Role of the manganese efflux system *mntE* for signalling and pathogenesis in *Streptococcus pneumoniae*

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Summary

The ability of bacteria to sense and respond to both environmental and intracellular metal concentrations plays an important role in pathogenesis. The acquisition of manganese is vital for the virulence of several bacterial species. Although manganese uptake systems have been well studied in bacteria, no manganese efflux system has yet been identified. In this study we have identified a cation diffusion facilitator (CDF) protein (Sp1552) of unknown substrate specificity that functions as a manganese export system in *Streptococcus pneumoniae*. We designated the gene for this manganese efflux system *mntE* and found that the mutant strain was highly sensitive to manganese stress. Although the mutant was more resistant to oxidative stress and produced more H_2O_2 and pili, it had reduced virulence in a murine model of infection, indicating that manganese export plays a role in host pathogenesis. There was a distinct differential transcriptional response to extracellular and intracellular manganese accumulation. Our study indicates that manganese efflux is required for invasive disease and may provide a useful antimicrobial target to devise future therapeutics.

Introduction

Bacterial pathogens must acquire all the necessary cofactors for survival and replication from the host environment during the course of infection. One such limiting factor is the availability of various metals, including iron, zinc and manganese, which are required by both the host and the bacteria for essential cellular processes. Acquisition of metals is also essential for pathogenesis. Bacteria sense cation levels to respond to their environment via two-component regulators or transcriptional regulators that require a specific cation cofactor for promoter recognition. Hence, metal homeostasis is important not only for enzymatic function but also for appropriate transcriptional control of regulatory networks that govern gene expression under various environmental conditions.

Bacterial acquisition of manganese plays important roles in pathogenesis in a number of bacterial species. This is highlighted by the multiple strategies various species of bacteria have evolved to acquire manganese from the environment. Two main classes of manganese transporters have been identified in bacteria, Nramp H^+-Mn^{2+} transporters and the ATP-binding cassette (ABC) Mn^{2+} permeases, with nearly all sequenced bacterial genomes containing transporters of one or both of these classes (Papp-Wallace and Maguire, 2006).

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Manganese transporters can also transport other ions including Fe^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Cd^{2+} and Ni^{2+} (Kehres *et al*., 2000; Low *et al*., 2003). Furthermore, some predicted metal importers have been shown to function for export, underscoring the need to experimentally confirm bioinformatics predictions (Brenot *et al*., 2007). No Nramp transporters have been characterized in pneumococcus, although an ABC transporter specific for manganese import, PsaABC, has been identified and found to play a role in many transcriptional pathways indicating that perturbation of manganese homeostasis by disrupting import elicits a specific cellular response (McAllister *et al*., 2004; McCluskey *et al*., 2004; Johnston *et al*., 2006). Manganese influx systems have also been characterized in many bacterial species (Kehres *et al*., 2000; Makui *et al*., 2000; McAllister *et al*., 2004). The converse experiment, disruption of a manganese export system, to ascertain the effect of intracellular manganese accumulation has not been performed as no export system whose primary substrate is Mn^{2+} has yet been identified in any bacteria to our knowledge.

Although many studies have focused on influx systems, efflux of cations is also a potential mechanism for regulating genes and maintaining homeostasis, particularly in an environment replete with certain cations. Bacterial efflux systems have been characterized for arsenic (ArsB), cadmium (CadA), zinc (CzcD), copper (CzcD and CopZA), cobalt (CzcD) and nickel (CzcD) (Moore and Helmann, 2005). One of the most ubiquitous classes of metal transporters is the cation diffusion facilitator (CDF) family of proteins, which transports various metals in both prokaryotes and eukaryotes (Haney *et al*., 2005). Members of this family have broad capabilities of metal transport; for example, FieF from *Wautersia metallidurans* is primarily an iron-detoxifying transporter but can also mediate resistance against other divalent cations such as Zn^{2+}, Co^{2+}, Cd^{2+} and Ni^{2+} (Munkelt *et al*., 2004). Studies on substrate specificity based on sequence homology or computation predictions need to be experimentally confirmed.

Bacteria tightly control the intracellular levels of cations such as zinc and manganese under dynamic environmental conditions (Chapuy-Regaud *et al*., 2001; Outten and O'Halloran, 2001). A selective efflux system is required to transport calcium out of the cell in order to maintain a 3-log difference between intra- and extracellular calcium concentrations (Rosch *et al*., 2008a). Zinc efflux systems have also been recently identified in *Streptococcus pyogenes* and *Streptococcus pneumoniae* (Brenot *et al*., 2007; Kloosterman *et al*., 2007). Elucidation of the structure of the CDF protein YiiP, which is responsible for efflux of both iron and zinc in *Escherichia coli*, has provided insights into the structure determinants of metal ion selectivity (Grass *et al*., 2005; Wei and Fu, 2005; Lu and Fu, 2007). Putative manganese exporters have been identified and characterized in eukaryotes, particularly plants (Delhaize *et al*., 2003; Hall and Williams, 2003). To our knowledge, evidence of bacterial manganese exporters has remained elusive, although there is some evidence of active efflux from bacterial cells (Fisher *et al*., 1973).

In this study, we describe a predicted CDF protein (Sp1552) of unknown substrate specificity that functions as a manganese efflux system in *S. pneumoniae*. We have designated the gene for this manganese efflux system *mntE*, and used the mutant strain (*mntE*–) that accumulated higher levels of intracellular manganese to study resistance to oxidative stress, production of hydrogen peroxide, pilus expression, host pathogenesis in a murine model, and differential transcriptional response to extracellular and intracellular manganese. Our results suggest that although the mutant is more resistant to oxidative stress and produces more H_2O_2 and pili, it has reduced pathogenicity in the host. The global transcriptome response indicates that *S. pneumoniae* has distinctly different responses to intracellular and extracellular manganese accumulation, providing a unique perspective on the bacterial response to environmental manganese.

Results

Identification of the manganese exporter

On the basis of sequence homology, Sp1552 has been predicted to be a CDF inorganic cation transport system of unknown substrate (Tettelin *et al.*, 2001). We found the homologous gene to be conserved among several Gram-positive pathogens such as *Staphylococcus aureus, Clostridium botulinum, Bacillus anthracis* and *S. pyogenes*, and more distant homologues were identified in Gram-negative bacteria as well as Archaea (data not shown). The CDF family has been extensively studied in both eukaryotes and prokaryotes, and bacterial homologues have been implicated in zinc efflux (Grass *et al.*, 2001). Members of this family can selectively export cadmium, cobalt, nickel, zinc, manganese or iron (Moore and Helmann, 2005). One hallmark of eukaryotic CDF manganese transporters is the presence of a DXXXD motif at transmembrane domain 5 (Montanini *et al.*, 2007). This feature is conserved in Sp1552, indicating that Sp1552 may be functionally analogous to the CDF family of manganese transporters. The gene *sp1552* is highly conserved in *S. pneumoniae*: comparative genomic hybridization (CGH) analysis has shown that it is part of the core pneumococcal genome and it is also present in all sequenced strains to date (Obert *et al.*, 2006). Export systems for both zinc and calcium have been identified in *S. pneumoniae*; however, the substrate of the transporter encoded by *sp1552* has not been experimentally determined (Kloosterman *et al.*, 2007; Rosch *et al.*, 2008a).

To identify the substrate and function of *sp1552* in *S. pneumoniae*, we generated a deletion mutant of *sp1552* by allelic replacement and confirmed it by sequencing. We could not achieve complementation of the deletion mutant on a plasmid, as the gene product appeared to be toxic to *E. coli* during cloning (data not shown) into various streptococcal vectors, including pIB163, pIB165, pIB167, pIB169 and pABG5 (Hanski *et al.*, 1992; Biswas *et al.*, 2008). The mutation was nonpolar, as evidenced by the normal transcription of both upstream and downstream genes (data not shown). A revertant strain was generated by crossing the parental locus into the *sp1552*– mutant and regenerating the parental TIGR4 genotype, as confirmed by PCR and sequencing. This mutant, *sp1552*–, and the TIGR4 and the wild-type revertant were exposed to various compounds to determine the substrate of the transporter. Additional substrate was expected to rescue an influx system mutant and inhibit an exporter mutant.

Cation sensitivity profiles of *sp1552*– revealed that it was selectively sensitive to manganese ($P < 0.01$), as evidenced by a significant increase in its zone of inhibition compared with the parental TIGR4 and wild-type revertant (Fig. 1A). Switching of the cognate anion indicated this sensitivity was selective for manganese. TIGR4 and *sp1552*– did not significantly differ in their sensitivities for any other cation tested, including cadmium and zinc. Sensitivities to cobalt and cadmium were also tested using 100 mM solutions infused into the disc, but no differences were observed between the strains (data not shown). Growth of the *sp1552*– mutant was severely restricted in media supplemented with manganese (Fig. 1B) with total growth inhibition observed at a manganese concentration of 1 mM. Growth of parental TIGR4 was not inhibited under all concentrations of manganese used in the study. We tested the ability of several metals, including cobalt, nickel, calcium and magnesium, to inhibit the growth of *sp1552*–, but none of them caused a significant growth defect (Fig. 1B) compared with that in TIGR4. These data indicate that the *sp1552*– strain has increased sensitivity for manganese, suggesting a possible role of this gene in manganese export.

To confirm that the *sp1552*– mutant was deficient for manganese export, we measured the intracellular levels of manganese in both parental TIGR4 and *sp1552*– by inductively coupled plasma mass spectrometry (ICP-MS). The *sp1552*– strain accumulated five times more intracellular manganese than parental TIGR4 did (Fig. 2A). There was no difference between the TIGR4 and *sp1552*– strain with regard to intracellular concentrations of calcium or zinc

(Fig. 2A) or those of magnesium [12.2 ± 0.6 p.p.m. for TIGR4 and 12.8 ± 0.3 p.p.m. for *sp1552*– per 10^7 colony-forming units (cfu)]. For cadmium, a slight decrease from 1.16 ± 0.04 p.p.m. for TIGR4 to 0.83 ± 0.06 p.p.m. for *sp1552*– per 10^9 cfu was observed, which might be the result of downregulation of manganese import because many manganese transporters can also transport cadmium (Kehres and Maguire, 2003).

We further confirmed the accumulation of manganese in the *sp1552*– strain by the $^{54}Mn^{2+}$ uptake assay, wherein cultures were supplemented with radioactive manganese. The *sp1552*– mutant rapidly accumulated high levels of manganese, and continued to accumulate it over the course of 1 h (Fig. 2B). In contrast, the TIGR4 strain rapidly accumulated lower levels of manganese and subsequently maintained homeostasis. Re-suspending the bacteria in a medium not supplemented with $^{54}Mn^{2+}$ (arrow in Fig. 2B) resulted in a gradual decrease of approximately 50% in intracellular radioactive manganese in the TIGR4 stain but not in the *sp1552*– strain. It should be noted that although the acidic media used in these experiments prevents most autolysis, we cannot eliminate the possibility that cell lysis mediates part of the manganese efflux. Data from the cation sensitivity and uptake assay indicate that Sp1552 functions as a manganese efflux system and that a mutant deficient in export accumulates significantly higher levels of intracellular manganese. In light of these data, we propose naming the gene *mntE* to be in accord with previously identified bacterial manganese import systems, *mntA* and *mntH* (Bartsevich and Pakrasi, 1995;Makui *et al*., 2000).

Role of manganese export in resistance to oxidative stress

Manganese has long been known to play an important role in bacterial defence against reactive oxygen species (Inaika *et al*., 1999; Jakubovics *et al*., 2002; Papp-Wallace and Maguire, 2006). As the *mntE*– strain is deficient in manganese export, we hypothesized that it would be more efficient at handling reactive oxygen species because of an increase in available cellular manganese. Parental TIGR4 and *mntE*– strains were subjected to both superoxide and nitric oxide stress for 60 min and viability assessed by serial dilution and colony enumeration. Interestingly, the *mntE*– mutant showed a fourfold increase in survival over the parental TIGR4 in response to both oxidative and nitric oxide stresses (Fig. 3). One possible reason for this protection could be an increase in the manganese-dependent superoxide dismutase activity (Yesilkaya *et al*., 2000); however, there was no significant difference in superoxide dismutase activity between these two strains (data not shown). Manganese (II) bicarbonate complexes have been shown to reduce reactive oxygen species, providing an antioxidant defence independent of enzymatic factors (Archibald and Fridovich, 1981; Al-Maghredi *et al*., 2002; Daly *et al*., 2004). Our data also indicate that accumulation of high levels of intracellular manganese protects against reactive oxygen species.

Dysregulation of hydrogen peroxide production

Species of streptococci produce prodigious amounts of H_2O_2, which plays important roles in processes such as competing with other bacterial species and as a potential quorum-sensing molecule in *S. pneumoniae* (Battig and Muhlemann, 2008; Kreth *et al*., 2008). We monitored the production of H_2O_2 by the *mntE*– mutant by an indicator plate assay (Brenot *et al*., 2005). Streptococci produce abundant H_2O_2 at low cell densities on solid media, but their H_2O_2 production is limited at high cell densities (Brenot *et al*., 2005). Both TIGR4 and *mntE*– produced equivalent amounts of H_2O_2 at low cell densities (Fig. 4A and B, far right). However, when cells were plated at high cell densities, H_2O_2 production by TIGR4 was dramatically reduced but that for the *mntE*– mutant continued to remain high (Fig. 5A and B, compare leftmost lanes). This phenotype is similar to that of an *ahpC*– mutant in *S. pyogenes* (Brenot *et al*., 2005). Although pneumococci do not encode a homologue of *ahpC*, it is interesting to note that a putative thiol peroxidase is immediately downstream of the psaABC manganese uptake system, indicating potential cross-talk between manganese signalling pathways and

bacterial antioxidant defences (McAllister *et al*., 2004). TIGR4 and *mntE–* showed no difference in H_2O_2 production in liquid culture when cultured in ThyB media (Fig. 4C). However, under high manganese concentrations, the *mntE–* mutant showed a dramatic increase in H_2O_2 production (Fig. 4C). The high standard error of the *mntE–* mutant grown in manganese may be due in part to the measured absorbance approaching the upper limit of the linear range of the measurement assay at the dilution that all samples were measured for consistency. These data indicate that the availability of manganese may play a role in the ability of pneumococci to produce H_2O_2.

Role of manganese export in host pathogenesis

As the *mntE–* strain was significantly better at resisting reactive oxygen and nitrogen species normally encountered in the host, we expected it to be more virulent than the parental strain. The parental TIGR4 and *mntE–* mutant were assessed for their ability to sustain nasal colonization and establish invasive disease in an intranasal murine model of pathogenesis. The *mntE–* strain showed a significant defect in both nasal colonization ($P = 0.042$) and invasion into the blood ($P = 0.017$) (Fig. 5B). Overall survival data showed that mice infected with *mntE–* had a significant ($P = 0.003$) delay in time to death compared with the parental TIGR4 (Fig. 5A). These results indicate that although the *mntE–* mutant was more resistant to oxidative stress, loss of manganese export impaired pathogenesis in the host.

Disruption of manganese export influences pilus regulation

As the *mntE–* mutant was attenuated in a murine model of pathogenesis, we investigated whether this phenotype could be explained by differential regulation of important virulence determinants. Disruption of PsaR, which regulates the PsaABC manganese import system, has been shown to significantly upregulate the genes encoding the pilus subunits and the associated sortases (Lauer *et al*., 2005; Barocchi *et al*., 2006; Johnston *et al*., 2006). Hence, we expected the *mntE–* mutant to show decreased amounts of pilus expression and thereby reduced virulence because of accumulation of intracellular manganese. We used quantitative RT-PCR (qRT-PCR) to measure transcript abundance of the *rlrA* regulator, which is required for pilus transcription along with the major pilus subunit *rrgB*. There was an increase in both the *rlrA* and *rrgB* transcripts in the *mntE–* mutant as compared with parental TIGR4 (Fig. 6A), and this increase corresponded to an increase in the amount of pilus protein, as measured by both Western blot and ELISA using RrgB-specific antibodies (Fig. 6A and B). These results indicate that perturbation of intracellular manganese homeostasis may be a key cue for pilus expression. As manganese is highly abundant in the nasal and lung mucosa, differences in intracellular manganese levels may be an important cue for pathogenesis, particularly in the lung where pili play a role in invasive disease (Nelson *et al*., 2007; Rosch *et al*., 2008b). We expected that an increased amount of pili would increase the pathogenesis of the mutant, but the *mntE–* strain was not more virulent than parental TIGR4 in the murine model of infection. Additional factors may be responsible for this attenuation, necessitating a more global view of the transcriptional response due to intracellular manganese accumulation.

Differential transcriptional response to extracellular versus intracellular manganese

Perturbation of manganese homeostasis has been shown to have an impact on the expression of many genes in *S. pneumoniae*, most notably the *rlrA* locus encoding the pilus and the *psaABC* locus encoding the manganese uptake system (Johnston *et al*., 2006). The global transcriptional response to environmental manganese is complex and includes the action of metalloregulatory proteins, perturbation of cellular iron availability, and differential activity of enzymes that require manganese as a cofactor (Guedon *et al*., 2003). We hypothesized that *S. pneumoniae* has separate transcriptional pathways to differentiate between high extracellular and intracellular manganese concentrations, which could help pinpoint the role of manganese

in gene regulation. To elucidate this, global gene transcription in response to high (500 µM) manganese in parental TIGR4 as well as the transcription in response to intracellular manganese accumulation, as represented by the *mntE–* mutant, was examined.

We compared the transcriptome of *S. pneumoniae* grown in ThyB versus that grown in ThyB supplemented with 500 µM manganese. Table S1 lists all genes showing significant differences in transcript abundance and Table 1 indicates the number of genes in specific functional categories showing differential regulation. As expected, the operon downregulated the most was the PsaABC manganese uptake system, *sp1647–sp1650*, which has previously been shown to be repressed under conditions of high manganese (Johnston *et al.*, 2006). Other loci that were significantly downregulated included *sp0043*, encoding the ComB protein involved in competence. The *sp1869–1872* locus encoding an iron-compound ABC transporter was significantly upregulated in response to high levels of manganese, which agrees with previous results in *Bacillus subtilis* indicating that genes controlled by Fur (ferric uptake regulator) can be dysregulated by elevated concentrations of manganese (Guedon *et al.*, 2003). Two predicted surface proteins, choline-binding protein D (*sp2201*) and a predicted cell wall-anchored protein (*sp1992*), were upregulated. We confirmed the expression patterns observed in the microarray by qRT-PCR, and found that both methods agreed (Table 2). These data indicate that *S. pneumoniae* elicits a specific transcriptional response to high levels of manganese in the environment, which includes repression of the manganese uptake system and upregulation of an iron import system.

To differentiate between extracellular and intracellular manganese-induced signalling, we determined the global transcriptome response when manganese efflux was impaired (Table S2) by comparing transcripts of TIGR4 and *mntE–* when both strains were cultured under conditions of high manganese. Table 1 indicates the number of genes in specific functional categories showing differential regulation under these conditions. These conditions cause *mntE–* to accumulate approximately five times more intracellular manganese than TIGR4 does. Several loci were significantly upregulated, including the *sp0461–sp0467*, which encodes the pilus and associated sortases, confirming the qRT-PCR data. Several genes involved in central metabolic pathways were downregulated in response to manganese accumulation, including operons involved in lactose metabolism (*sp1189–1193*), glycosyl transferases (*sp1764–1771*) and sugar transporters (*sp1895–1897*). Manganese has been implicated to play a role in various steps of the sugar catabolism pathway in bacteria, and accumulation of intracellular manganese may therefore affect such pathways (Kehres and Maguire, 2003). It has been suggested that a high intracellular accumulation of manganese could effectively compete for magnesium, altering the activity of Mg^{2+}-requiring enzymes involved in the carbon flux (Papp-Wallace and Maguire, 2006). The *caxP* gene (*sp1551*), recently identified to encode a calcium/manganese efflux protein, is also significantly upregulated when manganese accumulates intracellularly (Rosch *et al.*, 2008a). The *sp1857* gene, which has been recently demonstrated to be involved in zinc tolerance in *S. pneumoniae*, is also upregulated, indicating that various metal export systems may be co-regulated (Kloosterman *et al.*, 2007). A brief comparison of various genes of interest showing differential regulation in response to high extracellular and intracellular manganese is detailed in Table 3. It should be noted that the transcriptional analysis was not exhaustive, as there was no detectable signal in a significant number of genes (16.8%), probably due to low expression or poor hybridization. These data indicate that *S. pneumoniae* elicits a specific response to the intracellular accumulation of manganese that is distinct from extracellular sensing pathways.

Discussion

Manganese plays an important role in the virulence of many species of bacterial pathogens that have Nramp and ABC transporters for its uptake (Papp-Wallace and Maguire, 2006). Nramp orthologues have been identified in many bacterial species such as *S. aureus, B. subtilis* and *Mycobacterium tuberculosis* (Agranoff *et al*., 1999; Que and Helmann, 2000; Hornsburgh *et al*., 2002). The pneumococcus relies on the ABC-type transporter PsaABC to import manganese into the cytosol (McAllister *et al*., 2004). Numerous other streptococcal species (e.g. *S. pyogenes, S. mutans, S. gordonii, S. parasanguis*) also use a specialized ABC transporter for manganese uptake (Papp-Wallace and Maguire, 2006). However, no manganese efflux system yet has been identified in bacteria. Our data indicate that pneumococcus encodes a CDF protein that functions as a manganese efflux system, the gene for which we have designated *mntE*, which provides a unique insight into the role of intracellular manganese in transcriptional regulation and virulence. It is interesting that MntE does not appear to transport cadmium, a characteristic shared by many other manganese transporters (Kehres and Maguire, 2003). Future studies need to focus on elucidating the amino acids that determine metal ion specificity for this channel. This transporter is conserved among a number of Gram-positive bacteria, including the staphylococci, streptococci, bacilli and clostridia, and has more distant homologues in Gramnegative bacteria and Archaea, indicating a potential role for manganese efflux in many different biological systems.

Manganese is important in providing defence against oxidative stresses *S. pneumoniae* (McAllister *et al*., 2004). A well-understood example is the manganesedependent superoxide dismutase (sodA), a cytosolic protein found in many bacteria that reduces oxygen radicals to protect against oxidative stress (Yesilkaya *et al*., 2000). Manganese bicarbonate complexes themselves can reduce reactive oxygen species directly, providing an antioxidant defence independent of enzymatic factors (Daly *et al*., 2004). Manganese regulates oxidative stress tolerance in streptococci (Jakubovics *et al*., 2002) and manganese supplementation restores phenotypic defects in knockout strains of superoxide dismutase (Al-Maghredi *et al*., 2002). Our data indicate that intracellular accumulation of manganese not only increases tolerance to oxidative stresses but also dysregulates H_2O_2 production by the pneumococcus. The intracellular concentration of manganese may be the key factor controlling the ability of the pneumococcus to produce high amounts of H_2O_2 and survive in the absence of catalase.

An important aspect of bacterial gene regulation is the ability to sense and respond to environmental conditions. The availability of various elements is an important factor in bacterial pathogenesis as both bacteria and host compete for scarce resources. The bacterial response to various metal ion stresses is complex and involves cross-talk between metalloregulatory pathways and some unexpected patterns that include upregulation of amino acid biosynthesis systems in response to specific heavy metals (Moore *et al*., 2005). A recent study has found that the host immune system exploits this competition by secreting calprotectin, which chelates zinc and manganese, rendering them unavailable for bacterial growth (Corbin *et al*., 2008). The manganese-responsive regulator, PsaR, regulates not only manganese uptake but also the loci involved in pathogenesis, such as pili (Johnston *et al*., 2006). Other species of streptococci use manganese as a signal to control the expression of virulence genes (Arirachakaran *et al*., 2007). *S. pneumoniae* upregulates manganese uptake in response to acidic stress (Martin-Galiano *et al*., 2005) as well as in the lung and intraperitoneal cavity (Marra *et al*., 2002). The environment of the lung contains relatively high concentrations of calcium (5 mM), manganese (34 µM) and zinc (200 µM) (Chicharro *et al*., 1999; Vanthanouvong and Roomans, 2004). The signalling pathways involved in the uptake and sensing of divalent cations are likely to be complex and interconnected, as evidenced by recent studies indicating PsaR can responded to both manganese and zinc (Kloosterman *et al*., 2008).

Differences in gene regulation between conditions found in the respiratory mucosa and standard culture media can provide insight into the transcriptional pathways important for establishing disease in the human host. Using the *mntE*– mutant, we were able to show that pneumococcus elicits a distinct transcriptional response to intracellular accumulation of manganese, a response that is different from extracellular manganese-induced signalling pathways. Such differences allow the bacteria to fine-tune a transcriptional response in response to both dynamic intracellular and extracellular conditions. Our findings indicate that *mntE*– is more fit in terms of resistance to oxidative stress and production of more H_2O_2 and pili, but this fitness benefit is abrogated in the host as the mutation reduced the pathogenicity of the bacteria. It is possible that disruption of manganese export also has a downstream effect on iron uptake and signalling, as both our transcriptional analysis and previous studies suggest a significant degree of cross-talk between these pathways (Guedon *et al*., 2003; Hanks *et al*., 2006). Although we did not observe any significant difference in iron sensitivity *in vitro*, more subtle differences may be evident *in vivo*. Also, downregulation of the PsaABC locus to almost undetectable levels could play a role in attenuation in the host, as this locus has been implicated in virulence (Marra *et al*., 2002). Downregulation of PsaABC is not sufficient to mediate protection against high levels of manganese in the environment, indicating that while downregulation of uptake does play an important role, an active efflux system is also required to maintain homeostasis. This indicates that manganese efflux is required at some stage of invasive disease, and provides a unique antimicrobial target for the development future therapeutics.

Experimental procedures

Media and growth conditions

Streptococcus pneumoniae TIGR4 and TIGR4R was grown on tryptic soy agar (EMD Chemicals, Gibbstown, NJ) supplemented with 3% sheep blood or in defined semi-synthetic casein liquid media supplemented with 0.5% yeast extract (C+Y) (Lacks and Hotchkiss, 1960; Rosch *et al*., 2008a). Erythromycin (1 µg ml^{-1}) and kanamycin (400 µg ml^{-1}) were added when appropriate. Todd Hewitt Broth (ThyB) and ThyB depleted of cations (ThyC) using Chelex resin (Bio-Rad, Hercules, CA) were formulated as previously described (Hanks *et al*., 2006). When appropriate, pH was adjusted to 6.5 using concentrated hydrochloric acid and subsequently sterile filtered. Cultures of *S. pneumoniae* were innoculated from frozen stock and incubated at 37°C in 5% CO_2.

Primers/mutant construction

Mutants in TIGR4 and TIGR4R were made by PCR-based overlap extension (Wurch *et al*., 1998). Briefly, regions flanking upstream and downstream of the target gene were amplified by PCR and spliced to an antibiotic cassette. The final PCR product was transformed into the pneumococcus by conventional methods, replacing the targeted gene with the antibiotic cassette. To confirm transformation, primers outside the transformed region were used for PCR and subsequent region sequencing. To construct *sp1552*–, the gene was replaced with an erythromycin resistance cassette. To generate the wild-type revertant strain of *sp1552*–, primers JR50 and JR53 of the list were used to amplify *sp1552* along with flanking sequence from TIGR4 chromosomal DNA. The PCR product was subsequently purified and transformed into *sp1552*– and grown on tryptic soy agar supplemented with 3% sheep blood and 1 mM $MnCl_2$ which is toxic to *sp1552*–. Of the approximately 10^5 colonies obtained, there were no spontaneous colonies in control transformations. PCR and sequencing were used to confirm reversion of *sp1552*– to the parental genotype. Oligos used and strains generated are listed in Table 4.

Cation sensitivity

To determine cation sensitivity, 1 M solutions of calcium chloride, calcium nitrate, cobalt chloride magnesium chloride, manganese chloride, manganese sulphate, iron chloride, nickel chloride and zinc chloride (Sigma-Aldrich, St Louis, MO) were prepared in Milli-Q water and sterile filtered. A freshly plated bacterial lawn was overlaid with a sterile, 5 mm filter disc with 10 µl of the cation solutions. Plates were incubated overnight, and the zone of inhibition was measured for each disc. Statistical significance was determined by Friedman test. For growth curves, the pH of standard ThyB was adjusted to 6.3 to facilitate solubility of the various cations. Cations were added to final concentrations of 1 mM for magnesium, manganese, calcium and nickel. Cobalt was added at to a final concentration of 100 µM and zinc to a final concentration of 500 µM. Higher concentrations of cobalt and zinc were found to have detrimental effects on the growth of TIGR4.

Elemental analysis

Intracellular elemental composition was measured by ICP-MS as previously described (Outten and O'Halloran, 2001). Cells were grown in chelexed ThyC supplemented with 1 mM calcium chloride, 300 µM manganese chloride, 1 mM magnesium chloride and 300 µM zinc chloride. To measure intracellular cadmium, this medium was further supplemented with 3 µM cadmium chloride. Bacteria were collected by centrifugation and the pellet washed five times in PBS + 10 mM EDTA. Pellet was dried overnight and analysed by the Bodycote Testing Group (Santa Fe Springs, CA). Bacterial cfu and dry weight were enumerated before sample analysis.

^{54}Mn uptake assay

Manganese uptake in live cells was measured as previously described (Maguire, 2007). Strains were grown in ThyB, pH 6.5 in a water bath at 37°C to $OD_{600} = 0.4$, after which 1 µCi of $^{54}Mn^{2+}$ (Perkin Elmer, Wellesley, MA) per millilitre of media along with 500 µM $MnCl_2$ was added. Aliquots were taken at indicated 15 min intervals, centrifuged to collect cells, and the pellet was washed twice in PBS + 10 mM EDTA to remove extracellular cations. After 1 h, bacteria were collected by centrifugation and re-suspended in ThyB medium lacking isotope to monitor efflux. Counts per minute were measured in the samples by a Perkin Elmer 1480 Wizard 3 Automatic Gamma Counter. A caesium standard and background control was used for all measurements. Data were normalized to OD_{600} to compensate for cellular growth.

RNA isolation and microarray analysis

To measure the global transcriptional response to high manganese, bacteria were cultured in ThyB supplemented with 500 µM manganese chloride. To measure transcriptional response to high intracellular manganese, both TIGR4 and the *sp1552*– mutant were cultured in ThyB supplemented with 500 µM manganese chloride. Bacterial RNA was harvested from mid-log-phase cultures by the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). Microarray experiments were performed as described previously (Orihuela *et al.*, 2004). Wholegenome *S. pneumoniae* oligonucleotide microarrays (version 6) obtained from the PFGRC at the J. Craig Venter Institute (http://pfgrc.jcvi.org/) were used for microarray experiments (lot numbers 6QSP11062007A and 6QSP11012007A). The array consisted of 70-mer oligonucleotide probes representing 2060 open reading frames from *S. pneumoniae* strain TIGR4 and 457 from strains G54 and R6, and each probe sequence was spotted at least five times on the array. Data have been submitted to NCBI under accession GSM358422, GSM358242, GSM358241, GSM358231, GSM358229 and GSM358101.

To analyse the microarray data, a series of filtration algorithms was applied to eliminate spots with poor-quality data. Spots flagged (as bad, absent or not found) by the image analysis software GenePix 6.0 (Axon Corp., Union City, CA) and spots having a signal-to-noise ratio

less than 1.5 or a background-corrected signal reading less than 20 in both Cy3 and Cy5 channels were excluded from analysis. To correct the intensity bias, loess normalization was applied to each microarray. Of at least five replicate spots on each array, only genes whose corresponding spots passed the filtering criterion at least 50% of the times were reported in further analysis. Of the 11 960 spots dedicated to TIGR4, approximately 16.8% were eliminated because of poor quality or low signal intensity. These were typically gene specific, with all five spots failing to give adequate signal intensity, indicative of either poor hybridization to the oligonucleotide probe or low abundance of the specific transcript. For each experiment, results were summarized from three independent microarray replicates, which included one dye flip experiment for adjusting the dye bias. The average of the fold changes from at least two replicated experiments for each gene was calculated, and those with a minimum twofold change were listed in respective tables. All genes included in the table had a *P*-value less than 0.05, using a *t*-distribution test.

Quantitative RT-PCR

Purified mRNA was quantified via NanoDrop with appropriate standards. cDNA synthesis and quantitative PCR were performed using the Superscript III Platinum SYBR Green Two-Step qRT-PCR kit (Invitrogen, San Diego, CA) on a ABI PRISM 7300. Relative transcript abundance of genes was normalized to *gyrA*, which served as an internal control. Primers used to confirm gene expression are listed in Table 4. The qRT-PCR experiments were performed in triplicate from independently isolated RNA samples.

Western blot and ELISA analysis of pilus expression

Logarithmically growing cells in C+Y media ($OD_{620} = 0.5$) were collected by centrifugation and lysed in 0.1% Triton X-100. To ensure equal loading, protein concentration was determined for each lysate via A-280 and loaded accordingly. Duplicate gels stained with Coomassie were used to confirm measured protein concentrations to confirm equivalent loading. Lysates were run on 4–12% NuPAGE Bis-Tris gels (Invitrogen) for 5–6 h to resolve higher-molecular-weight pilus polymers. Proteins were subsequently transferred to PVDF membranes by Western blot. Pilus proteins were detected using rabbit anti-RrgB (1:5000) in PBS + 0.1% Tween-20 + 5% non-fat dry milk.

ELISA analysis using equivalent numbers of bacteria, as determined by cfu, was used to confirm the Western analysis. Strains were grown in C+Y to an OD_{600} of 0.5, diluted in 0.1 M carbonate buffer (pH 9.6) and transferred in serial dilutions to 96-well ELISA plate. The plates were spun at 2000 *g* for 10 min, and the supernatant was removed. The plates were dried under a vent hood for 1 h before blocking in 10% FBS for 2 h. Rabbit polyclonal antiserum against Sp0463 (RrgB) was diluted 1:1000 in 10% FBS. The plates were washed five times with wash buffer (1% Tween 20, 1 mM Tris, 154 mM NaCl) and incubated with primary antibody for 1 h. The plates were washed five times and incubated with AP-anti-Rabbit IgG (Southern Biotech, Birmingham, AL) (1:2000) for 1 h. The plates were washed five times, incubated for 20 min in AP-yellow substrate (Sigma), and read at OD_{405} in a Spectramax 340 plate reader (Molecular Devices, Sunnyvale, CA).

Oxidant stress challenge

S-nitrosoglutathione (GSNO) killing was performed as previously described (Stoeher *et al.*, 2007). For superoxide killing, 60 mM methyl viologen (Sigma) was used as previously described (Tseng *et al.*, 2002). Briefly, triplicate samples of bacterial cultures were grown to an OD_{600} of 0.50 in C+Y media. Cells were centrifuged, re-suspended in PBS, and cfu were enumerated to quantify input. GNSO or superoxide was then added to the cultures at the desired concentrations and aliquots were taken at 15 min intervals for serial dilution and plating to enumerate bacteria.

Hydrogen peroxide measurement

Hydrogen peroxide (H_2O_2) indicator plates were made using standard ThyB plates supplemented with ABTS (Sigma, A1888) at a final concentration of 3 mg ml^{-1} in plates and horseradish peroxidase (Sigma, P8250) at a final concentration of 0.2 mg ml^{-1} to serve as a colorimetric indicator of H_2O_2 production. Plates were then dried for 24 h. Serial dilutions (1:10) of bacterial cultures were added to the plates and incubated anaerobically in GasPack anaerobic pouches at 37°C overnight. Plates were then exposed to room air and colour development on the indicator plates was monitored. Images were taken 30 min after exposure to atmospheric oxygen. For measurement of H_2O_2 production in liquid media, bacteria were cultured in C+Y media to an OD of 0.8 and subsequently back-diluted 1:20 into fresh ThyB or ThyB supplemented with 1 mM $MnCl_2$. Bacteria were allowed to grow for 2 h at 37°C/5% CO_2, and then 1 ml aliquots were taken, bacteria collected via centrifugation, and re-suspended in sterile PBS. Bacteria were then incubated at room temperature for 20 min to allow H_2O_2 production. H_2O_2 production was measured with a H_2O_2 assay kit from National Diagnostics (Atlanta, GA). A PBS blank and serial dilutions of a H_2O_2 standard solution were used as a background measurement and standard curve respectively. Bacteria were enumerated by serial dilution on TSA + 3% blood agar plates and respective values for H_2O_2 production were normalized for cfu.

Mouse challenge

Virulence studies were performed as previously described (Orihuela *et al.*, 2004). Exponential cultures were centrifuged, washed in sterile PBS, and re-suspended at the appropriate concentration in PBS as confirmed by serial dilution and plating on blood agar plates. Six-week-old female BALB/cJ mice (Jackson Laboratory, Bar Harbor, ME) were maintained in BSL2 facilities. All experiments were performed under inhaled isoflurane (2.5%). Bacteria were introduced by intranasal administration of 10^7 cfu in 25 µl of PBS. Mice were monitored daily for signs of infection. Nasal passages were lavaged and blood was extracted from the tail vein 24 and 72 h post infection, diluted, and plated on blood agar to ascertain bacterial colonization and bacteraemia. Statistical analysis was performed using Kaplan–Meier survival estimates for survival and Student's *t*-test for bacterial titres.

Acknowledgements

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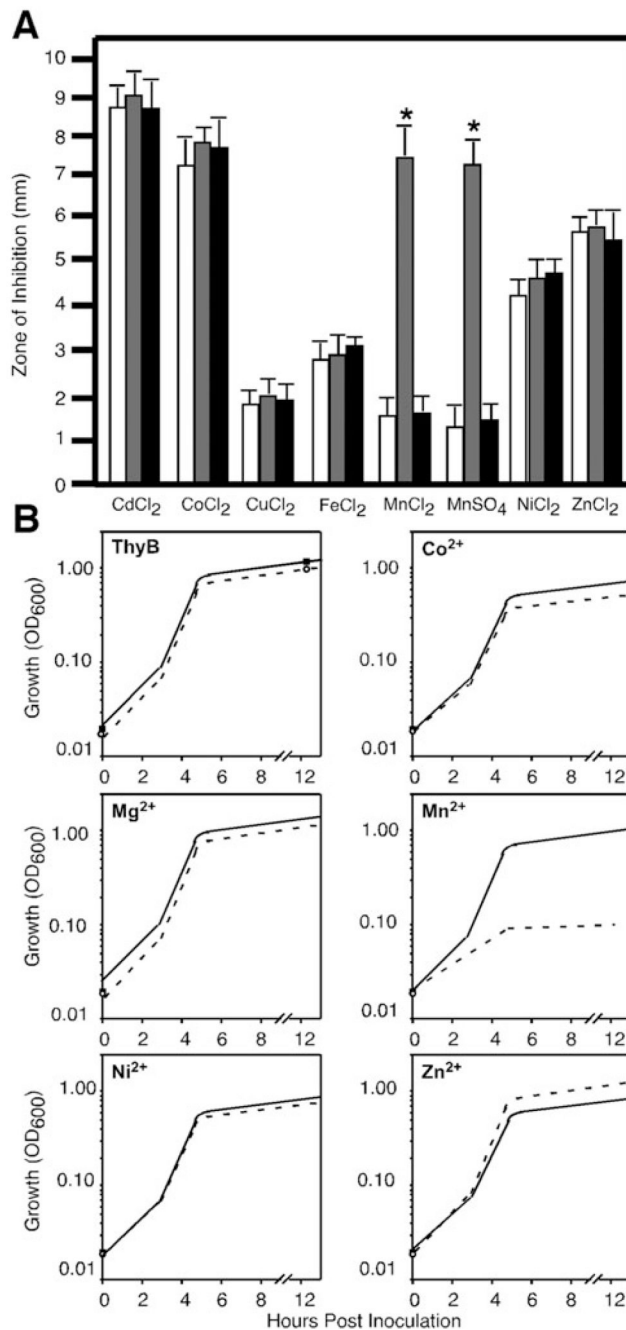
Fig. 1. Cation sensitivity profiles of TIGR4, *sp1552*– and the WT revertant
A. Bacterial lawns of TIGR4 (white bars), *sp1552*– mutant (grey bars) and WT revertant (black bars) were overlaid with filter discs saturated with 1 M solutions of the various cations tested. The zone of inhibition of growth was measured from the edge of the disc at 24 h. Data are representative of mean ± SD from four independent experiments. *P < 0.01.
B. Cultures of either TIGR4 (solid line) or the *sp1552*– mutant (dashed line) were grown in ThyB medium supplemented with cobalt, manganese, magnesium, nickel and zinc (concentrations given in *Experimental procedures*). Cultures were inoculated with 10^5 bacteria and growth was measured at OD_{600} over 12 h. Data are representative of one of three independent growth curves.
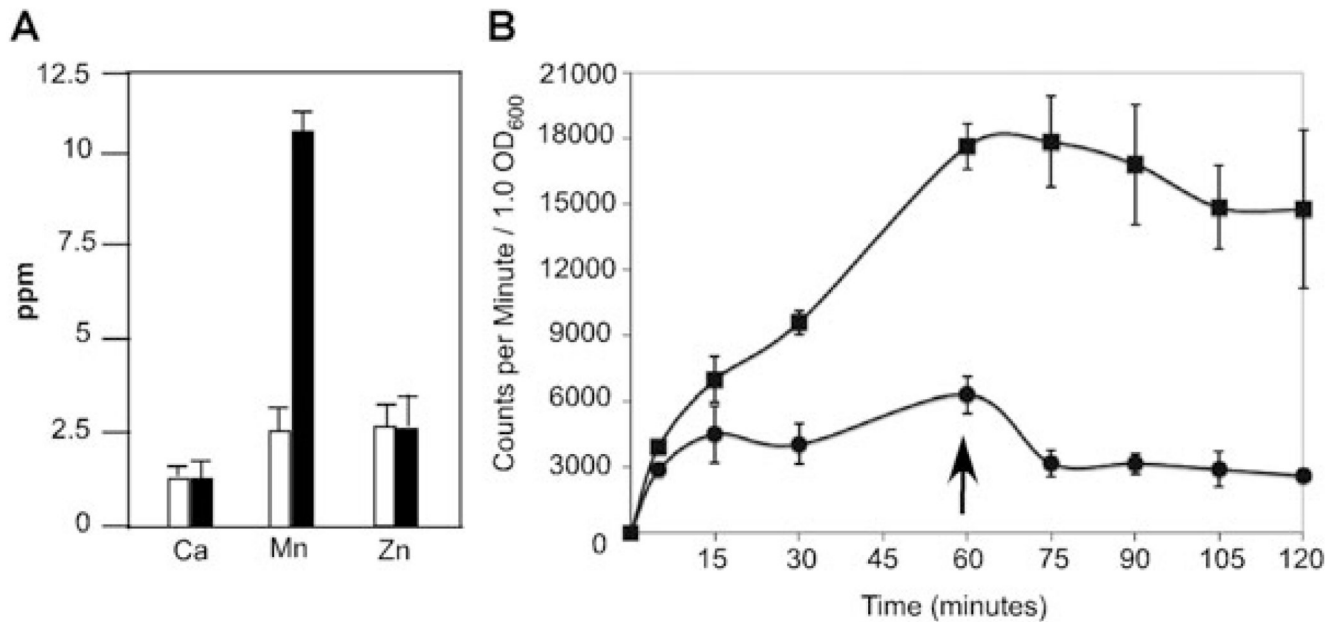
Fig. 2. Intracellular concentrations of cations

A. TIGR4 (white) and *sp1552*– (black) were grown in ThyC supplemented with calcium (1 mM), manganese (300 µM), magnesium (1 mM), zinc (300 µM) and cadmium (3 µM) to facilitate measurement of these specific cations. Cells were harvested and extracellular cations removed by washing in EDTA. Cation concentration was determined by ICP-MS in 10^8 bacteria (p.p.m., parts per million). Data represent mean ± standard deviation from at least two independent experiments. For all cations tested, an internal standard of known concentration was used to confirms measurements.

B. $^{54}Mn^{2+}$ uptake was measured in both TIGR4 (circles) and *sp1552*– (squares) in log-phase cultures spiked with 500 µM $MnCl_2$ and 1 µCi of $^{54}Mn^{2+}$. Parallel cultures were measured to normalize accumulation to cellular growth. Counts per minute were determined and expressed as a function of OD_{600}. After 1 h, cells were collected and re-suspended in media lacking isotope (arrow) to monitor efflux. Data represent mean ± standard deviation from three independent experiments.
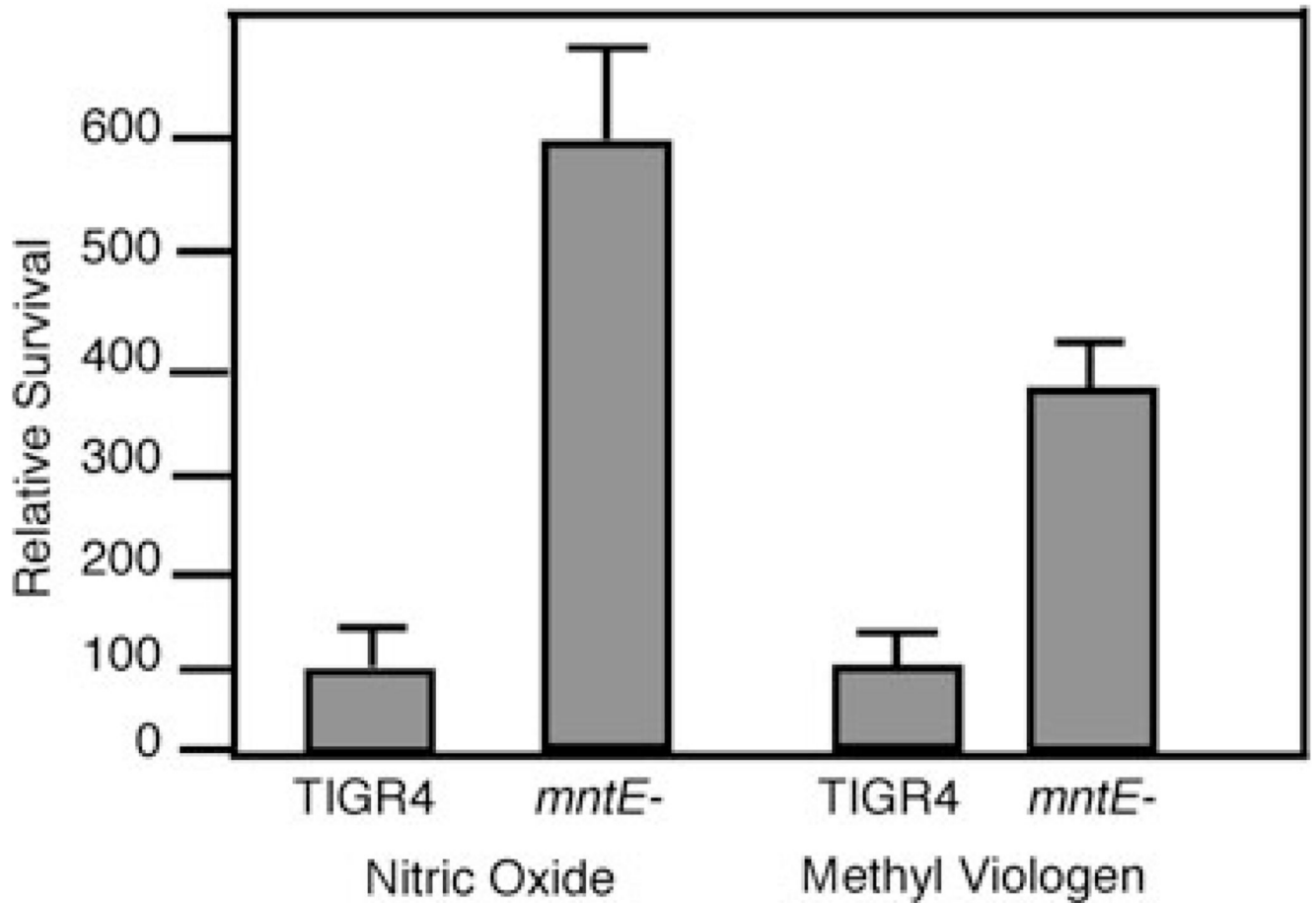
Fig. 3. Oxidative killing of TIGR4 and *mntE*–
Strains were subjected to either superoxide (methyl viologen) or nitric oxide (GNSO) stresses. Bacterial cfu were enumerated immediately before adding oxidants and at 15 min intervals thereafter via serial dilution and direct counts. Data represent mean ± standard deviation of relative survival (% of TIGR4) from three independent replicates after a 60 min exposure.
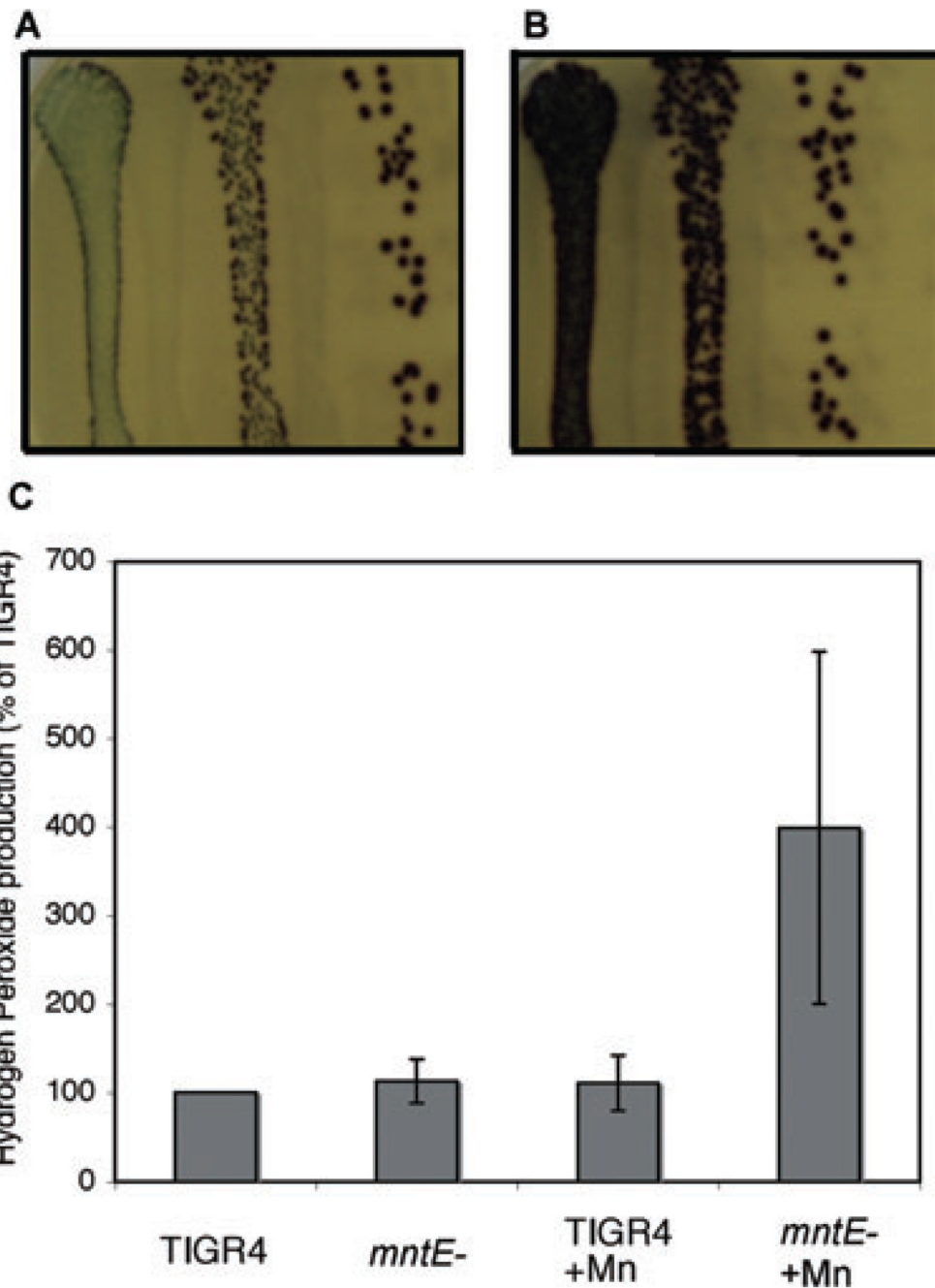
Fig. 4. Hydrogen peroxide production by TIGR4 and *mntE–*

A and B. TIGR4 (A) and the *mntE–* mutant (B) were serially diluted 1:10 (decreasing concentration from left to right) and streaked on to H_2O_2 indicator plates. Bacteria were grown under anaerobic conditions overnight on indicator plates, and images were taken after a 30 min exposure to atmospheric oxygen. Purple coloration indicates H_2O_2 production. Note that in the more dilute plating (far right of A and B) colonies produce equivalent amounts of H_2O_2, but in a plating that is 100 times more concentrated (far left of A and B) H_2O_2 production is restricted in TIGR4 but the *mntE–* mutant continues to produce prodigious amounts.

C. The amount of H_2O_2 produced by TIGR4 and *mntE–* mutant was measured after growth in ThyB and ThyB supplemented with manganese (100% = 10 µM H_2O_2 per 10^7 cfu).
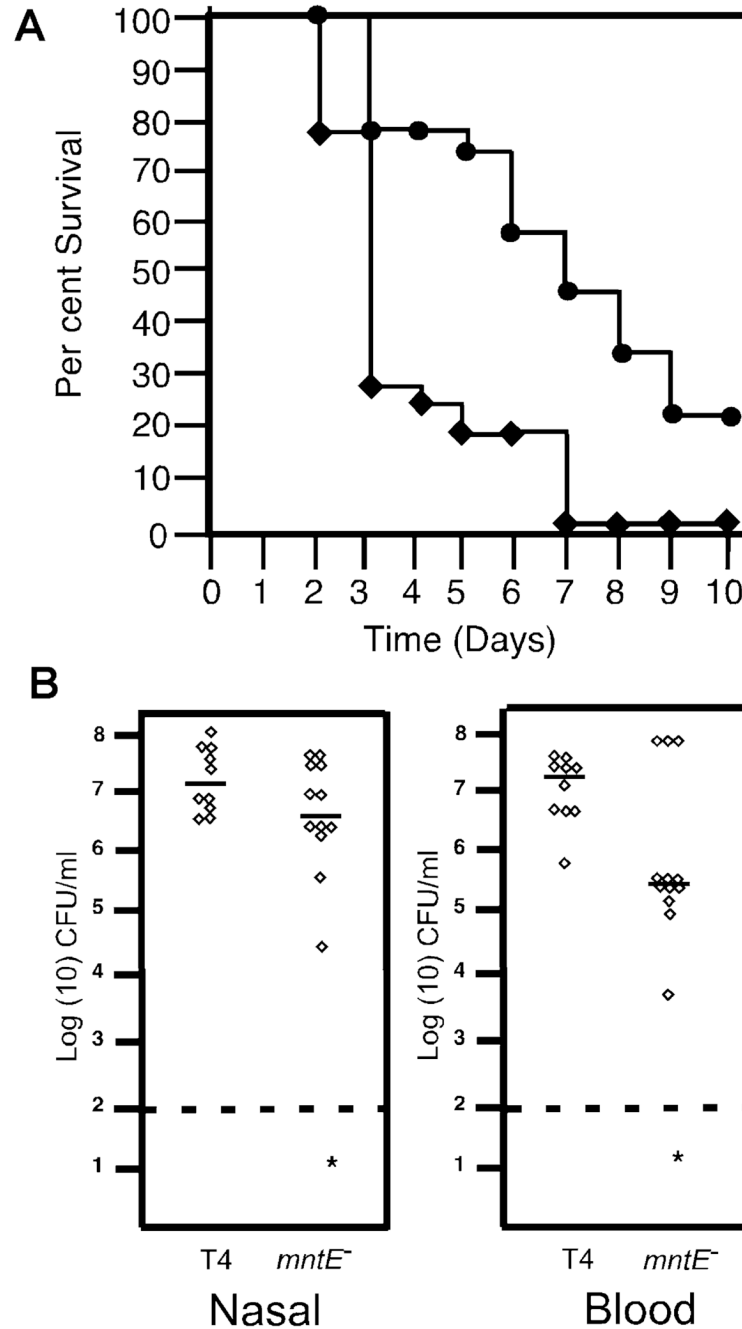
Fig. 5. Virulence of the manganese export mutant

A. Six-week-old female Balb/cJ mice were infected intranasally with 1×10^7 bacteria. Survival plot of mice infected intranasally with 1×10^7 TIGR4 (diamonds) or *mntE–* mutant (circles). Data are from 18 mice per group from two independent experiments.

B. Nasal and blood titres at 24 h are shown. Each symbol represents an individual mouse; five mice per experiment in duplicate are shown. The asterisk (*) indicates statistical significance of $P < 0.05$.
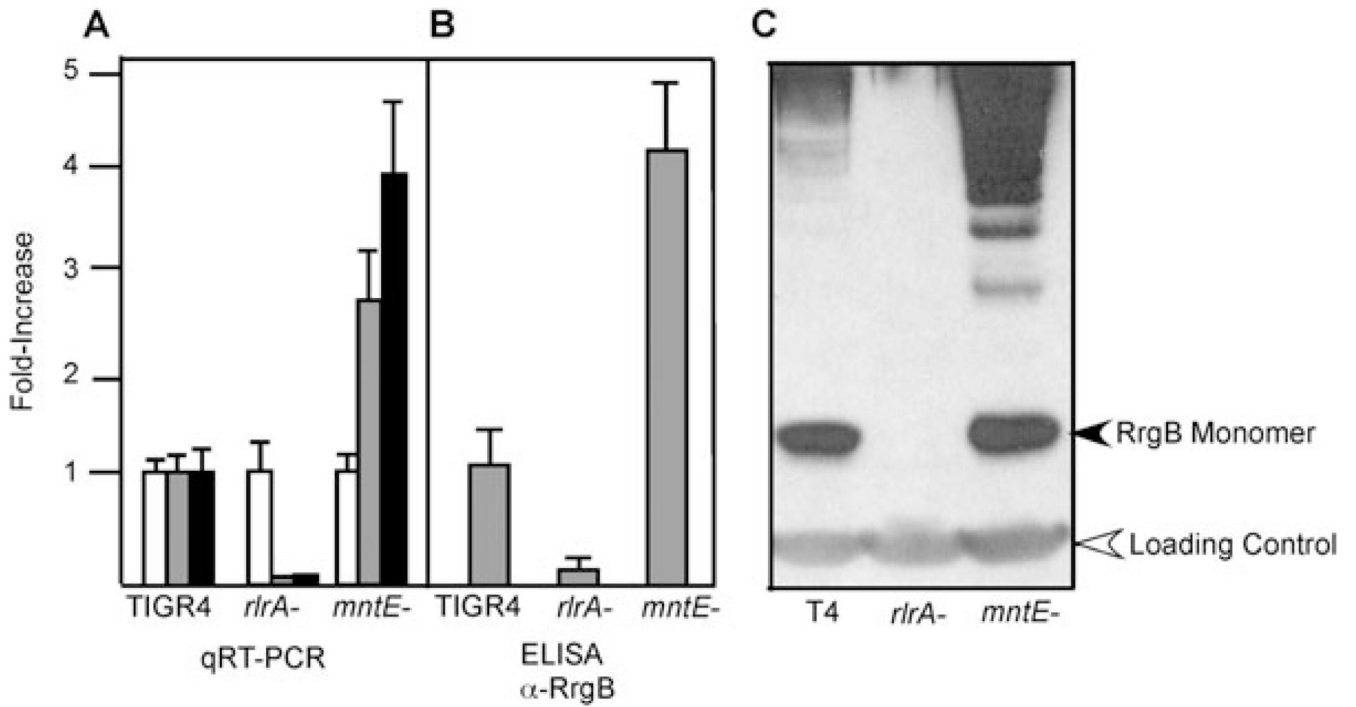
Fig. 6. Analysis of pilus expression
Relative expressions of *gyrA* (white bars), *rlrA* (grey bars) and *rrgB* (black bars) were measured in the TIGR4, *rlrA*– and *mntE*– strains by qRT-PCR of RNA isolated from logarithmically growing cells (A). Expression levels were normalized to *gyrA*, which served as an internal control. The increased level of the *rrgB* transcript resulted in approximately a fourfold increase in RrgB protein expression, as measured by ELISA (B) as well as by Western blot using RrgB-specific antisera (C). The RrgB monomer and loading control cross-reactive band are indicated by arrows. Data for qRT-PCR and ELISA represents mean ± standard deviation from three independent experiments. Western blot is representative of three independent experiments.

Table 1

Global transcript analysis of the bacterial response to extracellular and intracellular manganese.

Putative functional category	Number of genes responsive to high extracellular Mn	Number of genes responsive to high intracellular Mn
Transcriptional regulation	2	10
Carbohydrate transport	1	3
Amino acid and peptide transport	0	8
Carbohydrate metabolism	9	39
Protein metabolism	4	6
Metal transport/utilization	8	9
Other transport	4	10
Stress response and chaperones	1	5
Transcriptional regulators	7	32
Cell wall and surface proteins	3	13
Secretion	0	2
Competence	1	3
Electron transport, ATP synthesis	3	0
Hypothetical	7	34
Total genes	52	172

Middle column (TIGR4 : TIGR4 + Mn) is a summary of the comparison of TIGR4 transcripts from bacteria grown in standard ThyB versus ThyB supplemented with 500 µM manganese to determine bacterial response to extracellular manganese. The far right column compares transcript abundance of TIGR4 to *mntE–* when both strains were cultured in ThyB supplemented with 500 µM manganese to induce intracellular manganese accumulation. Full data set in Tables S1 and S2.

Table 2

qRT-PCR confirmation of gene expression patterns observed in microarrays.[a]

Gene/condition	Fold change (microarray)	Fold change (qRT-PCR)
TIGR4 versus TIGR4 + Mn		
SP0461 (*rlrA*)	NC	1.5
SP0463 (*rrgB*)	NC	1.3
SP1501	NC	1.2
SP1650	−30.3	−36.1
SP1992	2.0	3.4
TIGR4 + Mn versus *mntE*− + Mn		
SP0461 (*rlrA*)	4.0	4.4
SP0463 (*rrgB*)	2.6	3.4
SP1501	3.4	2.8
SP1650	−2.3	−30.5
SP1852 (*galT-2*)	−5.4	−3.7
Sp1992	3.6	4.3
SP2148 (*arcA*)	−5.2	−3.9

[a]Data represent average from three experiments, with each gene measured in duplicate. NC, no significant change.

Table 3

Representative transcripts of the bacterial response to extracellular and intracellular manganese.

Gene ID	Annotation	Fold change in response to high extracellular Mn	Fold change in response to high intracellular Mn
SP0043	Competence factor transport protein ComB	−2.3	NC
SP0285	Alcohol dehydrogenase, zinc containing	NC	−2.3
SP0461	Transcriptional regulator rlrA	NC	4.0
SP0463	Pilus subunit rrgB	NC	2.6
SP0519	dnaJ protein	−2.3	NC
SP0641	Serine protease, subtilase family	−14.7	−2.6
SP0877	PTS system, fructose-specific IIABC components	−2.1	12.7
SP1190	Tagatose 1,6 diphosphate aldolase	NC	−2.5
SP1649	Manganese ABC transporter, permease protein	−61.9	−3.8
SP1759	Preprotein translocase, SecA	NC	−2.8
SP1869	Iron-compound ABC transporter, permease protein	3.7	NC
SP1992	Cell wall surface anchor protein	2.0	3.6
SP2157	Alcohol dehydrogenase, iron containing	NC	−5.0
SP2201	Choline-binding protein D	2.1	3.8
SP2235	Response regulator ComE	NC	2.1
SP2236	Sensor histidine kinase ComD	NC	2.9
	Total number of differentially expressed genes	52	172

Middle column (TIGR4 : TIGR4 + Mn) is a representative sample of the comparison of TIGR4 transcripts from bacteria grown in standard ThyB versus ThyB supplemented with 500 µM manganese to determine bacterial response to extracellular manganese. The far right column is a representative sample of transcript abundance of TIGR4 and *mntE*– when both strains were cultured in ThyB supplemented with 500 µM manganese to induce intracellular manganese accumulation. NC, no change. Full data set in Tables S1 and S2.

Table 4

Primers and strains used in this study.

Strains/primers	Characteristics/sequence
Strains	
TIGR4	Pathogenic strain
mntE–	*sp1552* replaced by Erm cassette
WT revertant	*mntE–* crossed back to TIGR4 sequence
Primers	
ErmF	GGAAATAAGACTTAGAAGCAAAC
ErmR	CCAAATTTACAAAAGCGACTC
JR50	CAAGGGCAATGACCAAGATAGCATAGG
JR51	GTTTGCTTCTAAGTCTTATTTCCCTTGAGATTTGAGATAGATTGCTTCAT
JR52	GAGTCGCTTTTGTAAATTTGGCTTTCATCAAGAAACCAAAAAAGAATAG
JR53	CCAGTCGGATTGGGAAGAAAGTCATCG
Sp0461-RT1	GACTGCACAATCATATGTGTGACCC
Sp0461-RT2	CCATCGCAACAGGCTACCG
Sp0463-RT1	ATGCTGCTGTGATTGCTGCC
Sp0463-RT2	TCAATGCCTTGTCCAGTCGC
GyrA-RT1	TGTGACCCCAGACAAACCCC
GyrA-RT2	CTTGCTCATACGTGCCTCGG
Sp1501-RT1	CAATCATCATAGCACGCGCC
Sp1501-RT2	GCAGAAGCGCAATCTACTGGG
Sp1650-RT1	GTTCCGATTGGGCAAGACCC
Sp1650-RT2	TAGATAACATCAACGCCGTCGC
Sp1852-RT1	CACGCGCAAAGATAGCACCC
Sp1852-RT2	ATTGAGGTCATGGGCTTGGC
Sp1992-RT1	TGTTCCTGCAGTTGCTTGCG
Sp1992-RT2	ATGAAATCAAAGGCGCACCG
Sp2148-RT1	TTATGTTGCACCGTCCAGGC
Sp2148-RT2	TGACGATCACGGATGTTGGC