Dysregulation of transition metal ion homeostasis is the molecular basis for cadmium toxicity in *Streptococcus pneumoniae*

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Cadmium is a transition metal ion that is highly toxic in biological systems. Although relatively rare in the Earth’s crust, anthropogenic release of cadmium since industrialization has increased biogeochemical cycling and the abundance of the ion in the biosphere. Despite this, the molecular basis of its toxicity remains unclear. Here we combine metal-accumulation assays, high-resolution structural data and biochemical analyses to show that cadmium toxicity, in *Streptococcus pneumoniae*, occurs via perturbation of first row transition metal ion homeostasis. We show that cadmium uptake reduces the millimolar cellular accumulation of manganese and zinc, and thereby increases sensitivity to oxidative stress. Despite this, high cellular concentrations of cadmium (\(\sim 17\) mM) are tolerated, with negligible impact on growth or sensitivity to oxidative stress, when manganese and glutathione are abundant. Collectively, this work provides insight into the molecular basis of cadmium toxicity in prokaryotes, and the connection between cadmium accumulation and oxidative stress.
Globalm circumcidium (Cd) production has risen by >1,000-fold since the beginning of the twentieth century to ~20,000 tons per year. Correspondingly, anthropogenic release of Cd into the atmosphere now significantly outstrips natural fluxes and predominantly occurs via non-ferrous ore processing, combustion of fossil fuels, and manufacturing, use and disposal of Cd-containing products. As Cd cannot be degraded, its flux into marine and terrestrial ecosystems has increased the risk of exposure as it accumulates in the food chain, and it is estimated that humans ingest 30 µg of Cd every day. Cd, which occurs as the divalent cation in the natural environment, is acutely toxic to all forms of life, although there are exceptions to this rule for organisms that have evolved in environments with extremely low zinc abundance. Cd toxicity in humans and microbes is, at a cellular level, closely associated with oxidative stress, despite the inability of Cd to directly produce reactive oxygen species. As a consequence, understanding the molecular basis for how Cd causes toxicity is a crucial issue.

Cd²⁺ accumulation in microbes and humans primarily occurs by uptake via divalent metal transporters, such as manganese (Mn²⁺) transporters, although the molecular basis for this process remains unknown. We investigated Cd²⁺ toxicity in the Gram-positive bacterium Streptococcus pneumoniae (pneumococcus) as it has only a single Mn²⁺-specific uptake pathway. The pneumococcus acquires Mn²⁺ via the PsABC permease, which comprises the ATP-binding cassette (ABC) transporter, PsABC, and a cell-surface solute-binding protein (SBP), PsA. The PsA permease is essential for both Mn²⁺ uptake and in vivo virulence. Similar to other ABC permeases, the SBP PsA defines the specificity of the uptake pathway. However, our recent studies showed that the functional specificity of the PsA permease in Mn²⁺ acquisition arose not from the specificity of Mn²⁺ binding by PsA, but instead resulted from the concerted action of protein metal ion coordination chemistry and structural rearrangements that prevented release of other divalent transition metal ions, such as Zn²⁺, into the permease, despite their ability to bind to PsA. Thus, although it is conceivable that Cd may interact with the PsA permease, whether it causes injury to the cell by non-productively competing for metal ion uptake or by accumulation in the cytosol remains unclear.

Cd dysregulates transition metal homeostasis. Titration of Cd²⁺ into a cation-defined medium (CDM), with a constant concentration of Mn²⁺ (1 µM), showed that pneumococcal growth was perturbed (Fig. 1b and Supplementary Fig. 1a), but that this growth defect could essentially be abrogated by the addition of a stoichiometric concentration of Mn²⁺ to the Cd²⁺-supplemented CDM (Fig. 1c and Supplementary Fig. 1b). S. pneumoniae growth in the presence of 30 µM Cd²⁺ reduced the cellular concentrations of Mn²⁺ and Zn²⁺. Manganese accumulation was most affected, with a reduction of ~90% (Fig. 1a; 1 µM Mn²⁺ + 30 µM Cd²⁺, P < 0.0001 (one-way analysis of variance (ANOVA))), despite no significant effect on cell volume (Table 1; 1 µM Mn²⁺ + 30 µM Cd²⁺, P = 0.376 (one-way ANOVA))). Supplementation with Mn²⁺ restored pneumococcal Mn²⁺ accumulation to ~60% of the unchallenged levels (Fig. 1a and Supplementary Table 1; 30 µM Mn²⁺ + 30 µM Cd²⁺, P < 0.0001 (one-way ANOVA))). Zinc also showed a significant decrease in accumulation in the presence of Cd²⁺ (Fig. 1a; 1 µM Mn²⁺ + 30 µM Cd²⁺, P < 0.0001; 30 µM Mn²⁺ + 30 µM Cd²⁺, P < 0.0001 (one-way ANOVA))). Cd²⁺ accumulation increased in the presence of 30 µM Cd²⁺, with or without Mn²⁺ (Fig. 1a). The degree to which Cd²⁺ was accumulated was more than that

### Table 1: S. pneumoniae cell volume parameters.

| Strain | Cell volume (fl) |
|--------|-----------------|
| D39 1 µM Mn²⁺ | 0.113 ± 0.04 |
| D39 1 µM Mn²⁺ + 30 µM Cd²⁺ | 0.124 ± 0.07 |
| D39 30 µM Mn²⁺ + 30 µM Cd²⁺ | 0.127 ± 0.07 |

*The data correspond to the mean ± s.d. from >40 independent measurements.*
of any other transition metal ion in the pneumococcus, despite having no physiological role. At 1 mM Mn$^{2+}$; 30 mM Cd$^{2+}$ in the growth medium, intracellular Cd$^{2+}$ concentration was 46.4 ± 2.9 mM. Cd$^{2+}$ accumulation was reduced by nearly 70% on the addition of 30 mM Mn$^{2+}$ to the growth medium (16.5 ± 0.7 mM Cd$^{2+}$), but cellular accumulation of the ion still remained more than that of the other first row transition metal ions.

We then analysed the impact of Cd$^{2+}$ on transition metal ion homeostatic pathways. Transcriptional analyses revealed that psaA transcription increased by 8.4-fold (P = 0.0007 (two-tailed unpaired t-test)) and PsaA expression by 2.8 ± 0.3-fold (P < 0.0001 (two-tailed unpaired t-test)) when grown in the presence of 30 mM Cd$^{2+}$ (Fig. 1d,e), consistent with the Mn$^{2+}$-responsive transcriptional regulator, PsaR, sensing the Mn$^{2+}$ depletion.\textsuperscript{7,12} Transcription of psaA remained elevated during growth supplemented with 30 mM Mn$^{2+}$ albeit to a lesser, but still significant, extent (3.5-fold, P = 0.0135 (two-tailed unpaired t-test)), although PsaA expression did not appear to be significantly increased (Fig. 1d,e). Zinc accumulation in S. pneumoniae also occurs via an ABC permease, the Adc permease.\textsuperscript{9,13,14} Transcriptional analysis of the Zn$^{2+}$-responsive transcriptional regulator, adcR, and the Zn$^{2+}$-recruiting SBPs associated with the Adc permease, adcA and adcAII (refs 13,14,16), did not show significant changes in the presence of Cd$^{2+}$ (Supplementary Fig. 3). Despite this, the Zn$^{2+}$ accumulation levels (2.6 ± 0.4 mM) were comparable to strains without a functional Adc permease, AdcCBA (2.7 ± 0.3 mM) and ΔadcA/ΔadcAII (2.5 ± 0.3 mM).
The perturbation of Zn$^{2+}$ acquisition was not due to competition for the Adc permease as the dominant Zn$^{2+}$-recruiting SBP, AdCA, showed very poor interaction with Cd$^{2+}$ by comparison with Zn$^{2+}$ (Supplementary Fig. 3). Taken together, these findings implicate Cd$^{2+}$ as being able to exert an effect on the Zn$^{2+}$-responsive transcriptional regulators. *S. pneumoniae* uses two Zn$^{2+}$-responsive transcriptional regulators: AdCR, which regulates the Zn$^{2+}$-uptake machinery described above, and SczA, which regulates transcription of the cation diffusion facilitator efflux protein CzcD$^{15,17}$. Consequently, we examined whether CzcD was also affected by Cd$^{2+}$ abundance. Growth of *S. pneumoniae* in CDM with 1 μM Mn$^{2+}$; 30 μM Cd$^{2+}$ increased czcD transcription by ~70-fold (*P* = 0.0011 (two-tailed unpaired *t*-test); Fig. 1f), while supplementation with Mn$^{2+}$ (30 μM Mn$^{2+}$; 30 μM Cd$^{2+}$) reduced this to only ~12.7-fold more than cells grown in unsupplemented CDM (Fig. 1f). Although CzcD from *Cupriavidus metallidurans* is protective against Co$^{2+}$, Zn$^{2+}$, and Cd$^{2+}$ ions$^{18}$, there is no evidence to suggest that the *S. pneumoniae* orthologue has similar polyspecificity. Consequently, we examined an isogenic czcD mutant strain to ascertain whether pneumococcal czcD contributed to Cd$^{2+}$ resistance. The *S. pneumoniae* ΔczcD strain was hypersensitive to extracellular Zn$^{2+}$, consistent with a role in Zn$^{2+}$ homeostasis (Fig. 2). Furthermore, the Cd$^{2+}$-induced efflux of Zn$^{2+}$ observed in wild-type *S. pneumoniae* was abolished in the ΔczcD strain (ΔczcD 1 μM Mn$^{2+}$; 4.4 ± 0.3 mM Zn$^{2+}$; ΔczcD 1 μM Mn$^{2+}$; 30 μM Cd$^{2+}$ 4.2 ± 0.3 mM Zn$^{2+}$; *P* = 0.78 (two-tailed unpaired *t*-test)). However, although Cd$^{2+}$ accumulation in the ΔczcD strain showed a mild elevation of ~1.4-fold compared with the wild-type, the growth phenotypes of the ΔczcD strain were indistinguishable from the wild-type strain and also showed no increase in susceptibility to extracellular Cd$^{2+}$ (Fig. 2 and Supplementary Fig. 4). Taken together, these data indicate that, despite the upregulation of *S. pneumoniae* czcD in response to Cd$^{2+}$ exposure, CzcD is primarily a Zn$^{2+}$ efflux transporter and appears to serve, at best, only a minor protective role against Cd$^{2+}$.

In addition to czcD, the pneumococcal genome encodes four other putative metal ion efflux pathways. These are as follows: spd1384 (mntE), a paralogue of CzcD involved in Mn$^{2+}$ efflux$^{19}$; spd1438 (cadD), a membrane protein of unknown function associated with Cd$^{2+}$ tolerance$^{20}$; two P-type ATPases, spd0635 (copA), which is responsible for Cu$^{2+}$ efflux$^{21}$; and spd1927, which has homology to *pmtA*, a PerR-regulated gene in *S. pyogenes* associated with Zn$^{2+}$ resistance$^{22}$. The mntE gene has previously been reported to be constitutively expressed and not responsive to cellular Mn$^{2+}$ abundance$^{19}$. Here, we observed that extracellular Cd$^{2+}$ induced a 2.1-fold increase in mntE transcription (*P* = 0.0211 (two-tailed unpaired *t*-test); Supplementary Fig. 5a). On supplementation with 30 μM Mn$^{2+}$, mntE transcription was reduced to non-challenged levels (*P* = 0.3665 (two-tailed unpaired *t*-test) Supplementary Fig. 5a), suggesting that intracellular Cd$^{2+}$ concentrations modulated mntE expression via an unknown metal-responsive regulator. We then analysed an isogenic ΔmntE strain, which lacks the primary Mn$^{2+}$-efflux pathway$^{19}$, for sensitivity to Cd$^{2+}$. Consistent with Cd$^{2+}$ also perturbing Mn$^{2+}$ homeostasis via MntE, the ΔmntE strain retained a higher cellular Mn$^{2+}$ concentration (2.53 ± 0.1 mM) than the wild-type (0.78 ± 0.07 mM) (*P* < 0.0001 (two-tailed unpaired *t*-test)), when grown in 1 μM Mn$^{2+}$; 30 μM Cd$^{2+}$, and showed greater resistance to extracellular Cd$^{2+}$ (Fig. 2). The cadD gene showed a similar trend to czcD and mntE with a ~3.6-fold increase in transcription when challenged with 30 μM Cd$^{2+}$ (*P* < 0.0001 (two-tailed unpaired *t*-test); Supplementary Fig. 5b), followed by abrogation when supplemented with 30 μM Mn$^{2+}$.

**Figure 2 | Phenotypic impact of metal ions on *S. pneumoniae* growth.** Drop test analysis of *S. pneumoniae* wild-type (D39), ΔczcD, ΔmntE, ΔgshT, ΔgshT/ΔczcD and AsodA on blood agar (BA) supplemented with: 0, 100, 300 and 1,000 μM ZnSO$_4$ or 0, 10, 30 and 100 μM CdCl$_2$. Cells were grown and adjusted to A$_{600}$ of 0.4 and were serially diluted. Drops (5 μl) were spotted onto the BA plate, starting from the 10$^{10}$ dilution (top) down to the 10$^{-5}$ dilution (bottom).
However, the P-type ATPases, copA and spd1927, were transcriptionally unresponsive to Cd\(^{2+}\) (Supplementary Fig. S5c,d), suggesting that they were unlikely to contribute to Cd\(^{2+}\) management. Collectively, these data show that Cd\(^{2+}\) accumulation was associated with transcriptional activation of several metal ion membrane protein transporters. However, of those pathways with known efflux function, that is, CzcD and MntE, Cd\(^{2+}\) ions subverted their primary functional roles and, instead, further dysregulated transition metal ion homeostasis in S. pneumoniae via amplification of Zn\(^{2+}\) and Mn\(^{2+}\) efflux, respectively. The mechanism(s) of Cd\(^{2+}\) efflux in S. pneumoniae, if any, remains to be identified.

Cd\(^{2+}\) competes with Mn\(^{2+}\) for the Psa permease. To elucidate the hitherto unexplained molecular basis for Cd\(^{2+}\) uptake via Mn\(^{2+}\) transporters, we examined the interaction of Cd\(^{2+}\) with the Mn\(^{2+}\)-recruiting SBP PsaA. Although the physiological role of PsaA is in recruiting Mn\(^{2+}\), the metal-binding site of the protein has previously been shown to interact with other divalent transition metal ions\(^8\). However, coordination chemistry at the metal-binding site and structural dynamics prevent release of these other ions, and thereby maintain the functional role of the Psa permease in Mn\(^{2+}\) uptake\(^8\). Here the interaction of Cd\(^{2+}\) with PsaA was analysed by determining the structure of wild-type PsaA with Cd\(^{2+}\) at 2.0 Å resolution (Fig. 3a and Supplementary Fig. 6). The PsaA–Cd\(^{2+}\) complex revealed that Cd\(^{2+}\) was coordinated by the metal-binding site residues His67, His139, Glu205 and Asp280 in a structure that was highly similar (root-mean-square deviation (RMSD) \(\leq 0.3 \text{ Å}\) over 242 C\(^\text{x}\) atoms) to our previously reported Mn\(^{2+}\)- and Zn\(^{2+}\)-bound structures\(^{23,24}\). Nevertheless, small re-orientations of the side chains of the metal-coordinating residues allowed the bulkier Cd\(^{2+}\) ion (ionic radius of 95 pm for Cd\(^{2+}\) versus 84 pm for Mn\(^{2+}\)) to be accommodated into the protein–metal-binding site (Fig. 3b and Supplementary Table 2). In the PsaA–Cd\(^{2+}\) structure, the distances between metal-coordinating atoms and the metal ion were increased by \(\sim 0.2 \text{ Å}\) compared with those for PsaA–Zn\(^{2+}\) (Supplementary Table 2). Similar increases in metal–protein distances have been observed for the Cd\(^{2+}\)-thiolate-binding sites of Cd\(^{2+}\)-

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**Figure 3 | Structure of Cd\(^{2+}\)-bound PsaA.** (a) Cartoon illustration of Cd\(^{2+}\)-bound wild-type PsaA in closed conformation (PDB accession code 4UTP). The structure consists of N-terminal and C-terminal (β/α)\(_4\) domains with a domain-linking helix. The metal-binding site is situated between the two domains, in which a Cd\(^{2+}\) ion (pale-yellow sphere) is bound. (b) The metal-binding site of wild-type PsaA in the presence (light blue, PDB accession code 4UTP) and absence (green, PDB accession code 3ZK7) of Cd\(^{2+}\) (pale-yellow sphere), with the metal coordination shown as dashed lines. (c) Superposition of Cd\(^{2+}\)-bound (light blue, PDB accession code 4UTP) and apo (green, PDB accession code 3ZK7) wild-type PsaA structures (Cx atoms, residues 32–163, were used for the superposition). The linking helix is shown in cartoon representation, with other helices shown as cylinders. The crossing angle of the C-terminal (β/α)\(_4\) domains between the structures is marked. The bound Cd\(^{2+}\) ion is shown as a pale-yellow sphere. In the apo structure, backbone atoms in the flexible region of the linking helix (residues 184–194) are shown in stick representation, with hydrogen bonds between backbone atoms as dashed lines. (d) Comparison of the metal-binding sites between Cd\(^{2+}\)-bound (light blue, PDB accession code 4UTP) and Zn\(^{2+}\)-bound (purple, PDB accession code 1PS2) wild-type PsaA structures. The metal ions are shown as spheres (Cd\(^{2+}\), pale yellow; Zn\(^{2+}\), grey) and their coordination as dashed lines. (e) Comparison of the metal-binding sites between Cd\(^{2+}\)-bound (light blue, PDB accession code 4UTP) and Mn\(^{2+}\)-bound (yellow, PDB accession code 3ZTT) wild-type PsaA structures. The metal ions are shown as spheres (Cd\(^{2+}\), pale yellow; Mn\(^{2+}\), blue) and their coordination as dashed lines. In (a–e), the metal-coordinating residues are shown in stick representation. (f) In vitro metal binding of Cd\(^{2+}\) to wild-type or D280N variant apo-PsaA analysed by ICP-MS. Data correspond to the mean (± s.d.) molar ratio of Cd\(^{2+}\) to the PsaA isoform for apo-PsaA (black), Cd\(^{2+}\)-PsaA (light grey), EDTA–Cd\(^{2+}\)-PsaA (dark grey), apo–D280N–PsaA (black), Cd\(^{2+}\)-D280N-PsaA (grey) and EDTA–Cd\(^{2+}\)-D280N–PsaA (white).
substituted metallothionein and rubredoxin proteins, compared with the Zn\(^{2+}\)-bound equivalents\(^{25,26}\). Although Cd\(^{2+}\) and Zn\(^{2+}\) often display similar coordination spheres in proteins, the longer bond distances observed for Cd\(^{2+}\) usually result in an increase in the coordination number. Accordingly, the coordination geometry in the PsA–Cd\(^{2+}\) structure is an intermediate between a square-pyramid and trigonal-bipyramidal \((n = 5)\), in contrast to the 4- and 6-coordinate sites observed for the Zn\(^{2+}\)- and Mn\(^{2+}\)-bound PsA structures, respectively (Fig. 3d,e and Supplementary Table 3). A comparison of the observed coordination of PsA–Cd\(^{2+}\) with other metal-binding sites of metalloprotein structures in the PDB, composed of mixed N/O ligation atoms, revealed that Cd\(^{2+}\) is found in a variety of coordination geometries with coordination numbers ranging from 5 to 7 (Supplementary Table 4). Nevertheless, the only bona fide protein–metal-binding site for Cd\(^{2+}\) is in carbonic anhydrase from *Thalassiosira weissflogii*, which has been shown to be a cambialistic enzyme with Zn\(^{2+}\) in a tetrahedral geometry and Cd\(^{2+}\) in a trigonal-bipyramidal geometry\(^{3,27,28}\). Thus, the coordination geometry found in PsA–Cd\(^{2+}\) conforms to what has been observed for the only known naturally occurring Cd\(^{2+}\)-binding protein, and for other protein structures deposited in the PDB. We further examined the interaction of metal-free (apo) PsA with Cd\(^{2+}\) and observed that apo-PsA bound Cd\(^{2+}\) in a 1:1 molar ratio (Fig. 3f), with a *K*\(_D\) of 5.6 ± 1 nM (Supplementary Fig. 7). As the cognate ligand, Mn\(^{2+}\), has a *K*\(_D\) of 3.3 ± 1 nM for PsA, the relatively small (approximately twofold) difference in the *K*\(_D\)s is consistent not only with the competitive effect on Mn\(^{2+}\) acquisition but also the continued, albeit reduced, Cd\(^{2+}\) accumulation during growth in the presence of equimolar concentrations of both ions (30 μM Mn\(^{2+}\): 30 μM Cd\(^{2+}\)). Collectively, these findings are consistent with the observed impact of Cd\(^{2+}\) on Mn\(^{2+}\) accumulation in the wild-type and mutant *S. pneumoniae* strains and indicate that it is a competitive effect mediated via PsA. However, they also indicate that interaction of Cd\(^{2+}\) with PsA is distinct from the previously characterized Mn\(^{2+}\) and Zn\(^{2+}\) interactions.

PsA is permissive for Cd\(^{2+}\) binding and release. PsA uses a ‘spring-hammer’-like mechanism for metal ion binding, in which the dynamics of the metal ion-loaded protein are dictated by a combination of metal ion geometry and the distortion of a helix linking the N- and C-terminal lobes of the protein\(^{8}\). We further analysed the metal-binding site coordination geometry by use of an Asp280 variant isofrom (PsA-D280N), as it would be incapable of facilitating the trigonal-bipyramidal coordination observed in wild-type PsA\(^{8}\). The structure of PsA-D280N bound to Cd\(^{2+}\), refined to 1.7 Å resolution, revealed a partially solvated Cd ion loosely interacting with residue Glu205 (Fig. 4a,b). The C-terminal lobe of PsA showed minor movement (~1.0° relative to the open, metal-free conformation), but the inability of Cd\(^{2+}\) to ligate to residue Asp280 prevented further conformational changes. Consistent with these observations, PsA-D280N was greatly impaired in Cd\(^{2+}\) binding (Fig. 3f).

We then assessed the distortion of the linking helix by monitoring the number of disrupted backbone hydrogen bonds in its flexible region (residues 184–194) in wild-type PsA–Cd\(^{2+}\) (Figs 3c and 4a, and Supplementary Table 5)\(^{8}\). Both the hinge-bending angle between the N- and C-terminal lobes and the number of main-chain hydrogen bonds disrupted in the residue range 184–194 corresponded more closely to the Mn\(^{2+}\)-bound structure than to the ‘locked’ Zn\(^{2+}\)-structure. This indicated that Cd\(^{2+}\) binding was a reversible process (Supplementary Table 5), consistent with the observation that Cd\(^{2+}\) could be extracted (~40%) from the PsA–Cd\(^{2+}\) complex by using the chelating agent EDTA (Fig. 3f). Thus, these findings provide further support for the conclusion that both Mn\(^{2+}\) and Cd\(^{2+}\) compete for transport via the PsA permease, consistent with the cellular accumulation data, which shows that Mn\(^{2+}\) uptake occurs at the expense of Cd\(^{2+}\) import and vice versa.

Reduced Mn\(^{2+}\) levels result in sensitivity to oxidative stress. We then sought to assess how Cd\(^{2+}\) affected oxidative stress management in *S. pneumoniae*. Manganese has a prominent role in regulating the expression of superoxide dismutase (*soda*), where it also serves as a cofactor\(^{29–31}\). Transcription of *soda* was decreased under Cd\(^{2+}\)-induced Mn\(^{2+}\) starvation, but this was restored when supplemented with Mn\(^{2+}\) (Fig. 5a), and direct
measurement of SodA activity from *S. pneumoniae* showed a similar trend (Fig. 5b). The impact of Cd\(^{2+}\) on oxidative stress response was ascertained using paraquat, which causes oxidative damage by promoting a futile redox cycle in the cytoplasm. We observed that although growth in the presence of Cd\(^{2+}\) resulted in a significant decrease in survival (Fig. 5c, *P* = 0.0087 (two-tailed unpaired t-test)), the increased sensitivity was not due to Cd\(^{2+}\), but instead was a result of the decreased Mn\(^{2+}\) accumulation, as *S. pneumoniae* grown in 30 μM Mn\(^{2+}\); 30 μM Cd\(^{2+}\) exhibited wild-type levels of survival (Fig. 5c). Hence, the perturbation of Mn\(^{2+}\) homeostasis by Cd\(^{2+}\) heightens the sensitivity to oxidative stress, while Cd\(^{2+}\) could be accumulated to high intracellular concentrations with no apparent deleterious effect. This ability to accumulate Cd\(^{2+}\) without direct toxicity indicated that intracellular buffering of the ion was crucial.

Reduced thiol groups on small peptides, primarily glutathione in *S. pneumoniae*, have been implicated in having an essential role in intracellular transition metal ion management (Fe\(^{2+}\) and Zn\(^{2+}\)) and in ameliorating Cd\(^{2+}\) toxicity\(^{32}\). *S. pneumoniae* is incapable of de novo glutathione synthesis and acquires glutathione via a high-affinity ABC permease\(^{32}\). Here we investigated the contribution of glutathione to management of transition metal ion stress. In *S. pneumoniae*, the total glutathione content, in response to Cd\(^{2+}\) exposure, increased by approximately twofold to a mean (± s.e.m.) pneumococcal cell concentration of 19.0 ± 0.9 mM (Fig. 5d). Glutathione abundance is crucial for Cd\(^{2+}\) management, as a mutant strain incapable of glutathione acquisition, *S. pneumoniae* ΔsodT, was hypersensitive to Cd\(^{2+}\) stress (1 μM Mn\(^{2+}\); 30 μM Cd\(^{2+}\); Figs 2 and 5e) and, in contrast to the wild type, could not be rescued by addition of Mn\(^{2+}\) (Fig. 5e; 30 μM Mn\(^{2+}\); 30 μM Cd\(^{2+}\)). By contrast, the ΔsodA strain, which we have previously shown to be hypersensitive to oxidative stress challenge\(^{30,33}\), demonstrated a wild-type growth pattern.

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**Figure 5** The impact of Cd\(^{2+}\) on sensitivity to oxidative stress. (a) Relative transcription, corrected to 16S rRNA, of sodA by *S. pneumoniae* when grown in CDM supplemented with 1 μM Mn\(^{2+}\) (black), 1 μM Mn\(^{2+}\); 30 μM Cd\(^{2+}\) (light grey) and 30 μM Mn\(^{2+}\); 30 μM Cd\(^{2+}\) (dark grey). The data correspond to the mean (± s.e.m.) of three independent biological experiments. (b) SodA activity assay S. pneumoniae when grown in CDM supplemented with 1 μM Mn\(^{2+}\) (black), 1 μM Mn\(^{2+}\); 30 μM Cd\(^{2+}\) (light grey) and 30 μM Mn\(^{2+}\); 30 μM Cd\(^{2+}\) (dark grey). Activity was calculated as a percentage of SodA activity observed for *S. pneumoniae* cells grown in 1 μM Mn\(^{2+}\). The data correspond to the mean (± s.e.m.) of three independent biological experiments. (c) Paraquat killing of *S. pneumoniae* grown in CDM supplemented with 1 μM Mn\(^{2+}\) (black), 1 μM Mn\(^{2+}\); 30 μM Cd\(^{2+}\) (light grey) and 30 μM Mn\(^{2+}\); 30 μM Cd\(^{2+}\) (dark grey). Survival was calculated as a percentage of colonies at 30 min compared with cells not challenged with paraquat. The data correspond to the mean (± s.e.m.) of three independent biological experiments. (d) Total glutathione determination per mean cell volume for *S. pneumoniae* grown in CDM supplemented with 1 μM Mn\(^{2+}\) (black), 1 μM Mn\(^{2+}\); 30 μM Cd\(^{2+}\) (light grey) and 30 μM Mn\(^{2+}\); 30 μM Cd\(^{2+}\) (dark grey). The statistical significance of the differences in the mean data for a–d was determined by two-tailed unpaired t-tests (NS, not significant; *P* < 0.05, **P* < 0.01, ***P* < 0.001, ****P* < 0.0001). (e) *S. pneumoniae* ΔsodT (e) and ΔsodA (f) grown in CDM supplemented with metal ions as indicated. The data correspond to mean (± s.e.m.) absorbance 600 nm measurements from three independent biological experiments. Errors bars, where not visible, are overlapped by the representative symbols.
phenotype in the presence of Cd\(^{2+}\), with no apparent increase in sensitivity to metal ion stress (Figs 2 and 5f). Taken together, these findings indicate that glutathione serves a major role in Cd\(^{2+}\) buffering, whereas SodA appears to be dispensable, consistent with the lack of redox activity of Cd\(^{2+}\) ions.

To ascertain whether this was also the case for Zn\(^{2+}\), which is also presumed to be buffered by glutathione, we examined the response of the ΔgshT strain to Zn\(^{2+}\) exposure. Intriguingly, while the ΔgshT strain was insensitive to subinhibitory concentrations of Zn\(^{2+}\), similar to the ΔczcD strain, the combination mutant (ΔgshT/ΔczcD) showed significant attenuation of growth (100 μM Zn\(^{2+}\); Fig. 2). At higher concentrations of Zn\(^{2+}\), the growth of the ΔgshT/ΔczcD strain corresponded with the hypersensitive phenotype of the ΔczcD strain, while the ΔgshT strain showed a very mild impact, relative to wild type (300 μM Zn\(^{2+}\); Fig. 2). Taken together, these data show that intracellular management of Zn\(^{2+}\) occurs via the concerted actions of both glutathione and Zn\(^{2+}\) efflux, wherein the activation and efflux via CzcD is crucial to prevent Zn\(^{2+}\) intoxication. By contrast, management of Cd\(^{2+}\), which has no major efflux pathway, is predominantly dependent on cellular glutathione to prevent mismetallation of other proteins. Concordantly, when Cd\(^{2+}\) accumulation surpasses the buffering capacity of the glutathione pool (1 μM Mn\(^{2+}\), 30 μM Cd\(^{2+}\)), or in the absence of intracellular glutathione (ΔgshT), this leads to Cd\(^{2+}\) toxicity.

Discussion

Anthropogenic-facilitated entry of non-physiological elements, such as Cd\(^{2+}\), into the biosphere presents unique cellular challenges for biological systems. The prior absence of these elements from the biosphere obviated any selective pressure towards evolving mechanisms for managing their impact on biological processes. As a consequence, many non-physiological elements rapidly accumulate in the food chain leading to significant toxicity in higher organisms\(^{1,2}\). We observed that Cd\(^{2+}\) was toxic to S. pneumoniae and that this arose from acute dysregulation of the transition metal ion homeostatic mechanisms. Elucidation of the total cellular quotient of metal ions in S. pneumoniae not only showed the impact of Cd\(^{2+}\) but also revealed novel aspects of cellular transition metal homeostasis. S. pneumoniae is a Gram-positive organism, comprising only a single cellular compartment; thus the concentration of transition metal ions accurately reflects their total intracellular accumulation and encompasses metal ions occurring in labile exchangeable pools and those bound weakly or tightly to proteins, peptides, nucleic acids and other molecules.

Determination of the cellular quotient of metal ions in wild-type S. pneumoniae and mutant variants revealed the range over which cellular variation of transition metal ion concentrations is permissible. Manganese accumulation shows significant plasticity, with cells being viable at concentrations as low as ~3% of typical accumulation levels\(^{23,30}\). By contrast, Zn\(^{2+}\) accumulation is regulated in a much narrower window, with its minimal cellular quotient being between 20 and 40% of typical accumulation. Intriguingly, the higher total cellular quotient of Mn\(^{2+}\) ions suggests that the concentration ‘set-points’ for cellular accumulation of transition metal ions may also contribute to ensuring efficient acquisition of metal cofactors by proteins. Manganese, which has been reported to be buffered by molecules such as citrate, phosphate and histidines residues from proteins in the cytoplasm\(^{24,35}\), has fewer reported cellular roles. Hence, its presence at such high cellular concentrations suggests that in S. pneumoniae it could occur predominantly as a labile pool of cytoplasmic-buffered Mn\(^{2+}\). By contrast Zn\(^{2+}\), which is reported in numerous studies to be highly utilized in intracellular proteins\(^{36,37}\), has been predicted to have a significantly smaller labile pool\(^{10,36}\). Comprehensive intracellular metal ion speciation studies have been limited by a combination of technical and methodological challenges that remain to be surmounted.

Whether this is due to Cd\(^{2+}\) directly interacting with the Zn\(^{2+}\)-binding sites of intracellular metal sensors\(^{38-40}\) or via Cd\(^{2+}\)-mediated displacement of Zn\(^{2+}\) from thiol buffering sites\(^{41}\), which results in the non-physiological activation of the Zn\(^{2+}\)-responsive transcriptional regulators, remains to be determined. Irrespective of this fact, transcriptional activation of czcD is often construed as a mechanism to facilitate Cd\(^{2+}\)
efflux based on studies of the archetypal protein from C. metallidurans. However, in S. pneumoniae, similar to S. pyogenes, CzcD is primarily a Zn\(^{2+}\)-efflux pathway and does not provide a major protective role against Cd\(^{2+}\) ions. As a consequence, in S. pneumoniae, the Cd\(^{2+}\)-induced upregulation of czzD appears to preferentially deplete intracellular Zn\(^{2+}\), despite the greater abundance of Cd\(^{2+}\) ions.

Cd\(^{2+}\) accumulation is associated with oxidative stress, despite the redox-inert nature of the ion. The data here show that, in S. pneumoniae, the increased sensitivity to oxidative stress arises from the loss of sodA expression and activity due to Cd\(^{2+}\)-induced Mn\(^{2+}\) depletion. However, the loss of SodA was an indirect by-product of Cd\(^{2+}\) accumulation and, importantly, not associated with the ability of the pneumococcus to tolerate Cd\(^{2+}\) ions. This was demonstrated by the \(\Delta\)soda strain, which showed wild-type growth phenotypes in the presence of extracellular Cd\(^{2+}\), and in wild-type S. pneumoniae, which retained the ability to accumulate high cellular concentrations of Cd\(^{2+}\) without an increase in sensitivity to oxidative stress, when supplemented with Mn\(^{2+}\).

Although, SodA still has a major role in the \(\text{in vitro}\) and \(\text{in vivo}\) fitness of S. pneumoniae owing to a lack of other mechanisms to detoxify superoxide\(^{23,30}\). Here, in the presence of Cd\(^{2+}\), the loss of SodA also occurs in conjunction with dysregulation of Mn\(^{2+}\) and Zn\(^{2+}\) homeostatic mechanisms and the accumulation of very high concentrations of Cd\(^{2+}\). Hence, it is the combination of these factors that compromises pneumococcal viability in the presence of Cd\(^{2+}\).

Intracellular accumulation of Cd\(^{2+}\) in S. pneumoniae was crucially dependent on glutathione, which acts as a low-affinity metal ion buffer for certain transition metal ions (Fe\(^{2+}\), Zn\(^{2+}\) and Cd\(^{2+}\)), as phenotypic growth perturbations were seen when Cd\(^{2+}\) accumulation exceeded the total cellular glutathione pool (19–23 mM). Although it cannot be completely discounted that glutathione contributes to pneumococcal oxidative stress management\(^{32}\), the hypersensitivity of the \(\Delta\)gshT strain to extracellular Cd\(^{2+}\), in contrast to the \(\Delta\)soda strain, strongly implicates the major role for cellular glutathione is in metal ion buffering. The observed hypersensitivity of the \(\Delta\)gshT/\(\Delta\)czzD strain to Zn\(^{2+}\) stress further underscores how intracellular metal ion homeostasis relies on the contributions of both cellular glutathione and the efflux machinery\(^{13,43}\). The results are also consistent with the central role of low-molecular-weight thiols for metal ion homeostasis in other microorganisms. Bacilliithiol has recently been shown to have a crucial role in the buffering of Zn\(^{2+}\) ions in Bacillus subtilis\(^{44}\), while it also serves an undefined, but protective role in Cd\(^{2+}\) stress for B. subtilis and Staphylococcus aureus\(^{44}\). Although S. pneumoniae and other microorganisms lack a bacilliithiol biosynthetic pathway, the role of low-molecular-weight species for metal ion homeostasis is emerging as a crucial mechanism. Overall, our findings here highlight the role of glutathione as an intracellular metal ion buffer, in preference to oxidative stress management. This finding has broad ramifications for the assumed role(s) of cellular glutathione, and related thiol derivatives, in pyrokaryotes and potentially eukaryotes\(^{43,44}\).

In conclusion, Cd\(^{2+}\) accumulation in the food chain and its toxicity are crucial, but largely overlooked concerns. Our findings reveal the molecular basis by which Cd\(^{2+}\) is mislocated via a Mn\(^{2+}\) transporter and provides new insights into how Cd\(^{2+}\) dysregulates intracellular transition metal homeostasis. Disruption of multiple essential metal ion homeostatic mechanisms is highly damaging to biological organisms, such as S. pneumoniae\(^{44}\), and in this way Cd\(^{2+}\) acts to severely dysregulate both Mn\(^{2+}\) and Zn\(^{2+}\) homeostasis (Supplementary Fig. 8). By revealing the remarkably high (millimolar) cellular quotients of transition metal ions and glutathione, this work also shows how Cd\(^{2+}\), despite its inability to directly generate reactive oxygen species, is associated with oxidative stress. This work provides a new understanding of the mechanisms by which Cd\(^{2+}\) enters cells and causes toxicity, and thereby, opens the way to identifying new routes towards developing specific therapeutic agents capable of preventing Cd\(^{2+}\) toxicity.

**Methods**

**Growth experiments and whole-cell assays.** The S. pneumoniae D39 \(\Delta\)soda, \(\Delta\)gshT, \(\Delta\)gshT/\(\Delta\)czzD, \(\Delta\)mntE/\(\Delta\)czzD and \(\Delta\)soda/\(\Delta\)czzD strains have been generated previously\(^{13,32}\). The \(\Delta\)mntE and \(\Delta\)gshT/\(\Delta\)czzD strains were generated as described previously\(^{13}\), using primer sequences listed in Supplementary Table 6. S. pneumoniae D39, \(\Delta\)soda, \(\Delta\)czzD, \(\Delta\)mntE, \(\Delta\)gshT, \(\Delta\)gshT/\(\Delta\)czzD, \(\Delta\)mntE/\(\Delta\)czzD and \(\Delta\)soda/\(\Delta\)czzD were grown in CDM, which corresponded to the C + V media + metal ion supplementation\(^{23}\). The supernatant of the CDM was sampled by ICP–MS on an Agilent 7500cx ICP–MS (Adelaide Microscopy, University of Adelaide)\(^{23}\). Growth experiments were conducted in CDM supplemented with 1 mM MnSO\(_4\) and concentrations of 20, 30, 50 or 80 mM of CdCl\(_2\) or MnSO\(_4\) as specified. All chemicals used in this study were purchased from Sigma-Aldrich, unless otherwise specified. For bacterial growth experiments, an inoculum was prepared from overnight grown culture on a blood-agar (BA) plate and resuspended in CDM to an absorbance at 600 nm (\(A_{600}\)) of 1.0. The inoculum was then diluted in 200 \(\mu\)l of CDM in a 96-well flat-bottom plate (Greiner Bio One) to a final \(A_{600}\) of 0.05 and sealed with gas-permeable seal (Greiner Diversified Biosciences). The plate was then incubated at 37\(^\circ\)C, Omega spectrophotometer with an ACU gas controller (BMG Labtech) at 310 K in 5% CO\(_2\), \(A_{600}\) readings were recorded using the well-scan function every 30 min. Data from at least six independent growth experiments was averaged to ascertain the effect of CdCl\(_2\) on bacterial growth. Bacterial density at \(A_{600}\) was measured as the c.f.u. per ml determined by serial dilutions on BA plates.

Bacterial growth for ICP–MS, parquat killing and glutathione assays used identical growth parameters to the microplate experiments, with MnSO\(_4\) and CdCl\(_2\) supplementation as specified. For ICP–MS, 50 ml of culture was grown to \(A_{600}\) of 0.3–0.4, harvested and prepared for analysis by ICP–MS, as described previously\(^{23}\). For the paraquat killing assays, 1 ml of CDM supplemented with 1 mM MnSO\(_4\), grown until the \(A_{600}\) reached 0.3–0.4. Cells were then serially diluted 10-fold up to a 10–5 dilution, and 5 \(\mu\)l of each dilution were spotted on BA plates supplemented with varying concentrations of ZnSO\(_4\) (0, 100, 300 and 1,000 \(\mu\)M) or CdCl\(_2\) (0, 10, 30 and 100 \(\mu\)M). Plates were photographically documented following overnight incubation at 37\(^\circ\)C, 5% CO\(_2\).

**RNA extraction and RT-PCR analysis.** Pneumococci were grown as for the ICP–MS analyses, then 500 \(\mu\)l of the culture was mixed with 1 ml of RNA Protect (Qiagen). RNA was extracted and purified using an RNeasy Protect Bacteria Mini Kit (Qiagen) after enzymatic lysis using lysozyme and mutanolysin, all according to the manufacturer’s instructions. DNase I treatment (Roche) was performed following manufacturer’s reverse transcriptase (RT) protocol (dithiothreitol is added in a 50\(\mu\)l reaction), with a Roche LC480 Real-Time Cycler. The transcription levels of genes analysed were normalized to those obtained for 16S rRNA. Primer sequences are in Supplementary Table 6.

**Cell volume and concentration determination.** Scanning electron microscopy was used to determine the dimensions of the pneumococcal cell. Bacteria were grown as described above, harvested and fixed as prescribed for, and analysed by a Philips XL30 FEG scanning electron microscope as described in ref. 45. Cell measurements were obtained using instrument software. The cell dimensions were used to calculate volume assuming an ellipsoid:

\[
V = \frac{4}{3} \pi a b c
\]

where \(r_1\), \(r_2\), and \(r_3\) are the radii of the ellipsoidal cell in three dimensions determined by scanning electron microscopy (SEM). The total quotient of transition metal ions was then derived using cell volume (\(V\), litres), where the total
cell density is known (c.f.u.), and concentration of metal ions in a known sample volume and known number of cells (M, moles).

\[
C = \frac{M_{\text{in}}}{V_{\text{sample}}} \times \text{cells/L}
\]  

(2)

The derived concentration represents mean molarity of metal ion per cell of mean dimension.

Expression and purification of apo-PsaA. Recombinant PsaA was expressed in E. coli LEMO21(DE3) from the pCAMC1C10-PsaA construct. The dodecahistidine tag was removed from affinity-purified PsaA by enzymatic digestion by the human rhinovirus 3C protease and the protein purified further on a HiTrap HP column. Apo-PsaA was purified by dialyzing the dodecahistidine tag-cleaved protein in a 20-kDa molecular-weight-cutoff membrane (Slide-A-Lynx, Pierce) against 50 mM sodium acetate buffer, pH 4.0, with 20 mM EDTA at 300 K. The sample was then dialysed against 41 of 20 mM Tris-HCl, pH 7.2, and 100 mM NaCl, at 277 K and centrifuged at 18,000 g for 10 min to remove any insoluble material. Protein samples were analysed for metal content by heating 5 μM protein at 370 K for 15 min in 3.5% HNO₃ and the metal ion content was measured by ICP–MS.

Protein assays. Protein concentration determination was performed using the DC Bio-Rad protein determination assay. Metal-loading assays were performed on purified apo-PsaA (30 μM) by mixing with 300 μM CdCl₂ in a total volume of 2 ml in the assay buffer (20 mM MOPS, pH 7.2, and 100 mM NaCl) for 60 min at 277 K. The sample was desalted on a PD10 column (GE Healthcare) into the assay buffer and the protein concentration was quantified. Protein was then either kept for ICP–MS analysis or mixed with 3 mM EDTA in a total volume of 2 ml for 60 min at 277 K. Samples were then desalted on a PD10 column as before. Solutions (5–10 μM) of control, metal-loaded and EDTA-treated protein were prepared in 3.5% HNO₃ and boiled for 15 min at 370 K. Samples were then cooled and centrifuged for 20 min at 14,000 g. The supernatant was then analysed by ICP–MS and the protein-to-metal ratio was determined.

Immunoblot analyses of PsaA expression levels. Wild-type and mutant S. pneumoniae were grown under the same conditions as for ICP–MS. After reaching an A₅₆₀ of 0.4, cells were incubated with 0.1% sodium deoxycholate at 310 K for 60 min to induce lysis. Protein concentrations were determined and 10 μg of total protein was loaded into each lane. After electrophoretic separation by SDS–PAGE, proteins were transferred to a nitrocellulose membrane using the iBlot (Life Technologies) system. The blots were incubated with murine anti-PsaA serum (1:2,000; ref. 23), followed by anti-mouse IRDye 800 (LI-COR; 1:50,000), and were developed using log, inhibitory molecules were responses modeled, with the experimentally derived K₀ for Fluozin-3 with Cd²⁺, in GraphPad prism to determine the K₀ value for Cd²⁺ binding by PsaA.

Protein crystallization and structure determination. Cd-bound PsaA–D280N protein crystals were obtained in 12.5% (w/v) polyethylene glycol (PEG) 1000, 12.5% (w/v) PEG 3350, 12.5% (w/v) MPD, 0.1 M Trizma–Bicine, pH 7.2, and 0.01 M CdCl₂ using the hanging drop vapour diffusion method, Cd²⁺-bound wild-type PsaA crystals were grown in 26–36% PEG 400, 0.1 M NaCl, 0.1 M Trizma–HCl, pH 8.0, and 0.1 M CdCl₂ also using vapour diffusion. Before data collection, the crystals were flash-cooled by rapid immersion in liquid nitrogen. The diffraction data were collected on a single beamline using a synchrotron (MX) source. The crystals were screened iteratively using Phenix.AutoBuild47. The structures were refined initially using Phenix,Refine and Refmac5 manual model fitting in Coat48. Both structures had two molecules (chains A and B) in each asymmetric unit and chain A molecules were used in subsequent structural analyses. Structural analyses (superpositions, metal ion coordination and N–C-terminal domain-crossing angles) were performed in Chimera49. Secondary structure elements were assigned by DSSP (version 2.10; ref. 50). The WHATIF51 and PISA52 web services were used to identify hydrogen bonds and salt bridges. Data processing and refinement statistics can be found in Supplementary Table 7.

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Author contributions

S.L.B., R.A.E. and C.A.M. designed and executed all the biochemical studies. C.-L.Y.O. generated the mutant strains. Z.L., R.M.C. and M.J.M. designed and executed the crystallographic experiments. S.L.B., R.M.C., M.L.O. and C.A.M. drafted the manuscript. All authors contributed to the design, analysis, discussion of the research and writing of the final manuscript.

Additional information

Accession codes: Coordinates and structure factors for Cd2+–bound PsaA D280N, partially occluded state, and for Cd2+–bound wild-type PsaA, closed state, have been deposited in the RCSB Protein Data Bank under accession codes 4uto and 4up4, respectively.

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