

Inhibition of *Staphylococcus aureus* Growth on Tellurite-Containing Media by *Lactobacillus reuteri* Is Dependent on CyuC 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 cystine metabolism. Secreted products generated from *L. reuteri* cystine metabolism and thiols were shown to augment tellurite toxicity.**

Tellurite (TeO_3^{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 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-His₆-CFTR) and *L. reuteri* PNG201, which were grown in MRS containing 10 $\mu\text{g/ml}$ 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 rhamnosus* (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* cystine 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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TABLE 1. Strains used in this study

Strain	Details	Source or reference
<i>L. reuteri</i> strains		
BR11	Guinea pig vaginal isolate (formerly <i>L. fermentum</i> BR11)	13
PNG201	<i>L. reuteri</i> BR11 mutant defective in CyuC expression and cystine uptake (formerly <i>L. fermentum</i> PNG201)	19
BR11 (Mlp-His ₆ -CFTR)	<i>L. reuteri</i> BR11 containing pJRS233 (Mlp-His ₆ -CFTR) plasmid integrated in the <i>mlp</i> locus (not affected in cystine metabolism)	16
ATCC 55730	Probiotic strain isolated from commercial tablet (Forbiotic)	Blackmores Australia Ltd.
<i>L. lactis</i> MG1363	Plasmid-free <i>L. lactis</i> subsp. <i>cremoris</i>	6
<i>L. rhamnosus</i> GG	Commercial probiotic strain	ATCC 53103
<i>L. plantarum</i> ATCC 14917	Natural cabbage fermentation isolate	
<i>L. delbrueckii</i> ATCC 4797	<i>L. delbrueckii</i> subsp. <i>lactis</i> DSM 20076	DSMZ
<i>S. aureus</i> strains		
ATCC 49476	Methicillin-resistant <i>S. aureus</i>	Graeme Nimmo ^a
SS21c	Methicillin-sensitive <i>S. aureus</i> clinical isolate	Graeme Nimmo ^a
ATCC 35556	Methicillin-sensitive <i>S. aureus</i>	
<i>L. monocytogenes</i> ATCC 19112	Serovar 1/2c	ATCC

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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 thiocysteine, 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-

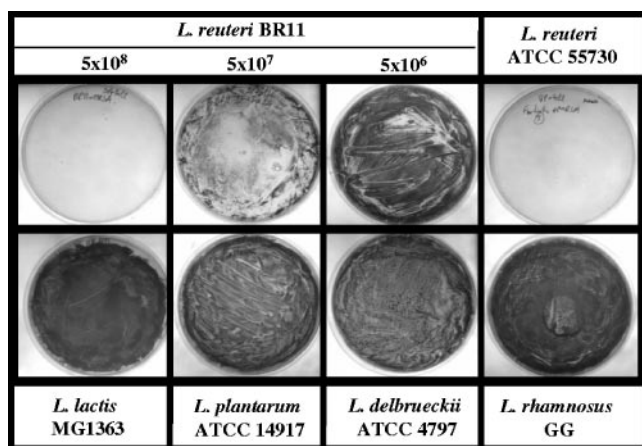


FIG. 1. Inhibition of growth of *S. aureus* on BPA in the presence of *L. reuteri*. Exponential-phase *S. aureus* ATCC 49476 (6×10^6 CFU) was plated onto BPA (containing 0.4 mM tellurite) along with washed exponential-phase *L. reuteri* BR11 (CFU indicated), *L. reuteri* ATCC 55730 (4×10^8 CFU), *L. lactis* MG1363 (5×10^9 CFU), *L. plantarum* ATCC 14917 (2×10^9 CFU), *L. delbrueckii* ATCC 4797 (2×10^8 CFU), or *L. rhamnosus* GG (1×10^9 CFU). Following 24 h of incubation, growth of *S. aureus* is seen as a black confluent lawn. The lactic acid bacteria were unable to grow on this medium (data not shown).

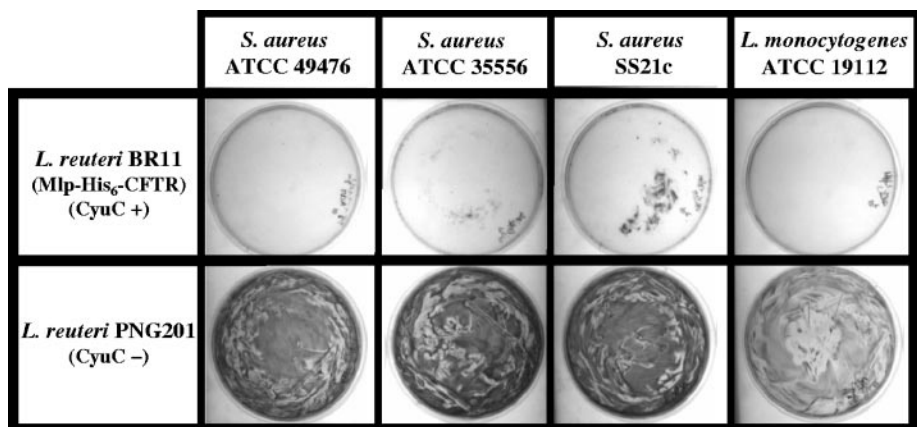


FIG. 2. The *cyuC* mutant of *L. reuteri* is unable to significantly inhibit *S. aureus* growth on BPA or *L. monocytogenes* growth on BHI containing tellurite. Exponential-phase *S. aureus* ATCC 49476 (7×10^6 CFU), *S. aureus* ATCC 35556 (3×10^6 CFU), and *S. aureus* SS21c (1×10^7 CFU) were plated onto BPA (containing 0.4 mM tellurite), while *L. monocytogenes* (5×10^6 CFU) was plated onto BHI (containing 0.2 mM tellurite). Also plated were washed exponential-phase *L. reuteri* BR11 (Mlp-His₆-CFTR; 3×10^8 CFU) or *L. reuteri* PNG201 (2×10^8 CFU) cells. Following 24 h of incubation, growth of *S. aureus* strains and *L. monocytogenes* is seen as black confluent lawns.

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 dithiothreitol, 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 cysteine 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

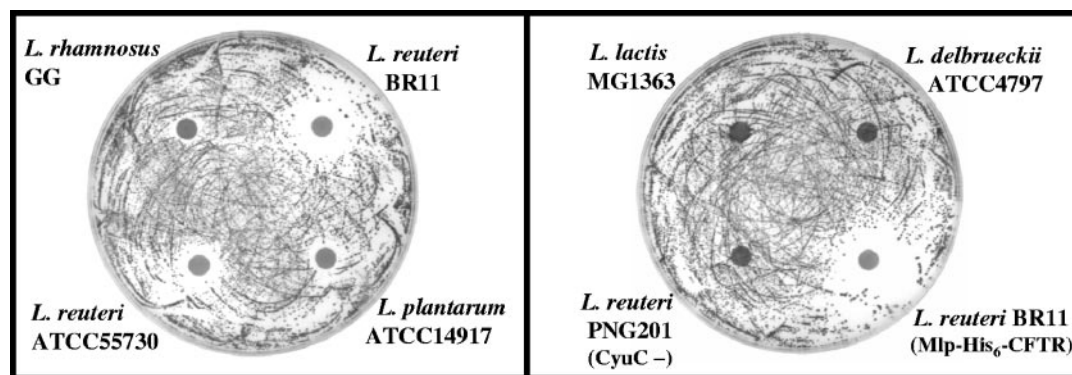


FIG. 3. Inhibition of *S. aureus* growth on BPA by lactic acid bacterial cells incubated with cystine and glucose. Following incubation of lactic acid bacteria with cystine and glucose, 5 μ l of this cell suspension was spotted onto filter paper disks (6-mm diameter) on top of BPA plates containing 0.4 mM tellurite that had been inoculated with *S. aureus* ATCC 49476 (9×10^4 CFU). Plates were incubated for 48 h. Significant *S. aureus* growth-inhibitory activity is seen for disks containing *L. reuteri* BR11, *L. reuteri* ATCC 55730, *L. reuteri* BR11 (Mlp-His₆-CFTR), *L. plantarum*, and *L. rhamnosus*, while no or little inhibition is seen for disks containing *L. lactis*, *L. delbrueckii*, and *L. reuteri* PNG201. No inhibition of growth of *S. aureus* was seen on BPA plates that did not contain tellurite.

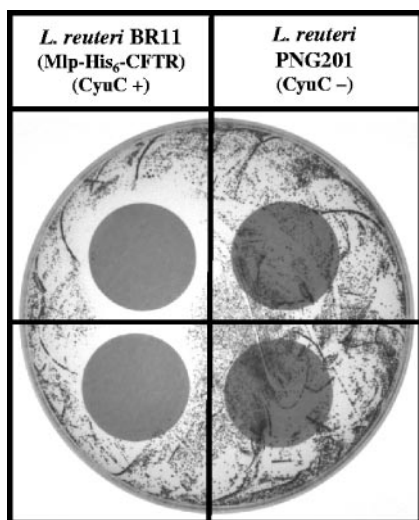


FIG. 4. The cystine-derived products secreted by *L. reuteri* BR11 (Mlp-His₆-CFTR), but not *L. reuteri* PNG201, inhibit *S. aureus* growth on BPA. *L. reuteri* strains were incubated with cystine and glucose, and the products from this reaction were obtained following centrifugation of the cells. Portions of the supernatant (100 μ l) were spotted before (top quadrants) or after (bottom quadrants) filtering through a 0.2- μ m syringe filter onto filter paper disks (24-mm diameter) on a BPA plate (containing 0.4 mM tellurite) inoculated with *S. aureus* ATCC 49476 (9×10^4 CFU). The plate was incubated at 37°C for 24 h. *S. aureus* growth-inhibitory activity is seen from the secreted products from *L. reuteri* BR11 (Mlp-His₆-CFTR) by the lack of colonies underneath and near the disks on the left side of the plate. No inhibition of growth of *S. aureus* was seen on BPA plates that did not contain tellurite.

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 dur-

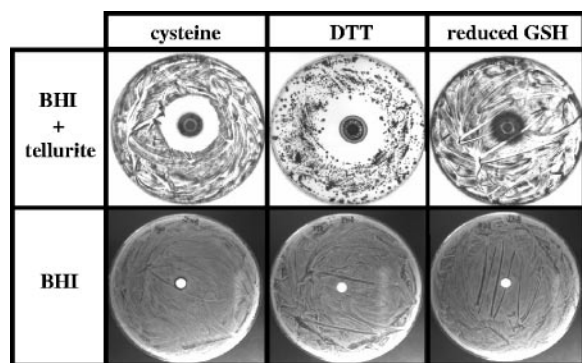


FIG. 5. Effects of various thiol compounds on growth of *S. aureus* in the presence of tellurite. Ten microliters of 0.45 M cysteine, dithiothreitol (DTT) or reduced glutathione (GSH) was spotted onto filter paper disks (6-mm diameter) placed on BHI agar containing 0.4 mM tellurite or BHI agar that had been inoculated with *S. aureus* ATCC 49476 ($\sim 1 \times 10^6$ CFU). Plates were incubated for 48 h at 37°C. The dark ring around the filter paper disks is likely due to tellurium formation, and within these black zones are some *S. aureus* colonies. Significant *S. aureus* growth inhibition is seen with cysteine and DTT, but not with reduced GSH on BHI containing tellurite. Little or no *S. aureus* growth inhibition is seen with the thiols on BHI without tellurite. DTT reduced the *S. aureus* lawn into discrete colonies on the BHI plate containing tellurite, even though the same number of *S. aureus* CFU was plated.

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

Species	Growth ^a with supplemented cysteine at (mM):									
	0	0.031	0.0625	0.125	0.25	0.5	1	5	10	
<i>S. aureus</i>	+	+	+	+	-	-	-	+	+	
<i>L. monocytogenes</i>	+	+	+	+	-	-	-	+	+	

^a +, growth; -, no growth.

ing 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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