible MepS1 in the background of ΔMepM ΔMepS1 (Table 1.2). The strain with a strong promoter and the native ribosomal binding site was IPTG-dependent, confirming that MepM and MepS1 are synthetically lethal (Figure 5).

Suppressor mutants were sequenced in which growth occurred despite lacking MepM and MepS1. The most common suppressor mutations, which also resulted in the highest amount of growth, were in CtpA (Table 2). Mutations in CtpA were also found in suppressor mutants of depletion strains, suggesting that these indeed had inadequate expression of the inducible gene. The mutations included frameshift and nonsense mutations, suggesting that loss of function of CtpA suppresses ΔMepM ΔMepS1. This was verified by constructing ΔMepM strains with CtpA Ser329Ala or CtpA Gln197*, then deleting MepS1. These strains grew

comparably to wild-type (Figure 6). When CtpA expression was induced through a plasmid in these suppressor mutant strains, growth was suppressed with wild-type CtpA but not a mutated CtpA, suggesting that loss-of-function mutations in CtpA are indeed what caused suppression of the synthetic lethality (Figure 7). Overexpression of CtpA in the wild-type background had no discernable effect. Transformation of the plasmid into suppressor strains with mutations in genes other than CtpA was unsuccessful and had growth on the selection media comparable to the negative control.

Growth of ΔMepM on solid LB no salt (LB0N) was initially comparable to wild-type after 16h, but after 40h appeared to lyse (Figure 8). MepS1 overexpression prevented this altered phenotype and was dependent on catalytic activity (Figure 9). This phenotype was not as apparent in liquid culture (Figure 10). Suppressors of the ΔMepM phenotype were isolated by myself (Figure 11) and other lab members and sequenced by other lab members. Mutations were found in genes encoding proteins related to reactive oxygen species.

There was no growth defect associated with overexpression of MepM or MepS1, nor was there any dominant negative effect from overexpression of catalytically inactive MepM or MepS1 (Figure 12).

Discussion

By showing that MepM and MepS1 are synthetically lethal, this study demonstrated that these genes comprise an essential set of endopeptidases in *P. aeruginosa*. Additionally, it shows that loss of endopeptidases can affect cell structure and morphology.

The Tn-seq results initially indicated that PA4404 and PA3472 may be putative endopeptidases while CarP, involved in sensing and regulating calcium levels, may be involved in endopeptidase regulation. However, deletion of these genes did not confirm that they were synthetically lethal as the Tn-seq suggested. The algorithm used to process the Tn-seq data may have been flawed as the confirmed synthetically lethal endopeptidases did not come up as hits on the screen.

Through suppressor mutation sequencing, CtpA was confirmed to be a regulator of endopeptidases. In *E. coli*, Prc regulates endopeptidases by proteolyzing MepS with the help of an adaptor protein NlpI.[10] Prc in *E. coli* is homologous to two genes in *P. aeruginosa*, Prc and CtpA. Thus, we hypothesized that CtpA, similar to Prc, regulates endopeptidases by degrading them. This is also supported by the suppressor mutations being loss-of-function. When CtpA is lost, it is likely that alternate endopeptidases can build up and compensate for the deleted endopeptidases. After these results were obtained, a paper was published by Srivastava et al showing that CtpA indeed regulates endopeptidases, including MepM, MepS1, MepS2, and PA4404, and requires the additional protein LbcA in order to carry out its proteolytic activity.[16] It is interesting that LbcA was not detected on the suppressor mutation screen. It is possible that mutations are simply more likely to occur in CtpA than LbcA or that perhaps there is a greater fitness defect associated with LbcA. It is also curious that PA4404 was shown to be an endopeptidase regulated by CtpA, as it did arise on the synthetic lethality screen but did not appear to be synthetically lethal when deleted in the ΔMepM background. Perhaps the fitness defect associated with the double deletion was minor, to the extent that it was detected on the screen but not on spot dilutions. Alternatively, there could have been suppressor mutations such as in CtpA that compensated for the associated growth defect.

It is interesting that ΔMepM initially grew on LB0N at 42^{o}C but then appeared to lyse after growing for a longer period of time. It is unclear whether this represents programmed cell death or killing of neighbours, but this could represent a novel bacterial pathway or a hitherto unknown trigger for established mechanisms such as the Type VI secretion system. Sequencing of suppressor mutant strains by labmates revealed that genes involved in reactive oxygen species are necessary for this lysis to occur. It is reasonable that ΔMepM strains may have an altered cell wall, suggesting that environmental stress in the context of a compromised cell wall may induce a form of bacterial apoptosis involving reactive oxygen species. Analysis of the cell wall composition would be helpful to determine exactly how deletion of MepM affects the cell wall. Additionally, co-culture with wild-type PAO1 could help determine whether this represents self-lysis or killing of neighbours.

Conclusions

Overall, this study demonstrates that *P. aeruginosa* requires endopeptidases to function normally, and that MepM and MepS1 comprise a minimal set of endopeptidases essential for survival. Thus, endopeptidases may be promising therapeutic targets. Additionally, strains lacking MepM appeared to lyse after a time under stressful environmental conditions. This mechanism requires genes involved in reactive oxygen species. Further elucidation of this pathway may reveal a novel mechanism of programmed cell death or bacterial killing. In addition to better understanding the basic biology of bacteria, this pathway could possibly be exploited to develop additional antibiotics.

Summary

Bioinformatic analysis was employed to identify orthologues of E. coli MepS and MepM in *P. aeruginosa*. Based on this analysis, P. aeruginosa PA0667 and two paralogous proteins, PA1198 and PA1199, were identified, respectively. I created strains with these genes deleted or under an inducible promoter. I characterized these strains, and used them to carry out unbiased genetic screens including suppressor mutant sequencing and transposon-sequencing. From the results of these screens, I identified additional proteins involved in endopeptidase regulation and function and characterized them. MepM and MepS1 were identified to be an essential set of endopeptidases. Furthermore, I identified a novel phenotype in the ΔMepM strain which appears to initially grow then lyse when grown on LB without salt at 42^{o}C. Based on further work by fellow lab members, this appears to require genes involving reactive oxygen species, and thus may represent a hitherto uncharacterized pathway of bacterial killing or programmed cell death. Additionally, the protease CtpA was identified to be a regulator of endopeptidase activity.

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Figures

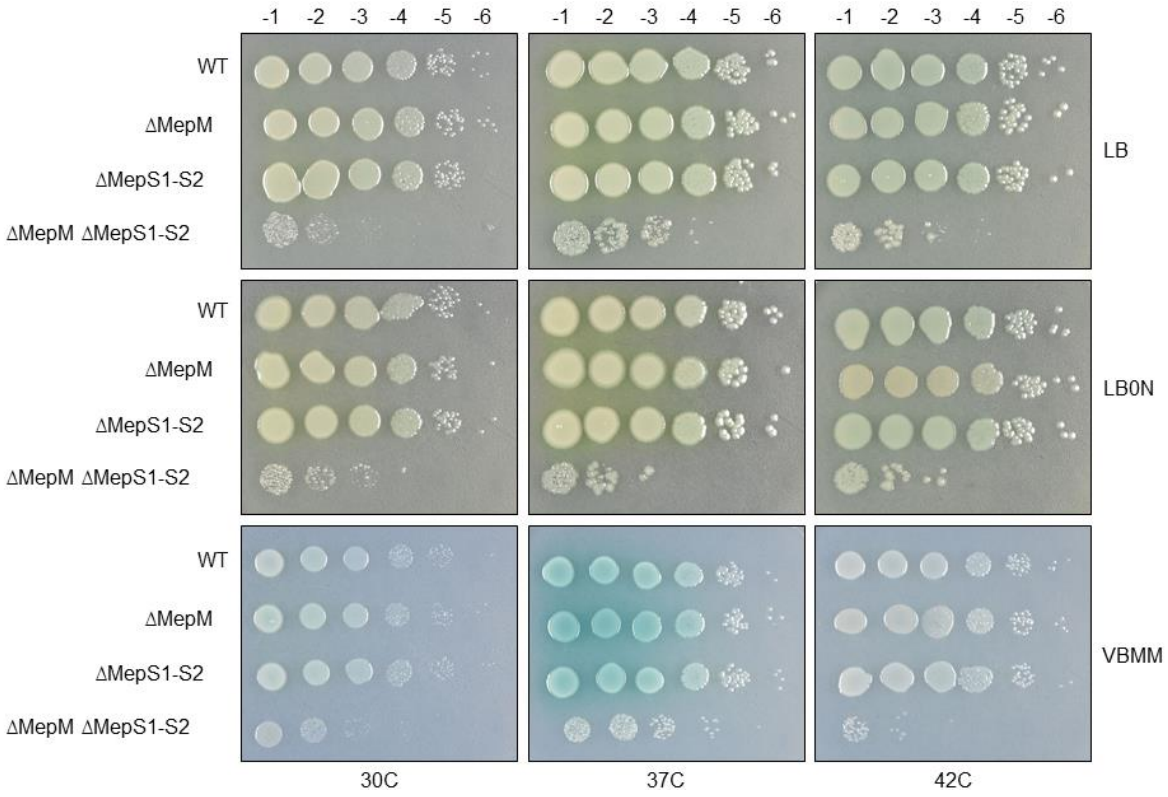

Figure 1. Deletion of MepM and MepS1-S2 leads to synthetic lethality. 10x spot dilutions were performed with dilution factor as labelled. Strains were grown overnight under media and temperature conditions as labelled. LB0N = LB without salt. Figure by N. Greene.

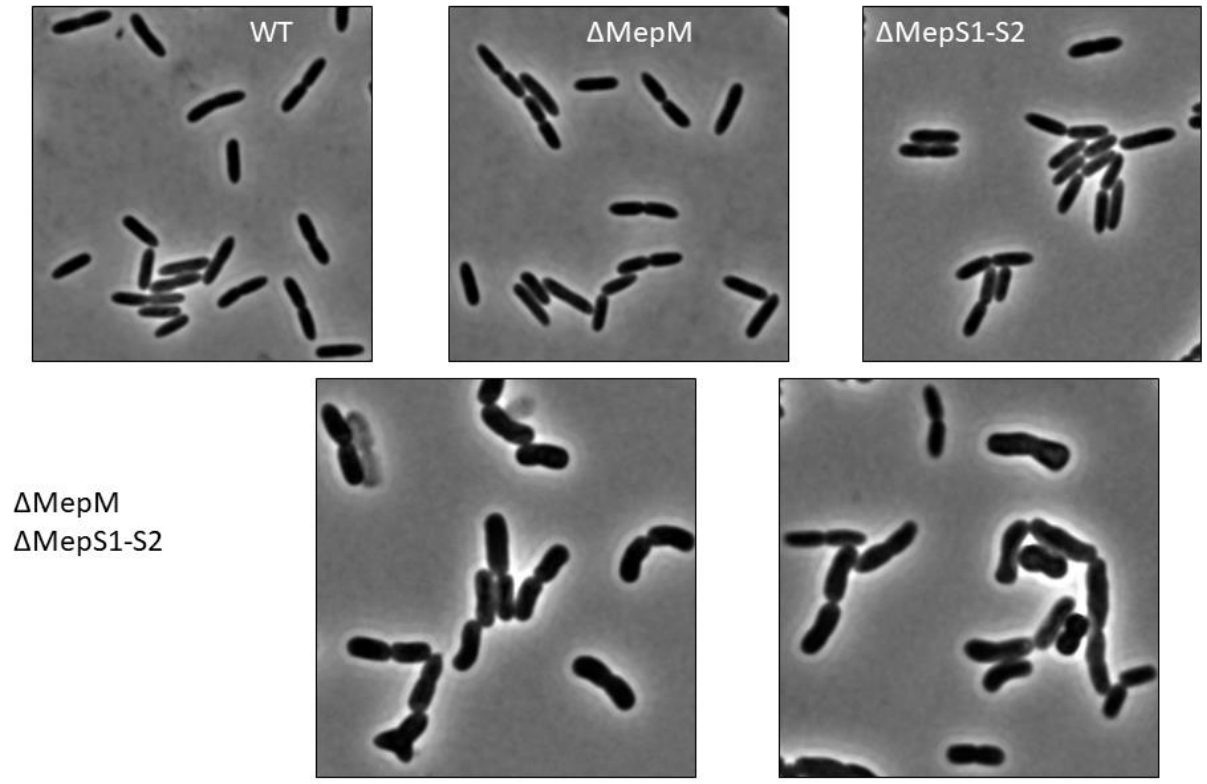

Figure 2. Deletion of MepM, MepS1, and MepS2 in combination alters morphology as seen under phase-contrast microscopy. Images are at the same magnification. Figure by N. Greene.

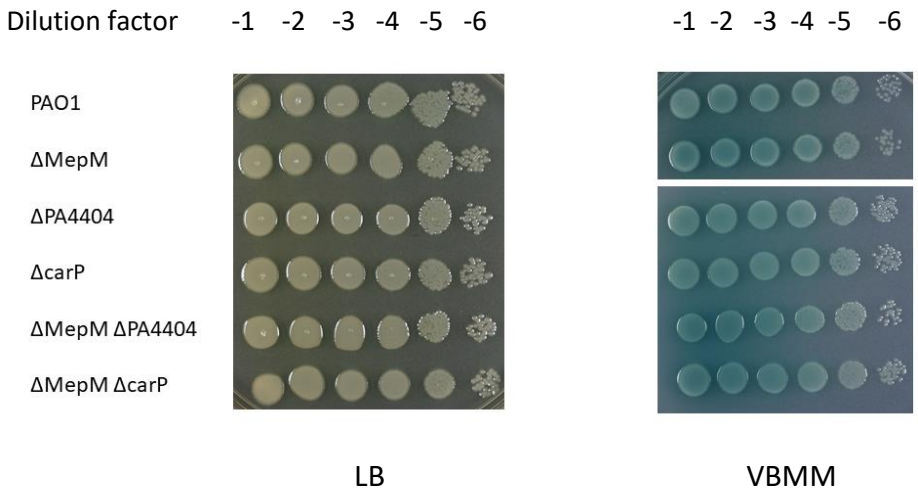

Figure 3. PA4404 and carP are not synthetically lethal with MepM. 10x spot dilutions were performed with dilution factors as labelled. Strains were grown for 19h at 37°C on media as labelled.

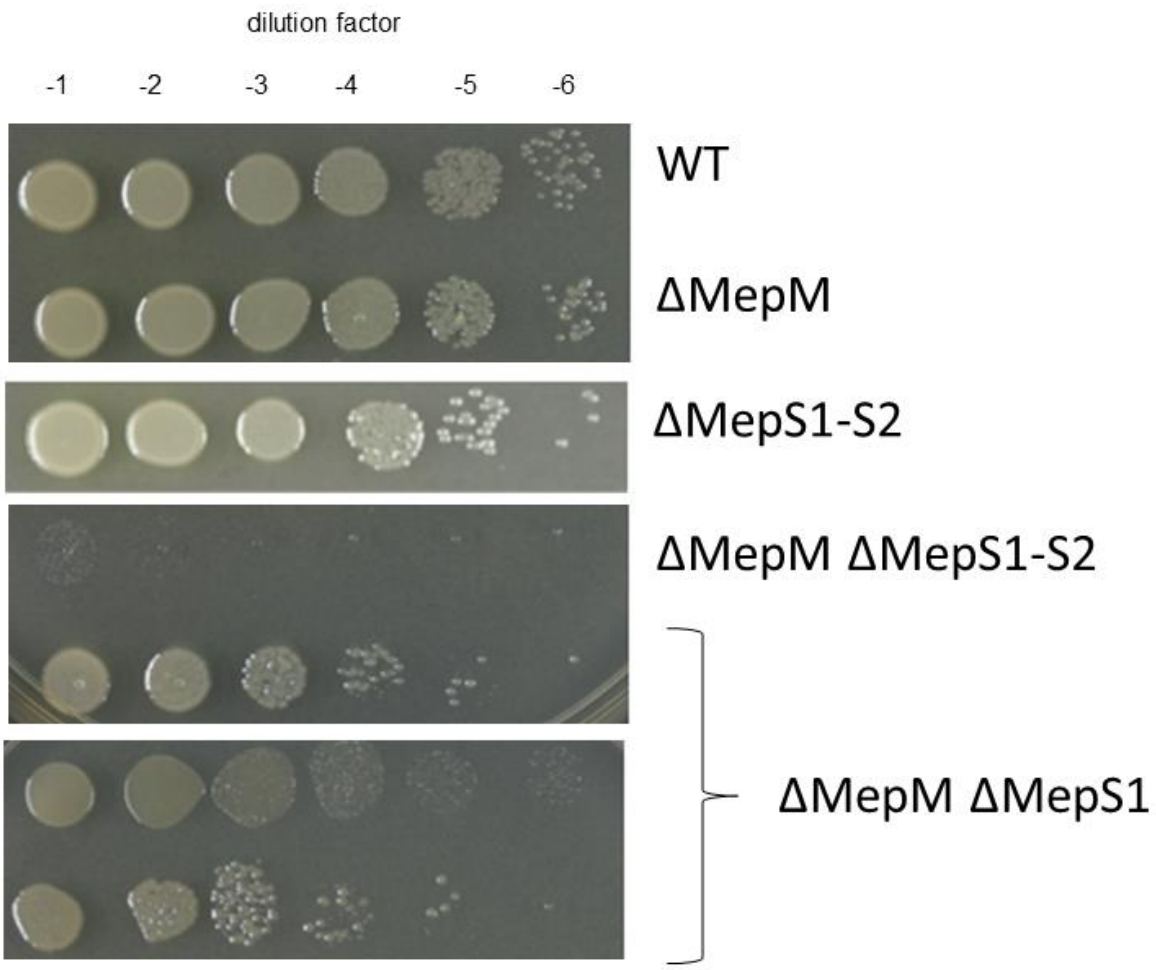

Figure 4. Deletion of MepM and MepS1 is sufficient to impair growth. 10x spot dilutions were performed with dilution factors as labelled. Strains were grown overnight on LB at 30^{o}C.

Gene	Promoter	RBS
MepM	Medium	Native (20 bp)
MepM	Weak	Synthetic
MepS1	Medium	Native (20 bp)
MepS2	Medium	Native (20 bp)
MepS1-S2	Medium	Native (20 bp)

Table 1.1. Depletion strains that were constructed with an inducible endopeptidase in the background of triple deletion ΔMepM ΔMepS1-S2. These strains all grew independently of the IPTG inducer except for MepS with weak promoter and synthetic RBS, which did not grow well with or without IPTG.

Gene	Promoter	RBS
MepS1	Medium	Synthetic
MepS1	Medium	Native (40 bp)
MepS1	Strong	Synthetic
MepS1	Strong	Native (40 bp)

Table 1.2. Depletion strains that were constructed with an inducible MepS1 in the background of double deletion ΔMepM ΔMepS1.

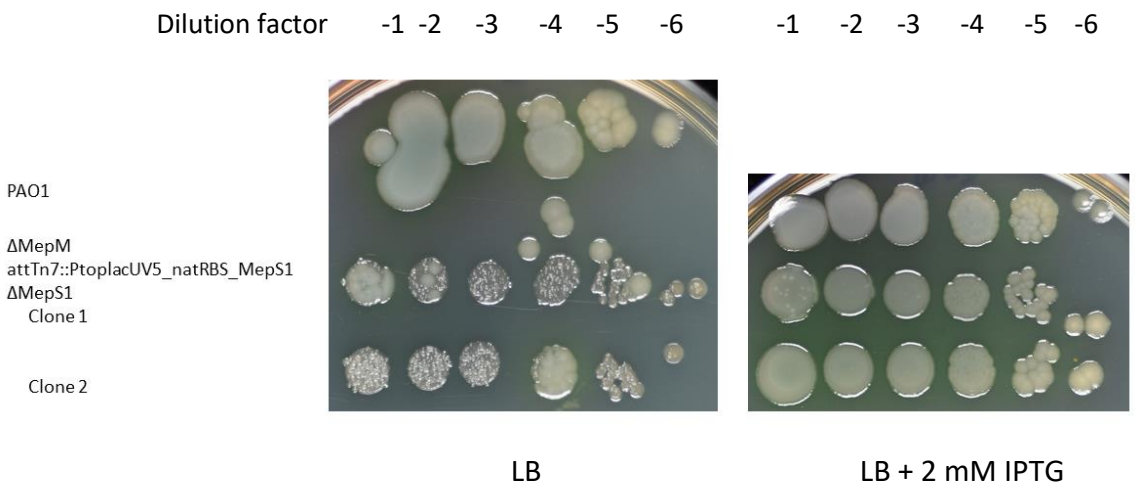

Figure 5. ΔMepM ΔMepS1 with inducible MepS1 is dependent on induction of MepS1 with IPTG to grow. 10x spot dilutions were performed with dilution factors as labelled. Strains were grown at 37°C for 2 days.

Gene	Mutation	Growth
CtpA	*Ser329Ala* *Gln197** *Gly236Asp (*2)* *Leu46Gln* *Asp82Ala* Ala204_Tyr205del Leu264_Gly270del Asp82Gly Ser300fs (*2)	+++
PagL	*Ala28Ser + Ser27Ala (?)*	+
BamB	*Ala164fs*	+
AlgC	*Asn515His*	+
Ssg	Val223fs	+
OprF	Phe147Ser	+
BphP	c.1947G>A	+

Table 2. Results of suppressor mutant screen, in which strains that grew despite lacking MepM and MepS1 were sequenced.

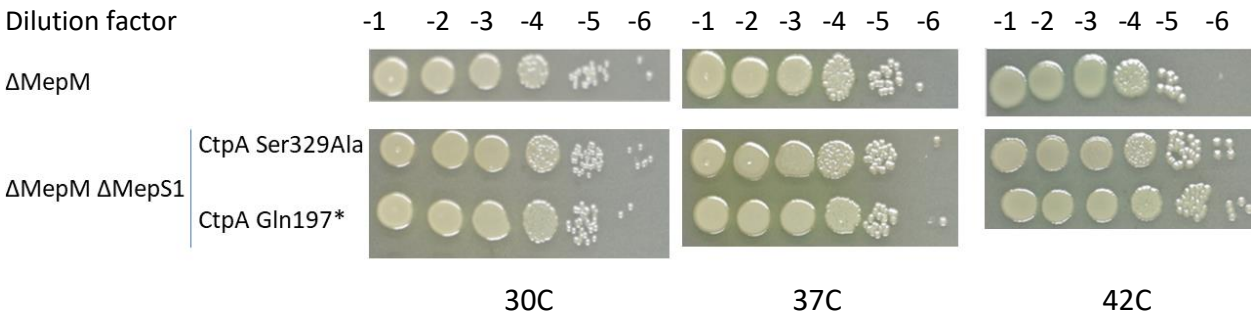

Figure 6. CtpA mutations suppress ΔMepM ΔMepS1. 10x spot dilutions were performed with dilution factors as labelled. Strains were grown overnight on LB at temperatures as shown above.

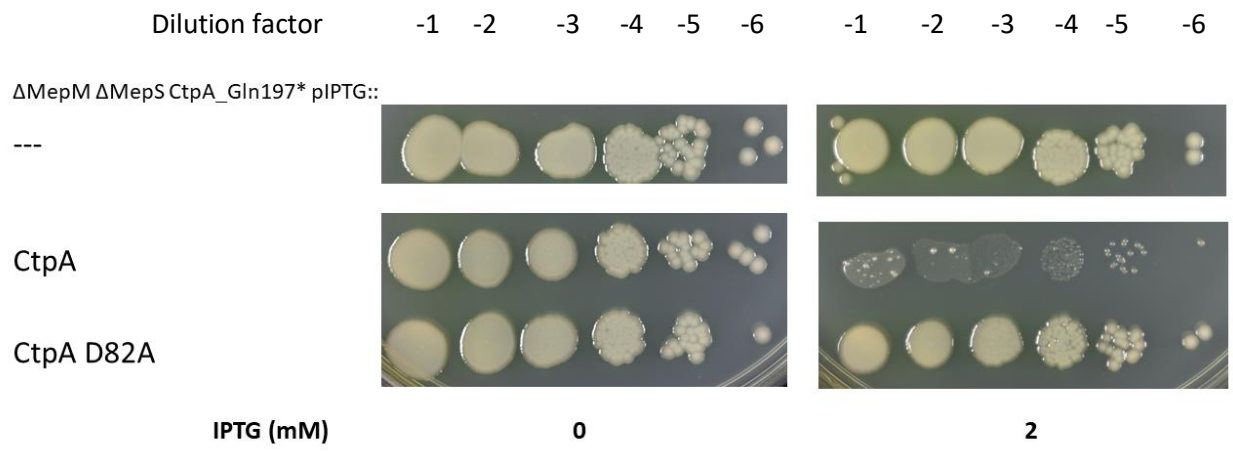

Figure 7. Induction of wild-type CtpA restricts growth of a ΔMepM ΔMepS1 strain with a suppressor mutation in CtpA. However, induction of CtpA D82A does not restrict growth, indicating that this is a loss-of-function mutation which suppresses the synthetic lethality of MepM and MepS1. 10x spot dilutions were performed with dilution factors as labelled. Strains were grown overnight on LB with 30 mM gentamicin at 37^{o}C.

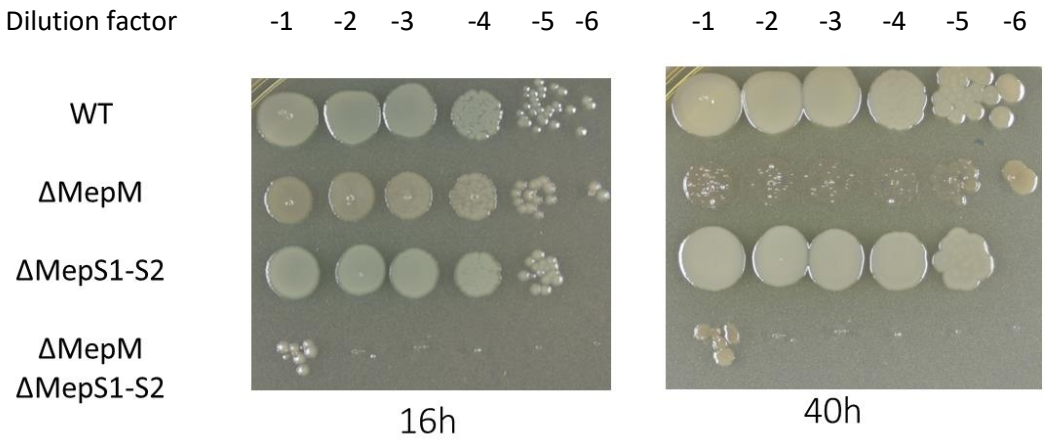

Figure 8. ΔMepM changes over time under high temperature and low osmolarity. 10x spot dilutions were performed with dilution factors as labelled. Strains were grown on LB without salt at 42°C. The same plate is pictured after 16h and 40h of incubation.

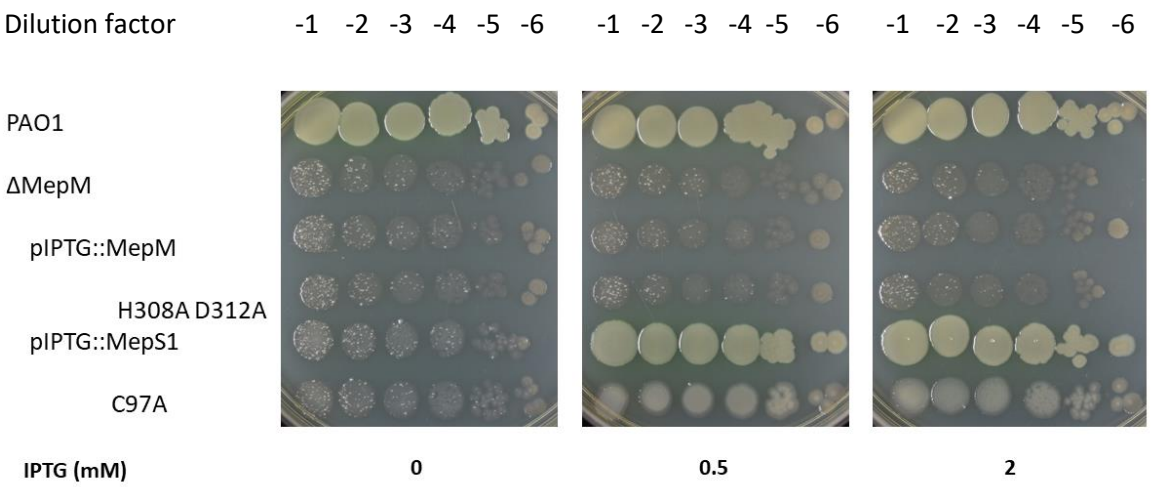

Figure 9. MepS1 overexpression can compensate for ΔMepM and is dependent on catalytic activity. 10x spot dilutions were performed with dilution factors as labelled. Strains were grown for 2 days on LB without salt at 42^{o}C.

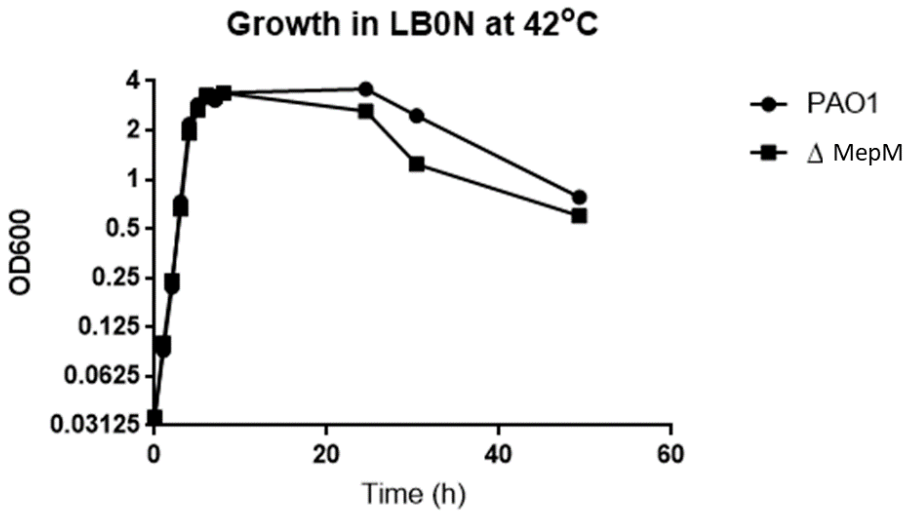

Figure 10. ΔMepM has comparable growth to wild-type PAO1 in liquid LB without salt at 42°C.

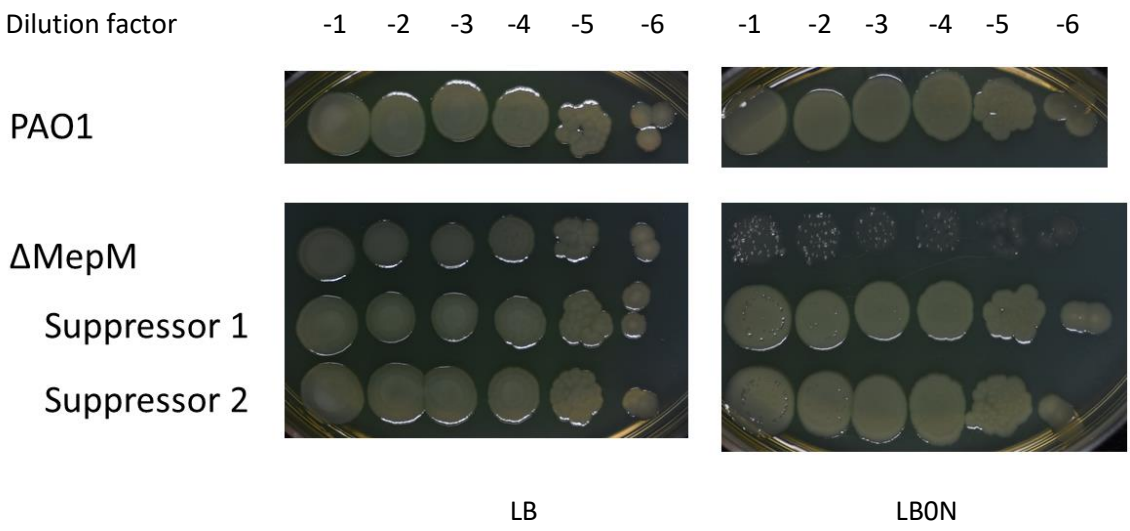

Figure 11. ΔMepM phenotype under high temperature and no salt can be at least partially suppressed by mutations. 10x spot dilutions were performed with dilution factors as labelled. Strains were grown for 43.5h on media as labelled at 42^{o}C.

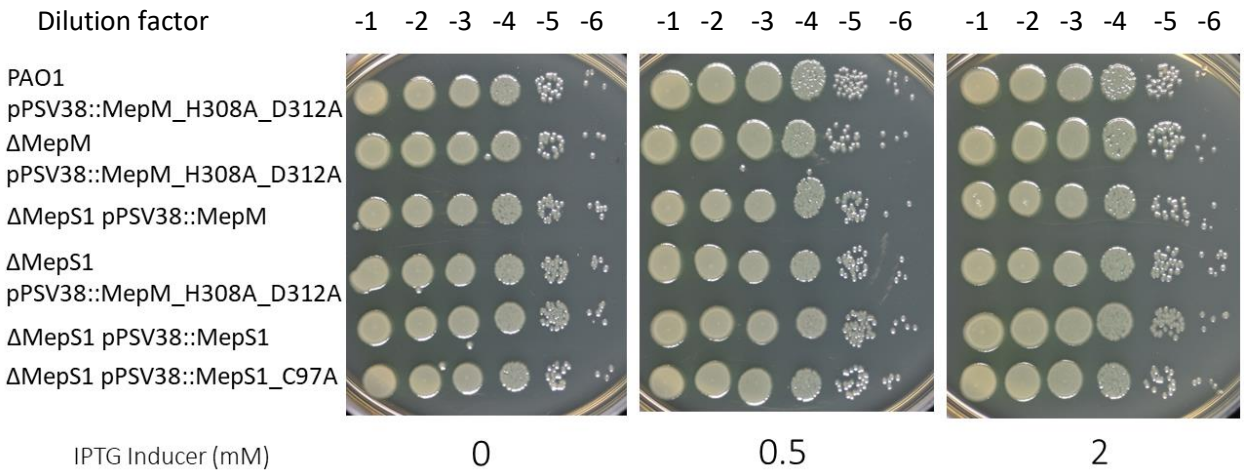

Figure 12. There is no dominant negative effect from overexpression of catalytically inactive MepM or MepS1. 10x spot dilutions were performed with dilution factors as labelled. Strains were grown at 37°C for 17h on LB with IPTG inducer as labelled.