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# Characterizing the role of the bacterial metallothionein, SmtA, in mammalian infection

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# **Characterizing the role of the bacterial metallothionein, SmtA, in mammalian infection**

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**ABSTRACT**

Mammalian metallothioneins (MT) are induced by various immunomodulatory molecules and are involved in a spectrum of immune processes such as essential metal homeostasis, detoxification of certain heavy metals, inflammation, and immune cell trafficking [1-3]. A bacterial metallothionein, SmtA, shares some sequence homology with mammalian MT as well as its metal-binding capabilities [4]. In addition to its ability to sequester heavy metals, eukaryotic MT has also been shown to scavenge free radicals such as reactive oxygen and nitrogen species (ROS, RNS), interfering with their toxic effects on cells and potentially influencing their regulatory roles in cell proliferation and differentiation [5, 6]. Over the course of a bacterial infection, innate immune cells undergo a respiratory burst where they release ROS as bactericides, during which mammalian MT protects the host cell against self-inflicted oxidative damage. We hypothesize that SmtA defends bacteria against inflammatory ROS in a manner similar to mammalian MT. We have shown (using transfected *Escherichia coli*) that SmtA provides protection to *E. coli* from some forms of oxidative stress and that SmtA can also protect *E. coli* when co-cultured with RAW264.7 macrophage cells. Further exploration will be necessary to determine whether SmtA provides protection against mammalian immune defense mechanisms *in vivo*. These initial *in vitro* results have provided motivation to continue these studies.

## INTRODUCTION

### *Immunomodulatory properties of mammalian MT*

Eukaryotic metallothioneins (MT) are highly conserved, small molecular weight proteins (~7kDa) rich in cysteine residues (Ca. 33 mol %). The cysteines present in MT provide the protein with many of its functional characteristics including the ability to bind heavy metals, scavenge reactive oxygen and nitrogen species (ROS, RNS), elicit chemotaxis, and influence immune cell trafficking. In mammalian cells, MT acts as a stress response protein and is induced by various immunomodulatory molecules such as divalent heavy metals, endotoxin, interferon, ROS, and RNS [7-12]. Its role as a stress response protein as well as its known interactions with various immune molecules indicate that MT is an important mediator of an effective immune response.

In total, four isotypes of mammalian metallothioneins have been identified, all of which are encoded in a gene cluster located on chromosome 8 in mice and chromosome 16 in humans [13]. MT-I and MT-II are widely expressed *in vivo* throughout development and throughout all cell types as well as in most cell lines. The remaining two isoforms, MT-III and MT-IV, have more restricted expression and are found predominantly in the brain (MT-III) or squamous epithelial cells (MT-IV) [14, 15].

MT is a major contributor in regulating essential metal homeostasis. Mammalian metallothioneins have been isolated naturally containing essential metals such as zinc and copper. The numerous thiol (SH) groups allow MT to bind 7-8 zinc and 9-11 copper ions [16]. Zinc is especially important to an effective immune response. In humans, zinc deficiency has been associated with thymic atrophy, decreased lymphoproliferation, decreased ratios of CD4+/CD8+ cells, decreased NK cell activity, and decreased monocyte cytotoxicity, all of which

result in diminished immune capacity [17]. Many studies have shown that MT regulates the amount of free zinc present in cells as well as the amount of zinc available for molecules important to immune function such as zinc-finger transcription factors, structural metalloproteins, and zinc-dependent apoenzyme [17, 18]. In this way, MT contributes to the effectiveness of the immune response by storing and providing elemental zinc to immune molecules requiring zinc for proper function.

MT may also contribute to the host's immune response during microbial infections by sequestering essential metals from infectious organisms. Bacteria incorporate zinc into a predicted 4-6% of their proteome. Zinc is required for bacterial gene expression, cellular metabolism, and as a cofactor of numerous virulence factors [19]. Therefore, it is no surprise that Zinc chelation and sequestration is one way host organisms have been shown to restrict infectious bacterial growth. For example, it has been shown that during infection zinc chelation by calprotectin (a host protein produced by neutrophils) inhibits the growth and survival of *Staphylococcus aureus* [2]. Aside from calprotectin, other studies have shown ZIP/Znt transporters are important in host zinc sequestration as well [19]. These results suggest metallothionein may also inhibit bacterial growth and aid the host's immune response during an infection by sequestering essential metals necessary for bacterial growth.

In addition to essential metals like zinc and copper, mammalian metallothioneins have been isolated naturally containing toxic heavy metals such as cadmium and mercury. MT also exhibits affinity for other reactive metal ions *in vitro* including Pb, Ni, Co, Fe, Ag, and Au [16, 20]. MT's ability to sequester cadmium and other toxic heavy metals has been shown to enhance cell survival by preventing toxic divalent metal cations from interacting with and damaging other parts of the cells. For instance, it has been shown that during cadmium exposure, MT binds

cadmium with high affinity, sequestering the toxic cationic cadmium in a CdMT complex that is relatively inert and prevents immediate cadmium cytotoxicity [16, 21].

Aside from its ability to bind metals, mammalian MT also participates in regulating the redox potential of cells. During times of oxidative stress, MT is able to scavenge free radicals such as ROS and RNS, interfering with their cytotoxicity [5, 6, 22]. In particular, MT has been shown to scavenge hydrogen peroxide, hydroxyl radicals, and nitric oxide [23-25]. In mammalian cells, free radicals are produced during both normal cellular activities and in the context of many pathological processes (Fig. 1). For instance, ROS are formed as byproducts during metabolism, from interaction with cationic Cd or other toxic heavy metals, and during inflammation.

During the course of a bacterial infection, innate immune cells undergo a respiratory burst in which they release bactericidal ROS into the extracellular space and phagocytic vesicles. Oxidant species known to be released during the respiratory bursts of macrophages and neutrophils include nitric oxide (NO), superoxide anions ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radicals ( $\cdot OH$ ), and hypochlorite ( $OCl^-$ ) [26]. Although ROS are released as bactericides, they can also cause unwanted damage to the host cell by leaking across host cell membranes. Mammalian MT, however, is able to scavenge and neutralize ROS in the cell, limiting oxidative damage to the host.

Three mechanisms operate in producing the respiratory burst. The first mechanism involves the NADPH oxidase located at the plasma membrane. The NADPH oxidase forms superoxide anions in a reaction with NADPH, molecular oxygen, and hydrogen ions. Superoxide radicals then form hydrogen peroxide in a secondary reaction, which can occur spontaneously or catalyzed by superoxide dismutase (SOD) (Fig. 2A). In a second mechanism, the products of the

NADPH oxidase system (e.g. superoxide anions and hydrogen peroxide) form the potent hydroxyl radical via the Haber-Weiss reaction (Fig. 2B). Finally, a third mechanism mediated by myeloperoxidase (MPO) results in the formation of hypochlorous acid (HOCl), which partially dissociates into hypochlorite radicals (Fig. 2C). The MPO enzyme is only present in granules of neutrophils and monocytes but disappears from differentiated macrophages. Although macrophages cannot synthesize MPO, they have been shown to take up neutrophil-generated MPO by endocytosis and phagocytosis. Together, these mechanisms account for the microbicidal release of ROS [27].

Mammalian MT expression is driven by a variety of cellular factors including the presence of metals ions and oxidative stress [28]. In the presence of zinc, the MRE-binding transcription factor 1 (MTF-1) binds to metal response elements (MREs) proximal to the MT promoter to initiate transcription. Upregulation of MT by other metals seems to be indirectly regulated. *In vitro*, the MT promoter (including relevant MREs and soluble MTF) is induced only in the presence of zinc; other metals do not initiate transcription. Upon introduction of ZnMT complexes, however, other metals are capable of activating transcription. This data supports the current hypothesis that one way metals other than zinc upregulate MT expression is by displacing zinc from the ZnMT complex, thereby releasing free zinc for MTF binding [29].

Similarly, an antioxidant response element (ARE) located upstream of the MT gene also participates in activating MT transcription in response to ROS [28]. In general, AREs positively regulate genes involved in the oxidative stress response such as superoxide dismutase, glutathione peroxidase, and glutathione-S-transferase [30]. Gene activation at the ARE occurs when the transcription factors, Nrf1 or Nrf2, are translocated to the nucleus in response to oxidative stress, subsequently binding to the ARE [31]. Mice deficient in Nrf1 or Nrf2 exhibited



severely decreased MT-1 gene expression (10-20% of wild-type) and MT-1 gene expression was undetectable in mice deficient in both Nrf1 and Nrf2, suggesting substantial involvement of Nrf1 and Nrf2 signaling at the MT promoter [32]. Furthermore, during oxidative stress, upregulation of MT is positively regulated by the release of MT-bound zinc, which acts at the MRE to additionally increase MT gene transcription. Studies suggest that other signal transduction cascades are also involved in MT expression [33].

*Bacterial metallothionein, SmtA, may share MT's immunomodulatory properties*

These observations regarding mammalian MT suggest a bacterial metallothionein, SmtA, may similarly influence the mammalian immune response. SmtA has been found in *Pseudomonas aeruginosa* and *Synechococcus* species where its role as a metal-binding protein is already established [34-36]. A sequence alignment between SmtA and mammalian MT shows possible alignments between various cysteine residues, which are known to be important in metal binding, as well as other sequence similarities (Fig. 3). In addition to cysteine motifs, it is thought that several histidine residues contribute to SmtA's ability to bind metals as well. A total of 8-9 cysteine and 3 histidine residues [37] allow SmtA to bind, on average, 3-4 zinc molecules [4, 38]. Further functional comparison of eukaryotic and prokaryotic metallothioneins is shown in Table 1.

SmtA is capable of binding heavy metals similar to the set known to bind mammalian MT (e.g. Zn, Hg, Cd, Cu, Co, Cr, and Ni) [39]. SmtA has been implicated in bacterial protection against either zinc or cadmium toxicity [34]. For example, *Synechococcus* SmtA mutants demonstrate a five-fold reduction in zinc toleration, which can be restored by exogenous recombinant SmtA [40]. SmtA mutants also demonstrate decreased accumulation of exogenous

zinc, suggesting SmtA functions in zinc sequestration and storage. This may indicate a selective advantage of SmtA-expressing cells in nutrient-deficient environments. Finally, SmtA-overexpressing cells do not demonstrate a detrimental phenotype, implying that SmtA in excess does not scavenge Zn or other essential ions from requiring proteins [41].

Like mammalian MT, SmtA is inducible in the presence of heavy metals. In contrast, however, SmtA expression is under a form of negative regulation. Its expression is controlled by the metalloregulatory protein SmtB, which normally binds the SmtA operator-promoter region in the absence of zinc, preventing transcription of the SmtA gene (Fig. 4). At low concentrations, active SmtB (apo) binds the operator-promoter site as a dimer. At higher concentrations, up to four SmtB molecules can bind the operator-promoter at one time, forming a tetrameric complex that physically hinders RNAP binding (Fig. 5) [42]. Similar to the regulation of mammalian MT, only zinc is capable of binding SmtB, yet other metals are known to activate SmtA expression. A similar mechanism to that proposed for eukaryotic MT expression by metals other than zinc has been suggested in the prokaryotic system [35]. Other factors contributing to SmtA expression have not yet been explored. It is likely, however, considering the large spectrum of molecules influencing mammalian MT expression that SmtA expression is also subject to additional modes of transcriptional activation or inhibition.

SmtA is known to share mammalian MT's metal-binding capabilities, and its induction through the metallo-regulated SmtB pathway is well understood. There is a lack of research, however, exploring the possibility of other roles for SmtA. Mammalian MT's ability to scavenge ROS is important to its interaction with the immune response, and if this property of MT were shared with its bacterial homolog, we hypothesize that SmtA could represent a virulence factor in specific bacterial pathogens.

Mammalian MT's ability to bind and neutralize ROS protects host immune cells from self-inflicted oxidative damage. Because SmtA shares cysteine motifs with MT, it may be able to scavenge ROS in a similar manner and protect bacteria from ROS during infection. Protection from oxidative stress could aid in bacterial survival and increase the virulence of an SmtA-expressing bacterium, such as *Pseudomonas aeruginosa*. *P. aeruginosa* infections commonly occur in the airways of immunocompromised individuals and account for 10.1% of hospital-acquired infections [43]. If SmtA indeed influences bacterial resistance to immune attack, it may represent a valuable target for therapeutic intervention.

Various microbial strategies exist to evade ROS-dependent killing. For example, *Staphylococcus aureus* exploits endogenous catalase levels in order to resist bactericidal oxidative damage (catalase neutralizes hydrogen peroxide by catalyzing its decomposition to water and molecular oxygen). Mandell et. al. (1979) demonstrated that *S. aureus* strains with high catalase levels were significantly more resistant to neutrophil-mediated killing and significantly more virulent in their mouse model when compared to *S. aureus* with low catalase levels [27]. Similar results were shown using *Listeria monocytogenes* [27]. We suspect, like catalase, high SmtA levels may increase bacterial resistance to ROS-mediated killing by neutralizing ROS such as hydrogen peroxide.

Further support that a bacterial metallothionein like SmtA may function in pathogenesis comes from the pathogen-host relationship between *Magnaporthe grisea*, a type of fungus, and *Oryza sativa* (rice). *M. grisea* infects rice plants by forming specialized structures, appressoria, that rupture the plant cuticle and allow entry of the infection. During infection, a Type I rice metallothionein expressed constitutively in healthy plants shows significant upregulation [44]. A separate study examining fungal pathogenicity implicated a *M. grisea* metallothionein, MMT1,

in functional appressoria formation [45]. *M. grisea* MMT1 mutants were unable to undergo appressoria-mediated penetration and failed to cause disease in rice plants. Reintroducing a functional MMT1 gene restored penetration and subsequent pathogenicity. This suggests that metallothioneins participate in host defense mechanisms and as pathogen virulence factors.

One source of oxidative attack against which SmtA might offer protection is the respiratory burst of innate immune cells. In addition to the concentrated release of ROS by host macrophage and neutrophils, airway pathogens such as *P. aeruginosa* are also subject to atmospheric concentrations of molecular oxygen, creating a relatively high oxidative environment. A further context in which the ability to bind and neutralize ROS would be beneficial to *Pseudomonas* is during biofilm formation. Studies have shown that biofilms form more readily in anaerobic microenvironments and that ROS can be used to disperse already formed biofilms in *Pseudomonas aeruginosa* culture [46]. Biofilms often exacerbate mammalian infections by increasing bacterial resistance to antibiotics. Antibiotics promoting the formation of ROS, however, have been shown effective in inhibiting the development of *E. coli* biofilms [47]. Thus, if able to scavenge ROS as we hypothesize, SmtA may play a role in promoting biofilm formation.

Given the sequence similarities (Fig. 1) and shared properties between eukaryotic and prokaryotic metallothioneins (Table 1), it is likely that SmtA shares properties with mammalian MT that have not yet been explored. The role of SmtA in metal sequestration has been well documented; however, its ability to scavenge and neutralize ROS is unknown. Our research is designed to establish whether or not SmtA has the capacity to bind and neutralize ROS and to explore whether the presence of SmtA is a factor in bacterial survival during infection. Our hypothesis is that SmtA binds and neutralizes ROS, protecting bacteria from oxidative stress

elicited during the inflammatory response and contributing to bacterial survival during the respiratory burst of innate immune cells.

## MATERIALS AND METHODS

### *Materials.*

Hydrogen peroxide (30%) was purchased from Fisher Scientific Inc. (Pittsburgh, PA). Tert-butyl hydroperoxide (70%), luminol, ampicillin, glutathione (GSH), and catalase were purchased from Sigma-Aldrich (St. Louis, MO). Sodium hypochlorite (2.5% w/w) was purchased from Ricca Chemical Company (Arlington, TX). Clear and white 96-well culture-treated plates were purchased from Nalge Nunc International (Rochester, NY). Black 96-well Microflour-2 plates were purchased from ThermoLabsystems (Franklin, MA). Hank's balanced salt solution (HBSS) was purchased from Thermo Scientific Hyclone (Logan, UT). Horseradish peroxidase (HRP) was purchased from Thermo Fisher Scientific (Waltham, MA). Isopropyl- $\beta$ -D-thiogalactoside (IPTG) was purchased from Gold Biotechnology (St. Louis, MO). N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM) was purchased from Invitrogen (Carlsbad, CA).

### *Cells.*

*E. coli* MC1061 cells (ATCC 53338) were cultured in YTA media. RAW267.4 cells (ATCC TIB-71) are a mouse macrophage cell line and were cultured in RPMI media supplemented with 1% penicillin-streptomycin, 25mM Hepes, 4mM L-glutathione, and 10% fetal bovine serum (FBS).

### *Plasmid construction and transformation of MC1061 E. coli.*

The *Pseudomonas aeruginosa* SmtA gene (YP\_791105) was cloned into the pGEX-6P-1 expression vector (Amersham) between the EcoRI and XhoI sites (pGEX-SMTA) (D. Laukens, U. Ghent) (Fig. 6). MC1061 *E. coli* + pGEX-SMTA were generously provided by D. Laukens

(sequence verified). MC1061 *E. coli* was transfected with the pGEX-6P-1 empty vector using the calcium chloride procedure and transfected cells were selected by plating on YTA plates containing 100ug/ml ampicillin. Successful transformation was verified by isolating the plasmid DNA from a 24-hour culture of MC1061 + pGEX-6P-1 according to Maniatis et. al. 1982. The miniprep was analyzed by gel electrophoresis on a 1% agarose gel containing 0.5ug/ml ethidium bromide (Figure 6). MC1061 + pGEX-SMTA and MC1061 provided by D. Laukens were analyzed as well. Under IPTG induction, the transfected *E. coli* express either a GST-tagged SmtA protein or GST (glutathione S-transferase) alone.

*In vitro exogenous oxidant exposure.*

The MC1061+pGEX-SMTA and MC1061+pGEX-6P-1 *E. coli* were subcultured from overnight cultures to log phase (0.2 OD<sub>600</sub>) in YTA media containing ampicillin (100ug/ml) and IPTG (100uM). The cultures were then exposed to the various concentrations of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), tert-butyl hydroperoxide (tBOOH), or sodium hypochlorite (NaOCl) ranging from 3mM to 15mM, aliquoted into 96-well plates, and maintained at 37°C with constant shaking. Survival was monitored at OD<sub>600</sub> and represented graphically as percent absorbance of untreated control. Samples were taken at 60 minutes post treatment and plated on YTA plates supplemented with ampicillin. Plates were then incubated inverted overnight at 37°C to determine CFU/ml.

*Infection of RAW267.4 macrophage.*

*E. coli* MC1061 expressing either plasmid construct were grown overnight and subcultured in rich media to log phase (0.2 OD<sub>600</sub>) with IPTG (100uM) and ampicillin (100ug/ml). The RAW264.7 cells were grown 72 hours, plated in 6 well plates, and allowed to adhere 2.5 hours in

5% CO<sub>2</sub>. The RAW264.7 cells were then infected with *E. coli* at an MOI of 5:1 and incubated in 10% CO<sub>2</sub> and 7% O<sub>2</sub> at 37°C. The supernatant was removed after 30 minutes and after 24 hours and plated on YTA plates supplemented with ampicillin and IPTG to determine CFU/ml.

#### *Long term MC1061 E. coli culture*

MC1061 *E. coli* cultures (Parent MC1061 *E. coli*, MC1061 + pGEX-SmtA, and MC1061 + pGEX-6p-1) were subcultured from overnight cultures in YTA media and allowed to grow at 37° under constant shaking. MC1061 + pGEX-SmtA and MC1061 + pGEX-6p-1 cultures were grown in media containing ampicillin (100ug/ml) and IPTG (100uM). The MC1061 parent strain was grown in media supplemented with IPTG (100uM). At the time points indicated, a sample of each culture was plated on YTA plates to determine CFU/ml. MC1061 cells were plated on YTA plates supplemented with IPTG and MC1061 + pGEX-SmtA or + pGEX-6p-1 cells were plated on YTA plates supplemented with ampicillin and IPTG.

#### *Luminol assay.*

GSH (0.32-10mM) and catalase (20U/well) were prepared in HBSS. Samples were exposed to 1mM H<sub>2</sub>O<sub>2</sub> in HBSS for 30 minutes at room temperature. The redox potential of each solution was then determined using a working solution of 0.1mM luminol and 50ug of HRP. Samples were aliquoted into a white 96-well plate and the luminescence immediately read at 420nm. The value of the blank (HBSS and luminol-HRP working solution) was subtracted from each well.



*CPM assay.*

Stock solutions of GSH and N-acetyl-L-cysteine (10mM) were freshly prepared in PBS and then diluted as needed. A stock and working solution of CPM was prepared as previously described by Ayers et. al. 1986 [48]. Sample solutions of GSH and N-acetyl-L-cysteine were prepared to yield 0.1-0.4nmol/well. The CPM working solution and sample solutions were added to a black Microflour-2 96-well plate in triplicate in a 1:1 ratio (100ul of CPM working solution and 100ul of sample solution). The plate was incubated in the dark at room temperature for 1 hour and then read immediately with a excitation wavelength of 387nm and an emission wavelength of 465nm. The value of the blank (100ul of CPM working solution and 100ul of PBS) was subtracted from each well.

*Statistics.*

The results for in vitro exogenous oxidant exposure and infection of RAW 267.4 macrophages were analyzed using Student's *t*-test. Where experiments were done in triplicate standard deviation values are included.

## RESULTS

### *In vitro exposure to exogenous oxidants*

MC1061 *E. coli* were transfected with a pGEX-6p-1 plasmid containing the *P. aeruginosa* SmtA gene under IPTG induction (pGEX-SMTA). A control pGEX-6P-1 plasmid (empty vector) was transfected into the same strain of *E. coli* for use as a negative control. In the presence of IPTG, the pGEX-SMTA plasmid expresses a GST-tagged SmtA protein while the pGEX-6P-1 plasmid expresses only the GST protein. Successful transformation was confirmed by isolating bacterial DNA from 24-hour cultures and analyzing the miniprep by gel electrophoresis on a 1% agarose gel containing 0.5ug/ml ethidium bromide (Fig. 7). The pGEX-SMTA and pGEX-6P-1 plasmids ran at the expected molecular weights, with the pGEX-SMTA band shifted slightly upward due to the additional 242 base pairs encoding the SmtA gene.

Bacteria expressing either the pGEX-SMTA or pGEX-6P-1 construct were sub-cultured from IPTG-induced overnight cultures in a rich media to early log phase (0.2 OD<sub>600</sub>) and treated with multiple concentrations of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), tert-butyl hydroperoxide (tBOOH), or sodium hypochlorite (NaOCl) as a source of oxidative stress. Both H<sub>2</sub>O<sub>2</sub> and tBOOH have been used previously in the literature as models of cellular oxidative stress [49]. We included NaOCl as well since hypochlorite radicals are present in the respiratory bursts of innate immune cells [26]. Cultures were aliquoted in triplicate into 96-well plates and bacteria were monitored at OD<sub>600</sub> every 30 minutes for 3 hours. Survival of the bacteria was expressed as percent of untreated controls.

When treated with H<sub>2</sub>O<sub>2</sub> at a range of concentrations, both SmtA-containing and SmtA-deficient strains showed a dose-dependent response. As the concentration of H<sub>2</sub>O<sub>2</sub> increased, the bacteria were less able to survive and grow. At each concentration of H<sub>2</sub>O<sub>2</sub>, the SmtA-expressing

*E. coli* were better able to survive the treatment than the control strain lacking SmtA (Fig. 8). Because the only difference between the two strains is the inducible SmtA gene present in the pGEX-6p-1 plasmid of the SmtA-expressing bacteria, the resistance towards H<sub>2</sub>O<sub>2</sub> was concluded to be due to the presence of SmtA.

In addition to using H<sub>2</sub>O<sub>2</sub> as a source of oxidative stress, parallel experiments were completed using two other oxidants, tBOOH and NaOCl, in order to further test the anti-oxidant capacity of SmtA. Testing multiple oxidants provides a more complete understanding of the extent to which SmtA offers protection to the bacteria. Unlike the effect observed after H<sub>2</sub>O<sub>2</sub> exposure, we did not find a significant difference between the responses of SmtA-expressing and SmtA-deficient bacteria when treated with either tBOOH or NaOCl (Fig. 8). This data suggests that the anti-oxidant protective effect of SmtA is not universal.

The pattern of bacterial survival when treated with tBOOH was strikingly different than the pattern of survival observed when treated with H<sub>2</sub>O<sub>2</sub> or NaOCl. When treated with H<sub>2</sub>O<sub>2</sub> or NaOCl, the bacteria exhibited a dose-dependent response to the oxidant. In contrast, when the bacteria were treated with tBOOH we did not observe a dose-dependent response; rather, the bacteria exhibited the same degree of survival at every concentration of tBOOH tested (final concentrations of 3-15mM) (Fig. 8).

To ensure that absorbance was representative of bacterial survival, we repeated the exposure to 10mM H<sub>2</sub>O<sub>2</sub> and measured viable bacteria by plating and counting colonies (CFU/ml). The bacteria expressing each plasmid construct were cultured and then treated with 10mM H<sub>2</sub>O<sub>2</sub> according to identical protocols. At 60 minutes post treatment, a sample was taken from both cultures (MC1061 + pGEX-SmtA and MC1061 + pGEX-6p-1) and plated to determine CFU/ml. We found that at 60 minutes post treatment with 10mM H<sub>2</sub>O<sub>2</sub>, the SmtA-

expressing bacteria were better able to survive and grow than the SmtA-deficient bacteria, supporting the results that relied on OD<sub>600</sub> as an indicator of bacterial survival (Fig. 9).

#### *In vitro infection of RAW 267.4 macrophages*

We were able to show that SmtA protects *E. coli* from exogenous H<sub>2</sub>O<sub>2</sub>. We also wanted to determine whether SmtA enhances the survival of *E. coli* when challenged with ROS released during the respiratory burst of macrophages. To do so, we measured the survival of *E. coli* MC1061 containing each plasmid construct after infection of RAW 267.4 murine macrophages. Macrophages are one of the first types of immune cells present at an infection site, and upon exposure to endotoxin, macrophages are known to undergo a respiratory burst during which ROS are released as bactericides [26].

Bacteria expressing each plasmid construct were grown overnight and subcultured to early log phase (0.2 OD<sub>600</sub>) in YTA media. RAW 267.4 cells were grown to confluency in 6-well plates, and infected with the *E. coli* at a 5:1 multiplicity of infection (MOI). After 30 minutes of infection, the supernatant was removed and plated on YTA plates to enumerate surviving bacteria. Surviving cell numbers were also determined after 24 hours of infection according to the same protocol. By taking our sample from the supernatant we assayed the survival and growth of extracellular bacteria exposed to the respiratory burst. We did not, however, determine the survival of intracellular bacteria that had been phagocytosed by the macrophage. The numbers of viable SmtA-expressing bacteria were larger than SmtA-deficient bacteria at 30 minutes and 24 hours post infection (Fig. 10). This data suggests that SmtA confers a survival advantage to *E. coli* in the oxidative environment produced during the respiratory burst of macrophages.

*Long-term culture*

In the previous experiments, we were most interested in how the presence of SmtA affects bacteria exposed to high oxidative stress environments (e.g. concentrated exogenous oxidant, respiratory burst of macrophage). In normal culture conditions, however, bacteria are constantly exposed to oxidative stress by way of atmospheric oxygen. Although less of an immediate danger to cells than the concentrated release of ROS during the innate immune response, atmospheric oxygen may be a factor in bacterial survival during long-term culture conditions. If so, the presence of SmtA, either exogenous or intracellular, may ameliorate gradual oxidative damage to cells, thereby increasing the survival of bacteria in long-term culture.

Wildtype MC1061, MC1061 + pGEX-SmtA, and MC1061 + pGEX-6p-1 *E. coli* were subcultured from overnight cultures in rich media and allowed to grow at 37° under constant shaking. Every 24 hours for 6 days, a sample of each culture was plated on YTA plates to determine CFU/ml. Preliminary results indicate a slight advantage from 72 hours on in SmtA-expressing bacteria when compared to SmtA-deficient bacteria (Fig. 11). Initially, however, bacteria expressing the pGEX-6p-1 plasmid were greater in number than wildtype MC1061 or SmtA-expressing bacteria. The advantage over wildtype MC1061 may be due to some factor inherent in the pGEX-6p-1 vector. The initial advantage over SmtA-expressing cells could be explained by protein burden: SmtA-expressing bacteria grow more slowly than the SmtA-deficient bacteria carrying the pGEX-6p-1 plasmid because additional protein (e.g. SmtA) must be transcribed and translated, which requires both time and energy. These preliminary results are interesting but must be repeated with replicate cultures.

## DISCUSSION

### *SmtA binds and neutralizes ROS*

SmtA-expressing MC1061 *E. coli* demonstrated increased survival and growth compared to MC1061 *E. coli* deficient in SmtA when exposed to exogenous H<sub>2</sub>O<sub>2</sub>. When treated with other ROS (e.g. tBOOH and NaOCl), we did not observe increased survival in SmtA-expressing bacteria. This suggests that SmtA plays a role in defending bacteria against ROS, but this protective effect is not universal to all forms of oxidative stress. This is not surprising, as it has been shown previously that mammalian MT scavenges certain oxidants, such as hydroxyl radicals, more efficiently than others. For instance, MT's ability to scavenge hydroxyl radicals is three hundred times that of GSH, the most abundant antioxidant present in cells. GSH, however, is better able to scavenge superoxide radicals [24]. Additional oxidants to be tested include superoxide anions and nitric oxide in order to further profile the scavenging potential of SmtA.

We were also able to show that SmtA-expressing bacteria are better able to survive and grow during RAW 267.4 macrophage infection. This evidence establishes further support for SmtA as a bacterial antioxidant. Macrophages are one of the first types of immune cells to respond to an infection. In addition to their phagocytic activity, macrophages undergo a respiratory burst during which various ROS are released into the extracellular environment as bactericides. By determining the survival and growth only of bacteria recovered from the supernatant, we limited our analysis to extracellular bacteria exposed to the respiratory burst. At this time, we do not know whether SmtA affects the effectiveness of phagocytosis or the survival of bacterial when phagocytosed. Because the phagosome also contains a high concentration of ROS, the presence of SmtA may increase the survival of phagocytosed bacteria as well, providing a further advantage to SmtA-expressing bacterium during infection.

From these initial experiments, it appears that SmtA shares eukaryotic MT's ROS scavenging capabilities in addition to its already established metal-binding function.

Demonstrating the antioxidant capacity of soluble SmtA would provide further evidence that the protective effect of SmtA is due to its ability to bind and neutralize ROS. We have begun to purify the GST-SmtA fusion protein from the MC1061 + pGEX-SMTA *E. coli* using GSH resin. Once purified, the GST tag can be removed from the SmtA protein using a GST precision protease.

Various biochemical properties could be explored with an available stock of soluble recombinant SmtA. For instance, if our hypothesis that SmtA binds and neutralizes ROS is valid, incubating different concentrations of soluble SmtA with H<sub>2</sub>O<sub>2</sub> should elicit a dose-dependent decrease in oxidant activity. ROS can be detected in solution using an enhanced luminol-based chemiluminescence assay [50]. Oxidation of luminol upon exposure to ROS such as H<sub>2</sub>O<sub>2</sub> results in chemiluminescence that is proportional to the amount of ROS in solution. The enhanced assay includes horseradish peroxidase (HRP) to increase sensitivity. To show this approach would be appropriate for use with SmtA, we optimized the assay using GSH. Different concentrations of GSH were incubated with 1mM H<sub>2</sub>O<sub>2</sub> in HBSS for 30 minutes. A working solution of luminol-HRP was added and samples were aliquoted into a 96-well white plate. Luminescence was immediately read at 420nm. As the concentration of GSH increased, less oxidation was detected (Fig. 12). Catalase, which catalyzes the decomposition of H<sub>2</sub>O<sub>2</sub>, was included in the experiment as a positive control. GSH, in the absence of H<sub>2</sub>O<sub>2</sub>, did not elicit chemiluminescence. These results indicate that the enhanced luminol-based chemiluminescence assay should be appropriate in analyzing the scavenging activity of SmtA.

In addition to showing that soluble SmtA is capable of neutralizing ROS in the absence of other cell material using luminol chemiluminescence, we would also like to quantify and compare the number of reactive thiols found in SmtA using the thiol-reactive probe, N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM) [48]. Similar to the enhanced luminol-based chemiluminescence assay, we first optimized the CPM assay using GSH, which contains one thiol group per molecule of GSH. In addition to GSH, we included N-acetyl-L-cysteine, which also contains one thiol per molecule. Our results corroborated those found previously, demonstrating a linear relationship between CPM reactivity and moles of GSH (Fig. 13). As expected, CPM reactivity with N-acetyl-L-cysteine was similar to that of GSH with no statistical significance between GSH and N-acetyl-L-cysteine samples at each concentration tested. Upon obtaining purified soluble SmtA, the CPM assay may be repeated to determine the number of reactive thiols per molecule of SmtA using a GSH standard curve.

#### *SmtA gene regulation*

We show here that SmtA protects transgenic *E. coli* from oxidative attack. Because our data suggests SmtA is beneficial to the organism during periods of oxidative stress, we hypothesize there may be a mechanism by which SmtA expression is increased by ROS signaling. The mammalian MT locus contains an ARE necessary for ROS-activated gene transcription. In addition, ZnMT complexes are known to release bound zinc in order to bind ROS, increasing cellular zinc levels available to act at the metal response element (MRE). Similar mechanisms may be at work in the prokaryotic system. If gene expression were to increase in response to oxidative stress, SmtA's role in protecting against ROS would be positively amplified.



*SmtA and chemotaxis*

Our research thus far has been focused on the interaction between SmtA and ROS released during the inflammatory response because the literature shows this is an important function of mammalian MT and an area of research that has not yet been addressed concerning bacterial metallothioneins. An additional immunomodulatory function of MT that SmtA may share is the ability to induce chemotaxis and influence immune cell trafficking.

MT has been found extracellularly in numerous circumstances, including in culture media, serum, urine, bronchoalveolar fluid, liver sinusoids, inflammatory lesions, and in other secretions and excretions [51-58]. Classical protein secretion occurs through ER targeting of a nascent polypeptide, which requires a N-terminal signal sequence. MT does not contain a signal sequence necessary for this type of classical secretion. With a growing repertoire of selectively secreted molecules that lack a signal sequence, several non-classical mechanisms have been suggested in the literature by which these molecules are released extracellularly. For example, both IL-1 $\alpha$  and IL-1 $\beta$  are associated with the cellular stress response and are thought to be secreted non-classically through interaction with specialized endolysosomes [59]. As yet, there is no conclusive evidence that MT is secreted selectively rather than as a byproduct of membrane compromise or necrotic cell death. MT does, however, share similarities with other proteins known to be secreted by non-classical pathways.

It is likely that MT has the same functional properties in the extracellular environment as it does inside the cell, namely its ability to bind heavy metals and free radicals due to its thiol-rich nature. More interestingly, however, there is evidence suggesting extracellular MT may have additional characteristics outside of cells that allow it to function as a chemotactic factor and influence immune cell trafficking. The primary amino acid sequence of MT contains

cysteines arranged in C, C-C, C-X-C, and C-X<sub>3</sub>-C motifs [13]. These motifs are characteristic of chemotactic cytokines and are used to differentiate between members of the chemokine superfamily [60]. In addition, the location of the MT gene cluster in both humans and mice is mapped closely to that of other chemokines such as CCL17 and CX3CL1 (Fig. 14). These observations suggest MT may display chemotactic activity [13].

Experiments done *in vitro* using the ECIS/taxis system show that Jurkat T cells and WBC 264-9C cells (macrophage-like cell line) chemotax in the presence of an MT gradient. This chemotactic response was blocked by treatment with monoclonal anti-MT antibodies. Chemotaxis was also blocked when the cells were first incubated with cholera toxin or pertussis toxin, indicating MT may elicit a chemotactic response via G-protein coupled receptor signaling [13]. It is well established that many chemokine receptors belong to a family of 7-transmembrane-spanning G-protein coupled receptors, providing further support for MT as a chemotactic factor [60].

Data indicating MT exists selectively outside of cells and acts as a chemotactic factor lead us to hypothesize that SmtA plays a similar role in cell trafficking. Like MT, SmtA does not carry an N-terminal signal sequence and may be secreted by a non-classical pathway. SecretomeP is a sequence-based prediction module for non-classically secreted mammalian and bacterial proteins [61]. After entering a protein sequence and indicating whether it is a mammalian sequence, gram-negative, or gram-positive bacterial sequence, the module gives the protein a SecP score. A SecP score above 0.5 indicates possible non-classical secretion. Analysis of the *P. aeruginosa* SmtA protein sequence by SecretomeP indicates it is likely to be non-classically secreted (Table 2). In addition to SmtA, we analyzed the protein sequences of the mammalian metallothionein, MT-1F, as well as *Mycobacterium tuberculosis* glutamine

synthetase (GlnA) and *Bacillus subtilis* superoxide dismutase (SodA), both of which are known to exist extracellularly and thought to exit the cell by a non-classical secretion pathway. *P. aeruginosa* RNA polymerase (RNAP) was included in the analysis as a negative control. If found extracellularly during infection and shown capable of inducing immune cell chemotaxis, SmtA may constitute a large and intricate role in bacterial pathogenesis.

SmtA as a chemotactic factor also has implications in the “danger signal” hypothesis. Studies suggest that stress response proteins such as MT can act as “danger signals”, alerting the immune response to action. The hypothesis proposes that sometimes non-self motifs will not elicit an effective response unless in the presence of tissue damage or other types of stress [62]. MT signals and responds to various types of stress (i.e. metal toxicity, oxidative stress, infection, etc.) and has been suggested to act as a danger signal [63]. From an evolution standpoint, MT may act as a danger signal because of its similarities to SmtA, which we have hypothesized may be secreted upon infection. The immune system may recognize similarities in mammalian MT to bacterial metallothioneins, falsely identifying metallothioneins as additional motifs of infection.

#### *SmtA in Pseudomonas infection*

We have shown that *E. coli* transformed with the *P. aeruginosa smtA* gene is resistant to some forms of oxidative attack. Further exploration is necessary to determine whether SmtA provides protection against mammalian immune defense mechanisms *in vivo*. These initial results, however, have provided promising incentive to continue these studies and move into a model system more similar to the natural infection.

*E. coli* do not naturally express SmtA, but the data suggests that the presence of SmtA is beneficial to the bacterium when challenged with ROS. Because the transfected *smtA* gene was

isolated from *P. aeruginosa*, the data also suggests that SmtA will have a protective effect against ROS attack in *P. aeruginosa* infection. *P. aeruginosa* are subject to a variety of oxidative assaults. As an airway pathogen, *P. aeruginosa* is exposed to high concentrations of atmospheric oxygen and must survive the respiratory bursts of alveolar macrophages. We have shown that SmtA protects MC1061 *E. coli* from the respiratory burst of RAW 267.4 macrophages. Similarly, we hypothesize that in an infection of alveolar macrophages, *P. aeruginosa* expressing SmtA will be better equipped for survival than *P. aeruginosa* deficient in SmtA.

*Pseudomonas* is also a biofilm-forming organism, and it is clear that biofilm formation is responsible for many of the antibiotic-resistant characteristics of *P. aeruginosa* infections. Barraud et. al. (2006) demonstrated that one form of ROS in particular, NO, induces biofilm dispersal in *P. aeruginosa* [46]. Although we did not test SmtA's ability to bind and neutralize NO specifically, we expect that SmtA is also capable of neutralizing this species of RNS. In addition, other types of oxidant species, such as H<sub>2</sub>O<sub>2</sub>, which we have shown to be neutralized by SmtA, may be capable of damaging or inhibiting the formation of pathogenic *P. aeruginosa* biofilms as well. If either were true, SmtA would further aid in the survival and persistence of *P. aeruginosa* by promoting biofilm formation.

#### *Proposed Pseudomonas aeruginosa system*

In our *E. coli* model, we utilized two constructs of the pGEX-6P-1 plasmid, one containing the *smtA* gene and a second identical to the first but without the *smtA* gene insert. We have already shown that *P. aeruginosa* SmtA provides protection to *E. coli* when challenged with H<sub>2</sub>O<sub>2</sub>. Moving to *P. aeruginosa* as a model organism, we would like to take advantage of the bacteria's

naturally present SmtA and compare its function at biological levels to *P. aeruginosa* experimentally lacking SmtA.

To assess the role of SmtA, future experiments should compare wildtype *P. aeruginosa* with *P. aeruginosa* treated with small interfering RNA (siRNA) to prevent the translation of the SmtA protein. The RNAi pathway occurs naturally in many eukaryotic cells and functions to regulate gene expression post-transcriptionally. Small interfering RNAs (siRNA) are short double-stranded RNA molecules with two nucleotide-long 3' overhangs. In the RNAi pathway, a single stranded siRNA assembles with Slicer, a RNase-like protein, to form an RNA-induced silencing complex (RISC). RISC then binds to mRNA sequences complementary to the bound siRNA sequence. Once bound, Slicer cleaves the target mRNA, which is now recognized by the cell as aberrant and is destroyed. Ideally, treatment with gene-specific siRNAs prevents translation of the target protein without many of the off-target effects experienced with gene knockouts.

If SmtA provides protection to *P. aeruginosa* during mammalian infection, targeting SmtA with siRNA may be applicable to treatment of *Pseudomonas* infections. The idea of RNA interference (RNAi) as a treatment for disease would not be novel to our studies; however, it is an innovative approach and constitutes a popular area of research that holds valuable therapeutic promise. Recently, RNA interference was highlighted as a treatment for HIV in humanized mice by blocking expression of CCR5 [64]. This research, along with other similar studies, provides the rationale for the use of RNA interference in our own research.

*Pseudomonas* infections treated with siRNA may stop the production of SmtA and render the organism less able to cause disease than *Pseudomonas* actively producing SmtA.

*Pseudomonas* infections often occur due to a temporary decrease in immune efficacy or

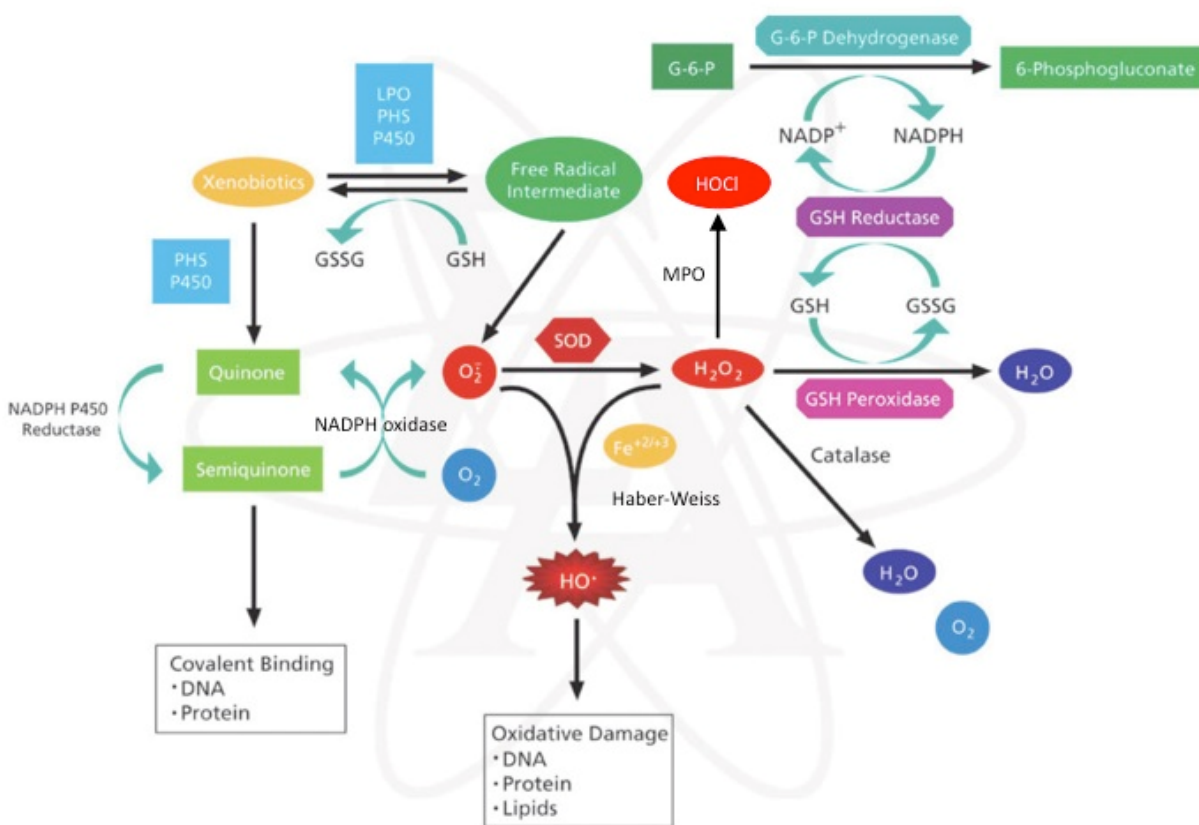
temporary damage to airway barriers. Typical treatment includes two antibiotics in combination: an anti-pseudomonal beta-lactam and an aminoglycoside. Beta-lactam antibiotics inhibit cell wall synthesis [65], while aminoglycoside antibiotics inhibit general bacterial protein synthesis [66]. *Pseudomonas* infections, however, have a tendency towards drug resistance, demonstrating a need for alternative therapies [67]. Developing a drug that would keep an infection under control until immune function is restored or until the airway repairs itself would allow the body, once healthy, to fight off the infection as it would normally in a healthy individual without the use of antibiotics. This would prevent multiple drug resistance infections and other undesirable side effects of antibiotic use. If SmtA proves to be a significant factor in bacterial defense against host immune mechanisms, therapeutic manipulation of SmtA may be a possible avenue of co-treatment for patients with *P. aeruginosa* infection. In addition, the experimental procedure, if successful, would indicate the ability to halt the production of SmtA with siRNA, providing a specific method of therapeutic intervention that could be explored further. Ultimately, our research may elucidate a new opportunity to fight *Pseudomonas* infection.

**Table 1.**

|                          | SmtA    | Mammalian MT |
|--------------------------|---------|--------------|
| Molecular weight (kDa)   | 8       | 7            |
| # amino acids            | 56-79   | 60-61        |
| # cysteines (histidines) | 8-9 (3) | 18-23        |
| # bound zinc             | 3-4     | 7-8          |
| Specificity to UC1MT     | +       | +            |
| Specificity to UC2       | -       | +            |

**TABLE 1. Comparison of *Pseudomonas aeruginosa* SmtA and eukaryotic MT.** It has been previously determined that SmtA binds less zinc molecules than MT and uses both cysteine and histidine residues to capture heavy metals. An immunodot blot was used to test the antigenicity of GST-SmtA extracts to a mammalian MT specific antibody, UC1MT, and UC2, an antibody known to bind MT but has not been tested for specificity to other metalloproteins. MT-I was used as a control in these experiments. The (+) indicates binding while the (-) indicates a failure to bind.

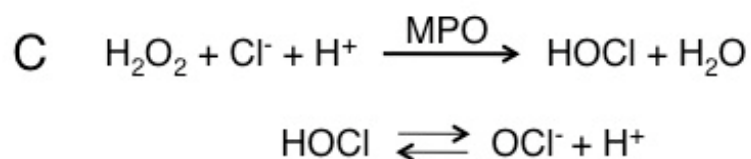
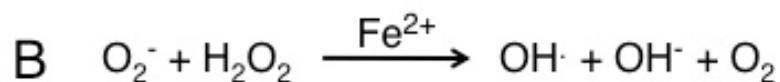
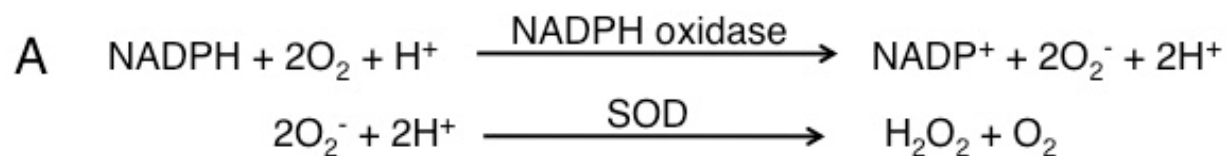
Figure 1.



**FIGURE 1. ROS and mammalian antioxidants.** In mammalian cells, ROS are produced during normal physiological processes (e.g. cellular respiration) and during disease states (e.g. inflammation). Under normal conditions, ROS are scavenged by superoxide dismutase (SOD), catalase, and glutathione (GSH) peroxidase. Oxidative stress occurs when there is an increase in oxidant species, a decrease in anti-oxidant activity, or when oxidative damage is not repaired. (Graphic provided by Sigma-Aldrich)



Figure 2.



**FIGURE 2. Respiratory burst enzymatics.** (A) NADPH oxidase catalyzes the formation of superoxide anions ( $\text{O}_2^-$ ). Superoxide anions can then react with hydrogen ions spontaneously or catalyzed by superoxide dismutase (SOD) to form hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). (B) In the Haber-Weiss reaction, superoxide anions react with hydrogen peroxide to form the potent hydroxyl radical ( $\text{OH}\cdot$ ). (C) Myeloperoxidase (MPO) catalyzes the formation of hypochlorous acid ( $\text{HOCl}$ ), which partially dissociates to form hypochlorite ( $\text{OCl}^-$ ).

**Figure 3.**

```

Homo sapiens+      -----MDPNCSCATGGGCTCTGSCCKCKECKCTSCCKS---C
Mus musculus**     -----MDPNCSCSTGGGCTCTSSCACKNCKCTSCCKS---C
Pseudomonas aeruginosa*** -----MNSETCACP-KCTCRPG-EDAVRD---G
SmtA, Synechococcus elongates* -----MTSTTLVKACE-PCLCNVDPSKAIDRN---G
                                     ^   ^   ^   ^

Homo sapiens+      CSCPMSCAKCAQGCICKGASEKSCCA-----
Mus musculus**     CSCPVGCSKCAQGCVCCKGAADKCTCCA-----
Pseudomonas aeruginosa*** -----MNSETCACP-KCTCRPG-EDAVRD---G
SmtA, Synechococcus elongates* LYYCSEACADGHTGGGKGC GHTGCNCHG-----

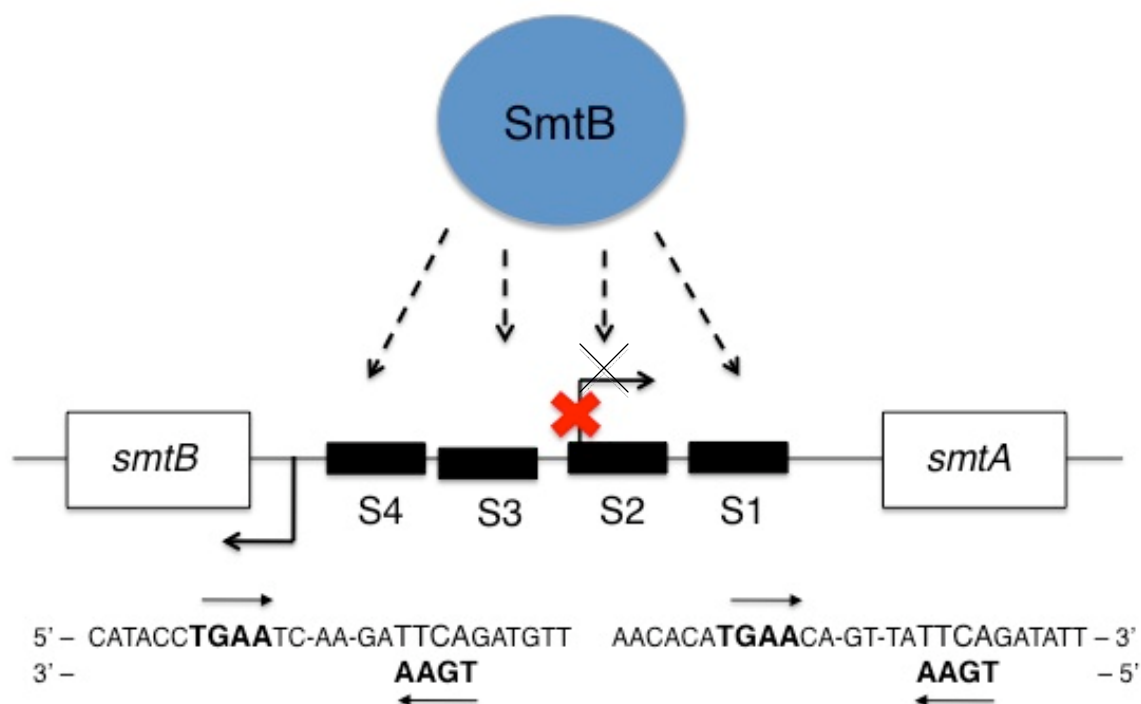
Homo sapiens+      -----
Mus musculus**     -----
Pseudomonas aeruginosa** SDPISP-----
SmtA, Synechococcus elongates* -----

+human, *cyanobacterium, **mouse, ***opportunistic gram negative bacterium

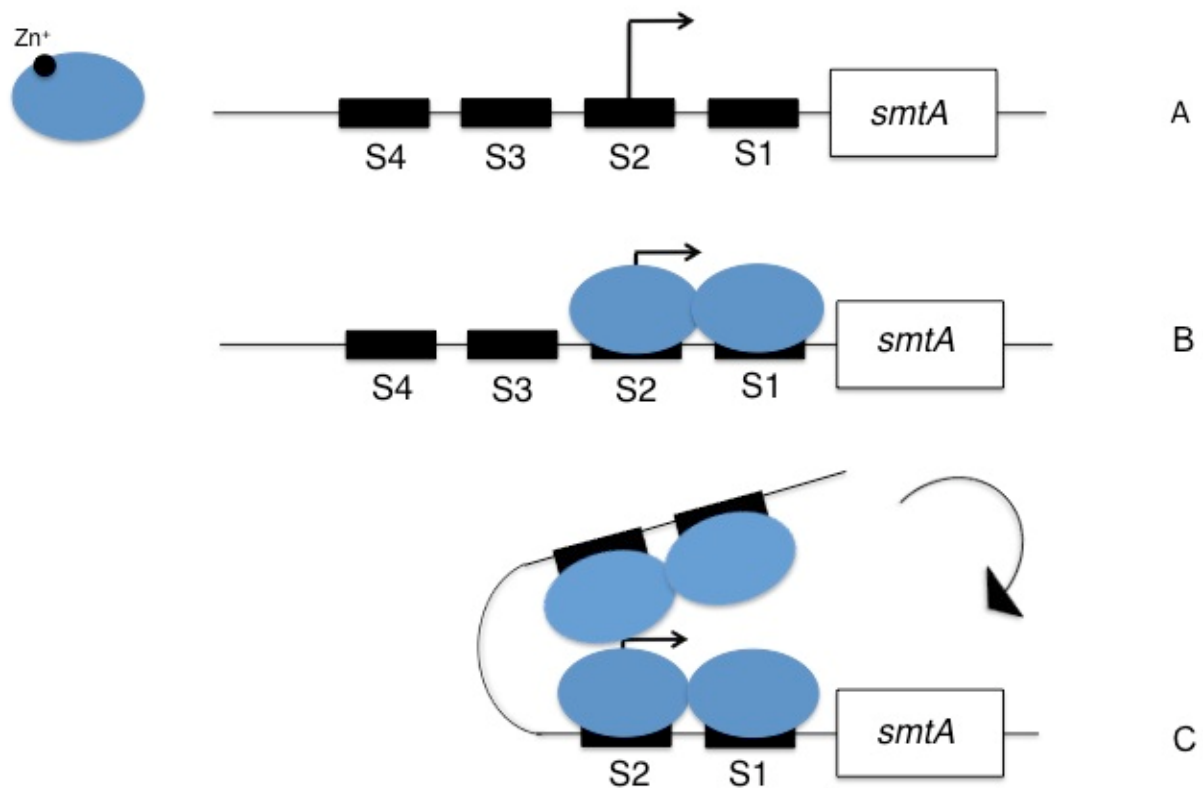
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**FIGURE 3. Comparison of cysteines in mammalian and bacterial metallothioneins.** This alignment of human and mouse MT-I and *Pseudomonas aeruginosa* and *Synechococcus* SmtA shows 4 cysteines conserved in all 4 sequences (indicated by red carets).

Figure 4.

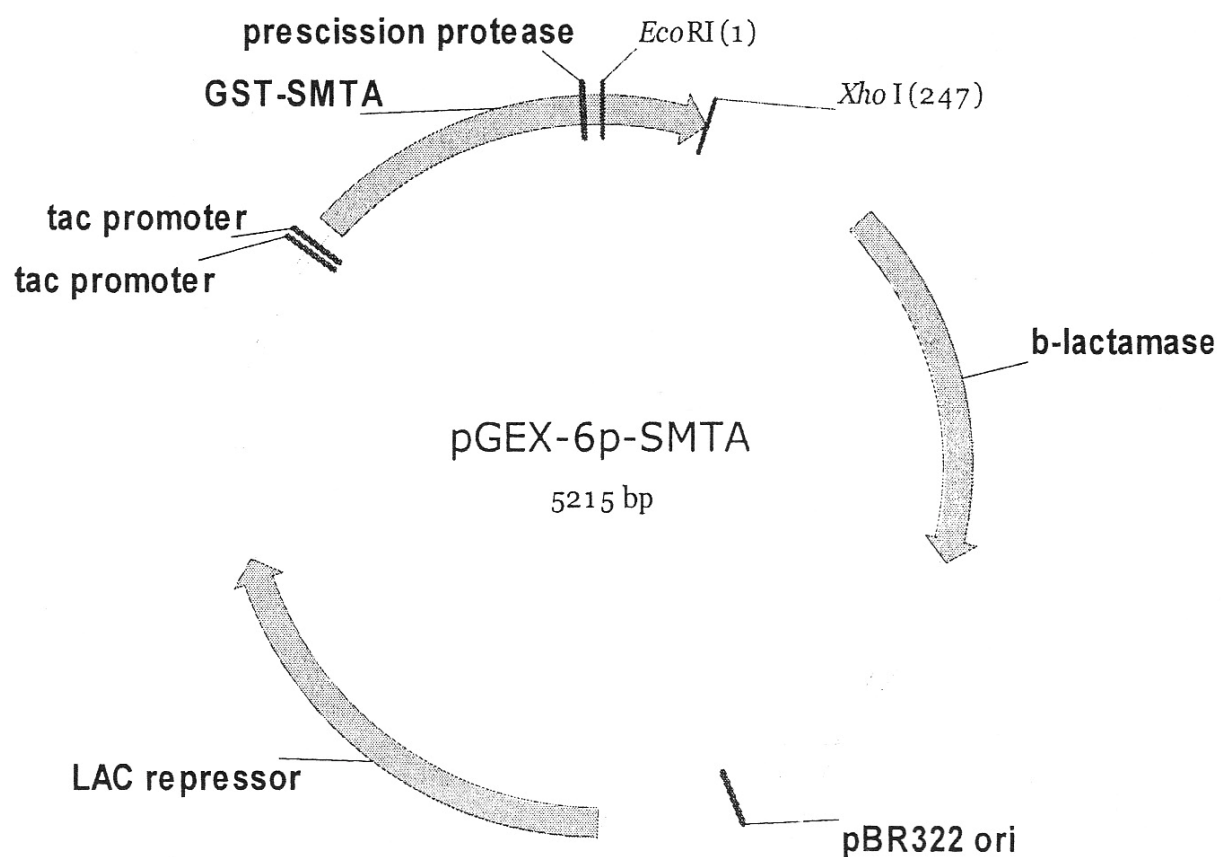


**FIGURE 4. SmtB action on the SmtA promoter.** Apo SmtB contacts the SmtA operator-promoter region at four sites: S1, S2, S3, and S4. The nucleotide sequence is shown below with nucleotide contact sites (sequence TGAA) in bold. (Adapted from Robinson et. al. 2001)

**Figure 5.**

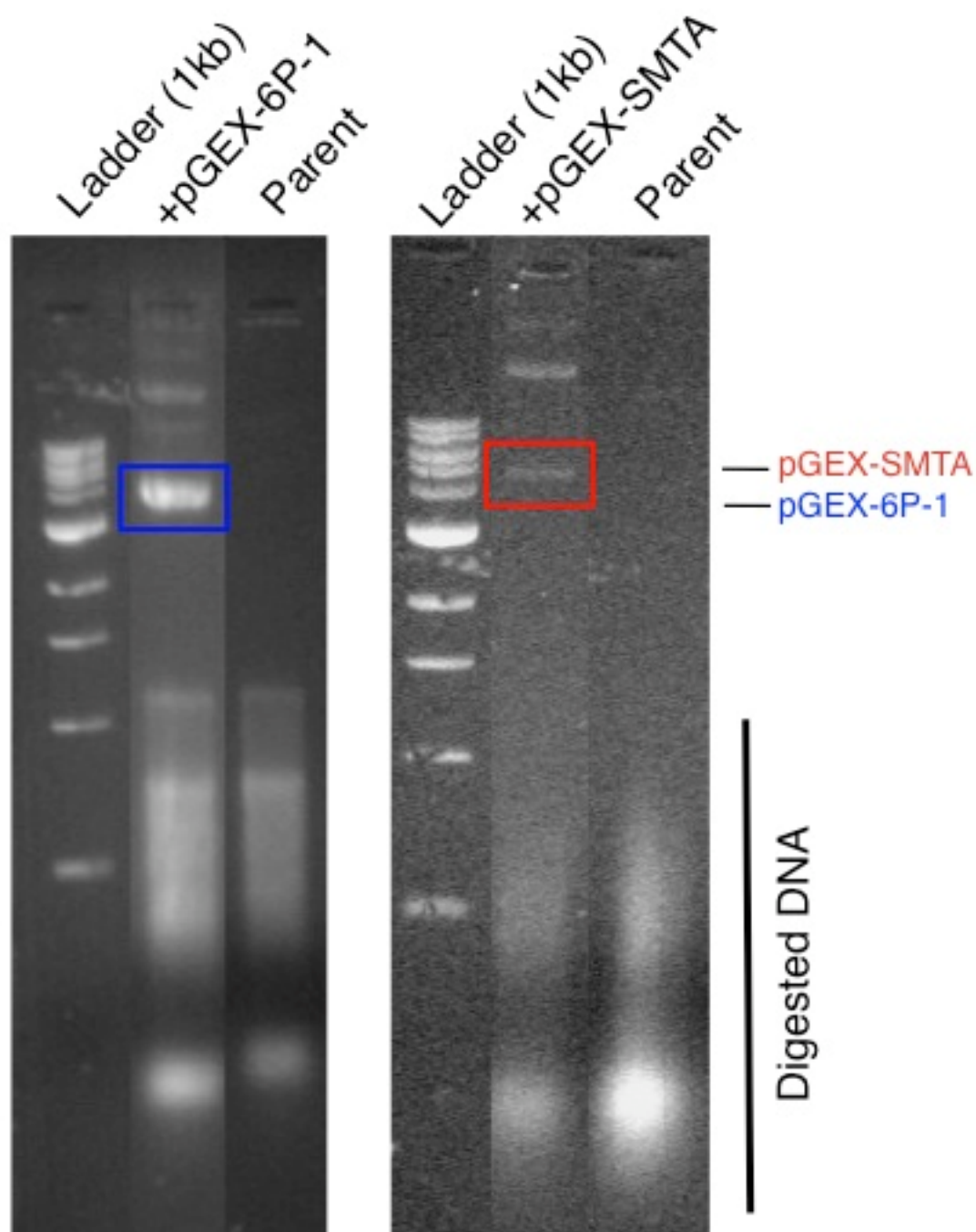
**FIGURE 5. Active SmtB binds at four sites to form a tetrameric complex at *smtA* promoter.** (A) SmtB (blue) is inactive when bound to Zn and will not contact the *smtA* promoter. (B) At low concentrations of Zn, active SmtB (apo) binds as a monomer or dimer (shown), which allows a low level of transcription. (C) When Zn is absent, SmtB forms a tetrameric complex, causing the DNA to bend, resulting in full inhibition of *smtA* transcription. (Adapted from Robinson et. al. 2001)

Figure 6.



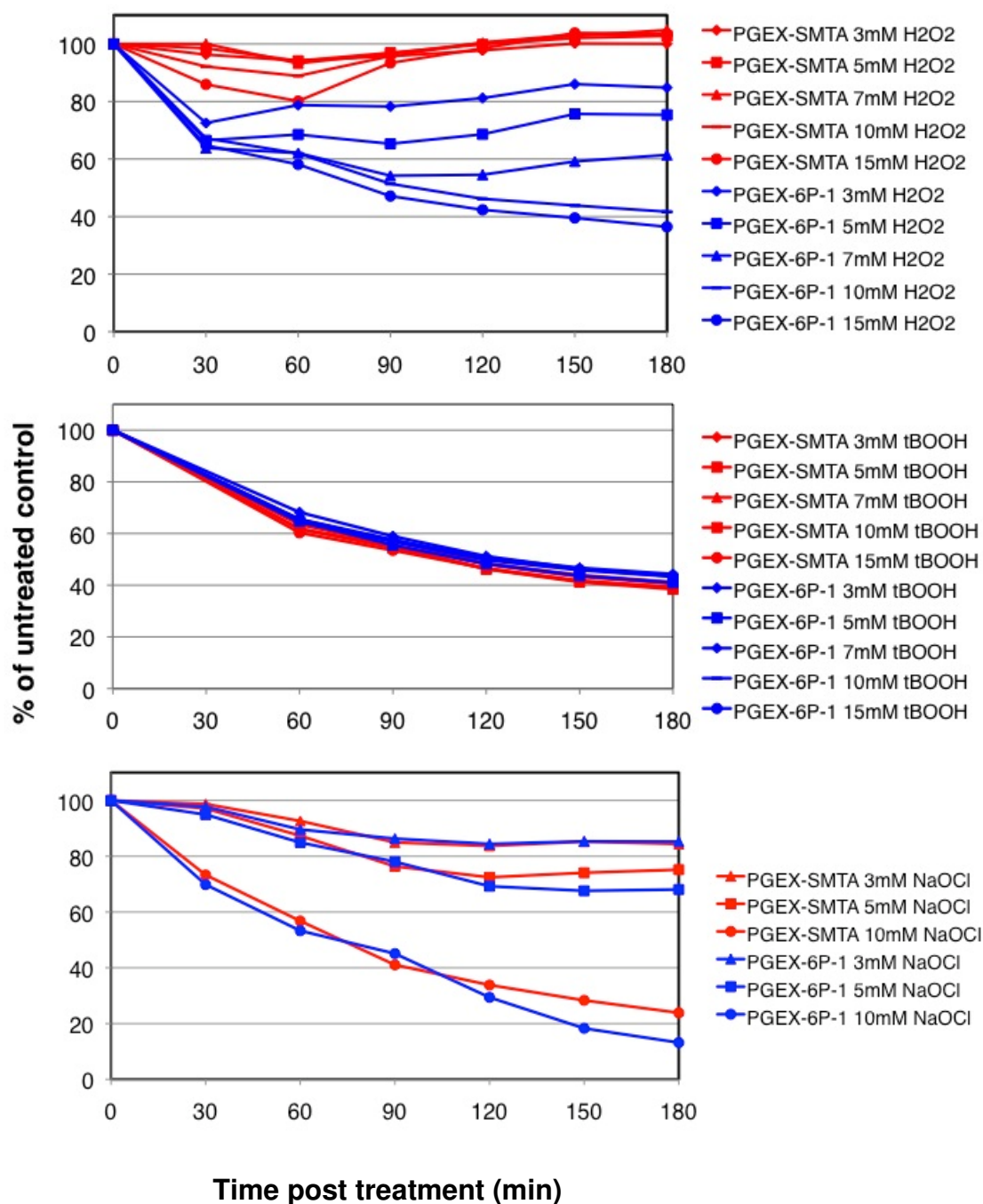
**FIGURE 6. pGEX-SMTA expression vector.** The SmtA gene was cloned into the pGEX-6p-1 expression vector between the between the EcoRI and XhoI sites. (Provided by D. Laukens, Ghent University)

Figure 7.



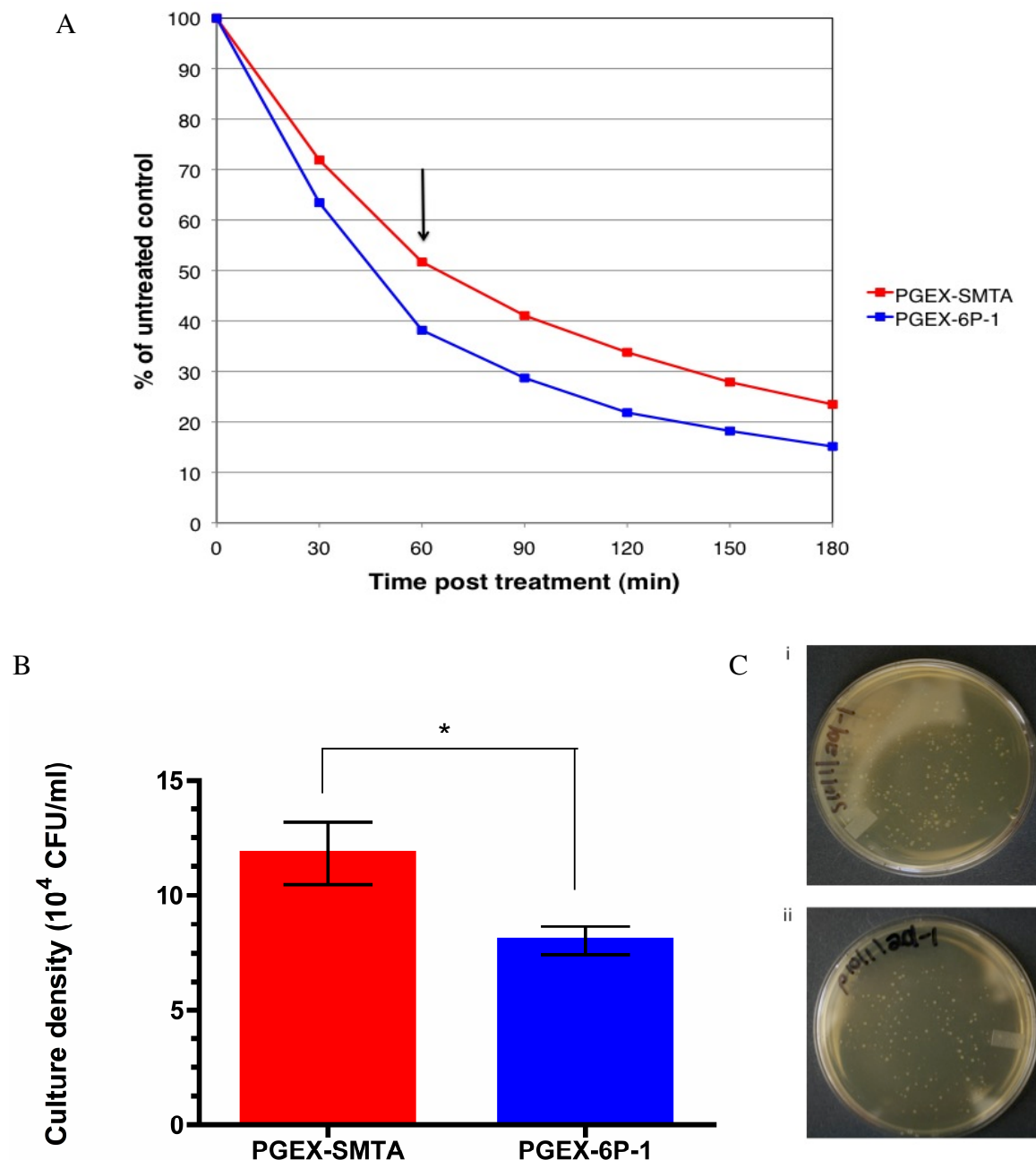
**FIGURE 7. Confirmation of pGEX-6P-1 transformation by gel electrophoresis.** Bacterial DNA was isolated from a 24-hour culture of MC1061 +pGEX-6P-1, MC1061 +pGEX-SMTA, or MC1061 according to Maniatis et. al. 1982. The miniprep was analyzed by gel electrophoresis on a 1% agarose gel containing 0.5ug/ml ethidium bromide. (SRD080210, SRD081310)

Figure 8.



**FIGURE 8. Survival of *E. coli* MC1061+pGEX-SmtA and MC1061+pGEX-6P-1 after exposure to exogenous oxidants.** *E. coli* MC1061 strains expressing either plasmid construct were sub-cultured from overnight cultures in a rich media containing IPTG to early log phase and monitored at OD<sub>600</sub>. The cultures were then aliquoted into 96-well plates and exposed to the various concentrations of H<sub>2</sub>O<sub>2</sub> (upper panel), tBOOH (middle panel), or NaOCl (lower panel) at time zero. Values are compared to an untreated control culture maintained for the same duration. (SRD071509, SRD082409, SRD100709)

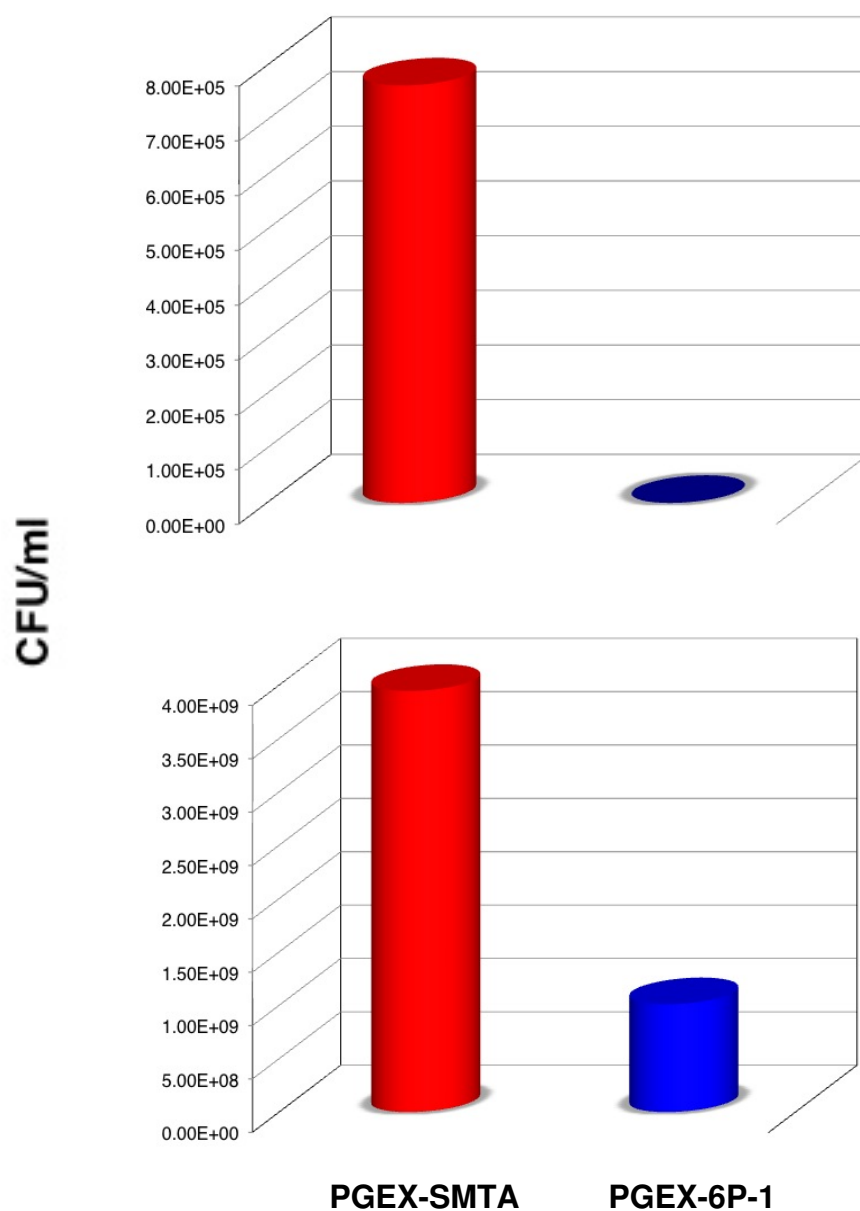
Figure 9.



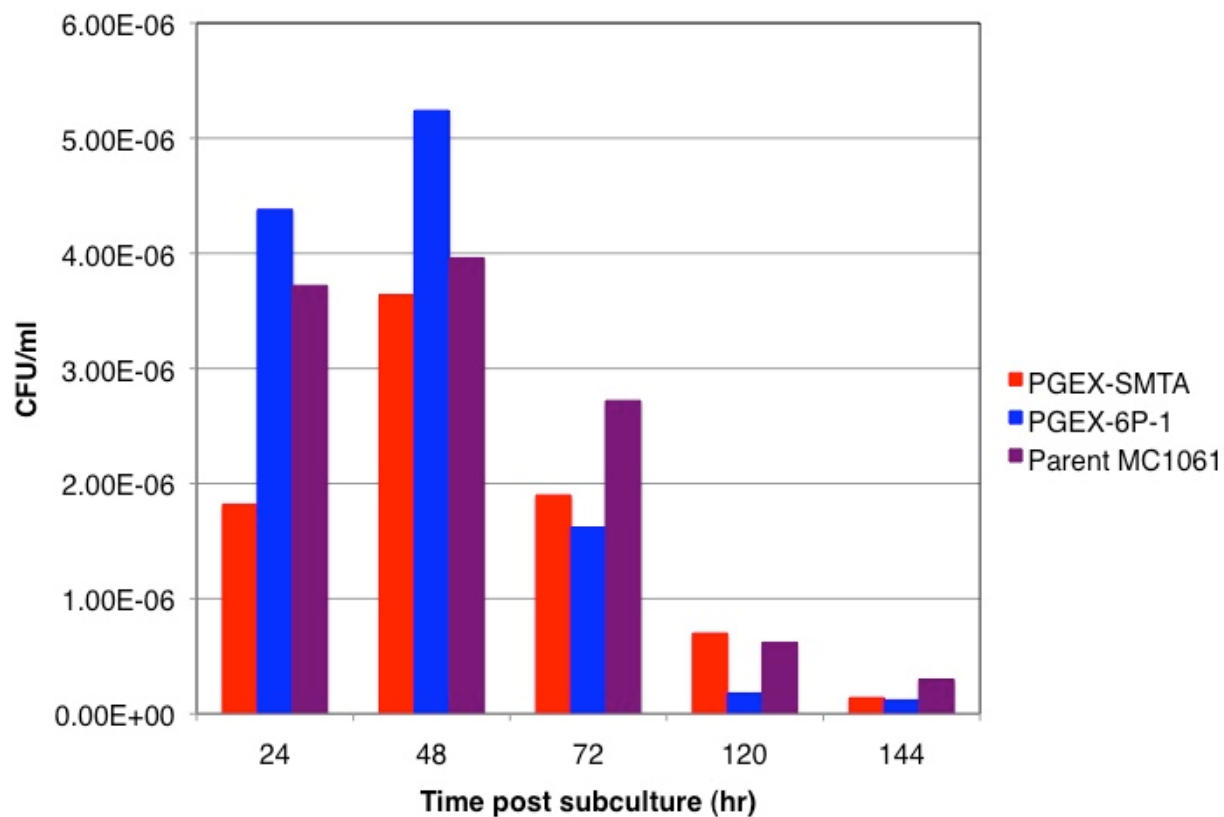
**FIGURE 9. Survival of *E.coli* MC1061+pGEX-SmtA and MC1061+pGEX-6P-1 after exposure to  $H_2O_2$ .** *E.coli* MC1061 strains expressing either plasmid construct were sub-cultured from overnight cultures in a rich media containing IPTG to early log phase and monitored at  $OD_{600}$ . (A) The cultures were then exposed to a final concentration of 10mM  $H_2O_2$  at time zero, aliquoted into a 96-well plate, and monitored at  $OD_{600}$ . Values are compared to an untreated control culture maintained for the same duration. (B) A sample was taken at 60 minutes (indicated by arrow) and plated to determine CFU/ml ( $p < 0.01$ ). (C) Plates shown. *E. coli* + pGEX-SmtA treated with 10mM  $H_2O_2$  was diluted  $10^{-1}$  (i) and *E. coli* + pGEX-6p-1 treated with 10mM  $H_2O_2$  was diluted  $10^{-1}$  (ii). 20ul of each diluted culture was plated. (SRD120810)



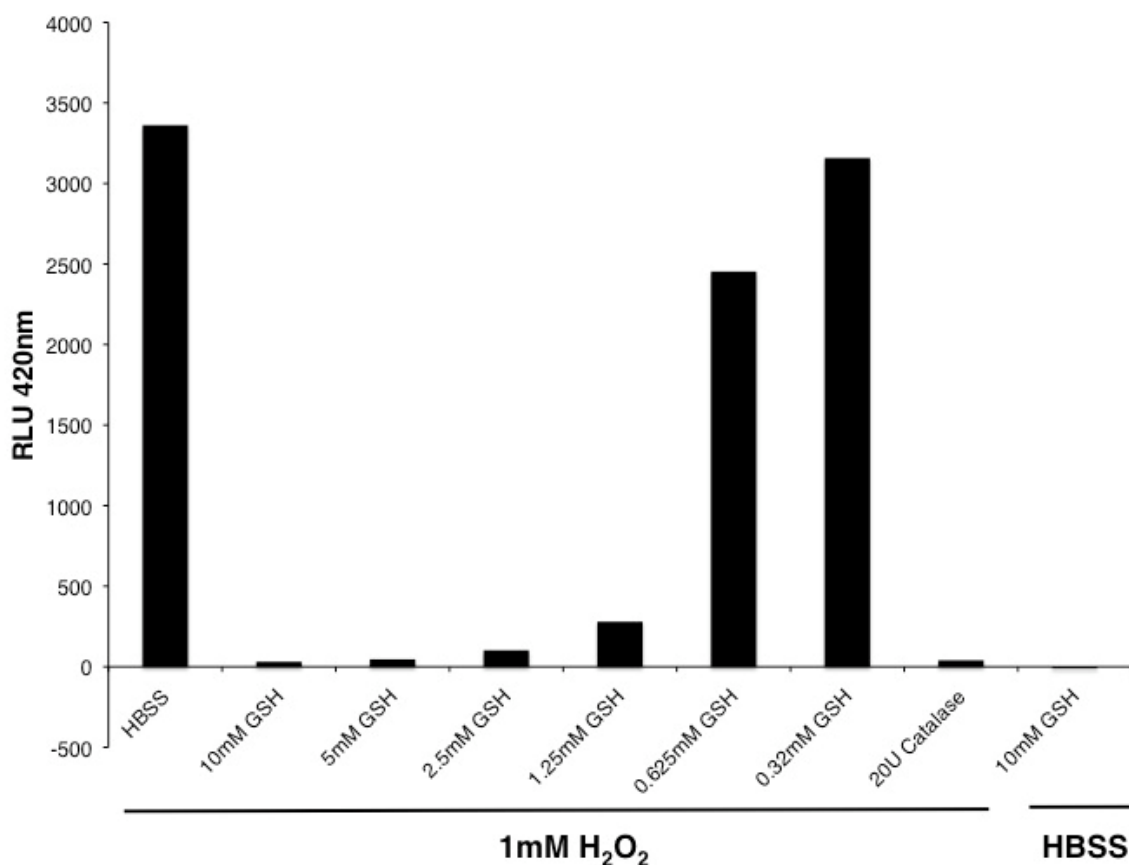
Figure 10.



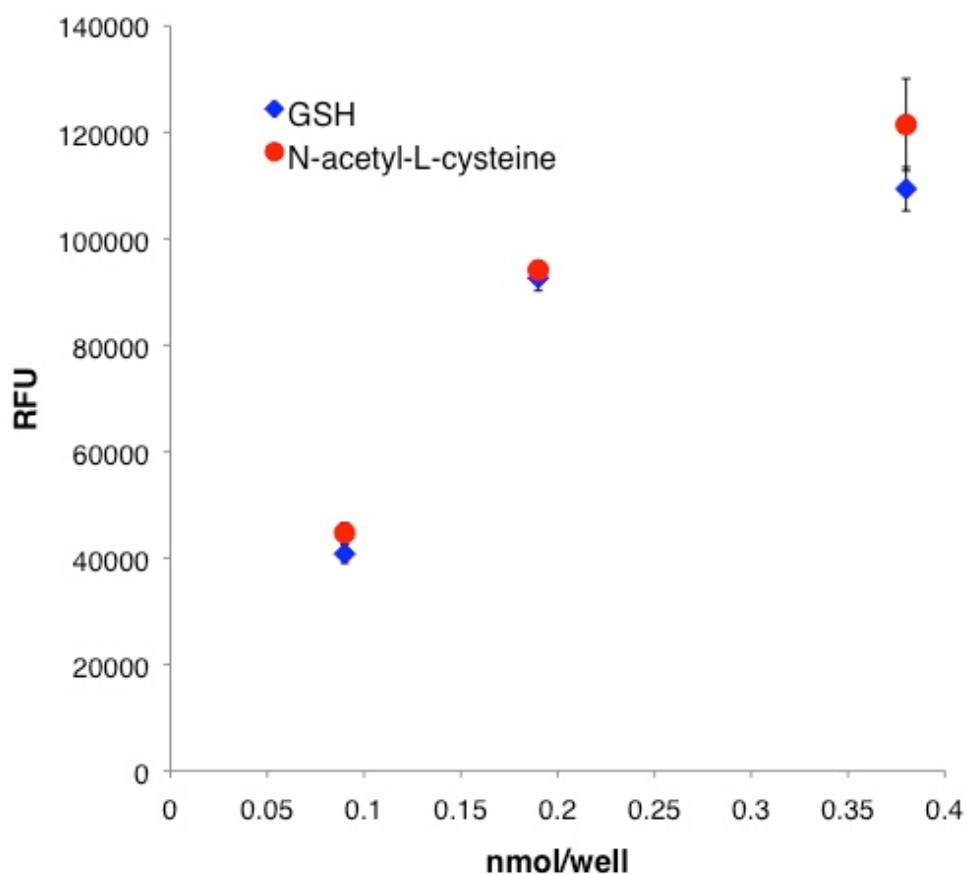
**FIGURE 10. *E. coli* MC1061+pGEX-SmtA and MC1061+pGEX-6P-1 infection of RAW267.4 macrophages.** The *E. coli* MC1061 strains expressing either plasmid construct were grown overnight and subcultured to log phase with IPTG present. The RAW264.7 cells were grown to confluency and plated in 6 well plates. The RAW264.7 cells were then infected with *E.coli* at an MOI of 5:1 and incubated in special gas at 37°C. The supernatant was removed after 30 hours (upper panel) and 24 hours (lower panel) and plated to determine CFU/ml. (KMP)

**Figure 11.**

**FIGURE 11. SmtA in long-term *E. coli* culture.** MC1061 *E. coli* cultures (Parent MC1061 *E. coli*, MC1061 + pGEX-SmtA, and +pGEX-6p-1) were subcultured from overnight cultures in rich media and allowed to grow at 37° under constant shaking. At the time points indicated, a sample of each culture was plated on YTA plates to determine CFU/ml. (SRD062810)

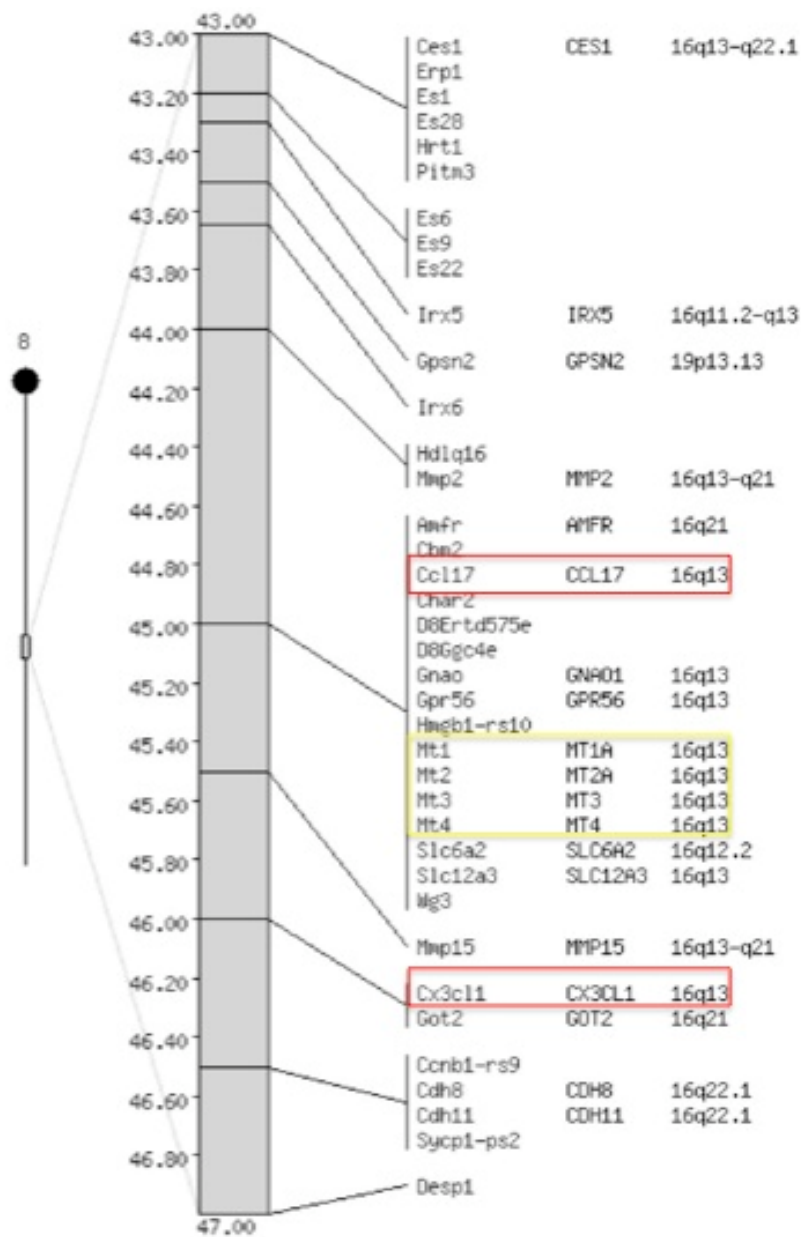
**Figure 12.****FIGURE 12. GSH decreases chemiluminescence of luminol in the presence of H<sub>2</sub>O<sub>2</sub>.**

Solutions of GSH and catalase were prepared in HBSS. H<sub>2</sub>O<sub>2</sub> was added to a final concentration of 1mM. Samples were incubated at RT for 30 minutes. A working solution of luminol-HRP was added to each sample and then immediately aliquoted into 96-well white plate and read at 420nm. The value of the blank (HBSS and luminol-HRP working solution) was subtracted from each well. (SRD020611)

**Figure 13.****FIGURE 13. GSH and N-acetyl-L-cysteine show similar linearity of reactive thiols.**

Working solution of CPM and sample solutions were added in a 1:1 dilution in triplicate to a black 96-well plate and incubated in the dark at RT for 1 hour. The plate was then read using an excitation wavelength of 387nm and an emission wavelength of 465nm. The value of the blank (100ul PBS and 100ul of CPM working solution) was subtracted from each well. (SRD032311)

Figure 14.



**FIGURE 14. Metallothionein gene shows proximity to chemokine genes in mice and humans.** Mouse chromosome eight is shown with the murine MT genes and homologous human MT genes highlighted in yellow. Chemokine genes highlighted in red. (Adapted from Yin et. al. 2005)

**Table 2.**

| Organism                      | Protein | SecretomeP score |
|-------------------------------|---------|------------------|
| <i>Pseudomonas aeruginosa</i> | SmtA    | 0.652            |
| <i>Homo sapiens</i>           | MT-1F   | 0.865            |
| <i>Bacillus subtilis</i>      | GlnA    | 0.539            |
| <i>Escherichia coli</i>       | SodA    | 0.701            |
| <i>Pseudomonas aeruginosa</i> | RNAP    | 0.219            |

**TABLE 2. Prediction of non-classical protein secretion by SecretomeP.** SecretomeP is a sequence-based prediction module for mammalian and bacterial non-classical secretion. Protein sequences were inputted into the module to obtain a SecretomeP score. A score above 0.5 indicates possible non-classical secretion.

## **ACKNOWLEDGEMENTS**

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