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Interplay between manganese and zinc homeostasis in the human pathogen *Streptococcus pneumoniae*†

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Abstract

ICP-MS analysis of *Streptococcus pneumoniae* reveals a high cell-associated Mn(II) concentration that is comparable to that of Zn(II). Stressing these cells with 100–200 μM Zn(II) leads to a slow-growth phenotype and a total Mn(II) concentration that is reduced, with no decrease of other metal ions. Supplementation of the growth media with as little as 10 μM Mn(II) fully restores the growth defect and cell-associated Mn(II) to normal levels. DNA microarray analysis reveals that zinc stress induces the expected upregulation of *czcD* (encoding a zinc effluxer), but also a pleiotropic transcriptional response suggestive of mild cell wall stress. Genes encoding a nitric oxide (NO) detoxification system (*nmlR*) and the Mn(II) uptake system (*psaBCA*) are also induced. We conclude that Zn(II) toxicity results in a cytoplasmic Mn(II) deficiency, possibly caused by competition at the Mn(II) uptake transporter protein PsaA.

Zinc is an essential metal ion in all organisms and is used as an essential cofactor in metalloenzymes and for structural and regulatory roles.[1,2] Cellular homeostasis of zinc is maintained by the coordination of uptake, efflux and intracellular sequestration and allows an organism to respond to large fluctuations in extracellular zinc availability. This may be particularly important during the course of an infection of a mammalian host by the pathogen *Streptococcus pneumoniae* since zinc concentrations are reported to range from 5 μM in the nasopharynx to ≈300 μM in lung tissue.[3]

In *S. pneumoniae* and other streptococci, inactivation of the *adc* (adhesin competence) operon leads to both a reduction in virulence and adhesion.[4] The *adc* operon encodes an ABC (ATP-binding cassette) transporter AdcCBA that is proposed to uptake zinc in *S. pneumoniae*[5] and is under the transcriptional control of AdcR.[6] Zinc efflux in *S. pneumoniae* requires the cation diffusion facilitator (CDF) protein CzcD,[7] regulated by the TetR family regulator SczA (see below).[8] Interestingly, a Δ*adcCBA* deletion strain is still able to acquire extracellular zinc, presumably through the PsaBCA ABC transporter,[5] which has been shown to uptake manganese. The CDF protein MntE, a paralog of CzcD, has recently been reported to function as a manganese efflux protein and appears to be constitutively expressed.[9]

In this study we evaluate the effects of defined perturbations of zinc homeostasis in *S. pneumoniae* on a metabolic and genetic level and in so doing, characterize a clear connection between zinc and manganese homeostasis in *S. pneumoniae* D39. ICP-MS

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analysis reveals that *S. pneumoniae* grown in limited aeration (static) liquid culture in brain heart infusion (BHI) broth is characterized by a cell-associated Mn concentration that is comparable to that of Zn and in excess of Fe (Figure 1). The total [Fe] is 3–4 fold lower than previous estimates in *S. pneumoniae* R6 strain and in *E. coli*,[10] for reasons that require further investigation. Other catalase-negative, hydrogen peroxide generating lactic acid bacteria are characterized by high intracellular Mn, and this could provide resistance to hydrogen peroxide toxicity.[11–13] *S. pneumoniae* also requires manganese for capsule production (tyrosine-protein phosphatase CpsB), metabolism (lactate dehydrogenase and pyruvate kinase) and for ROS detoxification (Mn superoxide dismutase).[3] Remarkably, the total cellular concentrations of Mn and Zn are comparable in *S. pneumoniae* despite the fact that Mn is present at ≈50-fold lower concentration in this growth medium (Figure S1); this suggests that PsaBCA is a highly efficient Mn transporter even in the presence of a highly competitive metal like Zn, which sits near the top of the Irving-Williams series.[14]

Growth of the parent wild-type, Δ*adcR* and *czcD::kan-rpsL* strains in the presence of increasing concentrations of zinc reveal a growth deficiency in the Δ*adcR* strain characterized by a slightly longer lag phase and lower growth yield (Figure S2).[6,15] In addition, a kinetic Zn^{67}/Zn^{68} uptake experiment reveals the Δ*adcR* mutant shows an increased uptake of zinc from the media (Figure S3), thus suggesting that zinc import through AdcR-controlled expression of AdcBCA is at least partially repressed in the wild-type strain under these conditions (120 μM Zn). In contrast, the *czcD::kan-rpsL* strain does not grow in media containing ≥120 μM Zn (Figure S2).[15] Metal contents as determined by ICP-MS show that the *czcD::kan-rpsL* and double mutant Δ*adcR czcD::kan-rpsL* strain in the presence of 100 μM added Zn contain ≈2-fold higher Zn than either the parent or Δ*adcR* strains under the same conditions (Figure S4). These data suggest that zinc efflux is primarily responsible for maintaining zinc homeostasis under these conditions of moderate zinc stress (100–200 μM).

Most significantly, all strains tested were characterized by a 40–50% decrease in cell associated Mn under these conditions (Figure 2). Addition of excess extracellular Mn rescued the growth phenotype of these strains (Figure S2 and not shown) while restoring the intracellular Mn concentration to above normal (Figure 2). In addition, Mn supplementation decreased the intracellular concentration of Zn (Figure S4). For the parent and Δ*adcR* strains as little as 10 μM manganese completely restores growth (other studies have found that as low as 3 μM manganese is sufficient).[16] For the *czcD::kan-rpsL* strain, 300 μM manganese was required for a full recovery of growth. This effect is highly specific for Mn since the metal concentrations for copper, nickel and iron did not decrease in response to high external Zn concentrations (Figure S5); in addition, none of these metals could rescue the growth phenotype of any of these strains induced by Zn (Figure S2).

Microarray analysis of the parent strain grown in BHI media (≈20 μM Zn) compared to BHI plus 200 μM zinc reveal a moderate number of transcriptomic changes (Table S3), some of which were previously described.[15] These changes are suggestive of mild cell envelope stress, given the up-regulation of members of the WalRK, CiaRH and TCS03 two-component system regulons.[17] It is important to note that SczA-regulated genes *czcD* (encoding a Zn effluxer) and *nmlR* are highly upregulated at high Zn, while the expression of the *adcR* regulon[6] is unchanged at 220 μM total Zn relative to 20 μM. This reveals that zinc efflux by CzcD is the primary homeostatic response to zinc stress under these conditions. This is fully consistent with microarray analysis of a strain harboring a nonfunctional *adcR* missense allele (AdcR H108Q)[6] in BHI with 200 μM zinc vs. the parent strain with zinc (Table S4). These data allow us to conclude that the expression of the *adcR* regulon is repressed in the wild-type strain in BHI (≈20 μM Zn),[6] at 120 μM (above) and at 220 μM total Zn in the growth media, in striking contrast to the *sczA* regulon (see Figure 3).

Although more studies are required, these data suggest that Zn-regulated *down*-regulation of zinc import through AdcCBA and *up*-regulation of efflux through CzcD occurs are distinct cytoplasmic $[Zn]_{free}$, in contrast to previous observations in *E. coli*.[1] This in turn, suggests distinct sensitivities of AdcR-mediated repression and SczA-mediated depression of transcription in the cytoplasm.

A major finding is that the entire *psaR* regulon is derepessed at high Zn thus revealing that *S. pneumoniae* is sensing cytoplasmic Mn deficiency at 200 μM added Zn (Table S3). As expected, a comparison of the parent strain grown in BHI with 200 μM zinc and 300 μM manganese versus that grown in BHI shows very few transcriptional changes compared to when manganese is not added (Table S5). Zinc stress is still sensed however, as *czcD* and *nmlR* are highly transcribed, and the *psaR* regulon is now repressed (Table S5). Thus, the transcriptomic and ICP-MS analyses are internally consistent and reveal that a major consequence of external Zn stress is a cellular Mn deficiency. Interestingly, Mn deficiency induced by deletion of *psaA* reveals the same five transcriptional changes, including the *psaR* regulon (*prtA*, *psaCBA*) and *czcD*.[16]

We next tested if the Zn-dependent Mn deficiency was caused by efflux through the manganese CDF protein MntE.[9] A deletion mutant of *mntE* was constructed and compared to the Δ*adcR* strain. A growth phenotype at 200 μM Zn was observed in the Δ*mntE* strain that was identical to the Δ*adcR* mutant (Figure S6A). ICP-MS reveals that Mn levels are similarly reduced by Zn stress in the Δ*mntE* mutant relative to Δ*adcR* or the wild-type strain (Figure S6B), indicating that the decrease in cell associated Mn is not due to Mn efflux. As expected, addition of a moderate dose of Mn (50 μM) was capable of rescuing the effects of zinc toxicity of the Δ*mntE* strains (Figure S6A).

These data therefore suggest by process of elimination that cell-associated Mn levels are decreased by Zn toxicity as a result of competition with Zn for transport by PsaBCA (Figure 3). Efforts to force Mn and Zn uptake through PsaBCA in a Δ*adcA* strain are complicated by the presence of an orphan Zn binding lipoprotein AdcAII[18] that may well function to stimulate metal uptake through AdcBC; as a result, this strain was not investigated further. Although a Δ*psaA* strain is sick due to manganese deficiency,[5,16] addition of 200 μM zinc causes a clearly reduced growth phenotype which is rescued by addition of 300 μM Mn (Figure S7A), a finding in opposition to competition between Zn and Mn at PsaA alone. A Zn^{67}/Zn^{68} isotope ratio experiment that compares the parent strain grown in 100 μM Zn^{67} to the Δ*psaA* strain cultured in 100 μM Zn^{67} and 300 μM Mn (which have identical growth kinetics) reveals that less Zn^{67} is taken up from the media by the Δ*psaA* strain. This finding is consistent with the observation that Δ*psaA* cells contain about half the total concentration of Zn (when grown in excess Mn) relative to a wild-type conterpart (Figure S7B).[16] This suggests competition between Zn and Mn for binding to AdcA lipoprotein and transport by AdcCBA. In contrast, Zn may be a competitive inhibitor of Mn transport by PsaCBA, further evidence for which is that the crystal structure of PsaA was solved with Zn bound,[19] suggesting a signficant affinity of the Mn transporter for its non-cognate metal (Figure 3). We stress, however, that we have no direct evidence for zinc competition for Mn uptake by PsaCBA, although this seems likely.

In addition to possible competition at the transporter, a high extracellular Zn/Mn ratio could potentially alter the activity of the *psaBCA* regulator, PsaR.[3,15] DNA binding experiments show that PsaR binds *psaBCA* operator DNA tightly in the presence of 1 μM Mn(II) or Cd(II); however, in the presence of 1 μM Zn(II) the binding is weak (Figure 4). Similar results have been reported for the Mn regulator from *S. gordonii*, ScaR.[20] This lack of co-repression by the non-cognate metal Zn may help to ensure that expression of the high affinity Mn uptake system is expressed under conditions of both extracellular and

intracellular Zn stress in an ongoing effort by *S. pneumoniae* to counteract the effects of cytoplasmic Mn deficiency (see Figure 3). This may provide a physiological advantage to *S. pneumoniae* during colonization of the lung or bloodstream where Zn concentrations are reported to be high (15–300 μM) and Mn is deplete (20 nM).[3,16] In fact, the extracellular zinc binding proteins Pht (pneumococcal histidine triad proteins) and AdcAII, which are regulated by AdcR, may favorably position *S. pneumoniae* to scavange zinc in a switch from a Zn deplete to Zn replete environment during invasion, while reducing competition at PsaA when extracellular Zn concentrations are high as a result of innate immune responses.[21] Detailed mechanistic and biochemical studies of the purified transporters will be required to obtain additional support for this model of metal competition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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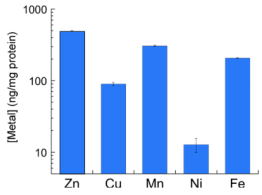

Fig. 1.

ICP-MS analysis of total WT *S. pneumoniae* cell-associated metal ion concentration (expressed as ng metal/mg protein) when grown in BHI media under microaerophilic conditions, 37 °C.

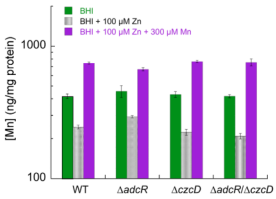

Fig. 2.

Mn content of the parent wild-type, Δ*adcR*, Δ*czcD::kan-rpsL* and Δ*adcR* Δ*czcD::kan-rpsL* strains grown in BHI alone (*green*), in the presence of 100 μM zinc added (*gray*) and in the presence of 100 μM zinc and 300 μM manganese added (*purple*).

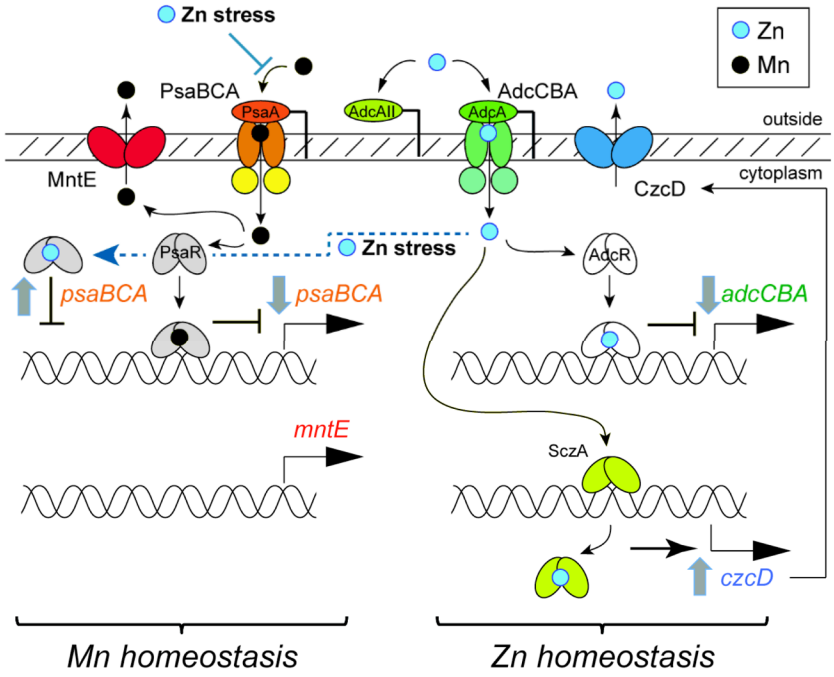

Fig. 3.

Schematic rendering of Zn and Mn homeostasis in *S. pneumoniae* highlighting the metal transporters and metal regulators discussed here and how Zn stress interferes with Mn homeostasis (see text for details).

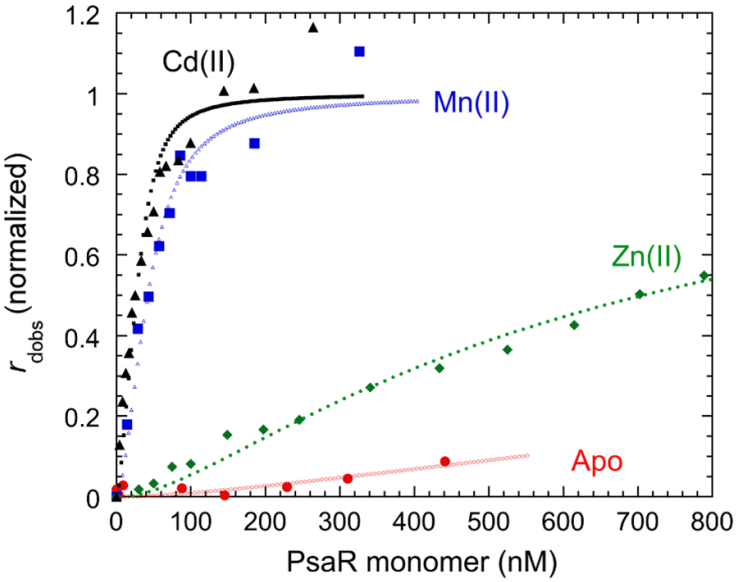

Fig. 4.

PsaR operator DNA binding curves for apo-Zn(II)-, Mn(II)-and Cd(II)-substituted PsaR. The anisotropy (r_{obs}) of a fluorescein-labeled *psaBCA* operator DNA fragment was measured as a function of total [PsaR] and fit to a model that invokes the binding of a single dissociable PsaR dimer ($K_{dimer}=2\times10^6$ M^{-1}) to the DNA.