CONCISE COMMUNICATION

Lactobacillus fermentum RC-14 Inhibits *Staphylococcus aureus* Infection of Surgical Implants in Rats

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***Staphylococcus aureus* is a common cause of community and hospital-acquired infections. Moreover, the clinical impact of *S. aureus* is on the rise because of the global increase in the incidence of multidrug-resistant strains and its growing prevalence as a major cause of surgical infections. As a result, there is a pressing need to identify new antistaphylococcal agents and preventative strategies that will help in the management of these types of infections. This report describes the successful use of a probiotic, *Lactobacillus fermentum* RC-14, and its secreted biosurfactant to inhibit surgical implant infections caused by *S. aureus*. *L. fermentum* RC-14 and its secreted biosurfactant both significantly inhibited *S. aureus* infection and bacteria adherence to surgical implants.**

Staphylococcus aureus is a major human pathogen that can cause a variety of infections, ranging from minor skin abscesses to more serious, potentially life-threatening infections, such as bone and soft tissue surgical infections, sepsis, and invasive endocarditis [1]. Unfortunately, treatment of these severe infections has become increasingly difficult because of the emergence of antibiotic-resistant strains of *S. aureus* [2]. As a result, researchers are devoting more time developing alternative and more effective antistaphylococcal agents. Some recent laboratory successes include AgrD analogues that can inhibit the global expression of virulence factors [3] and vaccines that target in vivo–expressed bacterial antigens [4].

In a radical departure from established treatment schemes, we hypothesized that specific probiotic strains of lactobacilli with known anti-infective properties [5] could inhibit *S. aureus*–induced surgical wound infection. Here, by use of a rat model of surgical implant infection, we sought to determine whether *Lactobacillus fermentum* RC-14 and its secreted biosurfactant (BSF) inhibit surgical implant infection caused by *S. aureus* and implant biofilms.

Received 24 July 2001; revised 5 November 2001; electronically published 5 April 2002.

Presented in part: annual meeting of the Wound Healing Society, Toronto, 2000; American Society of Microbiology, Biofilms 2000, Big Sky, Montana, 16–20 July 2000 (abstract 141); 46th annual meeting of the Plastic Surgery Research Council, Milwaukee, Wisconsin, 25 June 2001 (abstract 78); 40th annual meeting of the American Society for Cell Biology, San Francisco, 9–13 December 2000 (abstract 2035).

Canadian Council of Animal Care guidelines were followed.

Financial support: St. Joseph's Health Care (SJHC) Pooled Research Trust Fund, SJHC Imperial Oil Fund for Geriatric Medicine, and Plastic Surgery Education Fund (research grant) (to B.S.G.); National Science and Engineering Research Council (to G.R.); Canadian Institutes of Health Research (to J.C.H.).

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The Journal of Infectious Diseases 2002;185:1369–72

0022-1899/2002/18509-0024$02.00

Methods

Bacterial cultures. *S. aureus* (Oxford strain) was cultured in brain-heart infusion (BHI) broth (Oxoid) overnight at 37°C and on BHI agar plates. *L. fermentum* RC-14 and *Lactobacillus rhamnosus* GR-1 were cultured in Man-Rogosa-Sharpe (MRS) broth (Merck) and on MRS agar plates.

BSF production. BSF was collected from strains GR-1 and RC-14, as described elsewhere [6], and filter sterilized.

Animal housing. Eight-week-old (300 g) male Sprague-Dawley rats (Charles River) were housed in shoebox plastic cages in Lawson Health Research Institute animal facilities.

Animal surgery. Rats were anesthetized by intraperitoneal injection of a solution containing ketamine (100 mg/mL) and xylazine (10 mg/mL) (mixed 2:1, respectively) at a dose of 0.1 mL/100 g body weight. Each rat was clipped of dorsal hair and swabbed with a povi-iodine antiseptic solution. A single 2-cm dorsal incision was made to create a small subcutaneous pocket. A small (1 cm^2) sterile piece of silicone (SILK; Degania Silicone) was inserted into the subcutaneous compartment and inoculated with a 100-μL PBS solution containing bacteria (lactobacillus and/or *S. aureus*) and/or 100 μL of a filtered-sterilized PBS solution of BSF at the indicated colony-forming unit or protein concentrations, respectively. For the BSF experiments, implants were incubated overnight at 4°C in the BSF solutions. Surgical incisions were then sutured. A postoperative analgesic (buprenorphine hydrochloride, 0.01 mg/kg) was administered when needed. On day 3, the animals were killed with CO_2, and the surgical sites were reentered for visual assessment of the presence or absence of a subcutaneous abscess. This was used as a gross measure of surgical implant infection.

Animal experiments that optimized the challenge dose of *S. aureus* (9 animals/group) were given 5×10^5, 2.5×10^6, 5×10^6, $7.5 \times$

10^6, 10^7, or 5×10^7 cfu of *S. aureus*. The 50% (~7×10^6 cfu, ID_{50}) and 100% (~5×10^7 cfus, ID_{100}) infectious doses were used for further studies. One experiment that examined the effect of live lactobacilli on *S. aureus* infection required 9 animals per group. In total, 108 animals were inoculated with either *S. aureus* ID_{50} alone or coinoculated with 10^6, 10^7, 10^8, 10^9, or 10^{10} cfu of GR-1 or RC-14. An additional experiment was done for imaging implant bacteria by fluorescent microscopy. For this study, 6 animals in each group were inoculated with either *S. aureus* ID_{100} alone or coinoculated with *S. aureus* ID_{100} and either 10^{10} cfu of GR-1 or RC-14. Finally, for each of the 3 (observer blinded) BSF experiments, 6 animals per group were inoculated with *S. aureus* ID_{100} and either 100 μg of RC-14 BSF or control GR-1 BSF (total, 18 animals/group).

Colony-forming unit assay. We scored surgical implants for cfu activity. In brief, implants were washed in PBS, placed in a fresh PBS solution, sonicated for 30 s, and vortexed for 1 min. Gram staining of the implants confirmed that all of the bacteria were removed from the implant. Various PBS dilutions were then cultured on either MRS (lactobacilli) or BHI *(S. aureus)* agar plates and incubated at 37°C under anaerobic (5% CO_2) or aerobic conditions, respectively. Numbers of colony-forming units were scored the next day *(S. aureus)* or after 2 or 3 days of incubation for the lactobacillus strains.

Polymerase chain reaction (PCR) identification of implant colony-forming unit. Single colony-forming units were picked and genomic DNA prepared as described elsewhere [7]. DNA was then added to a reaction mixture containing 0.5 μM primers (randomly amplified polymorphic DNA [RAPD], 5′-ACGAGGCAC-3′ and 5′-ACGCGCC CT-3′ [8]), 0.3 m*M* dNTPs, 2 m*M* $MgCl_2$, and 1 U of platinum Taq polymerase (Gibco-BRL) in a final volume of 50 μL. PCR thermal cycling parameters included a denaturation cycle (94°C, 2 min), followed by 40 thermal cycles (94°C, 1 min; 35°C, 2 min; and 68°C, 2 min) and a final extension cycle (68°C, 10 min). The PCR products were separated by gel electrophoresis

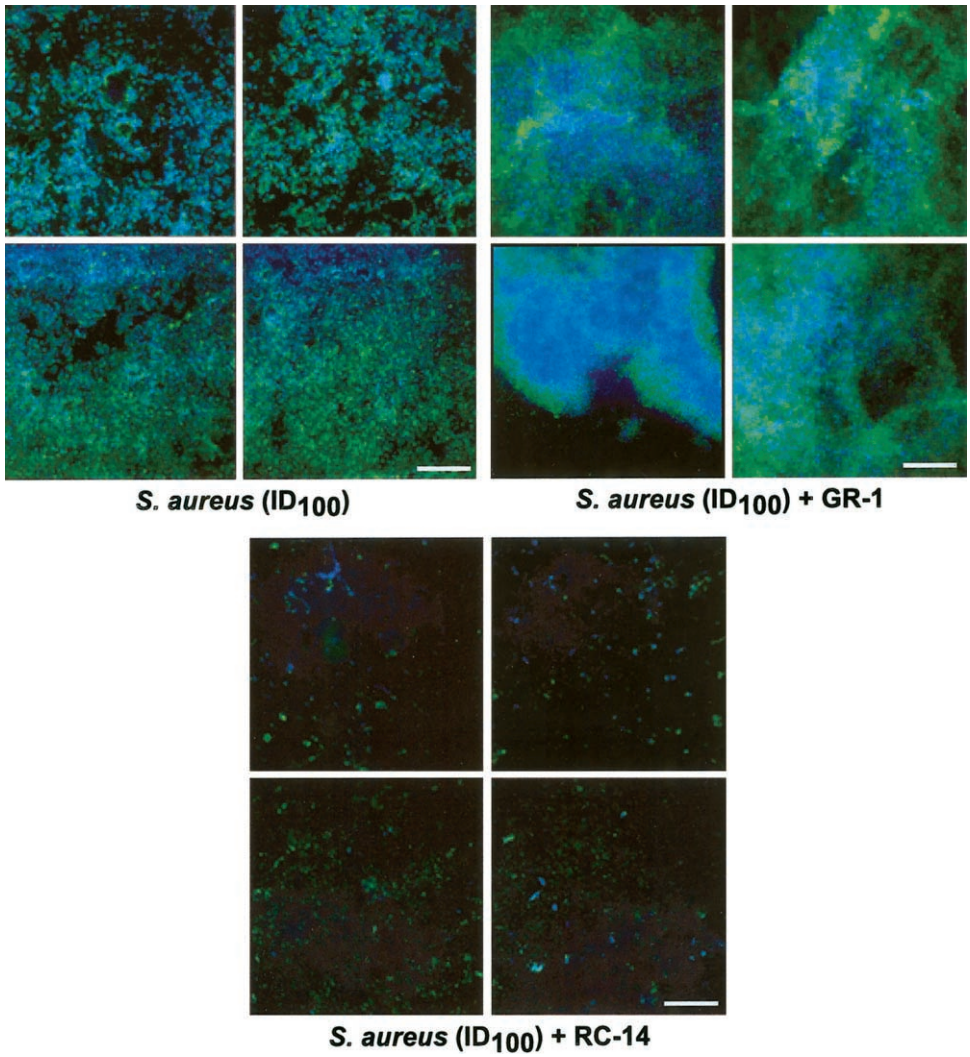

Figure 1. *Lactobacillus fermentum* RC-14 inhibits bacterial adhesion to surgical implants. Surgical sites were reentered and scored for the presence of a visible subcutaneous abscess 3 days after surgery. Implants were then retrieved, washed, and stained for bacteria with Oregon green wheat germ agglutinin and 4′,6-diamidino-2-phenylindole dihydrochloride. Shown are representative fluorescent microscopy images of implants collected from rats treated as indicated. *Bars,* 100 μm.

(2% agarose), stained with ethidium bromide, and photographed under UV transillumination.

Implant imaging with fluorescent microscopy. Surgical sites were reentered to retrieve the implants. Implants were briefly washed in PBS and then incubated in a PBS solution containing Oregon green wheat germ agglutinin (OG-WGA) for 20 min at room temperature (RT) per the manufacturer's instructions (Molecular Probes). Implants were then washed in PBS and stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes) for 20 min at RT. Implants were then washed in PBS and mounted onto glass slides by use of aqua mounting media (DAKO Diagnostic). Digital images were acquired on a Nikon Eclipse TE200 inverted microscope (Nikon Pan Apo 10×/0.45 neutralizing antibody) by use of a liquid cooled CH350 CCD camera (Photometrics). Fluorescence was quantified by using the data inspector tool of softWoRx v2.5 Resolve 3D software (Applied Precision). Data are shown as the total mean OG-WGA fluorescence ± SE for each microscope field ($n = 30$; 10 fields/implant, 3 implants/group).

Statistics. Statistical comparisons between groups were made by the Student's *t* test. Two-sided *P* values $< .05$ were considered to be significant.

Results

L. fermentum *RC-14 inhibits* S. aureus *infection of surgical implants.* We studied the antistaphylococcal activity of 2 probiotic bacterial strains, *L. fermentum* RC-14 and *L. rhamnosus* GR-1 [6], in a rat model of surgical implant infection. Initially, we determined the acute dose response of our infectious agent, *S. aureus* (Oxford strain). Three days after surgery, animals were killed, and the surgical sites were reentered. Visually apparent subcutaneous abscesses developed in 50% and 100% of the animals inoculated with ~7×10^6 and 5×10^7 cfu of *S. aureus,* respectively.

To test whether RC-14 and GR-1 could inhibit *S. aureus* infection, we performed a series of coinoculation experiments with *S. aureus* ID_{50} and various doses of lactobacilli. Treatment with strain RC-14 inhibited the development of subcutaneous abscesses, and the highest RC-14 dose (10^{10} cfu) completely inhibited abscess formation. Overall, inoculation doses of 10^6, 10^7, 10^8, 10^9, and 10^{10} cfu of RC-14 resulted in 4, 2, 1, 1, and 0 of 9 animals in each group developing abscesses, respectively (data not shown). By comparison, animals inoculated with *S. aureus* ID_{50} alone resulted in 5 of 9 animals developing subcutaneous abscesses. In contrast, treatment with strain GR-1 did not inhibit abscess development. In total, animals inoculated with *S. aureus* ID_{50} alone or together with 10^7, 10^8, 10^9, or 10^{10} cfu of strain GR-1 resulted in 5, 5, 4, 4, and 4 animals (9 per group) developing subcutaneous abscesses, respectively.

In a separate experiment, we examined the extent of bacterial colonization of the implants by fluorescent microscopy. Figure 1 shows representative images of the implants recovered from animals of each treatment group that were stained with OG-WGA (surface label bacteria) and DAPI. Quantification of the total mean OG-WGA fluorescence for the RC-14 and GR-1 treatment groups was markedly different from that of the control *S. aureus* treatment group ($n = 30$ fields; 10 fields/implant, 3 implants/group). Implants collected from the RC-14 group showed significantly less bacterial staining (308.8 ± 15.1; $n = 30$ fields; $P < .00001$, Student's *t* test) compared with *S. aureus*–inoculated animals (640.4 ± 37.7; $n = 30$ fields), while implants recovered from GR-1–treated animals showed significantly more bacterial staining (965.3 ± 93.3; $n = 30$ fields; $P < .003$, Student's *t* test). The identities of the implant bacteria were confirmed by PCR analysis by use of RAPD primers [8] (figure 2). Animals inoculated with PBS alone (negative control group) showed no signs of infection and had no detectable bacterial colony-forming unit (data not shown).

RC-14 BSF inhibits S. aureus *infection of surgical implants.* Lactobacilli strains RC-14 and GR-1 secrete a BSF that is capa-

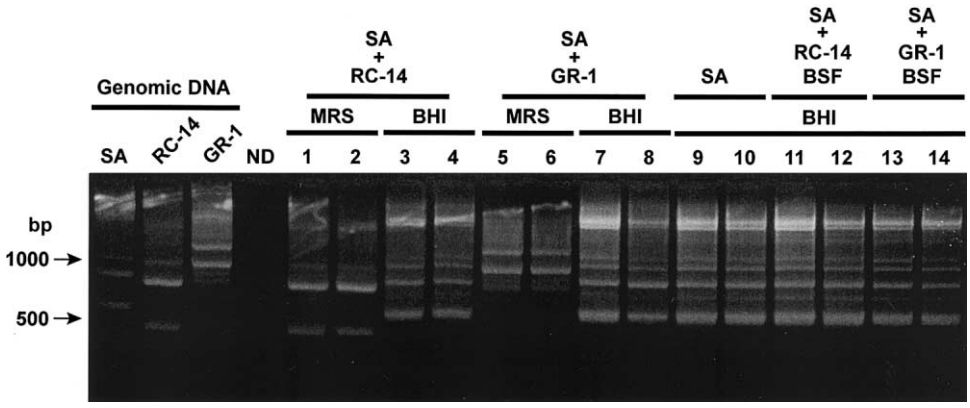

Figure 2. Identification of implant colony-forming unit by random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR). Representative bacterial colonies (1–14) derived from the indicated culture plates (brain-heart infusion [BHI] agar; Man-Rogosa-Sharpe [MRS] agar) for each treatment group (*Staphylococcus aureus* [SA] plus RC-14 *[Lactobacillus fermentum],* SA plus GR-1 *[Lactobacillus rhamnosus],* SA, SA plus RC-14 biosurfactant [BSF], and SA plus GR-1 BSF) were identified by RAPD PCR primers. Genomic DNA from each bacterial species served as positive controls. ND, no DNA.

ble of inhibiting the adhesion of a number of pathogenic bacteria to surfaces in vitro [6, 9]. Because the BSF may account, in part, for the anti-infective activity of RC-14, we reasoned that treating the surgical sites and implants with the RC-14 BSF would also confer protection against *S. aureus* infection. In 3 observer-blinded experiments (6 rats/group), implants were incubated overnight in BSF (1 mg/mL) and surgically inserted into animals. Surgical sites were then coinoculated with 10^8 cfu of *S. aureus* and 100 μg of BSF. In animals treated with RC-14 BSF, subcutaneous abscess formation was significantly inhibited (89%), as was the number of implant colony-forming unit (62%) (both $n = 18$; $P < .004$ and $P < .001$, respectively, Student's t test) when compared with the control GR-1 BSF-treated group (data not shown). The identities of the scored implant colony-forming unit were confirmed by RAPD PCR (figure 2).

Discussion

Our results show that viable *L. fermentum* RC-14 and its secreted BSF can dramatically inhibit surgical infection caused by *S. aureus*. The antistaphylococcal activity of strain RC-14 and its BSF is remarkable for a number of reasons.

To our knowledge, this is the first study to demonstrate the utility of a specific probiotic strain of lactobacilli and its BSF in prevention of surgical implant infection in vivo. To date, probiotic lactobacilli have been used exclusively to treat gastrointestinal and urogenital tract disorders. Therefore, based on our observations, it is reasonable to suggest that this probiotic concept may be applicable to other types of clinical infections.

Second, although probiotic lactobacilli protect against a number of infections, the exact mechanisms responsible for this activity are not entirely clear. However, we recently showed that the RC-14 BSF contains a number of collagen-binding (Cnb) proteins [10] including a 29-kDa Cnb (p29Cnb) that can inhibit the adhesion of *Enterococcus faecalis* 1131 in vitro [5]. Given that *S. aureus* often initiates host binding via cell surface extracellular matrix-binding proteins (ECMBPs) [11], it seems reasonable to suggest that ECMBPs present within RC-14 BSF may effectively compete with *S. aureus* ECMBPs for binding to host sites. This could explain, in part, the competitive exclusion mechanism of probiotic lactobacilli [12] in colonizing and protecting the urogenital and gastrointestinal tract from infections. Although this notion is consistent with bacterial adhesion being a critical step in the development of most *S. aureus* infections [11], other factors produced by RC-14 may account in large part for the observed antistaphylococcal activity. Nevertheless, the use of live probiotic lactobacilli at sites of foreign bodies does not seem feasible since it would likely pose some risk of infection.

The antistaphylococcal activity of the RC-14 BSF is rather intriguing, given the comparable activity to that of live RC-14. Although lactobacilli produce a number of substances that can inhibit the growth of bacteria [13], the RC-14 BSF does not appear to inhibit the growth of *S. aureus* in liquid culture (authors' unpublished data). However, the ability of the RC-14 BSF to inhibit *S. aureus* binding to implants undoubtedly impacts the in vivo growth of *S. aureus,* since bacteria adhesion to surfaces promotes their growth. Alternatively, other mechanisms may account, in part, for the antistaphylococcal activity of RC-14 BSF. For example, a recent in vitro study of *Lactobacillus plantarum* 299v and *L. rhamnosus* GG showed that these probiotic strains could inhibit the adhesion of *Escherichia coli* to intestinal epithelial cells by stimulating epithelial expression of mucins [14]. Taken together with our results, it is conceivable that RC-14 BSF may also contain signaling factors that interact with host and/or bacterial cells and inhibit infection. Further study is needed to determine the identity of the antistaphylococcal components of RC-14 BSF.

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