Inhibition of expression of a staphylococcal superantigen-like protein by a soluble factor from *Lactobacillus reuteri*

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Lactobacillus reuteri RC-14 has previously been shown to inhibit *Staphylococcus aureus* infection in a rat surgical-implant model. To investigate the basis for this, communication events between the two bacterial species were examined. *L. reuteri* RC-14 and *Staph. aureus* Newman were grown in a co-culture apparatus that physically separates the two species, while allowing the passage of soluble compounds. Using two-dimensional gel electrophoresis (2D-E), protein expression changes in *Staph. aureus* were analysed in response to co-culture with medium alone, *L. reuteri* RC-14, and a *Lactobacillus* strain that did not inhibit *Staph. aureus* infection in the rat model. It was observed that one protein in particular, identified as staphylococcal superantigen-like protein 11 (SSL11), showed a dramatic decrease in expression in response to growth with *L. reuteri* RC-14. Genetic reporters that placed both *gfp* and *lux* under the transcriptional control of the SSL11 promoter confirmed the 2D-E results. Interestingly, using similar reporter gene experiments, it was observed that the *Staph. aureus* P3 promoter from the staphylococcal accessory gene regulator (*agr*) locus also showed a decrease in expression in response to growth in the presence of *L. reuteri* RC-14. It was further demonstrated that *L. reuteri* RC-14 supernatant contained small unidentified molecules that were able to repress the SSL11 and P3 promoters, but the repression of SSL11 occurred independently of the *agr* system. These results suggest that *L. reuteri* RC-14 has the potential to alter the virulence of *Staph. aureus* via secretion of cell–cell signalling molecules.

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INTRODUCTION

Bacteria utilize chemical communication systems to control gene expression within populations (Waters & Bassler, 2005). Although most research has focused on communication within a single species, there has been increasing interest towards understanding interspecies bacterial communication in order to understand cell–cell communication in natural environments, and to identify novel ways to attenuate undesirable bacteria (Visick & Fuqua, 2005). The term ‘quorum sensing’ (Fuqua *et al.*, 1994) is used to

describe a communication system employed by many bacteria to co-ordinately change gene expression in response to microbial density and the environment. Gram-negative bacteria characteristically use acylhomoserine lactones (AHLs) for their communication systems, and these are generally able to diffuse through the cell membrane, and interact directly with their intracellular target proteins when present in sufficient concentrations (Bassler, 1999). Gram-positive bacteria typically employ small post-translationally modified peptides for inter- and intraspecies communication (Dunny & Leonard, 1997). These communication peptides are usually processed from larger propeptides, and most interact with two-component histidine kinase receptors to initiate an intracellular phosphorylation cascade. In addition, many bacteria produce the autoinducer-2 (AI-2) furanosyl borate ester signal, which is encoded by the *luxS* gene (Surette *et al.*, 1999). The *luxS* gene is found in approximately 50 % of sequenced bacterial genomes, and it may represent a conserved interspecies communication signal (Waters & Bassler, 2005).

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Abbreviations: *agr*, accessory gene regulator; AHL, acyl-homoserine lactone; AI-2, autoinducer-2; AIP, autoinducing peptide; ESI-MS, electrospray ionization mass spectrometry; 2-DE, two-dimensional gel electrophoresis; GFP, green fluorescent protein; HRP, horseradish peroxidase; SSL11, staphylococcal superantigen-like protein 11; RLU, relative light unit; ROT, repressor of toxins.

It is evident that there are additional mechanisms for interspecies communication in bacteria. For example, McKenney *et al.* (1995) demonstrated that the addition of concentrated cell-free culture supernatant from *Pseudomonas aeruginosa* increased the production of quorum-sensing-regulated virulence factors in *Burkholderia cepacia*, and this was attributed to AHLs in the added supernatant. The host microbiota have also been shown to have the ability to upregulate genes involved in the virulence of *P. aeruginosa*, via interspecies communication of AI-2 (Duan *et al.*, 2003). In addition to this, a recently characterized communication system between *Streptococcus gordonii* and *Veillonella atypica*, which are both colonizing members of early dental plaque, was shown to result in increased expression of α -amylase from *Strep. gordonii*, and when grown in an open system, the signal worked only when the bacterial species were in close proximity (Egland *et al.*, 2004). *Staphylococcus aureus* has been shown to produce a peptide in a cell-density-independent manner, termed *staph*-cAM373, and this may act as a sex pheromone for certain *Enterococcus faecalis* strains (Muscholl-Silberhorn *et al.*, 1997). The pAM373 plasmid-specific peptide may function to enhance gene transfer from *Ent. faecalis* to *Staph. aureus*, and this is a major concern for the transfer of antibiotic-resistance genes. Alternatively, interference with bacterial cell-cell communication has been termed 'quorum quenching' (Waters & Bassler, 2005); an example of this has been demonstrated in which *Bacillus subtilis* produces an AHL-lactonase called AiiA, which hydrolyses the lactone ring of AHLs, and destroys their function (Dong *et al.*, 2000, 2001). Expression of the *Bacillus anthracis aiiA* gene in *Burkholderia thailandensis* has been shown to inhibit the accumulation of the quorum-sensing molecules *N*-decanoylhomoserine lactone and *N*-octanoylhomoserine lactone, and alter motility in *B. thailandensis* (Ulrich, 2004).

Staph. aureus is a common hospital and community-acquired pathogen that causes infections ranging from relatively uncomplicated skin infections, such as boils and impetigo, to more severe and life-threatening deep tissue infections, including endocarditis and osteomyelitis. One well-characterized quorum-sensing system in *Staph. aureus* is the accessory gene regulator (*agr*) (Recsei *et al.*, 1986; Yarwood & Schlievert, 2003). The *agr* locus includes an operon (*agrBDCA*) encoding an autoinducing prepeptide (AgrD), which is exported and processed by AgrB to become the active autoinducing peptide (AIP), and this is sensed by a two-component system (AgrC/A). At high cell density, AIP accumulates, and results in activation of the *agr* locus. The effector molecule of the *agr* system is an RNA molecule termed RNAIII, which is under control of the P3 promoter, and is transcribed divergently from the *agrBDCA* operon (Novick *et al.*, 1993). Expression of RNAIII results in the up-regulation of many secreted proteins, and the simultaneous downregulation of many cell-surface-expressed factors (Dunman *et al.*, 2001; Recsei *et al.*, 1986), by a mechanism that has yet to be characterized. The ability of *Staph. aureus* to cause human disease of such a range of severity is

consistent with its ability to express a large number of surface and secreted virulence factors (Kuroda *et al.*, 2001). The emergence of highly resistant strains of *Staph. aureus*, including those resistant to last-resort antibiotics (Anonymous, 2002a, b), has led to an increased interest in identifying novel antimicrobial therapies to combat this pathogen (Ji *et al.*, 1997; Wright *et al.*, 2005).

The Gram-positive bacterium *Lactobacillus reuteri* RC-14 (formerly *Lactobacillus fermentum*) (Reid *et al.*, 2005) has previously been shown to inhibit *Staph. aureus* infection in a rat surgical-implant model (Gan *et al.*, 2002). To investigate this phenomenon, we hypothesized that the inhibition may result from specific communication events between the two bacterial species. To address this, we analysed staphylococcal protein expression changes when *Staph. aureus* was grown in the presence and absence of *L. reuteri* RC-14. We show that the expression of staphylococcal superantigen-like protein 11 (SSL11) is dramatically decreased in response to growth with *L. reuteri* RC-14. Furthermore, using gene-reporter systems, we show that *L. reuteri* RC-14 supernatant contains small molecules that are able to repress both the SSL11 and P3 promoters, with repression of SSL11 occurring independently of the staphylococcal *agr* system.

METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown in Luria-Bertani broth (Difco), *Staph. aureus* was grown in brain heart infusion (BHI) broth (Difco), and lactobacilli were grown in BHI medium or Man-Rogosa-Sharpe (MRS) medium (Merck Frosst Canada), as required. Solid media were obtained by the addition of 1.5% (w/v) Bacto agar (Difco). All strains were grown at 37 °C. For plasmid selection, chloramphenicol was used at 10 µg ml⁻¹ for *Staph. aureus* and *E. coli*, and ampicillin and kanamycin were used at 100 µg ml⁻¹ and 50 µg ml⁻¹, respectively, for *E. coli*. Mannitol salt agar (Difco) and Rogosa agar (Difco) were used as selective media for *Staph. aureus* and lactobacilli strains, respectively. To assay for α - and β -haemolysis, *Staph. aureus* was plated on sheep blood agar (Becton Dickinson). All reagents were made with water purified using a Milli-Q water-purification system (Millipore).

Preparation, manipulation and analysis of DNA. Standard DNA manipulations were performed as described (Sambrook & Russell, 2001), using enzymes supplied from New England Biolabs, in accordance with the manufacturer's instructions. Oligonucleotides were obtained from Invitrogen, and are described in Table 1. PCRs were performed in a Peltier Thermocycler (MJ Research), in 50 µl reaction volumes, with PFX DNA polymerase (Invitrogen). PCR products were purified using the QIAquick PCR purification kit (Qiagen). DNA sequencing was performed at the Sequencing Facility, Robarts Research Institute, London, Canada. Plasmids were introduced into *Staph. aureus* RN4220 using bacteriophage 80 α , as described (Sebulsky *et al.*, 2000), before they were transduced into other *Staph. aureus* strains.

Co-culture experiments. Co-culture experiments were performed using a two-chamber device containing two 30 ml glass compartments separated by a 0.45 µm mixed cellulose ester membrane (Millipore). Co-cultures were grown in BHI at 37 °C, with slow shaking (~30 r.p.m.) to improve diffusion of small molecules between the compartments. *L. reuteri* RC-14 or *Lactobacillus rhamnosus* GR-1 (5%, v/v,

Table 1. Bacterial strains, plasmids and oligonucleotides used in this study

Strain, plasmid or primer	Description	Source or reference
Strains		
<i>Staph. aureus</i>		
Newman	Wild-type	Dajcs <i>et al.</i> (2002)
RN4220	Restriction-deficient derivative of 8325-4; accepts foreign DNA, ($r_k^- m_k^+$)	Kreiswirth <i>et al.</i> (1983)
RN6390	Prophage-cured wild-type strain	Novick <i>et al.</i> (1993)
RN6911	<i>agr</i> -null mutant of RN6390 containing a 3 kb fragment with Tc ^r marker in place of the 3.4 kb <i>agr</i> <i>Clal</i> – <i>Hin</i> CII fragment	Peng <i>et al.</i> (1988)
MSSA476	Invasive community-acquired methicillin-sensitive strain	Holden <i>et al.</i> (2004)
<i>spa</i> -515	Protein-A-deficient derivative of 8325-4	Patel <i>et al.</i> (1989)
<i>E. coli</i>		
DH5 α	F [–] ϕ 80 Δ lacZ Δ M15 Δ (<i>lacZYA</i> – <i>argF</i>) <i>U169 endA1 recA1 hsdR17</i> ($r_k^- m_k^+$) <i>deoR</i>	Gibco-BRL
<i>Lactobacillus</i> species		
<i>L. reuteri</i> RC-14	Isolated from the urogenital tract of a healthy woman	G. Reid
<i>L. rhamnosus</i> GR-1	Isolated from the urogenital tract of a healthy woman	G. Reid
Plasmids		
pSB2034	P3 promoter controlling expression of both <i>luxABCDE</i> (from <i>Photorhabdus luminescens</i>) and translationally enhanced <i>gfp3</i> (red-shifted <i>gfp</i> variant); Cm ^r , Ap ^r	Qazi <i>et al.</i> (2001)
pJLED1	SSL11 promoter amplified by PCR from <i>Staph. aureus</i> Newman inserted into <i>Eco</i> RI- and <i>Xma</i> I-digested pSB2034; Cm ^r , Ap ^r	This study
Primers*		
P _{SSL11} :: <i>gfp/lux</i> (forward)	CACCGAATTCTAACTTTGATAAATACATAG	This study
P _{SSL11} :: <i>gfp/lux</i> (reverse)	CATCAACCCCGGGATTCTATGCTCCCAATT	This study
<i>ssl11</i> (forward)	CACTCAACATATGAGTACATTAGAGGTTAGATC	This study
<i>ssl11</i> (reverse)	CACCTCTGAGGCTCCCTCGAATAATTTTA	This study

*Restriction sites for subsequent cloning of the PCR products are underlined.

inoculum) were inoculated first, and grown to an OD₆₀₀ of 0.15 (~5 h), before inoculation of *Staph. aureus* Newman (1%, v/v, inoculum) in the second compartment. After overnight growth, co-cultures were examined using selective agar media to ensure that cross-contamination had not occurred.

Extraction of *Staph. aureus* cell-wall-associated proteins, and two-dimensional gel electrophoresis. Cell-surface-associated proteins were extracted from *Staph. aureus* Newman, essentially as described (Hermann *et al.*, 2000). Specifically, 30 ml cultures were harvested (5000 g, 10 min, 4 °C) at late-exponential phase, and subsequently washed in 50 mM Tris/HCl, pH 7.5. The final pellet was resuspended in 2 ml Sarcosyl buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl₂, and 2% w/v, *N*-lauroyl-sarcosine), and incubated on ice for 20 min. The cell wall suspensions were then centrifuged (10 000 g, 10 min, 4 °C), and the supernatant was recovered, aliquoted and stored at –80 °C prior to analysis. For two-dimensional gel electrophoresis (2D-E), the Sarcosyl-extracted proteins were precipitated with the Perfect Focus Kit (Geno Technology), following the manufacturer's specifications. Precipitated proteins were resuspended in 200 μ l rehydration buffer [9 M urea, 4% (w/v) CHAPS, 0.5% (v/v) Biolytes (3–10; Bio-Rad Laboratories)] and 20 mM dithiothreitol, and left at room temperature for 1 h, with occasional mixing. Insoluble material was removed by centrifugation (16 000 g, 1 h, 15 °C). The concentration of each protein sample was then determined by a modified Bradford procedure (Bio-Rad Laboratories). Aliquots of the solubilized proteins (6 μ g for analytical gels, and up to 200 μ g for preparative gels) were applied to immobilized pH gradient (IPG) strips (7 cm, pH

range 4–7; Bio-Rad Laboratories), which were rehydrated overnight at 50 V in an IEF cell (Bio-Rad Laboratories). The following day, first-dimension IEF was carried out with increasing voltage [200 V for 100 Volt-hours (Vh), 500 V for 250 Vh, 1000 V for 500 Vh, and 8000 V for 8000 Vh], and then the strips were immersed in an equilibrium buffer containing 6 M urea, 2% (w/v) SDS, 50 mM Tris/HCl (pH 8.8), 30% (v/v) glycerol, and 65 mM dithiothreitol. After 15 min, the strips were placed in the equilibrium buffer, which contained 135 mM iodoacetamide instead of dithiothreitol, for a further 15 min. The second-dimension SDS-PAGE was performed using the Mini-Protean III electrophoresis unit (Bio-Rad Laboratories). Stacking gels and separating gels were used at 4 and 10%, respectively. Following electrophoresis, the analytical gels were stained with SYPRO Ruby (Bio-Rad Laboratories), and preparative gels were stained with Coomassie blue R-250. Gel images were captured using an AlphaInnotech camera, and the 2D-E protein profiles were analysed using Phoretix-2D (version 5.1) software (Non-linear Dynamics). Relative volumes were estimated by calculating the ratio of the volume of a spot to the volume of the spots from the entire gel. Results are the means of at least three independent experiments. In experiments that compared two conditions, proteins were considered to be induced or repressed if the mean relative volume for an individual protein was at least twofold higher or lower than that for the control.

Identification of proteins of interest. Peptide mass fingerprints were obtained using facilities provided by The Biological Mass Spectrometry Laboratory at the Dr Don Rix Protein Identification Facility at The University of Western Ontario (London, Canada).

The proteins were prepared according to the protocol provided by the facility. Briefly, the excised gel pieces were washed and dried in acetonitrile, and the proteins were subjected to reduction/alkylation by using dithiothreitol (10 mM) and iodoacetamide (55 mM). After several washing steps in 100 mM ammonium bicarbonate, and dehydration in acetonitrile, a solution of trypsin (15 ng μl^{-1}) was added to each gel piece, and the digestions were performed overnight at 37 °C. The digested fragments were then recovered with a solution of acetonitrile:formic acid (50:5, v/v), and dried under vacuum. The dried peptides were stored at -80 °C until needed, and, as required, the peptide mixture was diluted 1:1 with α -cyano-4-hydroxycinnamic acid. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of the samples was performed using a Reflex III (Bruker), operating in a linear positive-ion mode with the N_2 laser, to obtain MS fingerprints and sequence tags. A Mascott Database search was then performed to identify proteins based on their pI, molecular mass and mass fingerprint (only protein databases were searched). The peptide sequence data (Table 2) were also used as query sequences in BLASTX searches of the complete *Staph. aureus* genomes. The ORFs within the contigs retrieved by the BLAST search were identified, and their theoretical tryptic peptide fingerprints were determined via the ExPASy web site (<http://www.expasy.ch>), and compared with the peptide mass fingerprints obtained by MALDI-TOF MS analysis of the proteins. For confirmation, the peptide sequence data were also used as query sequences in BLASTP searches of all complete *Staph. aureus* genomes.

Generation of anti-SSL11 antisera. The *ssl11* gene, lacking the DNA encoding the N-terminal signal peptide, was PCR amplified using primers *ssl11* (forward) and *ssl11* (reverse), and cloned into the multiple cloning site of pET28a. SSL11 was expressed and purified from *E. coli* BL21 (λ DE3), transformed with pET28::*ssl11*, using Ni^{2+} affinity chromatography, as specified by the manufacturer (Qiagen). The N-terminal His₆ tag was removed by cleavage with 1 unit thrombin (Sigma) per milligram of recombinant protein (16 h at 21 °C). The recombinant protein was purified from the His₆ tag and thrombin by anion-exchange chromatography using a DEAE column pre-equilibrated in 20 mM Tris/HCl buffer, pH 7.4 (Bio-Rad Laboratories). Polyclonal antisera to His₆-SSL11 were generated in a New Zealand white rabbit at the Animal Care and Veterinary Services facility at The University of Western Ontario.

Preparation of *L. reuteri* RC-14 supernatant. Cell-free supernatants were prepared by growing *L. reuteri* RC-14 in BHI to an OD₆₀₀ of 0.4–0.6, and then removing the cells by centrifugation (5000 g, 10 min, 4 °C). The remaining cell-free supernatant was filtered using 0.45 μm pore-size filters, checked for sterility by plating an aliquot on MRS agar, and assayed as described below. To rule out effects from hydrogen peroxide or pH, the supernatant was treated with catalase (1000 U ml^{-1} ; Sigma), or adjusted to neutral pH with 5 M NaOH. To examine the heat stability of the compound, the supernatant was boiled for 30 min, and the volume was adjusted to the original volume by the addition of sterile water. To examine the role of lactic acid, BHI medium was treated with lactic acid to a pH of 2 or 7 (at concentrations of 20 mM, 50 mM or 100 mM), and, to rule out nutrient limitation in the BHI medium, a 1:175 dilution of 200-fold concentrated *L. reuteri* RC-14 supernatant was added to fresh BHI medium. The supernatant was also treated with proteases (0.5 mg ml^{-1} Pronase, proteinase K or trypsin, or a combination of all three enzymes; Sigma) for 12 h at 55 °C.

Creation of the *gfp/lux* gene reporter construct. Plasmid pSB2034 is an *E. coli*/Gram-positive bacterial shuttle vector that contains both *gfp* and *lux* under the transcriptional control of the staphylococcal P3 promoter (Qazi *et al.*, 2001). The P_{SSL11}::*gfp/lux* fusion was constructed by PCR amplification of a 385 bp DNA fragment from *Staph. aureus* Newman, corresponding to the untranslated 5' end of

Table 2. Characteristics of proteins identified by 2D-E

Spot	Fold-change	Protein (<i>Staph. aureus</i> Mu50) (accession no.)	Sequence tag	Mass (measured/theoretical) (kDa)	pI (measured/ theoretical)	Percentage coverage
1	-5.6	SSL11 (NP_370957)	SGNTASIGGITK	24/22.3	5.9/6.0	64
2	-2.1	Cysteine synthase (NP_371037)	TIDAFLAGVGTGGTSLGVGK	34/33	5.4/5.5	21.29
3	+2.1	Superoxide dismutase (NP_372077)	LNAAVEGTDLESKEIEIVANL DSV PANIQTA VR	25/22.7	5.1/5.2	17.08
4	-2.6	Hypothetical protein (NP_372378)	LTLQVVSIDEQ GK	24/22.3	5.4/5.3	16.58
5	+2.1	30S ribosomal protein (NP_371780)	AGQFYINQR	34/29.1	5.6/5.4	7.84
6	+8.6	Formyltetrahydrofolate synthetase (NP_372256)	IVTEIYG GSK	60/59.8	5.8/5.7	4.8
7	-2.4	ABC transporter (ATP-binding protein) (NP_371366)	YLN EGFSGG EK	28/29.1	4.8/4.7	7.9

the *ssl11* gene (bases -1 to -385), using primers P_{SSL11}::*gfp/lux* (forward), and P_{SSL11}::*gfp/lux* (reverse). The PCR product was cloned as an *EcoRI*-*XmaI* fragment into the unique *EcoRI/XmaI* sites of pSB2034. The resulting plasmid created a transcriptional fusion (pJLED1), and was confirmed to contain the SSL11 promoter region directly upstream of the vector-borne *lux/gfp* by sequencing the promoter region and the insertion sites. Both pJLED1 and pSB2034 were recovered from *E. coli*, and introduced into *Staph. aureus* Newman as described above.

Green fluorescent protein (GFP) and Lux expression analysis.

For quantification of bioluminescence in the absence of exogenous aldehyde, overnight cultures of *Staph. aureus* Newman, harbouring either pSB2034 or pJLED1, were diluted 1:50 in medium containing the necessary antibiotics. Samples of each condition were prepared in triplicate, and loaded into a 96-well microtitre plate (320 µl), and incubated at 37 °C, without shaking, in a Luminoskan luminometer (Thermo). OD₆₀₀ and luminescence were measured every hour over a 48 h period, and results were calculated as relative light units (RLUs). The data were normalized by taking the maximum RLU detected, and dividing by the corresponding maximum c.f.u. value for each condition. GFP was detected using fluorescence spectroscopy (Olympus BX-61 light microscope with a FITC filter), and fluorescent images were analysed using the Pro-Image Plus program version 5.0.1 (Media Cybernetics). All media used to grow *Staph. aureus* Newman in the promoter expression analysis were diluted 1:1 in Milli-Q water. For repression of an active SSL11 promoter, a 1:150 dilution of concentrated RC-14 supernatant (in Milli-Q water) was added to a culture of *Staph. aureus* Newman that had been growing in BHI medium for 26 h, and, at the same time point, an equivalent volume of Milli-Q water was added to *Staph. aureus* Newman growing in BHI medium and *Staph. aureus* Newman growing in *L. reuteri* RC-14 supernatant.

Screening for cell-cell signalling compounds. *L. reuteri* RC-14 cell-free supernatant was lyophilized, and resuspended in 100% methanol (1:20 of the original supernatant volume). Centrifugation (5000 g, 10 min, 4 °C), followed by passage through Whatman No. 1 filter paper and a 0.45 µm pore-size filter, removed insoluble material. Rotary evaporation was used to concentrate the methanol-soluble material to 1/100 of the original volume of the culture supernatant. An aliquot (400 µl) of the concentrated supernatant was passed across a P-10 gel-filtration column (Bio-Rad Laboratories), and 2 ml fractions were collected. The fractions testing positive for biological activity in the luminescence 96-well plate assay were dried, resuspended in water, and further purified by HPLC on an Agilent 1100 HPLC, using a Zorbax 300SB-C8 (4.6 × 250 mm, 5 µm diameter) column (Agilent Technologies). Solvent A consisted of trifluoroacetic acid (0.1%) in water, and solvent B consisted of trifluoroacetic acid (0.085%) in acetonitrile. All solvents used were HPLC grade (Fischer Scientific). The chromatographic method used was as follows (flow rate 1.5 ml min⁻¹): 2% solvent B for 2 min, followed by a gradient of 2–80% solvent B over 30 min, and a final step of 80% solvent B for 2 min. Fractions were collected off the HPLC C8 column, and tested in the luminescence 96-well plate assay. In order to determine whether the repression of both the SSL11 promoter and the staphylococcal P3 promoter was dependent on the same compound from the *L. reuteri* RC-14 supernatant, we performed the 96-well luminescence assay with *Staph. aureus* Newman containing either pJLED1 or pSB2034, grown in BHI medium, *L. reuteri* RC-14 supernatant, and an HPLC fraction that had previously tested positive for the ability to repress the SSL11 promoter. Isolated HPLC fractions were further evaluated by electrospray ionization mass spectrometry (ESI-MS) and MS/MS analysis in the Q_{TOF} instrument (Micromass), in positive-ion mode at a cone voltage of 20 V. For controls, MS analysis was performed

on the two antecedent fractions, and the fraction collected directly after the active fraction (these fractions were shown to be biologically inactive).

Statistical analysis. Statistical comparisons between independent means were made by Student's *t* test, assuming unequal variances.

RESULTS

2D-E of the *Staph. aureus* Newman cell-wall-associated proteins produced in response to growth with *L. reuteri* RC-14

The goal of this study was to evaluate cell-cell communication events between *Staph. aureus* and *L. reuteri* RC-14, in order to elucidate the mechanism by which *L. reuteri* RC-14 inhibits *Staph. aureus* infection (Gan *et al.*, 2002). We initially analysed protein expression patterns from *Staph. aureus* in response to growth with *L. reuteri* RC-14. Since we initially reasoned that the inhibition may be due to an alteration in the adhesive properties of *Staph. aureus*, we focused on a subproteome enriched for surface proteins of *Staph. aureus*. Controls for the experiment included growth of *Staph. aureus* in medium alone, and growth with *L. rhamnosus* GR-1, a strain previously shown to lack the ability to inhibit *Staph. aureus* infection in the rat surgical-implant model (Gan *et al.*, 2002). The protein expression profiles of the cell-wall-associated preparations analysed by 2D-E were very similar for all the gels. The total number of spots detected varied between 118 and 130, and the number of spots per gel for a given condition remained constant. Specifically, when *Staph. aureus* Newman was co-cultured with BHI, *L. reuteri* RC-14 and *L. rhamnosus* GR-1, the gels had 122, 130 and 118 detectable spots, respectively (Fig. 1). In each of these co-culture conditions, there were no significant differences detected between the OD₆₀₀ values of *Staph. aureus* Newman when samples were taken during the late-exponential phase (results not shown). Each condition was repeated a minimum of three times, and the gels shown in Fig. 1 are from a representative experiment.

We were interested in staphylococcal proteins that either decreased or increased in expression in response to co-culture with *L. reuteri* RC-14, and, based on this, we selected seven of the spots in the preparative gels for further analysis (labelled in Fig. 1A, B). The identifications of the proteins are listed in Table 2, and the genes encoding each of the seven proteins were found in all complete *Staph. aureus* genomes published to date (Baba *et al.*, 2002; Gill *et al.*, 2005; Holden *et al.*, 2004; Kuroda *et al.*, 2001). We noted that expression of protein 1, annotated as SSL11, decreased considerably when co-cultured with *L. reuteri* RC-14. SSL11 belongs to a larger group of genetically clustered proteins known as the staphylococcal superantigen-like proteins (SSL proteins) (Lina *et al.*, 2004), which are proposed to be important virulence factors in *Staph. aureus* (Al-Shangiti *et al.*, 2004; Arcus *et al.*, 2002; Fitzgerald *et al.*, 2003; Langley *et al.*, 2005).

Fig. 1. 2D-E analysis of cell-surface-associated proteins expressed by *Staph. aureus* Newman when co-cultured with BHI medium (A), *L. reuteri* RC-14 (B), and *L. rhamnosus* GR-1 (C), as described in Methods. Spots were analysed using Phoretix 2D software, and only those spots with at least a two-fold change were analysed. Relative to growth with RC-14, proteins labelled in (A) showed decreased expression, and proteins labelled in (B) showed increased expression. Note that protein 1 showed a dramatic decrease in expression in response to growth with RC-14, but not in response to growth with GR-1. This protein had a pI of ~5.9, and a molecular mass of ~25 kDa, and MS analysis showed that it was homologous to a known staphylococcal superantigen-like protein. Molecular mass markers (kDa) are indicated on the right, and the pI gradient is shown on the bottom.

SSL11 is present in *Staph. aureus* supernatant, and is repressed by growth in *L. reuteri* RC-14 supernatant

To further confirm our findings, we cloned and over-expressed recombinant SSL11 from *E. coli*, and used the pure protein to raise polyclonal rabbit antisera against this protein. We then analysed the supernatant from *Staph. aureus* *spa-515*, a strain that is deficient in protein A, to prevent non-specific immunoglobulin binding. Supernatants were analysed from *Staph. aureus* *spa-515* grown to stationary phase in BHI medium, and in *L. reuteri* RC-14 supernatant. We observed a clear decrease in the expression of an immunoreactive band consistent with the predicted size of SSL11 when *Staph. aureus* was grown in the *L. reuteri* RC-14 supernatant (Fig. 2A). We also performed a Western blot experiment with extracted surface proteins from *Staph. aureus* using goat IgG conjugated to horseradish peroxidase (HRP) to detect protein A. Results from this experiment indicated no noticeable change in protein A expression when grown in the *L. reuteri* RC-14 supernatant (Fig. 2B). These experiments indicate that production of the SSL11 protein from *Staph. aureus* is markedly reduced when grown with supernatant from *L. reuteri* RC-14.

Staphylococcal SSL11 and P3 promoters are both repressed in the presence of *L. reuteri* RC-14 supernatant

Observing that a potential exotoxin decreased in expression in response to co-culture with *L. reuteri* RC-14 motivated us

Fig. 2. Western blot analysis of SSL11 and protein A expression from *Staph. aureus*. (A) Secreted proteins were separated by 12% SDS-PAGE, and SSL11 was detected using anti-SSL11 antisera. Molecular mass markers (kDa) are shown on the left. SSL11 had a predicted molecular mass of ~24 kDa (indicated by the arrowhead). *Staph. aureus* *spa-515* (a protein-A-deficient mutant) was grown to stationary phase in BHI medium (lane 1) and *L. reuteri* RC-14 supernatant (lane 2), and surface proteins were extracted as described in Methods. (B) Staphylococcal cell-wall-associated proteins were separated by 12% SDS-PAGE, and protein A was detected using goat IgG-HRP (protein A is an IgG-binding protein). *Staph. aureus* Newman was grown to stationary phase in BHI medium (lane 1) and *L. reuteri* RC-14 supernatant (lane 2). Molecular mass markers (kDa) are shown on the left. The expected molecular mass of protein A was ~54 kDa (indicated by the arrowhead).

to construct a dual-gene reporter construct to study *ssl11* gene expression, and to confirm our findings. Based on the *Staph. aureus* COL sequence (Gill *et al.*, 2005), PCR primers $P_{SSL11}::gfp/lux$ forward, and $P_{SSL11}::gfp/lux$ reverse, were used to amplify the SSL11 promoter region from *Staph. aureus* Newman. This region corresponds to a 385 bp region upstream of the *ssl11* gene, and it is immediately downstream of a putative type-IC restriction-modification system (Fig. 3). The nucleotide sequence of this region from *Staph. aureus* Newman was 100 % homologous to the same region in *Staph. aureus* strains COL (Gill *et al.*, 2005), Mu50 (Kuroda *et al.*, 2001), N315 (Kuroda *et al.*, 2001) and MRSA252 (Holden *et al.*, 2004), although, interestingly, in *Staph. aureus* strains MSSA476 (Holden *et al.*, 2004) and MW2 (Baba *et al.*, 2002), the first 79 bp of this sequence showed little or no homology to the Newman/COL sequence (Fig. 3). Despite this, we did not detect a difference in SSL11 protein expression between *Staph. aureus* Newman and *Staph. aureus* MSSA476, as detected by anti-SSL11 antibody in Western blot experiments (results not shown). The SSL11 promoter region replaced the staphylococcal P3 promoter in the Gram-positive expression vector pSB2034, thereby creating the gene reporter construct pJLED1. Since the expression of many exoproteins in *Staph. aureus* is regulated by the two-component *agr* signalling pathway (Dunman *et al.*, 2001; Recsei *et al.*, 1986), the expression of the P3 promoter (pSB2034 construct) was also monitored. P3 is one promoter of the *agr* system, whose untranslated gene product, RNAPIII, is the effector molecule of the *agr* system (Novick *et al.*, 1993). Reporter constructs pJLED1 and pSB2034 were both introduced separately into *Staph. aureus* Newman. *Staph. aureus* harbouring these gene reporter constructs was grown in two separate microtitre plates to monitor hourly luminescence and culture density. For optimum activation of both the SSL11 and P3 promoters, *Staph. aureus* was grown in BHI medium diluted 1 : 1 with Milli-Q water. As a result of this, all further media

used in promoter expression experiments were diluted 1 : 1 with Milli-Q water. The strains were grown in a 96-well plate in the presence or absence of *L. reuteri* RC-14 supernatant (Fig. 4A). The results of these experiments indicate that both P_{SSL11} and P3 are activated in the stationary phase when *Staph. aureus* Newman is grown in BHI, with a peak of luminescence detected at approximately 30 h of growth (Fig. 4A), while both P_{SSL11} and P3 are repressed when *Staph. aureus* is grown in the presence of *L. reuteri* RC-14 supernatant. The data obtained from the luciferase experiments were also confirmed by monitoring *Staph. aureus* grown in BHI, and in *L. reuteri* RC-14 supernatant, for the expression of GFP (results not shown). Importantly, growth of *Staph. aureus* was slightly inhibited when grown in *L. reuteri* RC-14 supernatant, compared with growth in BHI; this effect was probably due to a decrease in nutrients available in the spent supernatant, since *Staph. aureus* grows equally well in BHI and BHI supplemented with concentrated *L. reuteri* RC-14 supernatant (results not shown). To compensate for the decrease in growth observed for *Staph. aureus* grown in *L. reuteri* RC-14 supernatant, we measured the maximum RLU detected for each condition, and divided the result by the corresponding maximum c.f.u. under a variety of different conditions (Fig. 4B). Based upon normalized data, we determined that the SSL11 promoter remained repressed in the presence of the *L. reuteri* RC-14 supernatant, and that the observed effect was not due to a change in pH, or to the presence of lactic acid or hydrogen peroxide in the *L. reuteri* RC-14 supernatant (Fig. 4B, and results not shown). Also, we demonstrated that we could alleviate repression of the SSL11 promoter by adding decreasing amounts of *L. reuteri* RC-14 supernatant to *Staph. aureus* Newman (pJLED1) growing in BHI medium. We determined that the ability of the *L. reuteri* RC-14 supernatant to repress SSL11 promoter activation was insensitive to both protease activity (Pronase, proteinase K and trypsin, and a cocktail of all three enzymes) and heat

Fig. 3. Schematic representation of the *ssl11* locus in *Staph. aureus*. The arrows are representative of individual coding regions from *Staph. aureus* COL (Gill *et al.*, 2005). The *ssl11* gene is preceded by a 382 bp region lacking predicted ORFs that is directly downstream of SA0447, which is a gene whose product is predicted to belong to a restriction endonuclease complex. SA0479 is predicted to encode a lipoprotein of unknown function. The nucleotide sequence of the intragenic region between SA0477 and *ssl11* (nt 479807–480191 in COL) is shown, with the region cloned into pJLED1 shown in upper-case letters. The start of SSL11 is indicated with the corresponding amino acid sequence.

Fig. 4. Repression of the *ss11* and P3 promoters by *L. reuteri* RC-14. (A) RLUs detected from *Staph. aureus* Newman harbouring pJLED1 (left) and pSB2034 (right); strains were grown in BHI medium (●) and *L. reuteri* RC-14 supernatant (○). Experiments for each condition were performed in triplicate, and the results shown are from a representative experiment. (B) Maximum luminescence detected per maximum c.f.u. detected from *Staph. aureus* Newman harbouring pJLED1 (left) and pSB2034 (right). OD₆₀₀ and RLUs were measured every hour for 48 h. *Staph. aureus* harbouring either gene reporter construct was grown in BHI medium (BHI), *L. reuteri* RC-14 supernatant (SUP), decreasing concentrations of *L. reuteri* RC-14 supernatant (25 % SUP, 15 % SUP and 5 % SUP), pH-adjusted *L. reuteri* RC-14 supernatant (pH SUP), catalase-treated *L. reuteri* RC-14 supernatant (CAT SUP), BHI containing a small amount of concentrated *L. reuteri* RC-14 supernatant (CON. SUP), and *L. rhamnosus* GR-1 supernatant (GR1), in a 96-well plate. The suppression of SSL11 promoter activation by *L. reuteri* RC-14 supernatant was also insensitive to protease treatment, thermal treatment, and a lactic-acid effect (data not shown). Error bars represent the standard errors from three separate experiments done in triplicate. **P* < 0.05 versus BHI. (C) RLUs detected from *Staph. aureus* Newman harbouring pJLED1 grown over a 48 h time period. *Staph. aureus* was cultured in BHI medium (●) and *L. reuteri* RC-14 supernatant (○), and BHI medium supplemented with concentrated *L. reuteri* RC-14 supernatant at 26 h (▲). The vertical arrow denotes the time period at which concentrated supernatant was added to the BHI medium.

(Fig. 4B and results not shown). The addition of concentrated *L. reuteri* RC-14 supernatant to the BHI medium also resulted in SSL11 promoter repression, ruling out the lack of nutrients or other compounds from the BHI medium as a

cause of the repression. Of particular note, supernatant from *L. rhamnosus* GR-1 was not capable of repressing SSL11 or P3 promoter activity, consistent with the proteomic analysis (Fig. 4B).

Since *L. reuteri* RC-14 supernatant repressed transcription of the SSL11 promoter, we were interested in determining if spent culture supernatant could repress an active SSL11 promoter. To accomplish this, an aliquot of concentrated *L. reuteri* RC-14 supernatant was added to *Staph. aureus* Newman (pJLED1) that had been growing in BHI medium for 26 h. The assay was performed in triplicate, and an equivalent volume of water was added to the same strain of *Staph. aureus* growing in either BHI medium or *L. reuteri* RC-14 supernatant. We observed a repression of the SSL11 promoter within 1 h following the addition of concentrated *L. reuteri* RC-14 supernatant (Fig. 4C), indicating that it was able to repress an activated SSL11 promoter. No repression of the SSL11 promoter was observed upon addition of an equivalent volume of water to *Staph. aureus* growing in BHI.

Expression and repression of the SSL11 promoter is independent of the *agr* system in *Staph. aureus*

The expression of many virulence factors in *Staph. aureus* is controlled by *agr*, a quorum-sensing system that upregulates the expression of many secreted proteins upon entering the late-exponential phase, while simultaneously repressing the expression of many cell-wall-associated proteins. We were interested in determining if the observed decrease in *ssl11* expression was a direct consequence of the decrease in activation of the P3 promoter. In order to analyse this, pJLED1 was introduced into *Staph. aureus* RN6390, and its isogenic *agr* mutant, RN6911. SSL11 promoter activation was observed when RN6390 and RN6911 were grown in control BHI medium, and repression was observed when each strain was grown in *L. reuteri* RC-14 supernatant (Fig. 5), indicating that both expression and repression of the SSL11 promoter are independent of P3 and the *agr* regulatory circuit in *Staph. aureus*.

Isolation of the signalling molecule

In order to begin characterization of the molecule(s) mediating the repression of the SSL11 and *agr* promoters, *L. reuteri* RC-14 supernatant was methanol extracted, and subjected to size-exclusion chromatography, followed by reverse-phase HPLC. Fractions were screened for activity using *Staph. aureus* Newman harbouring pJLED1, and luminescence was used as a measure of SSL11 and *agr* promoter activity. As shown in Fig. 6, an active HPLC fraction was able to completely inhibit activation of P_{SSL11}, but it had only moderate inhibitory activity against P3. We performed ESI-MS analysis to determine which ions were present in the biologically active fractions, but were absent, or significantly decreased, in the biologically inactive fractions from adjacent HPLC fractions. Using this process, we were able to restrict our analysis to two candidate ions with *m/z* values of 851 and 437. Because the larger of the two ions appeared to be a dimer of the smaller molecular mass ion, we conducted MS/MS analysis on the larger ion with *m/z* 851, and this fragmented into the smaller 437 Da

ion. Experiments are under way to elucidate the identity of the *L. reuteri* RC-14 signalling molecule.

DISCUSSION

Data obtained from the proteomic analysis of co-culture experiments, Western blot analysis of supernatant fractions, gene reporter analysis, and purification experiments, indicate that *L. reuteri* RC-14 is able to produce one or more molecules that inhibit the expression of both SSL11, a putative staphylococcal exotoxin, and RNIII, the effector molecule of the *agr* locus. This decrease in toxin synthesis

Fig. 5. Expression and repression of the SSL11 promoter is independent of *agr*. RLU were detected from the wild-type *Staph. aureus* parent strain (RN6390) (A), and an isogenic *agr*-null mutant strain (RN6911) harbouring pJLED1 (B); strains were grown in BHI medium (●), and *L. reuteri* RC-14 supernatant (○). SSL11 promoter activation was suppressed in *L. reuteri* RC-14 supernatant in both the parent wild-type strain and the *agr* mutant strain, indicating that expression and suppression of the SSL11 promoter occur independently of P3 and the *agr* system. Repression of the SSL11 promoter was observed for a 48 h time period, and the first 20 h is shown in the figure.

Fig. 6. Differential repression of P_{SSL11} and P_3 by HPLC-fractionated *L. reuteri* RC-14 supernatant. Representative graphs of normalized RLUs detected from *Staph. aureus* Newman harbouring (A) pJLED1, and (B) pSB2034, over a 48 h time period. *Staph. aureus* Newman harbouring either gene reporter construct was grown in BHI medium (●), *L. reuteri* RC-14 supernatant (○), and BHI containing the HPLC-purified cell-cell signalling compound that was shown to be able to repress the $SSL11$ promoter in the 96-well luminescence assay (▲). Datasets for each gene reporter construct were normalized individually, such that for each time point, the RLU detected is shown as a percentage of the maximum RLU detected from *Staph. aureus* harbouring either gene reporter construct grown in BHI. The maximum RLU value detected from *Staph. aureus* (pSB2034) grown in BHI medium was 0.0092, while the maximum RLU value detected from *Staph. aureus* (pJLED1) grown in BHI medium was 0.0515.

may explain, at least in part, the attenuated ability of *Staph. aureus* to cause infection in the rat surgical-implant model (Gan *et al.*, 2002). Since *L. rhamnosus* GR-1 was unable to repress these promoters, this provides additional indirect evidence that these putative communication events may have contributed to the decreased pathogenesis of *Staph. aureus* in the rat model. While several researchers have shown that inhibiting RNAIII expression through the use of peptides that inhibit RNAIII transcription *in vitro* inhibits

Staph. aureus infection *in vivo* (Balaban *et al.*, 1998, 2000; Mayville *et al.*, 1999; Wright *et al.*, 2005), others have shown that, depending on the model, expression of RNAIII *in vivo* may not be required for virulence (Yarwood *et al.*, 2002). We were somewhat surprised to observe the repression of the staphylococcal P_3 promoter, since we did not detect a corresponding increase in multiple cell-surface-expressed factors in the 2D-E experiments. Because the surface extracts were analysed at the late-exponential phase, it is possible that RNAIII was not yet fully active. Also, it is not clear whether other global regulators have also been affected, and these could override some of the *agr*-dependent factors. For example, the repressor of toxins (ROT) has been shown to at least partially restore regulation in an *agr* mutant, although mutation of ROT has not been reported to significantly affect the expression of *ssl11* (Said-Salim *et al.*, 2003). Although regulation of protein A expression is complex, the unaltered expression of protein A in the presence of *L. reuteri* RC-14 supernatant supports our findings (Fig. 2).

Our data indicate that the addition of concentrated *L. reuteri* RC-14 supernatant to a *Staph. aureus* culture in stationary phase can repress an activated $SSL11$ promoter within 1 h (Fig. 3C), implying that this is a specific regulation event. In the reporter experiments, we further showed that the inhibition was independent of the luciferase system, since GFP from pJLED1/pSB2034 was also inhibited. By subjecting *Staph. aureus* Newman harbouring pSB2034 to the potential communication signal from RC-14 supernatant, we demonstrated that an active HPLC fraction capable of completely inhibiting P_{SSL11} had only moderate inhibitory activity on P_3 (Fig. 6). This result implies that the inhibition of P_3 may be mediated by an independent signalling factor, or, alternatively, that the P_3 promoter may be much less sensitive to repression by this compound. Although it is well known that many staphylococcal virulence factors are regulated by *agr*, our results indicate that $SSL11$ is expressed and repressed independently of the *agr* locus (Fig. 5). This is not uncommon, since other exotoxins, including staphylococcal enterotoxin A and staphylococcal enterotoxin J (SEA and SEJ), have also been shown to be expressed independently of *agr* (Tremaine *et al.*, 1993; Zhang *et al.*, 1998). As a consequence, therapeutic agents that decrease toxin production in *Staph. aureus* via interference with *agr* are likely to have no effect on the expression of $SSL11$. It will be of interest to determine if other SSL proteins are also regulated independently of the *agr* locus.

Despite the clear repression of $SSL11$ by *L. reuteri* RC-14, the role of $SSL11$ in staphylococcal virulence is unclear. The cluster of staphylococcal SSL proteins was first identified by Williams *et al.* (2000), and these proteins (formerly known as the staphylococcal enterotoxin-like proteins, or SET proteins) (Arcus *et al.*, 2002; Fitzgerald *et al.*, 2003; Williams *et al.*, 2000) share the C-terminal β -grasp domain, and the N-terminal β -barrel domain, which are characteristic of known superantigens, yet they appear to have lost the low-affinity MHC class II binding (Al-Shangiti *et al.*, 2004; Arcus

et al., 2002). Although SSL1 (formerly Set1) has been shown to induce proinflammatory cytokines from peripheral blood mononuclear cells (Williams *et al.*, 2000), none of the recombinant SSLs examined to date has exhibited key properties expected of superantigens, including MHC class II binding or T-cell activation (Arcus *et al.*, 2002; Langley *et al.*, 2005; Williams *et al.*, 2000). We determined that recombinant SSL11 from *Staph. aureus* Newman lacks the ability to proliferate T cells (unpublished data), and this provides further evidence that the lack of superantigen activity is not a result of inactive allelic variants present in a select group of strains. However, SSL7 was recently shown to bind to IgA and complement C5 from human serum, inhibit the ability of IgA to bind to its receptor, and block complement-mediated killing of bacterial cells (Langley *et al.*, 2005); none of the other SSLs tested shared this activity, and those authors suggested that the SSL proteins have distinct and possibly non-redundant functions. We were able to detect SSL11 in *Staph. aureus* supernatant by Western blot (Fig. 2), and, because SSL11 contains a typical 30 aa signal sequence, it is clear that it is a secreted protein. SSL11 was originally detected as a *Staph. aureus* cell-wall-associated protein, possibly due to its high level of expression, which may have resulted in some protein remaining associated with the cell surface. Contradictory to previous reports examining *Staph. aureus* COL (Fitzgerald *et al.*, 2003), we found that SSL11 was expressed by *Staph. aureus* Newman. This difference could be attributed to differences in the regulatory circuits of *Staph. aureus* Newman and COL strains, since *Staph. aureus* Newman is a clinical methicillin-sensitive isolate (Dajcs *et al.*, 2002), while *Staph. aureus* COL is an early methicillin-resistant *Staph. aureus* (MRSA) isolate (Shafer & Iandolo, 1979). Furthermore, recombinant SSL11 reacted with all five convalescent human sera samples from patients with previous *Staph. aureus* infections (unpublished data), consistent with previous reports (Arcus *et al.*, 2002; Fitzgerald *et al.*, 2003), although some SSL proteins have been shown to be non-immunoreactive with such sera (Fitzgerald *et al.*, 2003).

The potential communication system that we have uncovered may have evolved as a result of the bacteria sharing similar niches within the host microbiota, since *L. reuteri* RC-14 is a vaginal isolate from a healthy woman (Gardiner *et al.*, 2002), and *Staph. aureus* is commonly found on the mucosa of the vagina. A bacterial species able to detect and respond to competitors in a microenvironment would have the advantage of being able to alter its gene expression appropriately (Federle & Bassler, 2003). This potential communication system may actually represent a quorum-quenching mechanism, and whether or not it is broadly conserved, or highly species-specific, remains to be determined. It is interesting to speculate that the compound responsible for the repression of P3 could be similar to an *agr* AIP, since at least four *Staph. aureus* *agr* groups exist, such that an AIP produced from one group inhibits *agr* expression in other groups (Novick, 2003). Indeed, *Lactobacillus plantarum* produces a compound that is similar to

Staph. aureus AIPs; this compound is produced by the *lam* operon, which shows similarities to the *agr* system in *Staph. aureus* (Sturme *et al.*, 2005). Unfortunately, the *L. reuteri* genome sequence is currently not available, and we were unable to detect homologues of either the *agr* or *lam* operons in other sequenced lactobacilli. We were, moreover, unable to inactivate the *L. reuteri* RC-14 compound using various protease treatments, leading to the conclusion that this compound may not be a simple peptide. We do not believe that AI-2 is the key mediator in the response we have described, given that *Staph. aureus* is a producer of AI-2 (Schauder & Bassler, 2001; Sun *et al.*, 2004), and it is unlikely that *Staph. aureus* would respond differently to a similar compound also produced by *L. reuteri* RC-14. Alternatively, different bacteria may produce different forms of the AI-2 signalling molecule (Miller *et al.*, 2004), and this is thought to be dependent upon the environment in which 4,5-dihydroxy-2,3-pentanedione, the precursor to AI-2, is produced (Chen *et al.*, 2002). It is possible that an altered form of AI-2 acts as an inhibitor.

Confirmation of a bona fide signalling cascade controlling the expression of SSL11 that is inhibited by a compound produced in *L. reuteri* RC-14 supernatant will require the identification of the putative signalling molecule(s), and this will also be necessary to evaluate the role of this compound in interfering with the virulence of *Staph. aureus*. The research reported in this study is an important step in the quest to identify novel antimicrobial agents that inhibit *Staph. aureus* infection, and it also provides insight into natural inhibitors of *Staph. aureus* infection. The research will also serve to further characterize communication signals produced from non-pathogenic commensal bacteria that may alter the expression of virulence factors in pathogenic bacteria, and it will also aid in further understanding interspecies cell-cell communication.

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