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Inhibition of *Staphylococcus aureus* Growth on Tellurite-Containing Media by *Lactobacillus reuteri* Is Dependent on CytuC and Thiol Production

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*Lactobacillus reuteri* inhibits *Staphylococcus aureus* growth on Baird-Parker agar. This activity required the presence of tellurite and was not shared with other lactic acid bacteria or an *L. reuteri* mutant defective in cysteine metabolism. Secreted products generated from *L. reuteri* cysteine metabolism and thiols were shown to augment tellurite toxicity.

Tellurite (TeO$_2^{2-}$)-containing Baird-Parker agar (BPA) is used as a selective and differential medium for the isolation and enumeration of *Staphylococcus aureus* from foods (2). Tellurite is also used in media for the selection of pathogens, including *Corynebacterium diphtheriae*, *Vibrio cholerae*, *Shigella* spp., and *Escherichia coli* O157 (14). In previous work, *Lactobacillus reuteri* BR11 (formerly *Lactobacillus fermentum* BR11) was engineered to express the *S. aureus*-killing bacteriocin lysostaphin (18). *L. reuteri* and *S. aureus* coculture experiments were performed to investigate the killing effect of secreted lysostaphin (18). In the course of that study, a result was obtained that was not fully understood. It was found, using the methods described in reference 18, that when an undiluted aliquot of an overnight coculture in MRS (buffered with potassium phosphate, pH 7) containing a non-lysostaphin-expressing *L. reuteri* BR11 (9 × 10$^8$ CFU/ml) and *S. aureus* (9 × 10$^8$ CFU/ml) was plated onto BPA, no *S. aureus* grew. Here we report the investigation of the basis of this phenomenon.

*L. reuteri* (BR11 and ATCC 55730) but not other lactic acid bacteria inhibit the growth of *S. aureus* and *Listeria monocytogenes* on tellurite-containing media. The first questions addressed in this study were whether the *L. reuteri*-mediated *S. aureus* growth inhibition or killing is occurring in the coculture or on BPA and the extent of the dependence of the phenomenon on the number and species of *Lactobacillus* cells. All strains used are shown in Table 1, and all growth media and growth additives were obtained from Oxoid (Basingstoke, United Kingdom). Culture conditions were as described previously (18) except for *L. reuteri* BR11 (Mlp-His$_6$-CFTR) and *L. reuteri* PNG201, which were grown in MRS containing 10 µg/ml erythromycin at 40°C overnight and then diluted and grown to log phase at 37°C. Dilution of the *L. reuteri*-*S. aureus* coculture by 100-fold or greater resulted in *S. aureus* growth on BPA, indicating that *S. aureus* is viable in the coculture (data not shown). Growth inhibition was found to take place on BPA, since adding aliquots of separately grown and washed *L. reuteri* BR11 and *S. aureus* ATCC 49476 resulted in a lack of *S. aureus* growth (Fig. 1). Inhibition of *S. aureus* growth on BPA was less effective, with reduced numbers of *L. reuteri* BR11 cells, and did not occur when *L. reuteri* BR11 was replaced with *Lactobacillus plantarum*, *Lactococcus lactis*, *Lactobacillus delbrueckii*, or *Lactobacillus casei* (Fig. 1). Another *L. reuteri* strain (ATCC 55730) was also found to significantly inhibit *S. aureus* growth (Fig. 1). Growth inhibition caused by *L. reuteri* BR11 was found to be entirely dependent upon the presence of tellurite, since omission of this compound from BPA resulted in a confluent lawn of *S. aureus* growth (*L. reuteri* BR11 is unable to grow on BPA with or without tellurite). *L. reuteri* BR11 was able to significantly inhibit *S. aureus* growth on other growth media supplemented with tellurite, including *Luria-Bertani* agar (containing 0.2 mM tellurite) and brain heart infusion (BHI) agar (containing 0.4 mM tellurite). *L. reuteri* BR11 was also found to inhibit other *S. aureus* strains (ATCC 35556 and SS21c) and *L. monocytogenes* on tellurite-containing medium (Fig. 2).

Lactic acid bacteria can produce antimicrobial substances, including bacteriocins, lactic acid, and hydrogen peroxide. It is likely that the inhibitor is not lactic acid, since inhibition of *S. aureus* growth by *L. reuteri* BR11 also occurred on BHI agar containing tellurite that was buffered with 0.2 M potassium phosphate (pH 7). *L. reuteri* BR11 produces hydrogen peroxide in the presence of oxygen but not under anaerobic conditions (8). However, hydrogen peroxide is not likely to be the inhibitor, since *S. aureus* growth was inhibited by *L. reuteri* BR11 in both the presence and absence of oxygen and on medium containing catalase (500 U/ml; from bovine liver [Sigma-Aldrich]). Therefore, our model at this point indicated that *L. reuteri* secretes a substance that potentiates tellurite toxicity.

An *L. reuteri* cysteine uptake-deficient mutant does not inhibit *S. aureus* growth on tellurite-containing media. In the case of *S. aureus*, it is known that cysteine metabolism is important for tellurite resistance (12). It has also been shown that tellurium compounds interact with thiols (1), and tellurite decreases the level of reduced thiols in *E. coli* and *Pseudomonas* spp. (15, 20). It was hypothesized that *L. reuteri* BR11 inhibits the growth of *S. aureus* on tellurite-containing media by modifying the extracellular thiol or disulfide levels. *L. reuteri* and

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close relatives possess a unique operon whose products are devoted to the uptake and conversion of the disulfide amino acid cystine (9). One of the products of this operon is a very abundant high-affinity cystine binding protein, CyuC, which is essential for cystine uptake (9, 17, 19). A mutant (L. reuteri PNG201) defective in CyuC expression is unable to import cystine or generate extracellular thiol from cystine (19).

It was found that L. reuteri PNG201 is unable to inhibit S. aureus or L. monocytogenes growth on tellurite-containing medium (Fig. 2). This suggests that the tellurite-mediated S. aureus growth-inhibiting activity of L. reuteri BR11 is either due to the depletion of cystine in the growth medium or the production of a compound derived from cystine. To test this, we investigated whether cystine potentiates or lessens the S. aureus-inhibitory activity of L. reuteri. L. reuteri cells were washed and resuspended in potassium phosphate magnesium (KPM) buffer (19), and 1.5 ml of a suspension with an optical density (OD) of ~1.5 was incubated with 0.4 mM cystine and 21 mM glucose at 37°C for 75 min. Five microliters of this suspension was spotted onto filter paper disks on BPA inoculated with S. aureus (Fig. 3). L. reuteri BR11 cells, but not L. reuteri PNG201 (CyuC mutant) cells, incubated with cystine plus glucose inhibited growth of S. aureus on tellurite-containing medium (Fig. 3). Also, L. reuteri ATCC 55730, L. plantarum, and L. rhamnosus cells that were incubated with cystine and glucose were able to inhibit growth of S. aureus (Fig. 3). L. lactis and L. delbrueckii cells incubated with cystine and glucose had no significant S. aureus-inhibiting activity (Fig. 3). These experiments suggest that products of Lactobacillus cystine metabolism heighten the toxicity of tellurite to S. aureus.

Thiols inhibit the growth of S. aureus and L. monocytogenes in the presence of tellurite. We hypothesized that cystine-derived thiol(s) heightens the toxicity of tellurite. To test this, the products secreted by L. reuteri BR11 (Mlp-His6-CFTR) and L. reuteri PNG201 (CyuC mutant) during incubation with cystine and glucose (as described above) were obtained following removal of the cells by centrifugation. Under these conditions, the only difference between the strains should be the amount of thiols secreted. One hundred microliters of the supernatant (either filtered or not filtered) was spotted onto disks on BPA inoculated with S. aureus (Fig. 4). The products secreted by L. reuteri BR11 (Mlp-His6-CFTR) but not L. reuteri PNG201 (CyuC mutant) were found to inhibit S. aureus growth in the presence of tellurite (Fig. 4). Cystine is a likely substrate for the L. reuteri BR11 cystathionine γ-lyase (which is encoded in the same operon as cyuC [17]), and the predicted products of such a reaction would be thioctypeine, pyruvate, and ammonia (4). Thiocysteine is unstable and can spontaneously degrade to form cysteine and elemental sulfur (7) and can react nonenzymatically with thiols to generate H₂S. Previously, H₂S was found to be produced by L. reuteri BR11 cells when incu-

### TABLE 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Details/Source or reference</th>
</tr>
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<tbody>
<tr>
<td>L. reuteri strains</td>
<td></td>
</tr>
<tr>
<td>BR11</td>
<td>Guinea pig vaginal isolate (formerly L. fermentum BR11) 13</td>
</tr>
<tr>
<td>PNG201</td>
<td>L. reuteri BR11 mutant defective in CyuC expression and cystine uptake ATCC 4797 19</td>
</tr>
<tr>
<td>BR11 (Mlp-His6-CFTR)</td>
<td>L. reuteri BR11 containing pRS233 (Mlp-His6-CFTR) plasmid integrated in the mlp locus (not affected in cystine metabolism) 16</td>
</tr>
<tr>
<td>ATCC 55730</td>
<td>Probiotic strain isolated from commercial tablet (Forbiotic) Blackmores Australia Ltd.</td>
</tr>
<tr>
<td>L. lactis MG1363</td>
<td>Plasmid-free L. lactis subsp. cremoris 6</td>
</tr>
<tr>
<td>L. rhamnosus GG</td>
<td>Commercial probiotic strain ATCC 53103</td>
</tr>
<tr>
<td>L. plantarum ATCC 14917</td>
<td>Natural cabbage fermentation isolate</td>
</tr>
<tr>
<td>L. delbrueckii ATCC 4797</td>
<td>L. delbrueckii subsp. lactis DSM 20076</td>
</tr>
<tr>
<td>S. aureus strains</td>
<td></td>
</tr>
<tr>
<td>ATCC 49476</td>
<td>Methicillin-resistant S. aureus Graeme Nimmo*</td>
</tr>
<tr>
<td>SS21c</td>
<td>Methicillin-sensitive S. aureus clinical isolate Graeme Nimmo*</td>
</tr>
<tr>
<td>ATCC 35556</td>
<td>Methicillin-sensitive S. aureus Graeme Nimmo*</td>
</tr>
<tr>
<td>L. monocytogenes ATCC 19112</td>
<td>Serovar 1/2c ATCC</td>
</tr>
</tbody>
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* Queensl Land Pathology Services, Microbiology Department, Princess Alexandra Hospital, Brisbane, Australia.
bated with cystine and glucose (8). Here, a significant quantity of cysteine was found to be secreted by *L. reuteri* BR11, but not *L. reuteri* PNG201 (CyuC mutant), during incubation with cystine and glucose (data not shown) as determined using the cysteine-specific acid ninhydrin assay (5). Therefore, at least two different thiols (H2S and cysteine) are secreted by *L. reuteri* BR11 following incubation with cystine and glucose, and it was concluded that either or both of these increase the toxicity of tellurite.

Next, we determined if specific thiols can inhibit the growth of *S. aureus* on tellurite-containing medium. Cysteine and di-thiothreitol, but not reduced glutathione, were found to inhibit the growth of *S. aureus* on BHI medium containing 0.4 mM tellurite but did not inhibit growth on BHI alone (Fig. 5). To quantify thiol concentrations needed for this inhibitory effect, growth of *S. aureus* and *L. monocytogenes* in liquid BHI containing 0.4 mM tellurite and various concentrations of cysteine was investigated (Table 2). Ten microliters of exponential-phase-grown *S. aureus* cells (OD at 600 nm [OD600], ~0.01) or *L. monocytogenes* (OD600 ~0.8) was inoculated into 5 ml liquid medium, incubated overnight at 37°C without shaking, and then examined for growth. Concentrations of cysteine between 1 mM and 0.25 mM prevented growth of both *S. aureus* and *L. monocytogenes* in the presence of tellurite. Addition of higher concentrations of cysteine (5 mM and 10 mM) to BHI containing tellurite caused a rapid reduction of tellurite to black elemental tellurium. *S. aureus* and *L. monocytogenes* growth occurred under these high concentrations of cysteine (Table 2).

The results presented here clearly demonstrate that thiols, including those produced by cystine metabolism of *L. reuteri*, can augment the toxicity of tellurite. This was unexpected, since it was predicted that thiol-mediated reduction of tellurite would lead to detoxification. This was the case with higher thiol levels, which caused the abiotic production of nontoxic black elemental tellurium. However, in the presence of lower levels of thiols, the toxicity of tellurite was increased. This finding has important implications for the development of new strategies to control *S. aureus* infections, particularly in environments where tellurite is used for biocontrol.
of thiols, little or no abiotic production of tellurium occurred, and tellurite toxicity was increased. It is possible that the increase is due to \( S.\ aureus \) having heightened thiol levels, which result in an increase in reactive oxygen species generated during intracellular tellurite reduction. It has recently been shown that intracellular tellurite reduction causes production of reactive oxygen species and that this is linked to its mechanism of toxicity (15). Selenite toxicity appears to have a similar basis (10). Glutathione- and thioredoxin-deficient mutants of \( E.\ coli \) or \( Salmonella \) are hyper-resistant to selenite, which suggests that the intracellular interaction of selenite with thiol results in toxic oxidative by-products (3, 11). Also, selenite mutagenicity is the result of an intracellular but not an extracellular reaction with thiols, possibly due to proximity with DNA (11).

The results of the current study have clear implications in the development and application of tellurite-containing selective media for the isolation of pathogens and provide insight into the role of thiols in tellurite detoxification and toxicity.

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**TABLE 2. Growth of \( S.\ aureus\) ATCC 49476 and \( L.\ monocytogenes\) in BHI containing 0.4 mM potassium tellurite and various concentrations of supplemented cysteine**

<table>
<thead>
<tr>
<th>Species</th>
<th>Growth* with supplemented cysteine at (mM):</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>( S.\ aureus )</td>
<td>+</td>
</tr>
<tr>
<td>( L.\ monocytogenes )</td>
<td>+</td>
</tr>
</tbody>
</table>

* +, growth; -, no growth.


