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Targeting Cell Membrane Adaptation as a Novel Antimicrobial Strategy

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Abstract

Emergence of antibiotic resistance is an example of the incredible plasticity of bacteria to survive in all environments. The search for new antibiotics active against traditional targets is more challenging due not only to the lack of novel natural products to fulfill the current clinical needs against multidrug-resistant (MDR) bacteria, but also for the possible “collateral” effects on the human microbiota. Thus, non-traditional approaches to combat MDR bacteria have been proposed. Here, we discuss the possibility of targeting the membrane response to the antibiotic attack (cell membrane adaptation) as a viable strategy to increase the activity of current antimicrobials, enhance the activity of the innate immune system and prevent development of resistance during therapy using the three-component regulatory system LiaFSR of enterococci as a model.

Introduction

It has long been recognized that the bacterial response to antibiotics is one of the most important evolutionary phenomena with major clinical consequences in the history of medicine. Antibiotics are rapidly disappearing from the clinical armamentarium posing immense challenges in the care of patients. The dwindling pipeline of new antimicrobial compounds available to treat multidrug-resistant (MDR) bacteria suggests that the current approaches to develop antibacterial agents are not sustainable [1, 2]. In recent years, the use of next-generation sequencing, combined with rational drug design, has broadened the search for novel compounds and targets [3, 4]. This approach could potentially open the

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door to new therapeutic strategies by targeting novel bacterial systems involved in essential cellular processes such as cell division or maintaining membrane integrity. Here, we review the rationale to target a signal transduction system involved in protecting the cell envelope against the antimicrobial attack. We postulate that this approach may restore the activity of currently used antibiotics and also likely favor clearance of the organism by the innate immune system.

LiaFSR system as a master regulator of the cell membrane stress response to the antimicrobial peptide attack

The LiaFSR system is a major player in the response against *l*ipid II *i*nterfering *a*ntibiotics (particularly bacitracin and vancomycin) in *Bacillus subtilis* [5]. This system has subsequently been shown to orchestrate the response to membrane active agents including organic solvents, detergents and cationic antimicrobial peptides (e.g. daptomycin [DAP]) [6–9]. In general, LiaFSR is organized into an operon with at least three genes, ***i)*** *liaS*, which encodes a bifunctional sensor histidine-kinase [HK]/phosphatase, ***ii)*** *liaR*, encoding the response regulator of the system and ***iii)*** *liaF*, which encodes a transmembrane domain protein that negatively regulates the transcriptional effects of LiaR, presumably by locking LiaS into a “phosphatase mode” [10, 11]. Of interest, the LiaFSR system is highly conserved across the low G+C bacteria, a group that contains many relevant human pathogens including *Staphylococcus aureus*, enterococci, streptococci and *Listeria monocytogenes* (Table 1).

VraSR is the homolog of LiaSR in *S. aureus*, and is part of a 4 gene operon, *vraUTSR* [12], that is induced by exposure to cell-acting antibiotics such as β-lactams, bacitracin, DAP, glycopeptides, D-cycloserine and antimicrobial peptides [13, 14]. It was first observed that VraSR was upregulated in vancomycin-intermediate *S. aureus* (VISA) strains [15], and subsequent work has shown that this system upregulates targets including penicillin binding protein 2, teichoic acid synthesis, *prsA* (a chaperone), and *murZ* (UDP-N-acetylglucosamine enolpyruvyl transferase), presumably to repair cell wall damage [13, 16–20]. Interestingly, serial passage of *S. aureus* in sub-inhibitory concentrations of DAP generated a derivative in which VraSR was upregulated and exhibited both VISA and DAP-nonsusceptible (DNS) phenotypes, further suggesting that VraSR responds to broad perturbations of the cell wall and cell membrane [21]. Similarly, mutations in *vraSR* can influence susceptibility to both vancomycin and β-lactams [22]. Moreover, dysregulation of the *vraSR* operon have been shown to affect methicillin and DAP susceptibility in clinical isolates of methicillin-resistant *S. aureus* (MRSA) [12, 23]. Collectively, VraSR is an important mediator of cell response to a variety of antibiotics in *S. aureus* and its dysregulation could lead to a MDR phenotype.

LiaSR is also implicated as a major determinant in biofilm formation and acid resistance in *S. mutans* [24] and two other streptococcal species, *S. gordonii* and *S. uberis* [25, 26]. A study by McCormick et al. demonstrated that expression of the *dlt* operon is influenced by LiaSR in *S. gordonii* [26]. Mutants defective of LiaS or LiaR were unable to upregulate expression of the *dlt* operon to counteract envelope stress when exposed to polymyxin B.

These observations suggest that LiaR plays a critical role in responding to global stressors to maintain membrane integrity.

The role of LiaFSR in enterococci has only been described recently in the context of DNS. A substitution in the transmembrane protein LiaF was first encountered in a clinical strain of DNS *E. faecalis* [27] and subsequent investigations identified mutations in LiaFSR as the most frequent genetic changes associated with DNS in *E. faecium* [28, 29]. A quantitative experimental evolutionary approach also demonstrated that alterations in *liaFSR* were the initial pivotal step leading to development of DNS in *E. faecalis* [30]. Mutation in *liaF*, presumably resulting in activation of the LiaSR system, can decrease DAP susceptibility against *E. faecalis* and abolished DAP bactericidal activity in time-kill assays [27, 31]. Interestingly, this mutation also contributed to remodeling of the cell membrane by redistributing cell membrane cardiolipin microdomains away from the division septa in *E. faecalis* [32]. In *E. faecium*, alterations in LiaFSR are associated with DNS and loss of bactericidal activity in “susceptible” strains [28, 29]. These data provide evidence to suggest that the LiaFSR system is important for cell membrane homeostasis and antibiotic resistance [32]. Recent structural and biochemical studies indicate that substitutions affecting LiaR are associated with DNS and constitutively activate the response regulator by mimicking phosphorylation (independent of LiaS), locking the protein in an “on” state by altering the oligomerization state [33] (Figure 1).

LiaR as a novel target for anti-adaptation antibiotics

Since the LiaFSR system seems to play a prominent role in the mechanism of bacterial protection against the antibiotic attack in Gram-positive bacteria, a logical hypothesis is that inactivation of this system may make bacteria more vulnerable to antibiotics that target the cell envelope (Figure 2). Moreover, since one of the mechanisms by which the innate immune system responds to invading bacteria is through the production of antimicrobial peptides by immune cells (which also target the cell membrane), disruption of the mechanism of cell membrane protection is likely to enhance the ability of the host to clear bacteria *in vivo*.

In order to support these hypotheses, a series of *liaR* deletion mutants in MDR enterococci were tested for their ability to survive the antimicrobial peptide challenge. A non-polar deletion of *liaR* in both DAP-resistant and –susceptible *E. faecalis* markedly increased susceptibility of the mutants to DAP, decreasing the MIC beyond wild-type levels (hypersusceptibility). This effect was seen independently of the presence of mutations in other genes that have been associated with DNS [34], confirming that LiaR plays a major role against the antibiotic attack. Interestingly, loss of functional LiaR also resulted in a marked increase in activity of other antibiotics and cationic antimicrobial peptides such as LL-37 (produced by human neutrophils), strengthening the concept that targeting LiaR could positively modulate the innate immune system making bacteria more vulnerable to clearance. Furthermore, the DAP hypersusceptibilty that occurs after deletion of *liaR* was also observed in strains of *E. faecium* regardless of the presence of mutations in *liaFSR* or the genetic background [35], indicating that alterations of LiaR have a “universal” effect in all species of enterococci. Of note, null *vraR* mutants of clinical DNS MRSA also exhibited

increased susceptibility to DAP [23]. Moreover, deletion of *vraR* reversed methicillin and non-β-lactam [36] resistance and had better outcomes with β-lactam therapy (namely oxacillin) in mouse infection models [12, 37].

Novel target: the pathway forward

The current era of MDR presents clinicians with the challenges of limited therapeutic efficacy and drug toxicity where safe, effective therapies once existed. To be accepted as "novel", the design of compounds should consider the following characteristics: ***i)*** novel chemical class, ***ii)*** new target, ***iii)*** a novel mechanism against an existing target and ***iv)*** low likelihood of developing resistance [38]. As discussed previously, search for compounds that are directed against unconventional targets are at the frontline of the war against antibiotic resistance. An understanding of the molecular events that lead to bacterial cell adaptation after antibiotic challenge is likely to open new avenues of targets with molecules that enhance the activity of current antimicrobials, reverse resistance and also enhance the immune system. While traditional antibiotics target proteins with essential functions, a focus on signal transduction systems can disrupt many cellular processes and even lead to a generalized arrest of cellular function. It is interesting to note that the modification of signaling pathways has been a remarkable success in anticancer therapies but has not been exploited in the development of antimicrobial strategies. As mentioned, LiaSR TCS is activated by a variety of stimuli, including many clinically useful antibiotics with different mechanisms of action (Figure 2). However, each of these signals converges to the response regulator LiaR, which serves as the courier to activate expression of downstream genes. Thus, by inhibiting the response regulator we could prevent the expression of a resistance phenotype, even in the presence of the resistance genes (Figure 2).

LiaR or its homologs emerge as an appealing target for novel antimicrobial development due to several characteristics. *First*, the LiaFSR system is conserved and specific to many low G +C Gram-positive bacteria, including many clinically relevant pathogens (Table 1). The high degree of identity between LiaR response regulators in these species suggests that development of an inhibitor targeting LiaR with broad range of activity against pathogenic organisms is feasible. *Second*, while LiaFSR does not appear to be essential in all organisms studied [10, 17, 34, 35, 39, 40, antimicrobial activity from the compound itself is not expected. Rather, LiaR has proven to be the "Achilles heel" of the membrane adaptive response and inactivation results in susceptibility to several antimicrobials and possibly enhancement of the innate immune system (see below) (Figure 2). Inhibition of LiaR can "resurrect" antimicrobial agents that are bactericidal, less toxic and more cost effective. *Third*, deletion of LiaR demonstrated parallel enhancement of the activity of host derived cationic antimicrobial peptides. Thus, we can augment the organism's susceptibility to the patient's own immune system by weakening the ability of bacteria to defend against antimicrobial peptide produced by immune cells. This concept will potentially allow those with weaker immune response to mount a more robust mucosal defense and possibly decrease colonization with MDR organisms that are the harbingers of infection [34, 35]. *Lastly*, emergence of resistance in LiaR-deficient mutants has been evaluated in two clinically important enterococcal species. In one study, *E. faecalis* was shown to incorporate exogenous fatty acids which circumvented the loss of LiaR and improved survival to a

variety of membrane stressors, including DAP [41]. In a serial passage, the same hypersusceptible isolate developed resistance to DAP after 19 days of exposure to sub-inhibitory concentrations. In *E. faecium*, the species that is by far a greater clinical challenge, resistance was not found after 19 days of adaptation [42]. Of note, response to antibiotics via LiaSR has been shown to begin within 10 minutes of induction [13]. Thus, with appropriate dosing and prompt management of the infectious source, the opportunities to develop resistance can be limited.

The anti-adaptive concept may have several limitations. The effectiveness of these nonconventional agents has not been demonstrated yet. The activity of conventional antibiotics is predicted by bacterial growth inhibition assays. However, anti-adaption agents are not expected to affect growth suggesting that standard minimum inhibitory concentrations would not be practical. Additional assays such as time-kill assay and animal infection models will be necessary to determine activity of these agents, which are time consuming and costly. As stated, anti-adaptation drugs are expected to synergize with existing antimicrobials. Thus, combinations of anti-adaptive and antimicrobial agents are needed for treatment. Yet, different genus of Gram-positive bacteria display heterogenoeus phenotype(s) in LiaR-depleted mutants (see above) and the optimal combinations are not known. Moreover, the potential for pathogens to acquire tolerance/resistance to anti-adaptive agents or become more virulent by bypassing LiaR-dependent responses needs to be evaluated. Thus, careful characterization of this approach to increase our understanding of the evolutionary risks for anti-adaptive agents will require a multidisciplinary approach.

Concluding remarks

The alarming increase in resistance to antibiotics in pathogenic bacteria urges researchers to identify innovative and unconventional strategies for antibiotic discovery. We provide the rationale for targeting cell membrane adaptation systems which could be an interesting approach to deal with MDR organisms. Based on data derived from targeting the LiaR response regulator in clinical strains of MDR enterococci, a LiaR inhibitor may be used to disarm clinically relevant Gram-positive organisms and restore activity of currently available antibiotics and we believe that pursuing this avenue should yield novel insights into bacterial physiology and drug development.

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Highlights

- The Lia system mediates response to cell membrane stressors in Gram-positive bacteria
- Disruption of the Lia system rendered bacteria susceptible to a variety of antibiotics
- LiaR or its homologs emerge as an appealing target for novel antimicrobial development

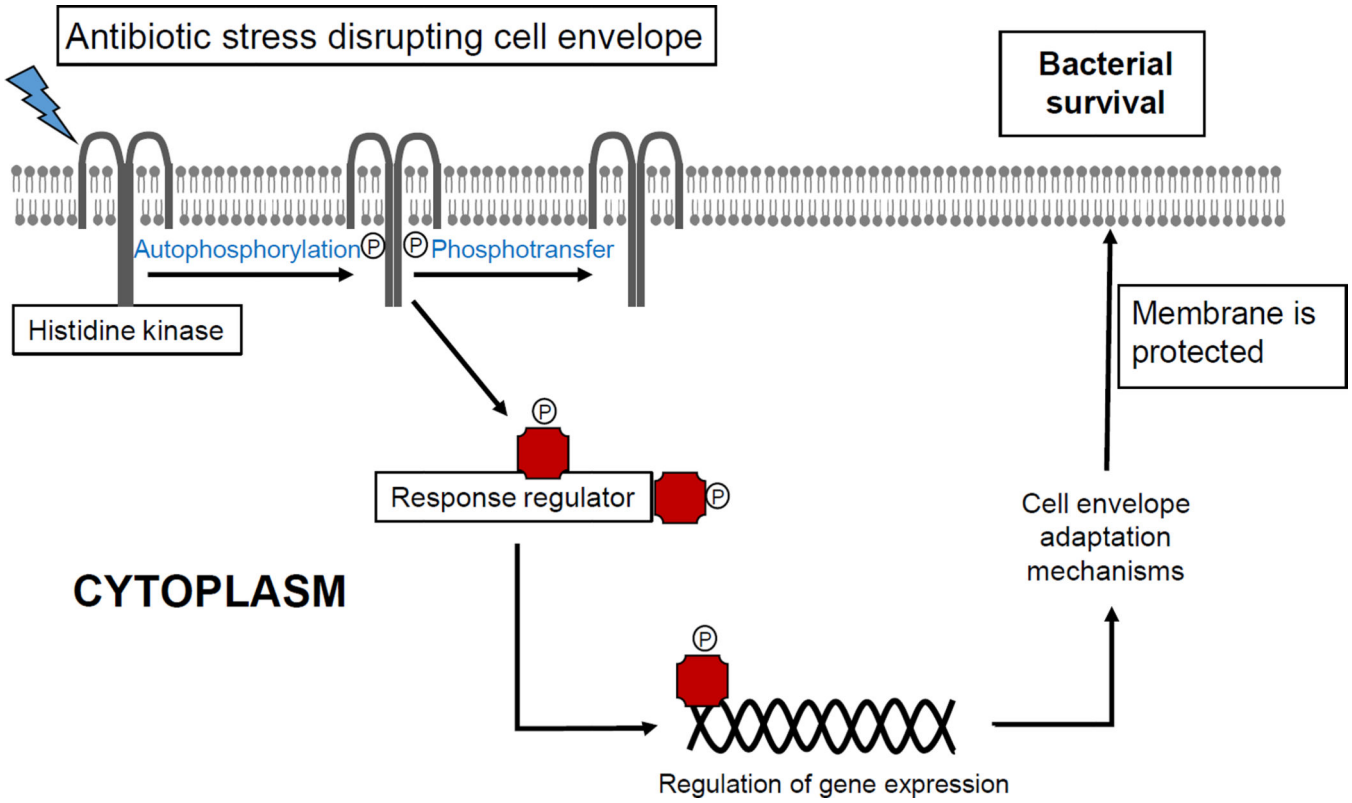

Figure 1. Bacterial cell membrane adaptation via two-component signal transduction systems

Protection of cell membrane is of paramount importance for bacterial survival. Several mechanism to protect the cell membrane against environmental stressors (including antibiotics) are available. The participation of signal transduction systems (two-component regulatory systems) is one of the most important strategies to mediate the cell membrane adaptive response.

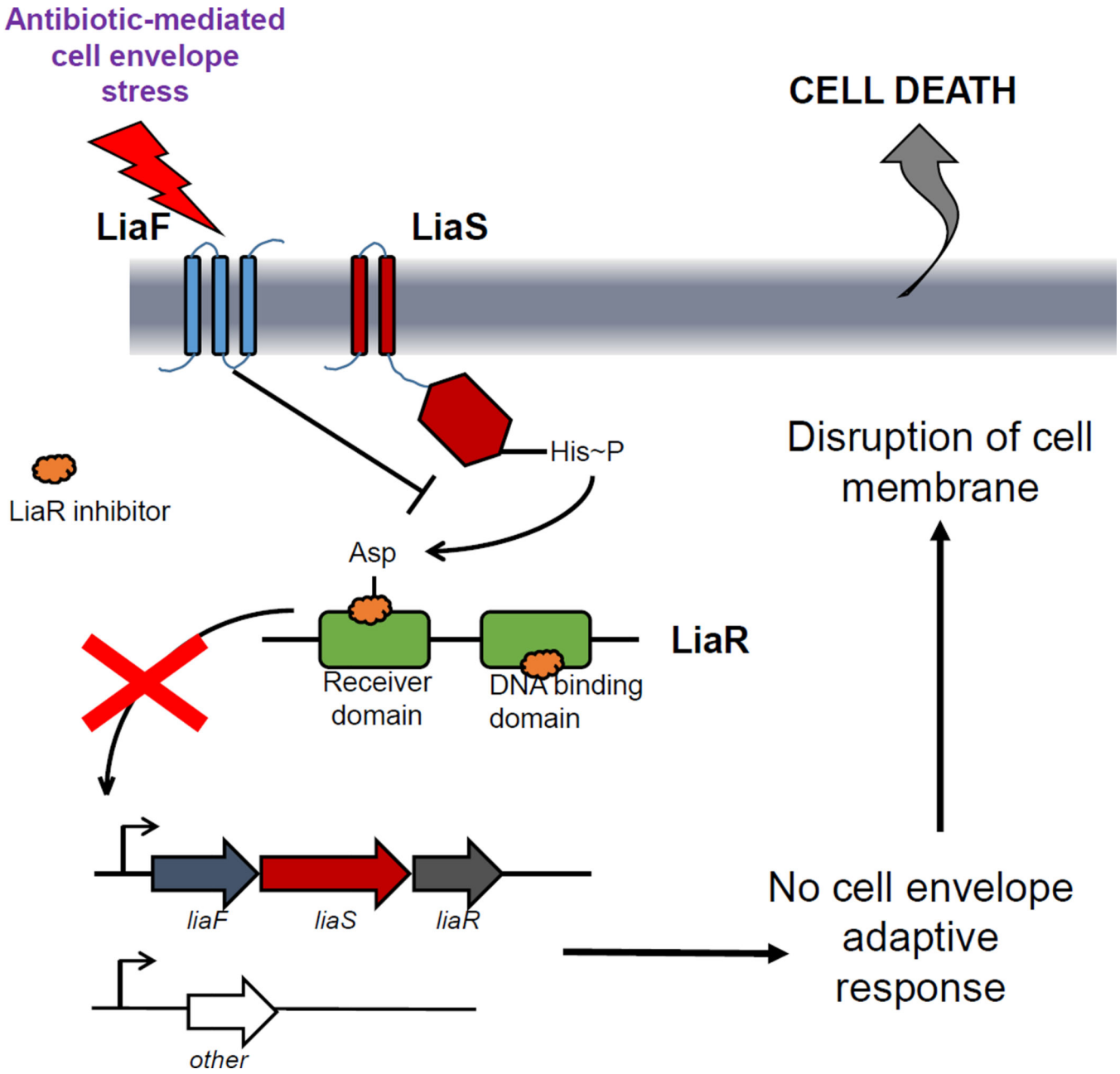

Figure 2. A model of LiaR inhibitors

Inhibiting the LiaR response regulator (green) would prevent the activation of the cell membrane adaptive response triggered by antibiotics targeting the cell envelope.

Table 1

Amino acid identity of *Bacillus subtilis* LiaR homologs in clinically relevant Gram-positive organisms

Organism	Identity (%)
Enterococcus faecalis	62
Enterococcus faecium	64
Lactococcus lactis	53
Staphylococcus aureus	53
Staphylococcus epidermidis	51
Staphylococcus lugdunensis	52
Streptococcus mutans	55
Streptococcus pneumoniae	63
Streptococcus pyogenes	56