Staphylococcus aureus Cell Wall Stress Stimulon Gene-lacZ Fusion Strains:
Potential for Use in Screening for Cell Wall-Active Antimicrobials

Running Title: lacZ-cell wall stress stimulon gene fusions

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lacZ fusion strains were constructed using the promoters of five cell wall stress stimulon genes: \textit{pbp2}, \textit{tcaA}, \textit{vraSR}, \textit{sgtB}, and \textit{lytR}. All fusion strains were induced only in the presence of cell wall-active antibiotics suggesting the potential of these strains for high throughput screening for new cell wall-active agents.

\textit{Staphylococcus aureus} is a medically important bacterium responsible for a number of diseases and the leading cause of both noscomial and community-acquired infections (1, 13, 14). Antibiotic resistance has developed rapidly in \textit{S. aureus}, and now methicillin-resistant strains of \textit{S. aureus} (MRSA) are encountered worldwide. Various strategies have been employed to search for new antibacterial drugs (5, 6, 7, 8, 11, 12, 17, 18, 23, 25). We propose a gene-expression assay as a strategy for screening and identification of potential cell wall-active antimicrobial agents. This approach is based on a fusion between a target gene promoter and a reporter gene (2, 3, 7, 9, 21, 24, 28, 31). In the past decade, microarray techniques have been used to pinpoint bacterial genes as potential novel targets for antibiotic discovery. Recently, several laboratories have used DNA microarray analyses to show that various genes involved in the cell wall synthesis are upregulated by cell wall-active antibiotics (15, 30, 33). Here we fused promoters of 5 genes (\textit{pbp2}, \textit{tcaA}, \textit{vraSR}, \textit{lytR}, and \textit{sgtB}) which were significantly upregulated by cell wall-active antibiotics (30), to a promoterless \textit{lacZ} genes (4) in \textit{S. aureus} SH1000 (10). To demonstrate the potential utility of these strains for drug discovery, we determined the specificity of gene induction by measuring \(\beta\)-galactosidase activity after treatment with various chemicals and incubation under different environmental conditions.
S. aureus cells were grown in tryptic soy broth/agar at 37°C with appropriate antibiotics. The promoter and lacZ gene transcriptional fusions of pbp2 (Ppbp2::lacZ), vraSR (PvraSR::lacZ), tcaA(PtcaA::lacZ), ltyR(PlytR::lacZ), and sgtB(PsgtB::lacZ) were constructed as described earlier (22, 27). The promoter fragments were subcloned upstream of a promoterless lacZ gene in the shuttle vector pAZ106 (4), and transferred into S. aureus RN4220 by electroporation (20). The lacZ fusion constructs were transferred by phage 80α-mediated transduction from S. aureus RN4220 into S. aureus SH1000 (22). Overnight cultures of the fusion strains were diluted 100-fold in TSB and grown to an OD$_{600}$ of about 0.3 at 37°C. Potential inducing agents were added to cultures and they were incubated for an additional 2 hours followed by β-galactosidase activity determination. β-galactosidase activity was measured colorimetrically using o-nitrophenyl-β-D-galactoside as the substrate (19). For potential high throughput screening we have modified assay for 96-well plates in a total reaction volume of 50 µl using MUG as substrate (29). Other molecular techniques were performed as described by Novick (20) and Sambrook and Russell (26).

To demonstrate that the expression of the lacZ was dose-dependent, all promoter::lacZ fusion strains were incubated with various concentrations of oxacillin (Fig. 1A). Overnight cultures of the Ppbp2::lacZ clone were diluted 1:100 in TSB and grown to an OD$_{600}$ of approximately 0.3. Various concentrations of oxacillin ranging from 0.075 µg/ml to 8.0 µg/ml were then added and the cultures were incubated with shaking at 37°C. Cultures were collected after 0.5, 1, and 2 h and β-galactosidase assays were performed. The induction of β-galactosidase could be seen after 0.5 hr with an oxacillin concentration as low as 0.3 µg/ml and the highest substantial pbp2 induction was seen after two hours with an oxacillin concentration of 1.2 µg/ml, which is also the MIC of oxacillin for SH1000. Similarly, dose-dependent β-galactosidase
assays were performed for all of the promoter::lacZ fusion strains with oxacillin as the inducing agent (Fig. 1B). For all strains, induction was shown with oxacillin concentrations as low as 0.3 µg/ml. Most strains exhibited approximately a 4-fold induction, with the exception of the PsgtB::lacZ strain, which exhibited a much lower basal β-galactosidase activity and a 13-fold induction (Fig. 1B). All strains also exhibited an increase in β-galactosidase expression as the oxacillin concentration was raised from 1.2µg/ml to 8 µg/ml oxacillin. Induction was seen in all fusion strains by all cell wall-active antibiotics tested, i.e., D-cycloserine, bacitracin, and vancomycin (Table 1). The largest overall induction was found using D-cycloserine as the inducing agent with the PsgtB::lacZ clone. Induction with bacitracin was modest but present in all strains, resulting in at least a two-fold induction.

To test whether the induction of promoters was specific to cell wall-active antibiotics, various classes of antibiotics, such as the translational inhibitors erythromycin, chloramphenicol, streptomycin, and tetracycline, the transcriptional inhibitor rifampin, the cell membrane permeability altering antibiotic nisin, and the inhibitor of folic acid biosynthesis trimethoprim, were added to growing cultures of the promoter::lacZ fusion strains at MIC concentrations. As shown in Table 1, no significant induction was observed. Northern blot analysis further confirmed that protein synthesis inhibitors did not result in transcription of cell wall stress stimulon genes (data not shown). These results clearly suggested that the induction of β-galactosidase in fusion strains is highly specific to cell wall-active antibiotics.

To show that the induction was not a general stress response, the effect of various environmental conditions were tested. As shown in Table 1, no induction was seen by the cell wall lytic enzymes lysozyme (5µg /ml) and lysostaphin (0.25µg /ml). Unlike cell wall-active antibiotics, which inhibit the synthesis of the cell wall, both of these agents cause enzymatic
degradation of peptidoglycan. Also, no induction was seen with Triton X-100 (1%), a mild detergent (cell membrane disrupter). Other environmental stress conditions, such as pH (5-9), temperature (25- 42 C), osmotic stress (1.5 M NaCl) and oxidative conditions (20 mM hydrogen peroxide or 50 µM paraquat) did not lead to induction (data not shown). Thus, the induction of all lacZ fusion strains was found to be unaffected by general stress conditions.

Among the clones we studied, PsgtB::lacZ showed a five- to fifteen-fold induction by cell wall-active antibiotics, with small standard deviations and a consistent basal level of β-galactosidase activity. sgtB is a member gene of the cell wall stress stimulon, under the control of the VraSR system in S. aureus (15, 16) and catalyzes peptidoglycan transglycosylase activity (32). The sgtB promoter strain may offer the best potential whole cell screen for cell wall-active agents with the advantages of screening directly in S. aureus, and avoiding compounds active against cell free targets that are inactive against whole cells (33).

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REFERENCES


Figure legend

Figure 1. A) Effect of oxacillin on β-galactosidase expression in Ppbp2::lacZ clone when incubated for 0.5, 1, and 2 h. Analysis of the β-galactosidase expression from SH1000 Ppbp2::lacZ fusion strains in response to oxacillin. Overnight cultures of Ppbp2::lacZ were grown to an OD$_{600}$ of 0.3, various concentrations of oxacillin were added, and incubated with shaking for 0.5 (■), 1 (□) and 2 h (▲). Cells were harvested and β-galactosidase activities were determined. Error bars represent the standard deviations of triplicate experiments.

B) Effect of oxacillin on β-galactosidase expression in all lacZ fusion clones. Analysis of the β-galactosidase expression from SH1000 promoter::lacZ fusion strains in response to oxacillin. Overnight cultures of Ppbp2::lacZ (□), PvrasR::lacZ (■), PtcaA::lacZ (▲), PlytR::lacZ (■) and PsgtB::lacZ (▲) fusion reporter strains were incubated with various concentrations of oxacillin for 2h. After antibiotic treatment, cells were harvested and β-galactosidase activities were determined as described in material and methods. Error bars represent the standard deviations of triplicate experiments.
Table 1. Effect of various inducing agents on the expression of β-galactosidase in lacZ fusion constructs.

<table>
<thead>
<tr>
<th>Inducing agents (concentration)</th>
<th>MIC µg/ml</th>
<th>Fold induction of β-galactosidase*</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Pphp2::lacZ</td>
</tr>
<tr>
<td>Oxacillin (1.2µg/ml)</td>
<td>1</td>
<td>3.67</td>
</tr>
<tr>
<td>D-cycloserine (150µg/ml)</td>
<td>1</td>
<td>3.41</td>
</tr>
<tr>
<td>Bacitracin (13.4µg/ml)</td>
<td>2</td>
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</tr>
<tr>
<td>Vancomycin (4µg/ml)</td>
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<td>2.69</td>
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<tr>
<td>Erythromycin (2.5µg/ml)</td>
<td>2</td>
<td>1.15</td>
</tr>
<tr>
<td>Chloramphenicol (3µg/ml)</td>
<td>0.5</td>
<td>0.94</td>
</tr>
<tr>
<td>Tetracycline (2µg/ml)</td>
<td>0.5</td>
<td>0.52</td>
</tr>
<tr>
<td>Streptomycin (3µg/ml)</td>
<td>1.5</td>
<td>0.30</td>
</tr>
<tr>
<td>Rifampin (0.05µg/ml)</td>
<td>0.05</td>
<td>1.90</td>
</tr>
<tr>
<td>Nisin (1µg/ml)</td>
<td>ND</td>
<td>1.02</td>
</tr>
<tr>
<td>Trimethoprim (1µg/ml)</td>
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<td>0.88</td>
</tr>
<tr>
<td>Lysozyme (5µg/ml)</td>
<td>ND</td>
<td>1.02</td>
</tr>
<tr>
<td>Lysostaphin (0.25µg/ml)</td>
<td>ND</td>
<td>0.65</td>
</tr>
<tr>
<td>Triton-X 100 (1% )</td>
<td>ND</td>
<td>0.52</td>
</tr>
</tbody>
</table>

*Represent the average ratio of β-galactosidase activity (treated versus untreated cultures) for three independent experiments. Values less than one indicate no induction of the promoter. ND, not determined.
Figure 1.