A New Direction for Manganese Homeostasis in Bacteria: Identification of a Novel Efflux System in *Streptococcus pneumoniae*

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Abstract

The ability to control intracellular levels of transition metals such as Mn^{2+}, Fe^{2+} and Zn^{2+} is critical for the virulence of many pathogenic bacteria. In this issue of *Molecular Microbiology*, Rosch *et al*. describe the first identification of a Mn^{2+} efflux system in bacteria, MntE of *Streptococcus pneumoniae*, and demonstrate that it is required for virulence in an animal model. Disruption of the *mntE* gene leads to widespread transcriptional changes that are distinct from responses to extracellular Mn^{2+}. These findings reveal, for the first time, that a bacterial trace metal efflux system plays a role in disease. Thus, MntE represents a new lead for the development of antimicrobials specifically aimed at disrupting microbial metal ion homeostasis.

Transition metals are essential for all living cells. Approximately a quarter to a third of all proteins are metalloproteins. However, metals are invariably toxic at high concentrations and, therefore, intracellular levels of metal ions must be tightly controlled. Metal ion homeostasis is maintained principally through the regulation of import and export across the cell envelope.

In human and animal hosts, essential metals are generally in short supply and bacterial growth depends upon the production of high-affinity metal ion scavenging systems. High affinity uptake systems for metal ions such as Fe^{2+} and Mn^{2+} are well-characterised in bacteria. In contrast, relatively little is known about the contribution of efflux systems to Fe^{2+} or Mn^{2+} homeostasis. Recently, a cation diffusion facilitator (CDF) family member, FieF (YiiP), was shown to export Fe^{2+} from *E. coli* cells (Grass *et al.*, 2005). In this issue of *Molecular Microbiology*, Rosch *et al.* describe a new member of the CDF family from *Streptococcus pneumoniae*, MntE, and show that it is selective for Mn^{2+}. This represents the first Mn^{2+} efflux system identified in bacteria. Furthermore, MntE is shown to be critical for host colonisation and virulence of *S. pnuemoniae*.

The existence of systems for Mn^{2+} efflux was demonstrated over 35 years ago in experiments tracking the accumulation of radiolabelled manganese (^{54}Mn) in *Bacillus subtilis* cells. When transferred to Mn^{2+}-replete medium, cells that had previously been incubated under Mn^{2+}-limitation rapidly imported Mn^{2+} to levels that inhibited protein and RNA synthesis (Fisher *et al.*, 1973). Within an hour, approximately 90% of the accumulated

Mn^{2+} was lost from cells, indicating that Mn^{2+} homeostasis depends upon an active efflux system.

Evidence for the presence of a Mn^{2+} efflux system in *S. pneumoniae* came from the analysis of a knockout mutant of locus Sp1552, encoding a predicted CDF family export system. The mutant was sensitive to Mn^{2+}, but not to other metal ions including Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+} or Zn^{2+} (Rosch *et al.*, 2009). Further investigation revealed that the Sp1552 (*mntE*) mutant accumulated >3-fold more intracellular Mn^{2+} than the isogenic wild-type, providing powerful evidence that MntE effluxes Mn^{2+} (Rosch *et al.*, 2009). It now seems likely that Mn^{2+} efflux from *B. subtilis* cells is due to a CDF family protein homologous to *S. pneumoniae* MntE. Four proteins predicted to be encoded in the *Bacillus subtilis* 168 genome share >20% amino acid identity with *S. pneumoniae* MntE. Of these, only the most distantly related protein, CzcD, has been characterised. It is an exporter of Zn^{2+}, Cu^{2+} and Co^{2+} (Guffanti *et al.*, 2002). Thus, the contributions of other CDF proteins to metal ion homeostasis in *B. subtilis* remain to be determined.

Members of the CDF family are found in virtually all bacteria, archaea and eukaryotes (Nies, 2003). In mammals, all characterized CDF proteins predominantly transport Zn^{2+}. Currently, there is little information on the mechanism of transport by mammalian CDF transporters. However, these proteins seem to be involved in regulating physiological homeostatic control, rather than in responses to the presence of pathogens. Most bacterial CDF proteins that have been characterised to date are involved in efflux of Zn^{2+} (Nies, 2003). In some cases, other cations might also be transported at physiological concentrations. Recently, the *E. coli* CDF protein FieF (YiiP)

was shown to export ferrous iron (Fe^{2+}) from cells (Grass *et al.*, 2005), which represents the first description of a bacterial iron exporter. Although Mn^{2+} efflux systems have not been reported previously in bacteria, there is evidence that CDF proteins are involved in Mn^{2+} efflux from eukaryotic cells. Thus, heterologous expression of MTP11 CDF protein from *Arabidopsis* or poplar in Mn^{2+}-sensitive *Saccharomyces cerevisiae* mutants restores Mn^{2+} tolerance to wild-type levels (Peiter *et al.*, 2007). In their native hosts, MTP11 proteins localise to the trans-Golgi network, suggesting that they mediate Mn^{2+} export by pumping Mn^{2+} cations into the Golgi and out of cells by exocytosis (Peiter *et al.*, 2007). However, transporters that efflux Mn^{2+} cations directly from the cytoplasm into the extracellular milieu in either prokaryotes or eukaryotes have not been identified. Knowledge gleaned from bacterial systems will inform studies on CDF proteins from plants or animals.

There is abundant evidence that bacterial Mn^{2+} homeostasis is important during a range of infections. Manganese uptake systems are indispensible weapons for the virulence of many Gram-negative and Gram-positive pathogens, including *S. pneumoniae* (reviewed by Papp-Wallace and Maguire, 2006). A key function of Mn^{2+} in streptococci is for protection against oxidative stress. In *S. pneumoniae*, disruption of the PsaBCA Mn^{2+} scavenging system results in hypersensitivity to superoxide and H_2O_2 (Johnston *et al.*, 2004). The identification of the MntE Mn^{2+} efflux protein allowed Rosch *et al*. (2009) to investigate the role of Mn^{2+} hyperaccumulation on oxidative stress and virulence. In line with previous studies, high levels of intracellular Mn^{2+}, accumulated in a *mntE* mutant, protected against oxidative stress induced by nitric oxide or superoxide. Interestingly, H_2O_2 production

was increased in the *mntE* mutant compared with the isogenic wild-type, specifically at high cell densities. This might reflect increased survival and, hence, prolonged metabolic turnover in the mutant under elevated H_2O_2 stress. Nevertheless, increased tolerance of oxidative stress did not lead to enhanced virulence of the *mntE* mutant; conversely, this strain was less pathogenic than wild-type bacteria in a mouse model (Rosch *et al.*, 2009).

The ability of *S. pneumoniae* to cause disease is profoundly influenced by gene regulation pathways (Hava *et al.*, 2003). Expression of the virulence-related pilus gene locus is down-regulated under high Mn^{2+} in a PsaR-dependent manner (Johnston *et al.*, 2006). Since the *mntE* mutant accumulates more Mn^{2+} than wild-type, it seemed likely that pilus genes might be further down-regulated in this strain, thus providing a possible explanation for the reduced virulence of the *mntE* mutant. However, assessment of pilus gene expression by quantitative RT-PCR and Western blotting demonstrated that these genes were up-regulated in the *mntE* mutant (Rosch *et al.*, 2009). These data prompted Rosch *et al.* to investigate the global transcription responses of *S. pneumoniae* to high extracellular Mn^{2+} and to hyperaccumulation of Mn^{2+} in the *mntE* mutant (Fig. 1).

Surprisingly, perhaps, the transcriptional responses to high extracellular Mn^{2+} and to accumulation of Mn^{2+} within cells were quite distinct (Rosch *et al.*, 2009). In total, 52 genes were regulated >two-fold in wild-type *S. pneumoniae* TIGR4 following exposure to high (500 μM) exogenous Mn^{2+}. The most strongly regulated genes included the *psaBCA* Mn^{2+}-scavenging system and a ferric iron uptake system (Fig. 1B). On the other hand, comparison between *S. pneumoniae* TIGR4 and the *mntE* mutant, both

cultured in high (500 μM) Mn^{2+}, identified 172 genes whose expression was changed >two-fold. Carbohydrate metabolism was apparently restructured in the *mntE* mutant and several insertion sequence elements were activated, possibly indicating a stress response. The expression of several known or putative virulence factors was altered, including neuraminidase (*nanB*), serine protease (*prtA*), and a homologue of the *S. gordonii* platelet-binding protein Hsa/GspB (Xiong *et al.*, 2008). Only six genes were regulated in response to both extracellular and intracellular Mn^{2+}, four of which (*psaBCA* and *prtA*) are known targets of the PsaR Mn^{2+}-dependent regulator (Kloosterman *et al.*, 2008).

The above data indicate that *S. pneumoniae* possesses mechanisms for sensing extracellular Mn^{2+} that are different from intracellular Mn^{2+}-sensing. How could this be achieved? One possibility is that the Mn^{2+}-dependent regulator PsaR interacts with the Mn^{2+} import system PsaBCA to sense flux through the transporter. Alternatively, a surface-exposed Mn^{2+} sensor might be involved in the response to extracellular Mn^{2+}. A candidate for this function is the two component system TCS04, which has been shown to control the expression of *psaBCA* and, in at least one strain of *S. pneumoniae*, *mntE* (McCluskey *et al.*, 2004).

The study by Rosch *et al.* (2009) emphasises the importance of metal ion homeostasis for bacterial virulence. To date, the roles of microbial metal ion efflux systems in bacterial pathogenesis have received little attention. However, given the ubiquitous presence of putative metal efflux genes in the genomes of almost all organisms (Nies, 2003), it seems likely that metal ion efflux systems are central to cellular physiology. It is imperative to

characterise the roles of these transporters in the pathogenic processes of different bacteria, since microbial metal ion homeostasis is potentially a highly promising target for the rational development of novel antimicrobial agents.

Figure legend

Schematic model of changes in Mn^{2+} transport (A) and gene transcription (B) in *S. pneumoniae* under high extracellular or high intracellular Mn^{2+} (based on data from Rosch *et al.*, 2009). A. In wild-type *S. pneumoniae* TIGR4 under low Mn^{2+}, the PsaBCA transporter (blue) mediates Mn^{2+} uptake, whilst MntE exports Mn^{2+} to maintain homeostasis. Under high extracellular Mn^{2+} concentrations, Mn^{2+} might enter the cell via an unknown low affinity transporter such as that proposed by Dintilhac *et al*. (1997). PsaBCA is down-regulated, preventing over-accumulation of Mn^{2+} within cells. PsaBCA is further decreased in a *mntE* knockout mutant. Manganese enters cells through a low affinity transporter and/or via low residual levels of PsaBCA (not shown). Higher Mn^{2+} concentrations are indicated by a more intense pink colour either side of the membrane. B. Key features of the transcriptional responses of *S. pneumoniae* to high extracellular Mn^{2+} or high intracellular Mn^{2+} (*mntE* mutant). Accumulation of Mn^{2+} within cells leads to more wide-ranging transcriptional changes, and a different set of genes regulated, compared with adding Mn^{2+} to the growth medium. Only a small number of genes (e.g. the *psaBCA* Mn^{2+} permease genes) were regulated in response to both extracellular and intracellular Mn^{2+}.

References

Dintilhac, A., Alloing, G., Granadel, C., and Claverys, J.P. (1997) Competence and virulence of *Streptococcus pneumoniae*: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases. *Mol Microbiol* **25**: 727-739.

Fisher, S., Buxbaum, L., Toth, K., Eisenstadt, E., and Silver, S. (1973) Regulation of manganese accumulation and exchange in *Bacillus subtilis* W23. *J Bacteriol* **113**: 1373-1380.

Grass, G., Otto, M., Fricke, B., Haney, C.J., Rensing, C., Nies, D.H., and Munkelt, D. (2005) FieF (YiiP) from *Escherichia coli* mediates decreased cellular accumulation of iron and relieves iron stress. *Arch Microbiol* **183**: 9-18.

Guffanti, A.A., Wei, Y., Rood, S.V., and Krulwich, T.A. (2002) An antiport mechanism for a member of the cation diffusion facilitator family: divalent cations efflux in exchange for K^+ and H^+. *Mol Microbiol* **45**: 145-153.

Hava, D.L., LeMieux, J., and Camilli, A. (2003) From nose to lung: the regulation behind *Streptococcus pneumoniae* virulence factors. *Mol Microbiol* **50**: 1103-1110.

Johnston, J.W., Myers, L.E., Ochs, M.M., Benjamin, W.H., Jr., Briles, D.E., and Hollingshead, S.K. (2004) Lipoprotein PsaA in virulence of *Streptococcus pneumoniae*: surface accessibility and role in protection from superoxide. *Infect Immun* **72**: 5858-5867.

Johnston, J.W., Briles, D.E., Myers, L.E., and Hollingshead, S.K. (2006) Mn^{2+}-dependent regulation of multiple genes in *Streptococcus pneumoniae* through PsaR and the resultant impact on virulence. *Infect Immun* **74**: 1171-1180.

Kloosterman, T.G., Witwicki, R.M., van der Kooi-Pol, M.M., Bijlsma, J.J., and Kuipers, O.P. (2008) Opposite effects of Mn^{2+} and Zn^{2+} on PsaR-mediated expression of the virulence genes *pcpA*, *prtA*, and *psaBCA* of *Streptococcus pneumoniae*. *J Bacteriol* **190**: 5382-5393.

McCluskey, J., Hinds, J., Husain, S., Witney, A., and Mitchell, T.J. (2004) A two-component system that controls the expression of pneumococcal surface antigen A (PsaA) and regulates virulence and resistance to oxidative stress in *Streptococcus pneumoniae*. *Mol Microbiol* **51**: 1661-1675.

Nies, D.H. (2003) Efflux-mediated heavy metal resistance in prokaryotes. *FEMS Microbiol Rev* **27**: 313-339.

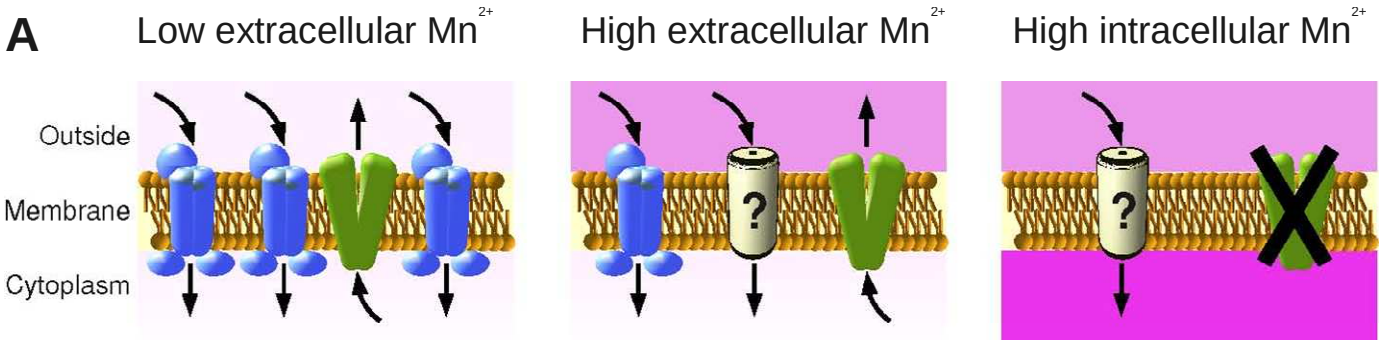
Papp-Wallace, K.M., and Maguire, M.E. (2006) Manganese transport and the role of manganese in virulence. *Annu Rev Microbiol* **60**: 187-209.

Peiter, E., Montanini, B., Gobert, A., Pedas, P., Husted, S., Maathuis, F.J.M., Blaudez, D., Chalot, M., and Sanders, D. (2007) A secretory pathway-localized cation diffusion facilitator confers plant manganese tolerance. *Proc Natl Acad Sci USA* **104**: 8532-8537.

Rosch, J.W., Gao, G., Ridout, G., Wang, Y.-D., and Tuomanen, E.I. (2009) Role of the manganese efflux system *mntE* for signaling and pathogenesis in *Streptococcus pneumoniae*. *Mol Microbiol*.

Xiong, Y.Q., Bensing, B.A., Bayer, A.S., Chambers, H.F., and Sullam, P.M. (2008) Role of the serine-rich surface glycoprotein GspB of *Streptococcus gordonii* in the pathogenesis of infective endocarditis. *Microb Pathog* **45**: 297-301.

A



B

Low Mn^{2+}:high Mn^{2+}

- Mn^{2+} uptake (*psaBCA*) ↓
- Ferric iron transport ↑

Wild-type:*mntE* mutant

- Mn^{2+} uptake (*psaBCA*) ↓
- Carbohydrate metabolism ↑ & ↓
- Insertion sequence elements ↑
- Virulence factors (e.g. *nanB*, *hsa*, pilus genes) ↑ & ↓