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A role for the DtxR family of metalloregulators in gram-positive pathogenesis

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SUMMARY

Given the central role of transition metal ions in a variety of biochemical processes, the colonization, survival, and proliferation of a bacterium within a host hinges upon its ability to overcome the metal ion deprivation that characterizes nutritional immunity. Metalloregulatory, or “metal-sensing” proteins have evolved in bacteria to mediate metal ion homeostasis by activating or repressing the expression of genes encoding metal ion transport systems upon binding their cognate metal ion. Yet increasing evidence in the literature supports an additional role for these metalloregulatory proteins in pathogenesis. Herein, we survey studies on the DtxR family of metalloregulators, namely DtxR (*Corynebacterium diphtheriae*), SloR (*Streptococcus mutans*), MtsR (*Streptococcus pyogenes*), and MntR (*Staphylococcus aureus*) to describe how metalloregulation enables adaptive virulence gene expression within the mammalian host. This research has important implications for drug design, as the generation of hyper-repressive metalloregulatory proteins may represent a mechanism by which to attenuate bacterial pathogenicity. The fact that metalloregulators are unique to prokaryotes makes these proteins especially attractive therapeutic targets.

Keywords

metalloregulation; gram-positive; pathogenesis; evolution; ecology

INTRODUCTION

Transition metals, serving as centers of enzyme catalysis, structural constituents of proteins, and stabilizers of protein structure, enable many of the biochemical processes fundamental to cellular metabolism and oxidative defense. Yet the very redox potential that facilitates catalysis can be detrimental if unmanaged (Agranoff and Krishna, 1998; Jakubovics and Jenkinson, 2001), for a surfeit of unbound metal ions within the cell can lead to the production of toxic reactive oxygen species by Fenton chemistry (Agranoff and Krishna, 1998). Thus the opposing forces of excess and paucity necessitate the tight regulation of intracellular metal ion concentrations. In the absence of the internal compartmentalization that defines eukaryotes, bacteria achieve metal ion homeostasis principally through the import and export of divalent metal cations across the cytoplasmic membrane (Jakubovics and Jenkinson, 2001).

The mammalian host poses unique challenges to invading bacteria, as mammals sequester transition metal ions, namely iron, zinc, and manganese, in response to infection (Kehl-Fie and Skaar, 2010). This “nutritional immunity”, intended to impede bacterial growth, encompasses a diversity of mechanisms, including the appropriation of metal ions by host

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binding proteins, such as lactoferrin and transferrin, as well as the intracellular localization of metal cations (Wakeman and Skaar, 2012). Such an immune response not only deprives invading pathogens of micronutrients essential to metabolism, but the dearth of available metal ion cofactors simultaneously increases bacterial susceptibility to the oxidative and nitrosative stress of macrophage phagosomes (Giedroc and Arunkumar, 2007). The survival and proliferation of a pathogen within a host thus becomes contingent upon its ability to acquire micronutrients in spite of host-mediated metal ion withholding through the use of chelating agents called siderophores, transferrin/lactoferrin receptors, and haemophores (Giedroc and Arunkumar, 2007; Skaar, 2010). Metalloregulatory proteins control the expression of genes responsible for the biosynthesis of these acquisition systems, amongst others involved in metal ion homeostasis, metabolism, and virulence, by acting as transcriptional activators or repressors upon binding specific metal ions (Que and Helmann, 2000; Rolerson *et al.*, 2006). By coordinately regulating virulence gene expression, metal ions can serve as an environmental signal, inducing an adaptive response via metalloregulation following entry into a mammalian host.

In bacteria, three types of metal-sensing transcriptional regulators control gene expression in response to metal ion concentrations: de-repressors (ArsR-SmtB, CopY, and CsoR-RcnR families), activators (MerR family), and co-repressors (Fur, NikR, and DtxR families). The early identification of Fur (ferric uptake regulator) as a regulator of iron uptake in *Escherichia coli* led to a spate of discoveries of Fur homologues in *Yersinia pestis*, *Salmonella typhimurium*, *Vibrio cholera*, *Pseudomonas aeruginosa*, *Helicobacter pylori*, and other gram-negative human pathogens (see Escobar *et al.*, 1999 and Carpenter *et al.*, 2009 for comprehensive overviews of Fur metalloregulation in gram-negative bacteria). This review, by contrast, examines metalloregulation in the context of gram-positive pathogens, focusing primarily on the DtxR family of proteins that forms a phylogenetic class distinct from the Fur family (Fig. 1). Since Boyd *et al.* (1990) first described the iron-responsive diphtheria toxin repressor (DtxR) in *Corynebacterium diphtheriae*, extensive sequencing of bacterial genomes has revealed the details of multiple DtxR-like repressors, including IdeR (*Mycobacterium tuberculosis*), ScaR (*Streptococcus gordonii*), MntR (*Staphylococcus aureus* and *Bacillus subtilis*), TroR (*Treponema pallidum*), MtsR (*Streptococcus pyogenes*), SirR (*Staphylococcus epidermidis*), and SloR (*Streptococcus mutans*) (Table 1). The most recent studies on DtxR and its homologues have delved into the structural specifics of these proteins, often using crystal structures to explore mechanisms of metal ion selectivity, metal ion binding, and DNA consensus sequence binding. As a wealth of literature has been dedicated to these matters (see Glasfield *et al.*, 2003; Guedon and Helman, 2003; Chou *et al.*, 2004; D'Aquino *et al.*, 2005; Wisedchaisri *et al.*, 2007; Stoll *et al.*, 2009; Waldron *et al.*, 2009; Haswell *et al.*, 2013; McGuire *et al.*, 2013), this review instead approaches metalloregulation from a pathogenesis perspective, with an evolutionary and ecological emphasis. While recognizing that metalloregulation represents just one dimension of virulence gene control, we nonetheless collate a number of studies on DtxR, SloR, MtsR, and MntR to draw broad conclusions about the role of metalloregulation in bacterial pathogenesis.

***Corynebacterium diphtheriae*: The evolution of DtxR-regulated virulence**

The pathogenicity of *C. diphtheriae* can be attributed, in large part, to a corynebacteriophage-acquired gene that encodes diphtheria toxin (*tox*). Accordingly, only through lysogenic conversion can a strain of *C. diphtheriae* become toxigenic. Upon colonization of the host's nasopharynx or skin, toxigenic strains synthesize and secrete the two-component AB exotoxin. The B subunit facilitates delivery of the A subunit across the host plasma membrane into the cell, where the latter catalyzes the NAD⁺-dependent ADP-

ribosylation of elongation factor 2 to ultimately and irrevocably inhibit RNA translation (Murphy, 1996).

Pappenheimer and Johnson (1936) first established the iron-dependent nature of diphtheria toxin production, finding a reduction of toxin synthesis by *C. diphtheriae* in the presence of iron salts. A number of hypotheses, postulated in the following decades, sought to explain the mechanism by which iron exerts its effects on *tox* expression. Only in 1990 did a study identify the iron-responsive aporepressor responsible for regulating *tox*. Boyd *et al.* (1990) fused the diphtheria *tox* promoter/operator (*toxPO*) to a *lacZ* reporter gene. Subsequent transformation of *E. coli* with the recombinant construct enabled the authors to systematically assay genomic libraries isolated from nontoxicogenic nonlysogenic *C. diphtheriae* for *lacZ* repression. Further analysis of apposite restriction fragments indicated the common presence of an ORF encoding a 25-kDa protein with modest sequence homology to *fur*. Follow-up assays, undertaken after isolating and subcloning the ORF into the plasmid harbouring *toxPO*, confirmed the regulatory role of the encoded putative protein. Moreover, Boyd *et al.* observed de-repression of β -galactosidase expression upon the addition of an iron chelator to a growth medium inoculated with the recombinant *E. coli* strain. Taken together, these results attested to the role of the protein, thereafter named diphtheria toxin repressor or DtxR, in the iron-dependent regulation of *tox* expression.

That the genomic libraries screened by Boyd *et al.* derived from a nontoxicogenic and nonlysogenic *C. diphtheriae* strain speaks to the chromosomal origin of DtxR, compelling us to contemplate how the expression of a phage-encoded virulence gene, *tox*, came to be regulated by a chromosomally-encoded transcription factor. Indeed, later genome-wide analyses revealed the existence of several chromosomally-encoded regulons subject to DtxR control, such as a haem oxidase encoded by *hmuO* and ferric siderophore transport systems encoded by *irp1* and *irp6* (Schmitt and Holmes, 1994; Yellaboina *et al.*, 2004). The preponderance of regulons involved in iron acquisition and transport suggests that DtxR, like other Fe^{2+} -dependent metalloregulatory proteins, initially evolved to maintain iron homeostasis.

To elucidate the provenance of DtxR-regulated *tox* expression, then, we must first consider the mechanism of lysogenic conversion. In *C. diphtheriae*, the process entails the integration of corynebacteriophage into the chromosome by a Campbell-type insertion event, as site-specific recombination occurs at phage (*attP*) and bacterial (*attB*) attachment sites (Rappuoli *et al.*, 1983). The acquisition of *tox* alone does not suffice, however, for DtxR regulation necessitates an appropriately situated consensus sequence, or DtxR box, upstream of the *tox* gene. On one hand, the DtxR box could have been corynebacteriophage-derived, acquired in tandem with *tox*. Yet this explanation relies on the presence of an adventitiously positioned sequence recognizable to DtxR on the phage genome. Alternatively, a DtxR box might have been located upstream of the *attB* site on the chromosome, allowing the regulation of *tox* following integration of the corynebacteriophage. Having said this, Rappuoli and Ratti (1984) identified only two *attB* sites on the *C. diphtheriae* chromosome. That one of these two sites could be preceded by a consensus sequence to which DtxR binds, again, seems rather fortuitous.

Ultimately, however, experimental evidence supports the plausibility of either derivation. The characterization of two other genes regulated by DtxR, *irp1* and *irp2*, demonstrated that the DtxR box does not comprise a strictly conserved stretch of nucleotides. In fact, comparison of the *tox*, *irp1*, and *irp2* promoter/operator sequences protected from DNase I digestion in DNA footprinting experiments revealed only 52% sequence identity amongst their respective DtxR boxes (Schmitt and Holmes, 1994). Furthermore, from a crystal structure of DtxR complexed with duplex DNA, Chen *et al.* (2000) noted a predominance of

nonspecific interactions between the DNA phosphate backbone of the DtxR box and amino acid side chains present in the recognition and stabilization helices of DtxR's DNA binding domain. Only three residues within DtxR's DNA binding domain appear to make base-specific contact with DNA, either in the form of hydrogen bonds or van der Waal interactions (Chen et al., 2000). This wobble in the sequence to which DtxR binds increases the likelihood that a corynebacteriophage or *C. diphtheriae* bacterium could have once possessed an appropriately situated consensus sequence. The ability to fine-tune toxin expression in accordance with metal ion concentrations would have conferred the bacterium with an obvious advantage, as regulated expression of virulence attributes enables the host-microbe interaction to be sustained in the long-term. Indeed, Brussow *et al.* (2004) describe striking similarities between DtxR regulation of *tox* in *C. diphtheriae* and Fur regulation of Shiga-toxin, *stx1*, in *E. coli*, as both cases exemplify the regulation of a phage-encoded virulence gene by a chromosomally-encoded *trans* element. The convergent evolution of this type of genomic crosstalk underscores the fitness advantage yielded by metalloregulatory control of virulence genes.

***Streptococcus mutans*: SloR-mediated adaptation to stresses in the oral cavity**

While much can be gleaned from examining the ultimate etiology of *C. diphtheriae* virulence, an understanding of *S. mutans*-induced cariogenesis warrants a more proximate perspective. The "ecological plaque hypothesis", postulated by Marsh (2003), ascribes the derivation of dental caries to an ecological perturbation within the oral cavity. An estimated 600 species of bacteria colonize the mouth, yet most live commensally within the host, adapted to the "feast-famine" existence that results from intermittent food intake over the course of the day (Burne, 1998). An increase in both the frequency and quantity of sugar consumption by the host, however, alters the community composition of the plaque microflora. Metabolism of fermentable carbohydrates by acidogenic bacteria such as *S. mutans* yields acidic byproducts, which decrease the pH at the tooth surface. As these bacteria are also often aciduric, an intermittently low pH environment exerts strong selective pressure, favouring their proliferation to the demise of more innocuous, acid-sensitive microbes (Marsh, 2003). If sustained over time, acid-induced demineralization of the enamel can lead to the decay defined by dental caries. The pathogenicity of *S. mutans* thus stems from its ability to simultaneously create and withstand various stresses in the mouth. The concentration of divalent metal cations serves as a proxy for the environmental conditions of the oral cavity, modulating the appropriate expression of virulence factors in *S. mutans* via SloR, a DtxR homologue that acts as a negative transcriptional regulator of so-called "Class I" genes and a positive transcriptional regulator of so-called "Class II" genes (O'Rourke *et al.*, 2010).

A number of Class I and Class II genes encode proteins involved in bacterial adherence, energy metabolism, and signal transduction that enable *S. mutans* to proliferate in spite of the stresses posed by the oral cavity. For example, salivary flow and mastication discourage formation of the plaque biofilm (Hamada and Slade, 1980), as particular components of saliva, such as lysozyme and secretory immunoglobulin A, serve to inhibit adhesion and encourage agglutination, ultimately facilitating the removal of microorganisms by swallowing (Jenkinson and Lamont, 1997; Burne, 1998). Successful colonization of the dentition therefore entails tenacious attachment to saliva-coated surfaces, mediated in *S. mutans* by sucrose-independent and/or -dependent adherence. Ionic interactions between bacterial surface antigens and host-derived factors present in the coat pellicle comprise the more easily reversible attachments of sucrose-independent adherence. By contrast, the synthesis of water-soluble and water-insoluble extracellular glucans from sucrose by cell-associated glucosyltransferases in sucrose-dependent adherence creates an irreversible interaction (Hamada and Slade, 1980). In expression profiling experiments, O'Rourke *et al.*

(2010) identified several genes involved in sucrose-dependent adherence subject to positive regulation by SloR, namely a glucosyltransferase (*gtfB*) and a glucan binding protein (*gbpB*). Given that metal ion concentrations rise during mealtimes, the coordinate upregulation of proteins involved in sucrose-dependent adherence by SloR enables *S. mutans* to utilize the concomitant availability of dietary sucrose to its advantage.

Adaptation to the high-energy nutrient fluxes of the mouth also necessitates mechanisms for polysaccharide storage and mobilization (Ruby and Goldner, 2004). In *S. mutans*, SloR acts as an Mn^{2+} -dependent transcriptional repressor of the *glgBCDA* genes, the products of which code for the synthesis and accumulation of intracellular glycogen-like storage polymers (IPS). Since manganese in the oral cavity derives largely from the diet, Mn^{2+} concentrations in saliva vary between and during mealtimes, ranging anywhere from 1nM to 36 μ M (Green, 1970, Chicharro *et al.*, 1999). Given these fluctuations, O'Rourke *et al.* (2010) propose that manganese may serve as a nutritional stress signal for *S. mutans*, such that shortly after a mealtime when Mn^{2+} becomes limiting but an excess of carbon is still available, SloR-mediated expression of the *glgBCDA* operon is de-repressed. The mobilization of IPS during subsequent periods of famine provides *S. mutans* with substrate for homolactic fermentation long after the supply of dietary carbohydrates has been exhausted. In this way, SloR-mediated regulation of the *glgBCDA* operon contributes to the very acid production that renders *S. mutans* cariogenic. Although *glgBCDA*, *gtfB* and *gbpB* represent just a few of the genes affected by SloR, an examination of their role in metabolism and adherence makes apparent the impact of SloR-mediated gene regulation on pathogenesis.

A study investigating the cariogenic potential of a *S. mutans* SloR knockout mutant (GMS800) in a germ-free rat model more broadly attests to the role of SloR metalloregulation in virulence, as rats mono-infected with GMS800 presented with considerably more carious lesions than those mono-infected with the wild-type strain, UA130 (Spatafora *et al.*, 2001). Such a “hyper-cariogenic” phenotype likely derives from the effects of Class I gene (eg. *glgBCDA*) de-repression on *S. mutans* virulence that outweigh the impact of Class II gene repression (eg. *gtfB* and *gbpB*). Hence, the heightened cariogenic potential of GMS800 observed in rats reflects the interplay of both positive and negative gene regulation by SloR, which ultimately tips the balance toward a pathogenic outcome. The ability to constitutively repress the SloR regulon would therefore be expected to attenuate *S. mutans* cariogenicity without destroying the ecology of the bacterial niche. A hyper-repressive SloR mutant (E169A/Q170A) recently generated by Haswell *et al.*, (2013) awaits *in vivo* testing to address this hypothesis.

***Streptococcus pyogenes*: Strain-specific variation in MtsR regulation**

Unlike *S. mutans*- or *C. diphtheriae*-induced disease, which almost always exhibits a particular outcome in the human host, *S. pyogenes* causes a vast array of infections ranging from acute (pharyngitis, scarlet fever, cellulitis, erysipelas, impetigo) to invasive (necrotizing fasciitis, myositis, streptococcal toxic shock syndrome) (Patterson, 1996). The diversity of clinical manifestations of *S. pyogenes* infection reflects the complexity of the microbe's pathogenicity. Much of the literature has explored the role of CovRS, a stress-responsive two-component signal transduction system, as a number of virulence factors fall under its control (Graham *et al.*, 2002). More recently, however, an examination of the strain-specific intricacies of *S. pyogenes* pathogenicity led several authors to unequivocally implicate the MtsR metalloregulator in *S. pyogenes* virulence gene expression.

The curiously high prevalence of invasive disease amongst patients infected with *S. pyogenes* serotype M3 strains compelled Beres *et al.* (2006) to explore the relationship between strain genotype and patient phenotype. Of the M3 serotype strains isolated from

patients with invasive infections, one particular subclone, MGAS9887, displayed a markedly decreased ability to cause necrotizing fasciitis. Indeed, Beres *et al.* observed significantly higher survival rates amongst mice injected intraperitoneally with MGAS9887 as compared to those injected with the reference, wild-type strain. To identify the molecular basis for this phenotypic difference, Beres *et al.* pursued DNA-DNA microarray hybridization-based comparative genomic resequencing and polymorphism sequencing of M3 strain samples. In contrast to the other strains analyzed, MGAS9887 possesses a single base pair insertion in the *mtsR* gene just downstream of the start codon. The ensuing frame-shift yields a premature stop codon after 13 amino acids, resulting in a truncated MtsR protein. Yet the presence of 60 other SNPs in the genomic sequence of MGAS9887 precluded Beres *et al.* from definitively attributing the strain's disparate infection phenotype to its non-functional MtsR metalloregulator.

Accordingly, Olsen *et al.* (2010) generated an isogenic *mtsR* deletion-mutant strain, MGAS315 Δ *mtsR*, in an attempt to recapitulate the *mtsR* mutation possessed by MGAS9887. Intramuscular inoculation of the mutant strain into mice produced a phenotypic outcome similar to that of MGAS9887, with significantly reduced virulence as compared to the reference strain. More convincingly, complementation of MGAS315 Δ *mtsR* with wild-type *mtsR* rescued its virulence potential. Having thus validated a causal relationship between MtsR and necrotizing fasciitis, Olsen *et al.* next sought to identify those MtsR-regulated genes whose de-repression might account for the phenotypic differences observed. Comparison of the MGAS315 Δ *mtsR* transcriptome with that of the reference progenitor strain revealed differential expression of genes involved in metal ion transport and oxidative stress, consistent with the canonical role of metalloregulatory proteins in metal ion homeostasis. Yet differences in expression of one particular gene, *prsA*, warranted further investigation, as the encoded peptidyl-prolyl isomerase ensures proper functioning of streptococcal pyrogenic exotoxin B (SpeB), a virulence factor paramount to the pathogenesis of necrotizing fasciitis. As a secreted cysteine protease, SpeB catalyzes the degradation of host extracellular matrix proteins and the cleavage of immunoglobulin molecules (Olsen *et al.*, 2010). In the absence of a functioning MtsR regulator, Olsen *et al.* suggest that overexpression of *prsA* hinders the proper maturation of SpeB, thereby attenuating the "flesh-eating" capability of *S. pyogenes*. The construction of two more isogenic mutants enabled the authors to confirm this hypothesis, for both mice and cynomolgus macaques infected with an overexpressing *prsA* mutant or a *speB* deletion-mutant exhibited compromised necrotizing fasciitis phenotypes, comparable to those infected with MGAS315 Δ *mtsR*. Yet epidemiological studies ascribe a representative number of pneumonia, bacteremia, and synovitis cases to the naturally occurring MntR insertion-mutant, MGAS9987, indicating that the *mtsR* mutation has a negligible effect on virulence outside of the context of necrotizing fasciitis, at least in serotype M3 strains.

We temper the generality of these conclusions in light of the results from another transcriptome study, which seem to suggest that the genes subject to MtsR control may vary amongst *S. pyogenes* serotypes. Like Olsen *et al.* (2010), Toukoki *et al.* (2010) compared the transcriptome of an isogenic *mtsR* knockout strain to that of a wild-type strain. In this study, however, the isogenic knockout strain derived not from a serotype M3 strain, but rather from a serotype M49 strain (NZ131), originally constructed by Bates *et al.* (2005). As this knockout had earlier demonstrated significantly reduced virulence in a zebrafish model of intramuscular and intraperitoneal infection (Bates *et al.*, 2005), Toukoki *et al.* sought to characterize the genes subject to MtsR control. Microarray analyses revealed differential expression of 64 genes, many of which had likewise been established as MtsR regulons by Olsen *et al.* (2010). Despite some overlap, however, both Olsen *et al.* and Toukoki *et al.* identified a number of MtsR regulons that appeared to be exclusive to their respective studies. While Toukoki *et al.* did not observe significantly different expression of *prsA*

between wild-type and knockout strains, the authors assigned additional unique virulence genes to MtsR regulation. A better understanding of the MtsR regulon demands further study in order to definitively attribute the discrepancies between the transcriptome data to strain-specific differences in MtsR regulation, rather than to experimental artifact. Nevertheless, these studies once again substantiate the evolutionary advantage conferred by metalloregulation of *prsA* and other virulence genes: by dovetailing their expression with colonization of mammalian tissue, MtsR regulation minimizes unwarranted metabolic expenditures.

***Staphylococcus aureus*: Association of MntR with virulence**

The complexity of *S. aureus* pathogenicity rivals that of *S. pyogenes*, as it also possesses an arsenal of virulence factors that allow it to survive and inflict damage in various host microenvironments. Although *S. aureus* frequently colonizes the nares and axillae as a commensal, perturbations within the microbe or host, such as a compromised immune system, transform it into a pathogen capable of causing a gamut of infections, such as skin lesions, endocarditis, toxic shock syndrome, pneumonia, and food poisoning (Foster, 1996). The rising prevalence of methicillin-resistance amongst *S. aureus* strains has rendered it increasingly important to decipher the mechanisms that underlie the microbe's pathogenicity. In contrast to the other metalloregulators described in this review, however, the link between MntR and *S. aureus* virulence, while established, has not been as thoroughly investigated.

Horsburgh *et al.* (2002) first revealed a manganese-dependent regulatory role for MntR through their characterization of *mntABC* and *mntH*, genes that encode manganese uptake systems in *S. aureus*. Curious about the effect of a loss-of-function mutation, the authors evaluated the virulence of an *mntR* mutant strain in a mouse model of abscess infection. That the number of bacteria sampled from abscesses did not significantly differ between mice infected with the *mntR* mutant and the wild-type strain suggested a negligible effect of MntR regulon de-repression on virulence. Yet the use of a gain-of-function mutant, derived from the heterologous and constitutively active, iron-independent DtxR(E175K) protein (Sun *et al.*, 1999), conversely indicated a significant effect of hyper-repression (Ando *et al.*, 2003). To ensure expression of DtxR(E175K) in *S. aureus*, Ando *et al.* modified the first ten codons of the mutant protein so that they more closely resembled those of MntR. The authors then subcloned the altered gene, along with the *mntR* promoter, into a shuttle vector before transforming *S. aureus* with the recombinant construct. A strain transformed with the empty shuttle vector served as the control for the following real-time qRT-PCR experiment, which confirmed the hyper-repressive effect of DtxR(E175K) in *S. aureus* on *mntB* transcription levels. Although the mutant protein did not repress *mntB* expression absolutely, DtxR(E175K) nonetheless exhibited higher transcriptional repression than did the control. This hyper-repressive phenotype impaired the strain's pathogenic potential, as the authors isolated significantly fewer bacteria from the lesions of mice infected with recombinant strains than from those infected with the control. Such a result echoes the findings of Manabe *et al.* (1999), who had previously illustrated that a hyper-repressive DtxR(E175K) mutant attenuated *M. tuberculosis* virulence in a mouse model of tail vein injection. Together, then, the results of Horsburgh *et al.* and Ando *et al.* raise questions about the genes that comprise the MntR regulon. Can the disparate effects of hyper-repression and de-repression on *S. aureus* virulence solely be attributed to changes in expression of *mntABC* and *mntH*? Microarray or transcriptome analyses, like those used in the characterization of the SloR and MtsR regulons, would allow for the identification of other genes subject to MntR control, thereby opening the door for further investigation into how MntR (dys)regulation contributes to the phenotypes observed.

Conclusions and future directions

Accumulating reports in the literature have provided insight into the proximate and ultimate explanations for metalloregulatory control of bacterial virulence. Metal ion concentrations serve as an environmental cue, enabling DtxR, SloR, MtsR, and MntR to adaptively regulate the expression of virulence genes during infection of the mammalian host. Armed with a fundamental understanding of the role of metalloregulation in bacterial pathogenesis, we can now consider how metalloregulation relates to other facets of virulence gene control. Many of the aforementioned studies allude to an elaborate interplay between metalloregulatory proteins and two component signal transduction systems. For example, in *S. mutans*, the *vicRK* signal transduction system modulates the expression of the *gtfB* and *gbpB* genes, which are also subject to positive regulation by SloR (Senadheera *et al.*, 2005). Similarly, in *S. pyogenes*, the MtsR-regulated genes *speB* and *ska* fall under control of CovRS (Toukoki *et al.*, 2010). Future studies might investigate crosstalk between these regulatory networks to parse apart the hierarchy of virulence gene control.

Ultimately, a more nuanced understanding of metalloregulation can inform rational drug design. As studies of SloR and DtxR hyper-repressive mutants suggest, mutations that increase the affinity of a metalloregulatory protein for its consensus sequence can attenuate a pathogen's virulence. Recent pharmaceutical efforts have explored the viability of small molecule antimicrobial peptide mimetics, which, if designed properly, could cross the plasma membrane and bind those amino acids that lock the protein into a hyper-repressive state (Doan *et al.*, 2012). Importantly, these mimetics would not interfere with host cellular functioning, as eukaryotes employ disparate mechanisms for maintaining metal ion homeostasis. The suitability of metalloregulatory proteins as therapeutic targets thus calls for an investigation of metalloregulators in other clinically important pathogens. Indeed, a simple BLAST search reveals the existence of DtxR homologues in *Enterococcus faecalis*, *Listeria monocytogenes*, and *Clostridium botulinum* (Table 1). As many of these metalloregulatory proteins have yet to be characterized, let alone named, they represent a compelling avenue for future research.

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Figure 1. Dendrogram of DtxR homologues

The GenBank accession numbers listed in Table 1 were used to obtain the primary amino acid sequence of each metallorepressor protein. Sequences were aligned using ClustalW2 following truncation of the C-terminus to remove the FeoA domain, if present. Finally, we input the generated alignment into the ClustalW2 phylogeny programme to create the above dendrogram.

Table 1

DtxR homologues of gram-positive bacteria.

Name of bacteria	DtxR homologue	Cognate metal ion(s)	GenBank Accession Number	Reference(s) ^a
<i>Corynebacterium diphtheriae</i>	DtxR	Fe2+	NP_939766.1	Boyd et al., 1990
<i>Streptococcus mutans</i>	SloR	Mn2+/Fe2+	NP_720655.1	Rolerson et al., 2006; O'Rourke et al., 2010
<i>Streptococcus pyogenes</i>	MtsR	Mn2+/Fe2+	YP_005388451.1	Olsen et al., 2010; Toukoki et al., 2010
<i>Streptococcus gordonii</i>	ScaR	Mn2+	YP_001451085.1	Jakubovics et al., 2000
<i>Staphylococcus aureus</i>	MntR	Mn2+	YP_007867705.1	Horsburgh et al., 2002.; Ando et al. 2003
<i>Staphylococcus epidermidis</i>	SirR	Fe2+	YP_187889.1	Hill et al., 1998
<i>Bacillus subtilis</i>	MntR	Mn2+	NP_390332.2	Que and Helmann, 2000
<i>Mycobacterium tuberculosis</i>	IdeR	Fe2+	NP_337286.1	Gold et al., 2001; Manabe et al., 1999
<i>Treponema pallidum</i>	TroR	Mn2+/Zn2+	YP_007935974.1	Posey et al., 1999
<i>Listeria monocytogenes</i>	MntR	unknown	YP_007604319	none
<i>Clostridium botulinum</i>	unnamed	unknown	YP_001252858.1	none
<i>Enterococcus faecalis</i>	unnamed	unknown	YP_007152624.1	none

^aThe references are not related to the GenBank Accession number in the previous column, rather they refer the reader to literature pertaining to the characterization of each metalloregulatory protein.