



PerR-Mediated Oxidative Stress Response in *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus is a human bacterial pathogen, known to cause a variety of illnesses and infections in the hospital and community settings. It produces a large number of virulence factors used for host invasion, tissue colonization and destruction, leading to severe infections in both healthy and immunodeficient people around the world. In this paper, we studied the potential mechanisms related to methicillin resistant *S. aureus* (MRSA) infection, analyzed the significance of oxidative stress for survival of *S. aureus*, and re-identified the molecular structure, regulatory boxes, and regulons of PerR (peroxide responsive repressor) gene, a key modulator responsible for MRSA. Then, we further investigated how PerR functions in response to oxidative stress and how the PerR regulon affects *S. aureus* virulence. As a conclusion, we postulate that the regulation of PerR and its regulon indirectly influences the expression of virulence factors by prolonging the pathogen's survival under harsh conditions that may lead to the increased risk of *S. aureus* transmission, infection, and spread of the disease.

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► Implication for health policy/practice/research/medical education:

Infections caused by methicillin resistant *Staphylococcus aureus* (MRSA) in medical settings are common. In the 1990's, there has been an increase in skin and soft tissue infections due to MRSA strains originating from outside of the medical environment. These infections have been shown to occur mostly in healthy, young individuals. Due to the rise of MRSA, it is necessary to identify new therapeutic solutions to eradicate the pathogen and investigating unique metabolic pathways in the pathogen shows promise in potentially identifying new drug targets.

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1. Introduction

Staphylococcus aureus is a Gram-positive human pathogen, capable of causing a wide range of infections from local infections to metastatic infections in healthy and in immunodeficient patients. Minor skin infections include pimples, impetigo, boils, cellulitis, folliculitis, carbuncles, scalded skin syndrome, and abscesses (1). Serious diseases are pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, chest pain, bacteremia, and sepsis (2-4). Long-term efforts to control this disease worldwide

have been invested, but all efforts have not been fully effective to control this disease. Recent studies have provided experimental clues of development of some potential strategies to deal with this problem. One of the most significant advancements in this area is the discovery of the transcriptional regulation as the most efficient defense mechanism against oxidative stress due to reactive oxygen species (ROS) in *S. aureus* resulting from the attack by host phagocytes upon infection (5). Current evidence has shown that stress-responsive genes are activated as part of the cellular defense mechanism where transcriptional regulators sense ROS in many bacteria (3, 6). One of these stress-responsive proteins is a major transcriptional regulator PerR (peroxide responsive repressor), which regulates oxidative stress response and mediates the repression of its own gene and other genes on its regulon (7). In this paper, we will analyze

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and discuss the potential transcriptional regulation mechanisms of PerR and its regulon and how PerR regulation influences virulence in *S. aureus*.

2. *S. aureus* Infection and Emergence of MRSA

This pathogen is known to be the leading cause of nosocomial infections due to methicillin resistant strains of *S. aureus* (MRSA), often causing postsurgical wound infections (1). Indeed, an increase in MRSA strains has become a major public health problem (8). It was proposed previously that MRSA was typically originated from hospital sources where the patient had been hospitalized or a person was in contact with a hospitalized patient (9-12). A recent study further provided evidence that only a few critical genetic changes existed among the genomes of 10 *S. aureus* isolates collected across the U.S., suggesting that a single MRSA strain was originated through point mutations that then led to the variation of several isolates (13). Probably, some nosocomial cases of MRSA are potentially due to the isolates originating from community reservoirs (5, 14).

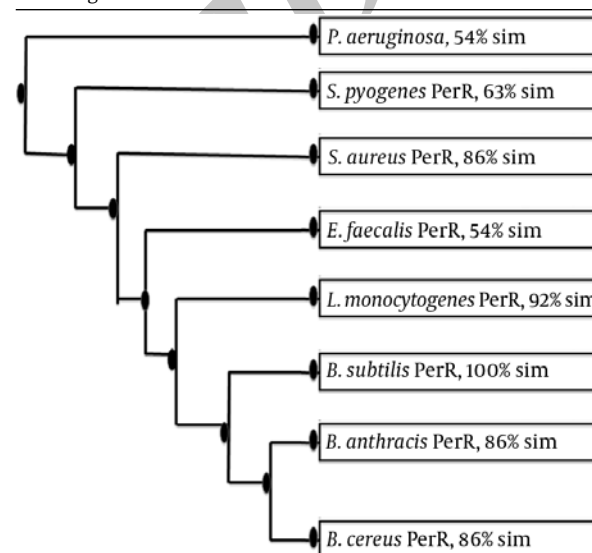
3. Significance of Oxidative Stress for Survival of *S. aureus*

The host immune system responds to the phagocytic attack of the pathogen during the initial stages of infection by *S. aureus*. These phagocytes include neutrophils and macrophages that migrate to the site of infection to engulf the pathogen and ultimately kill it in the phagolysosomal membrane (15). Subsequent to these reactions, the superoxide anion, hypochlorous acid and nitric oxide can react with other molecules to produce other ROS, such as hydrogen peroxide, peroxyxynitrite, and hypochlorous acid (15). In fact, production of various types of ROS is a common mechanism in many biological systems and is considered a general inducer of oxidative stress-related gene expression that leads to programmed cell death through several specified signal transduction pathways (16-18). Similarly, in order for the pathogen to protect itself against the ROS from host, genes involved in the regulation of molecular counter-defenses against ROS are subsequently expressed in the pathogen cells. In conjunction with the expression of the pathogen protecting itself against ROS, other genes that produce virulence factors are coordinately expressed to aid in the infection of the host (7). The gene expression of many virulence factors is controlled by regulatory systems with various transcription factors (5, 8, 15, 16, 18, 19). Several global regulators have been identified to be involved in the process of producing virulence factors, along with oxidative stress response in various pathogens including *S. aureus* (20, 21). One of the major transcription factors associated with oxidative stress response and *S. aureus* virulence is PerR(22).

4. PerR as a Universal Regulator

The *perR* gene found in *S. aureus* is 447 base pairs in length that encodes a functional protein with 148 amino acids (Gene ID 2859896, NCBI). In *S. aureus*, PerR has been shown to control an oxidative stress regulon, regulate iron storage proteins, and influence expression of virulence factors (22). This protein in *S. aureus* is a Mn (II) repressor and the PerR regulon responds to high levels of Fe (II) (23). One of the metal binding sites in PerR is involved in DNA binding and the second site involves zinc (Zn) coordination for the protein structure (24, 25). It appears that PerR could be a universal regulator that exists in various bacterial pathogens (26, 27). Our phylogenetic analysis revealed high ami-

Figure 1. Phylogenetic Tree Analysis Based Upon the Fast Minimum Evolution Algorithm.



The maximum sequence difference was set to 0.85. The evolutionary distance model used was the Grishin model. The PerR proteins from the different pathogens and *P. aeruginosa* Fur share a common evolutionary history. The % amino acid sim are included where the sequences were compared against *B. subtilis*.

Abbreviation: sim : Amino acid sequence similarity

Table 1. PerR Box Alignment¹ for PerR Regulon^a

Gene	PerR Box	PerR Box Location From -35 and -10 Elements
<i>kata</i>	ATTATAATTATTATA-A-	Upstream of -10
<i>ftn</i>	ATTATAATTATTATT-AT-	Upstream of -10
<i>bcp</i>	-TAAAATTATTATA-AT-	Upstream of -10
<i>fur</i>	-TTTAAATTATTAGT-A-	Upstream of -10
<i>ahpC</i>	ATTAGAATTATTATA-ATT	Upstream of -10
<i>trxB</i>	ATAATTATTATTATT----	Upstream of -10
<i>mrgA</i>	ATTAGAATTATTATA-AT-	Between -35 and -10
<i>perR</i>	ATAATAATTATTATA----	Upstream of -10

^a The green nucleotides are conserved among the PerR boxes. PerR boxes for regulon genes were analyzed using online sequence alignment tools (JCM and NCBI). *S. aureus* subsp. *aureus* MRSA252 was used for comparison.

no acid sequence homologies (54-100 %) in several representative bacterial pathogens (Figure 1), where PerR amino acid sequence alignment of *Bacillus subtilis* PerR (GenBank Accession NP_388753.1) vs other Gram-positive bacteria PerR strains showed that several pathogens (GenBank Accession YP_004876448.1 for *B. anthracis*, GenBank Accession AAN61567.1 for *B. cereus*, YP_003464888.1 for *Listeria monocytogenes*, AAY63967.1 for *Enterococcus faecalis*, ABF37117.1 for *Streptococcus pyogenes*, and YP_041326.1 for *S. aureus*) contain PerR with similarity above 60 %. *S. aureus* PerR has a sequence similarity of 86 % to *B. subtilis* PerR and 54 % to *P. aeruginosa* Fur. In fact, PerR and PerR homologues have been found in more Gram positive bacteria and less so in Gram negative bacteria (26, 27).

5. 3D Structure and Function of PerR Protein

PerR, like other Fur homologues, is a dimeric protein consisting of two monomers, each of which contains two metal ions (19). For the Zn binding sites on *B. subtilis* PerR, the Zn ion is bound by four cysteinates (19). Upon our amino acid sequence alignment analysis of *B. subtilis* PerR, *S. aureus* PerR, and *P. aeruginosa* Fur, the four con-

served cysteines are observed between *B. subtilis* PerR and *S. aureus* at similar positions and strictly organized as two Cys-(Xaa)₂ motifs with Cys-96, Cys-99, Cys-136, and Cys-139 for *B. subtilis* PerR and *S. aureus* PerR (Figure 2). It has been found that the Zn(Cys)₄ site in PerR-Zn joins the three S3, S4, and S5 beta-strands of each monomer to form the two dimmers, locking together the three strands of the beta-sheet (28). Due to the presence of the four cysteines in the *S. aureus* PerR, it is likely that these PerR proteins also contain a Zn(Cys)₄ site. This Zn(Cys)₄ site may be a unique feature of the PerR and PerR homologue regulators (4).

It was speculated that the metal binding to the His-37 causes the N-terminal domain to move towards the C-terminus which is the resultant conformational change capable of PerR binding to DNA (2). These residues may form a shape of an asymmetrical pyramid with the His-93 occupying the apical position and His-37, Asp-85, His-91 and Asp-104 forming the pyramid base for binding regulatory metals such as Fe(II) or Mn(II) ligands necessary for *in vivo* PerR repressor function in *B. subtilis* (29). Our further analysis of the *S. aureus* PerR structure show the Zn binding site consists of the four cysteines involved in the protein coordination (Figure 3A and 3B). The Mn(II) bind-

Table 2. A List of PerR Regulon

Gene	Gene Product	Function
<i>katA</i>	Catalase	Catalase activity, oxidative stress
<i>ftn</i>	Ferritin	Iron storage proteins
<i>bcp</i>	Thiol peroxidase	Antioxidant activity, oxidative stress
<i>fur</i>	Ferric uptake regulator	Transcriptional regulator, iron transport and metabolism
<i>ahpC</i>	Akyl hydroperoxide reductase	Peroxiredoxin activity, oxidative stress
<i>trxB</i>	Thioredoxin reductase	Thioredoxin-disulfide reductase activity, oxidation-reduction process
<i>mrgA</i>	Ferritin-like Dps homologue	Ferric ion binding, metalloregulation DNA-binding stress protein
<i>perR</i>	Peroxoide-responsive repressor	Transcriptional regulator

Table 3. Predicted Coding Region Based Upon PerR Box Sequence

Primary Locus ^a	Common Name ^a	Gene Length (bps)	Codons, No.	Protein Function	% Identity to PerR Box ^b , %
NWMN 0815	conserved hypothetical protein	1155	385	cellular component	94
NWMN 0731	TPR domain protein	1440	480	unknown	88
NWMN 0515	polysaccharide biosynthesis protein CapD	966	322	coenzyme binding	82
NMWN 2256	teicoplanin resistance associated protein B	1209	403	drug transmembrane transporter activity	82
NWMN 0892	serine protease HtrA	2325	775	protein fate: degradation of proteins, peptides, and glycoproteins	76
NWMN 1257	exonuclease SbcD	1122	775	exonuclease activity	76
NWMN 1754	D-isomer specific 2-hydroxyacid dehydrogenase family protein	951	317	NAD binding	76
NWMN 2397	fibronectin binding	2034	678	cellular component; biological process	76

^a Comprehensive Microbial Resource

^b NCBI BLAST with ATTATAATTATTAT as PerR Box (Morrissey, et al., 2004)

Figure 2. Clustal Sequence Alignment of *B. subtilis* PerR, *S. aureus* PerR and *P. aeruginosa* Fur.

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Bs_PerR      -----MAAHELKEALETLKETGVRI TPQRHAILEYLVNSMA-PTADDIYKALEGKFPN 53
Sa_PerR      MSVEIESIEHELEESIASLRQAGVRITPQRQAILRYLISSHT-PTADEIYQALSPDFPN 59
Pa_Fur       -----MVENSE-LRKAGLKVTLPRVKILQMLDSAEQRMSAEDVYKALMEAGED 48
              : *   *::*::** *   **. * .:  * :*::*::**   :

Bs_PerR      MSVATVYNNLRVRFRESGLVKELTYGDASSRF FVTS-DYFYAICCENCGKIV FHY PGLDE 112
Sa_PerR      ISVATIYNNLRVFKDIGIVKELTYGDSSSRF FNTH-NYEYIICEQC GKIV FQY PQLNE 118
Pa_Fur       VGLATVYRVLTQFEAAGLVVRHNF DGGH AVFELADSGHHDHMVCVDTGEVIEFMDAEIK 108
              ::**:*:. *  *.  *:* . . . . . : *::  . * * : * : *::** . :::

Bs_PerR      VEQLAAHVTGFKVSHRLEIYGVCQEC SKKENH 145
Sa_PerR      IERLAQHMTDFDVTHRMEIYGVCKECQDK--- 148
Pa_Fur       RQKEIVRERGFELVDENLVLYVRKKK----- 134
              ::  :  .*:. *:. : *  ::

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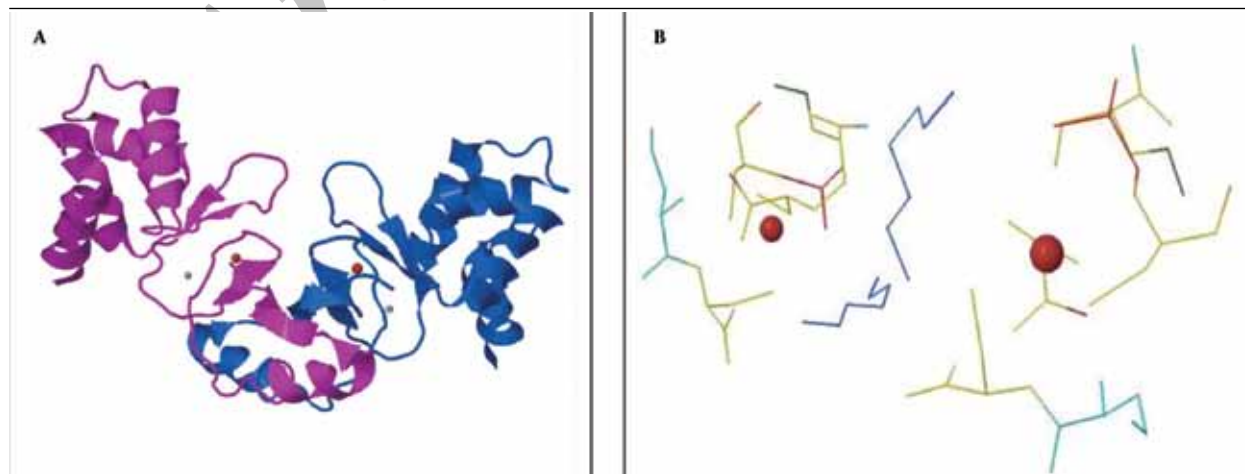
The GenBank accession numbers of fur gene sequences used in this analysis are NP_388753.1 for *B. subtilis* PerR, YP_041326.1 for *S. aureus* PerR and NP_253452.1 for *P. aeruginosa*, respectively. The yellow highlight indicates the four cysteines needed for Zn coordination. The green highlight shows the amino acids involved in the metal regulatory site. The blue highlight shows the three conserved amino acids involved in the third putative site.

ing site is located exterior to the protein, between the N- and C-terminal domains. This open area may be the site for hydrogen peroxide recruitment (29). The empty coordination site on Mn includes four chain carbonyls from Ala-36, Phe-86, Ser-89, and Val-103, a hydroxyl group from Thr-88, and an amino group of Lys-101. The best-fit model for the *B. subtilis* PerR Fe coordination is a similar square based pyramid orientation as the Mn coordination (2). Therefore, the *B. subtilis* PerR-Zn-Fe has the same penta-coordinate His₃Asp₂ environment where both Mn(II) and

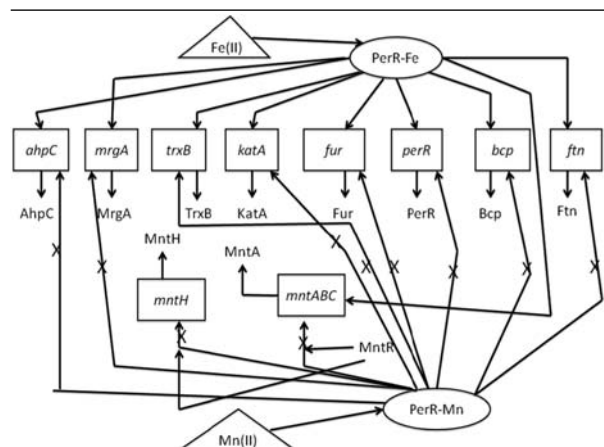
Fe(II) appear to compete for the same protein binding site (25). The *S. aureus* PerR is suspected to have a similar coordination pattern.

6. PerR Regulons and PerR Boxes

PerR controls the transcription of genes encoding the oxidative stress resistance proteins and also regulates transcription of genes encoding iron storage proteins. In recent years, the putative PerR binding sites have been

Figure 3. Molecular Structure of PerR-Zn-Mn Complex in *B. subtilis*

Both ribbon structure and Zn coordination site were predicted using the bioinformatics program of Interactive Structure Based Sequences Alignment Program Strap. The web link is <http://www.bioinformatics.org/strap/index2.html>. A. Ribbon structure, where the Zn(II) ions are red and Mn(II) are grey. The blue and violet ribbons represent the two protein chains. B. Zoomed in view of the Zn coordination site in PerR, where the red circle is Zn and the four cysteines (Cys-96, Cys-99, Cys-136, and Cys-139) are colored in yellow.

Figure 4. PerR Transcriptional Network

The rectangles are the genes regulated by PerR on the PerR regulon and the Mnt operon. The gene products are in black print. The ovals are the metalloregulators (PerR-Zn-Fe or PerR-Zn-Mn). The triangles are the metal ligands. The X represents that transcription of that gene is blocked.

determined to identify genes controlled by PerR (22). It has been reported that the PerR binding site for *perR*, *fur*, *ahpC*, *kata*, and *mrgA* genes are located in their promoter region near the -35 and -10 elements located either upstream from the -10 element or between the -35 and -10 elements as is the case for the *mrgA* gene, of which the *ftnA* and *mrgA* genes contain two PerR boxes each (23). Some of these genes are located on the same regulon as the gene that encodes the PerR protein, such as *fur*, *ftn*, *perR*, *trxB*, and *bcp* (11, 22).

A putative PerR box was identified at the upstream of the *nfrA* promoter where 9 of 14 nucleotides (TTCAATTATTAACTT) are the same as the consensus sequence (TATAAT-TATTATTA) for the PerR box that is responsible for the induced transcription of *nfrA* by hydrogen peroxide at high concentrations (12). Another PerR box that may regulate genes outside of oxidative stress response and Fe and Mn homeostasis may exist. In *S. aureus str. Newman*, many similar short sequences were identified with an identity ranging from 42-94 % against the conserved PerR box sequences of ATTATAATTATTATTAT (16). Eight of these short sequences with identities of 76-94 % can be predicted in the coding regions for proteins with known functions except for one where the function is unknown (Table 3).

7. Oxidative Stress Response by PerR and PerR Regulon in *S. aureus*

PerR may function as a major regulator of antioxidant defenses and storage regulator for Fe. PerR regulates a major oxidative stress regulon involved in intracellular thiol-disulfide balance, which include genes encoding for catalase, alkyl hydroperoxide reductase, thioredoxin-dependent thiol peroxidase (Bcp), and thioredoxin reductase (TrxR) (22). Upon our investigations, these genes

may provide reducing power to help eliminate ROS upon induction and help maintain their cellular redox balance, partially due to the presence of low molecular weight thiols (9, 10, 30). The presence of the putative PerR box near the *bcp* gene in *S. aureus* suggests that PerR has a regulatory role in transcription of thioredoxin and thiol peroxidases (28).

Other genes on the PerR regulon involve the Fe storage and Fe homeostasis regulator proteins (Fe storage protein ferritin, Ftn and ferritin-like Dps homologue, MrgA) which auto-regulates the expression of Fur (Fe homeostasis regulator) (22). The direct and indirect control of Fe homeostasis by the PerR regulon may allow *S. aureus* to maintain a non-toxic level of free Fe and redox stability to combat against ROS produced by Fenton reactions.

It is clear that there is a general transcriptional network for PerR, its regulon, and separate *mnt* operon as we proposed and illustrated in Figure 4. Within this network, the PerR protein is a Mn (II) repressor where *mntABC* transport gene is shut off in the presence of PerR-Mn and activated in the presence of PerR-Fe. The PerR regulon is induced by responding to high levels of Fe (PerR-Fe) and transcription of its genes are repressed with Mn (PerR-Mn). This simplistic network does not account for the complexity of how certain genes such as *fur* and *perR* are auto-regulated, how *ahpC* and *kata* have compensatory roles in their gene expression, and the bifunctional role of MntR to repress *mntH* when Mn (II) concentration is high and induces *mntABC* at low Mn (II) concentration.

8. Impact of PerR and PerR regulon on *S. aureus* Virulence

The PerR regulon contains at least four genes including *perR*, *fur*, *ahpC* and *kata* in *S. aureus*. A *perR* mutant demonstrated a lower degree of virulence in a skin abscess model of infection in mice (22). A *fur* mutant became less virulent potentially due to its reduced infection in a murine skin abscess model (20). The *kata* gene encoded catalase has also been considered to be an important virulence determinant in *S. aureus* due to their higher levels of superoxide dismutase (SOD) and catalase expression detected in clinical studies (21). *ahpC* compensates for the lack of *kata* where *ahpC* is expressed at a higher level, however, *ahpC* and *kata* do not likely play a role in protection from nasal secretions since similar proteins are found in neutrophils and *S. aureus* reduction by neutrophils was not observed (31). It is possible that *S. aureus* lacking *ahpC* and *kata* is compromised due to the lack of ability to survive under aerobic conditions.

The importance of *ahpC* and *kata* expression has been demonstrated for *S. aureus* to withstand harsh environmental conditions such as starvation or desiccation (32). *ahpC* and *kata* have demonstrated roles in desiccation tolerance as disrupted mutants (Δ *ahpC* Δ *kata*) showed a desiccation defect (33). Catalase has been shown to be

crucial for the longevity of starved *S. aureus*, where survival against ROS would be required (22). Therefore, PerR and its regulon appear to be involved in *S. aureus* virulence and longevity. *S. aureus* virulence and longevity.

9. Conclusions and Prospects

Genetic network analysis is a powerful tool to elucidate the gene-gene and protein-protein interaction involved in *S. aureus* infections. The PerR binding site exists in the promoter regions of *perR*, *fur*, *ahpC*, *kata*, and *mrgA* genes (23). Some of these genes are located on the same regulon as the gene that encodes the PerR protein, such as *fur*, *ftn*, *perR*, *trxB*, and *bcp* (11, 22). Our analysis has led to an identification of several novel PerR boxes present in *S. aureus* due to the existence of the consensus sequence ATTATAATTATTATTA at different coordinates throughout its genome. These PerR DNA binding sites for PerR gene regulation suggests a novel role for PerR in mediating stress responses and other cellular processes which potentially contributes to the pathogenic fitness of *S. aureus* surviving in the infected host.

Presently, the oxidative stress responses by PerR and the PerR regulon do play a major role related to the long-term survival of *S. aureus* under unfavorable conditions as desiccation and / or long-term starvation, which has been implicated in nosocomial *S. aureus* transmission and found to exacerbate the MRSA problem (34). Here, we propose that the oxidative stress resistance proteins are regulated by PerR as a major regulator of antioxidant defenses and storage regulator responsible for the long-term survival of *S. aureus*. These oxidative stress resistance proteins include catalase, alkyl hydroperoxide reductase, thioredoxin-dependent thiol peroxidase (Bcp), and thioredoxin reductase (TrxR).

To date, it appears that PerR may function as a universal regulator for many genes located in the cascade pathway of oxidative stress responses that further activate the expression of virulence genes upon infection of the host, a period, and spread in the communities. As a conclusion, we hypothesize that PerR and its regulon influences the expression of virulence factors by prolonging the pathogen's survival under harsh conditions that may lead to the increased risk of *S. aureus* transmission, infection, and spread of the disease. Therefore, several approaches should be taken to control and eventually minimize the incidences of *S. aureus* infections. First, several key minerals such as Mn, Fe, Zn could be formulated for medicinal use and optimized for testing in clinical trials; Second, it is possible to develop enzyme inhibitors against the PerR protein and other proteins in the PerR regulon; Third, anti-sense RNA (or RNAi) technology could be developed to control PerR gene expression using PerR box sequences. Consequently, it behooves us to further elucidate the PerR regulatory network in order to identify and characterize unique molecular targets for use in developing

therapeutic strategies for eradicating *S. aureus*.

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