Article Addendum

Role of two-component systems in the resistance of *Staphylococcus aureus* to antibacterial agents

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Two-component systems (TCSs) play important roles in the adaptation of bacteria to environmental changes and the regulation of virulence factor expression. In addition, the association of TCSs with susceptibility to antibacterial agents has been demonstrated in some bacterial species. *Staphylococcus aureus*, a major human pathogen that can cause serious problems due to nosocomial infections, possesses 16 TCSs. Here we report a TCS, designated BceRS (MW2 gene ID: MW2545-2544), which is related to bacitracin susceptibility. We found that BceRS regulates the expression of two transporters that determine susceptibility to bacitracin. One of these, BceAB (MW2543-2542), is located downstream of BceRS, while the other, VraDE (MW2620-2621), is more distant. With regard to other TCSs, VraRS and Aps/GraRS are reportedly associated with susceptibility to cell wall synthesis inhibitors and cationic antibacterial agents, respectively. Therefore, *S. aureus* possesses at least three TCSs that are involved in mediating its resistance to antibacterial agents.

*Staphylococcus aureus* is a major human pathogen that can cause serious problems related to nosocomial infections. This organism, and particularly methicillin-resistant *S. aureus* (MRSA), exhibits resistance to multiple chemotherapeutic agents, including β-lactams, quinolones and aminoglycosides.¹ Furthermore, vancomycin-intermediate or -resistant *S. aureus* has emerged in several countries, rendering successful chemotherapy problematic.²,³ Also, community-acquired MRSA has been reported to cause serious infectious diseases, sepsis and pneumonia.⁴ To date, many factors in *S. aureus* that confer resistance to antibacterial agents have been identified.⁵,⁶

Two-component systems (TCSs) have recently been shown to affect susceptibility to several antibacterial agents.⁶,⁷ TCSs are bacteria-specific signal transduction systems consisting of a sensor histidine kinase and cognate response regulator. In *S. aureus*, TCSs have been characterized mainly in terms of their effect on virulence factor expression and adaptation to environmental conditions.⁸ Sixteen TCSs have been identified in MRSA strain MW2. We previously investigated antibacterial agent susceptibility in a group of MW2 mutants that were gene-inactivated in 15 TCSs, with the exception of one essential TCS (*vicRK*).¹⁰ Among these mutants, one uncharacterized TCS (designated BceRS; MW2 gene ID: MW2545-2544) was associated with an increase in susceptibility to bacitracin, implying that it is involved in bacitracin sensing and resistance.

A genome database search revealed one uncharacterized ABC transporter (designated BceAB; MW2543-2542) located downstream of the TCS (BceRS) that shows homology with BceAB from *Bacillus subtilis*, which is responsible for bacitracin resistance.¹¹ Two other transporters in *S. aureus* strain MW2 (*vraDE*: MW2620-2621 and *vraFG*: MW0623-0624) show homology with *B. subtilis* BceAB. We constructed inactivation mutants of each transporter and evaluated...
the MIC of wild-type MW2 and all three mutants against bacitracin. The MIC for the ΔbceAB and ΔvraDE mutants showed 2- and 4-fold reductions, respectively, compared with wild-type, while the MIC for ΔvraFG was similar to that for wild type. The same result was obtained for the methicillin-sensitive S. aureus strain RN4220, suggesting that this phenomenon is not specific to MRSA. These data suggest that BceAB and VraDE are related to bacitracin susceptibility.

To determine whether BceRS senses bacitracin and induces expression of the transporters, we investigated their expression by quantitative PCR. In wild-type MW2, bceA and vraD expression was rapidly (5 min post-exposure) induced upon the addition of bacitracin to the medium. Interestingly, a low concentration of bacitracin (<1/64 MIC) significantly induced the expression of bceA and vraD up to 15 min post-exposure, whereas after 30 min, the expression of both genes decreased in a time-dependent manner. In contrast, higher bacitracin concentrations (>1/8 MIC) continued to induce expression after 30 min. vraD transcript levels after the addition of bacitracin increased by more than 100-fold, while the levels of bceA mRNA were nearly 10-fold higher. Only a slight induction of the TCS (bceR) transcript itself was observed upon the addition of bacitracin, suggesting that this signaling pathway does not lead to autocrine BceRS expression. In the ΔbceRS, but not the complemented strains, the induction of bceA and vraD expression upon the addition of bacitracin was completely inhibited. These results indicate that the BceRS TCS (MW2545-44) has the ability to sense bacitracin and positively regulates the two transporters responsible for bacitracin resistance. Interestingly, this TCS regulates not only a downstream operon encoding an ABC transporter (BceAB; MW2543-42), but also another operon encoding a transporter (VraDE; MW2620-2621), which is located separately from the TCS genes. In addition, transporters showing homology with BceAB have been reported in Gram-positive bacteria, including BcAB from Bacillus licheniformis, BcrAB from Enterococcus faecalis, and MbrAB from Streptococcus mutans. Recently, it was reported that the BceRSAB system was related to susceptibility to nisin, an antimicrobial peptide of the lantibiotic family that is produced by a number of strains of Lactococcus lactis subsp. Lactis. The mode of action of nisin involves an interaction with the membrane-bound cell wall precursor lipid II (undecaprenylpyrophosphoryl-MurNAc-pentapeptide-GlcNAc), leading to the formation of pores in the membrane of the target organism. Bacitracin is a polypeptide antibiotic produced by B. subtilis and B. licheniformis. Bacitracin binds to undecaprenyl pyrophosphate, which results in inhibition of the lipid cycle associated with cell wall biosynthesis. Since bacitracin and nisin both target membrane molecules related to cell wall biosynthesis, we believe that the BceRS system may be a mechanism of resistance to antibacterial agents that target cell wall biosynthesis in the membrane.

Previously, the bacitracin susceptibility-affecting gene bacA was reported in S. aureus. This gene was first identified on a multicopy plasmid in Escherichia coli, where it caused an increase in isopenol kinase activity and decrease in bacitracin susceptibility. Therefore, BacA from S. aureus (MW0645) likely possesses undecaprenyl kinase activity related to undecaprenyl pyrophosphate recycling. The inactivation of bacA resulted in an increase in bacitracin susceptibility (MIC = 4 μg/ml in the bacA mutant and 64 μg/ml in wild-type RN4220). We investigated whether bceRS regulates the bacitracin-induced expression of bacA, but found no such correlation. Therefore, S. aureus has two independent factors responsible for susceptibility to bacitracin.

The inactivation of another transporter, vraFG (MW0623-0624), that shows homology with bceAB from B. subtilis, did not affect bacitracin susceptibility. The gene that encodes this transporter, vraFG, is located downstream of the S. aureus TCS agrRS (MW0621-0622), and has been demonstrated to regulate vraFG expression. We found that agrRS inactivation did not affect the bacitracin-induced expression of vraDE and bceAB; thus, agrRS and vraFG are not associated with bacitracin susceptibility in S. aureus.

In this study, we demonstrated that one TCS, designated BceRS, is involved in bacitracin susceptibility. At least two other TCSs are also associated with the susceptibility of S. aureus to a number of antibacterial agents. AgrRS/GraRS is involved in susceptibility to cationic antibacterial agents, including vancomycin, gentamicin and nisin, and cationic antimicrobial peptides, including defensins and LL37. This system acts by regulating the expression of dlt and mprF. AdsRS/GraRS is reportedly involved in vancomycin-intermediate resistance owing to the increased expression of VraFG, an ABC transporter. Dlt and MprF are major contributors to a decreased cell surface negative charge by means of the addition of amino acids (dlt for alanine and mprF for lysine) to teichoic acids and phosphorylglycerol, respectively. In addition, Agr is associated with S. aureus cell surface charge through the regulation of apt. Agr is a quorum sensing system, the expression of which is dependent on cell density. Since Agr is a negative regulator of Ads, apt and dlt/mprF expression gradually decreases with increasing agr expression during growth.

Finally, cell surface charge becomes gradually more negative and susceptibility to cationic antibacterial agents increases during growth. These results indicate that the Agr system regulates not only the expression of virulence factors, but also susceptibility to antibacterial agents.

VraSR was originally identified as a factor that influences bacterial resistance to vancomycin. VraSR is a positive modulator in the regulation of cell wall biosynthesis (e.g., for pbpB, sbtB and murZ). The inhibition of VraSR leads to decreased resistance to cell wall inhibitors, including β-lactams, vancomycin, teicoplanin and fosfomycin. Bacitracin induces the expression of vraSR, implying a relationship between BceRS and VraSR. However, we found that vraSR expression was increased by bacitracin in a bceRS mutant. Also, in a vraSR mutant, the expression of bceA and vraD was significantly induced by bacitracin. These results indicate that BceRS has no effect on bacitracin-induced VraSR expression.

Our findings, together with those of other investigators, reveal that S. aureus possesses at least three TCSs that affect its susceptibility to antibacterial agents (Fig. 1). Interestingly, more than one TCS
is involved in bacitracin and nisin susceptibility. However, these TCSs are widely conserved among *S. aureus* strains, suggesting that these are not acquired factors, such as internal mutations or the result of external gene transfer. Also, TCS-mediated resistance is not sufficiently effective against high concentrations of antibacterial agents compared with other resistance factors such as *mecA* and *vanA*, suggesting that these TCSs are associated with resistance to relatively low concentrations of antibacterial agents. Considering that TCSs function in bacterial adaptation to altered environmental conditions, they may be involved in resistance to bacteriocins produced by other bacteria. Therefore, our analysis of TCS function highlights two distinct facets: its association with resistance to antibacterial agents, and as a survival strategy in the bacterial community.

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