NOTE

Liamocin oil from *Aureobasidium pullulans* has antibacterial activity with specificity for species of *Streptococcus*

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Aureobasidium pullulans is a yeast-like fungus that produces a number of industrially useful bioproducts including pullulan, xylanase, lipase and poly(β -L-malic acid).^{1–3} Certain strains produce dense ‘oils’ that accumulate in liquid cultures.^{2,4} Analysis of *A. pullulans* oils (subsequently named ‘liamocins’) identified four types with unique chemical structures consisting of a single mannitol headgroup partially O-acylated with polyester tails containing three or four 3,5-dihydroxydecanoic ester groups, some of which may be acetylated (Figure 1).⁵ Although some *A. pullulans* oils have been observed to exert an antiproliferative effect on certain cancer cell lines,^{6–7} these liamocins had only weak antibacterial activity against *Enterococcus faecalis*, and no antibacterial activity was observed against *Staphylococcus aureus*, *Lactobacillus fermentum*, *Escherichia coli* or *Pseudomonas aeruginosa*.⁷

Although over 20 strains of *A. pullulans* have been shown to produce the heavy oils,⁴ not all have been examined for antibacterial activity. *A. pullulans* strain NRRL 50380 is one of the best characterized strains with respect to liamocin production,⁵ but its oil has not previously been tested for antibacterial activity. In the present study, we tested oil from strain NRRL 50380 for antibacterial activity against the strains listed above and expanded the pool of target bacteria to include species of *Streptococcus*. Cultures of *A. pullulans* were grown in sucrose medium for 7 days at 28 °C,⁴ and liamocins were extracted with 2-butanone as described previously.⁷

Antibacterial activity of NRRL 50380 liamocin oil against selected bacteria was first measured qualitatively by agar diffusion. Bacteria (20 μ l at a density of 0.5 McFarland unit) were spread on tryptic soy agar media, and paper discs (6 mm diameter) were placed on the surface. Liamocin oil (10 μ l, 50 mg ml^{–1} in 2-butanone:dimethyl sulfoxide (DMSO) (1:1) solvent) was applied to each disc. Following incubation at 37 °C overnight, plates were scored for a zone of growth inhibition surrounding the disc. Qualitative analysis found that liamocins have strong antibacterial activity against six of seven *Streptococcus* species tested: *S. agalactiae*, *S. uberis*, *S. mutans*, *S. mitis*, *S. infantarius* and *S. salivarius* (Table 1). Only *S. sobrinus* was not

susceptible to liamocins. The oil also showed weak activity against *E. faecalis*, but no activity was observed against *S. aureus*, *L. fermentum*, *E. coli* or *P. aeruginosa* (Table 1).

MICs of liamocins were determined by a modification of the broth microdilution method.⁸ Serial twofold dilutions of liamocins in 2-butanone:DMSO (1:1) were added to tryptic soy broth (or brain heart infusion broth for *E. faecalis*) at a final solvent concentration of 1.25% in the wells of a 96-well plate. Each well was inoculated with the indicated target organism ($\sim 10^5$ CFU per ml), and plates were incubated at 37 °C. Plates were scored for the lowest concentration of liamocins that completely inhibited growth of the target strain. Results were consistent with those observed qualitatively, with MICs against *Streptococcus* species ranging from ≤ 10 to 78 μ g ml^{–1}, whereas MICs against *S. aureus*, *L. fermentum*, *E. coli* and *P. aeruginosa* were greater than 1250 μ g ml^{–1}, the highest tested concentration (Table 1). For comparison of liamocins potency with traditional antibiotics, we tested the *Streptococcus* strains for susceptibility to penicillin G, a drug commonly used to treat streptococcal infections. The MIC for penicillin G was 0.12 μ g ml^{–1} for all strains except *S. infantarius* NRRL B-41208, which had an MIC of 0.25 μ g ml^{–1}. Thus, liamocins do not appear to be as potent against streptococci as the broad-spectrum drug penicillin G. Nevertheless, the liamocins’ narrow spectrum of antibacterial activity may be advantageous over broad-spectrum agents, especially in prophylactic applications to target streptococcal pathogens, but avoid disruption of the beneficial normal flora or selection for antibiotic resistance in commensal bacteria.

Although the data reported here are for liamocins produced by *A. pullulans* NRRL 50380, oils from the following nine strains of *A. pullulans* were also tested for antibacterial activity: NRRL 62031, NRRL 62034, NRRL 62038, NRRL 62039, NRRL 62040, NRRL 62041, NRRL 62042, NRRL 50384 and NRRL 50382. In both qualitative antibacterial assays and broth dilution susceptibility tests, liamocins from all strains showed results similar to those observed for liamocins from strain NRRL 50380 (data not shown). Thus, the antibacterial

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Figure 1 Chemical structures of the liamocins and related exophilins. (a) Liamocin A1 and A2; (b) liamocins B1 and B2; (c) exophilin A. Note that liamocins A2 and B2 both contain a 3-O-acetyl (OAc) group.

Table 1 Antimicrobial susceptibility to liamocins produced by *A. pullulans* strain NRRL 50380

Strain	MIC ^a (μg oil per ml)	Qualitative assay ^b
<i>Streptococcus</i> species		
<i>S. agalactiae</i> NRRL B-1815	20	
<i>S. uberis</i> ^c	78	
<i>S. mutans</i> ATCC 25175	78	
<i>S. mitis</i> NRRL B-14574	20	
<i>S. infantarius</i> NRRL B-41208	78	
<i>S. salivarius</i> NRRL B-3714	≤ 10	
<i>S. sobrinus</i> NRRL B-4468	> 1250	
<i>E. faecalis</i> ATCC 29212	312	
<i>S. aureus</i> ATCC 29213	> 1250	
<i>L. fermentum</i> 0315-1	> 1250	
<i>E. coli</i> ATCC 25922	> 1250	
<i>P. aeruginosa</i> ATCC 27853	> 1250	

^aMICs of liamocin oil from *A. pullulans* NRRL 50380 were determined by a modification of the broth dilution method, with serial twofold dilutions ranging from 1250 to 10 μg ml⁻¹.

^bAntibacterial activity was measured qualitatively by agar diffusion assay.

^c*S. uberis* was a gift from David Donovan (USDA-ARS, Beltsville, MD, USA).

activity and *Streptococcus* specificity appear to be a general property of *Aureobasidium* liamocins.

The solvent extraction process used to prepare liamocins from culture media results in a heterogeneous mixture of liamocin structures⁵ (Figure 1). In an attempt to characterize the antibacterial activity of individual structures, components of *A. pullulans* NRRL 50380 liamocins were separated by reverse phase chromatography on a Brownlee Spheri 5 RP18 column (Waters Corporation, Milford, MA, USA) (Figure 2a). Fractions were analyzed by matrix-assisted laser desorption/ionization-MS (Figure 2b),⁵ and assayed for antibacterial activity in broth microdilution susceptibility tests. Fractions enriched for liamocin types A1, A2, B1 and B2 had MICs of 64, 128, 16 and 128 μg ml⁻¹, respectively. With respect to antibacterial activity, this suggests that liamocin B1, the non-acetylated type with four 3,5-dihydroxydecanoic acid (DDA) groups, is the most active type of liamocin.

Viability of *S. agalactiae* following exposure to liamocins was determined by a commercial two-color fluorescence assay (LIVE/DEAD BacLight Bacterial Viability Assay, Molecular Probes, Eugene, OR, USA) as well as by enumeration of surviving cells on agar media. *S. agalactiae* was chosen for this assay because it is an important agricultural pathogen, causing mastitis in cattle. Mid-log phase bacteria were suspended in sterile saline to an OD of 0.6 at 600 nm. Cells were treated with varying concentrations of liamocin oil from *A. pullulans* NRRL 50380 for 1 h at room temperature. For the fluorescence assay, cells were stained with SYTO 9 and propidium iodide, and fluorescence intensities at 530 nm (green) and 630 nm (red) were measured with an excitation wavelength of 485 nm. As specified by the manufacturer, a standard curve plotting the ratio of green:red fluorescence intensities versus percent live cells was constructed by staining bacterial suspensions of varying concentrations of saline-treated cells (live) and isopropanol-treated cells (dead). For enumeration of surviving bacteria, dilutions of treated cells were spread on tryptic soy broth agar plates and incubated at 37 °C.

Treatment of *S. agalactiae* NRRL B-1815 with 20 and 39 μg ml⁻¹ liamocin reduced the percentage of live cells to 50% and 12%, respectively (Figure 3). Values of percent live cells for the saline-treated and solvent-treated controls were not significantly different

Figure 2 HPLC purification and MALDI MS characterization of component liamocins. **(a)** Liamocins were fractionated on an RP18 HPLC column using a linear gradient of 50–100% acetonitrile in water. **(b)** Fractions collected from the HPLC run were pooled and assayed by MALDI-TOF MS. Molecular adduct ions ($[M+Na]^+$) are shown for liamocin dimers (m/z 577.65); trimers A1 (m/z 763.82) and A2 (m/z 806.80); tetramers B1 (m/z 949.84) and B2 (m/z 991.85); and pentamers C1 (m/z 1135.98) and C2 (m/z 1177.86). MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight.

($P > 0.05$). Similarly, exposure to $39 \mu\text{g ml}^{-1}$ liamocin reduced viable CFUs three log-fold compared with solvent-treated cells ($3.4 \log_{10}$ CFU per ml and $6.4 \log_{10}$ CFU per ml, respectively; Figure 3). Higher concentrations reduced viability below $2 \log_{10}$ CFU per ml. The fluorescence assay is based on the differential labeling of cells with intact and damaged membranes, that is the propidium iodide stain only penetrates cells with damaged membranes. The sudden decrease in the green:red fluorescence ratio following treatment with liamocins suggests a rapid loss of membrane integrity. Taken with the

corresponding decrease in recovery of viable cells on tryptic soy broth (TSB) agar plates following exposure, the liamocins appear to be bactericidal for *S. agalactiae*.

The DDA polyester tails of the liamocins are structurally similar to other antimicrobial compounds (Figure 1).⁵ Doshida *et al.*⁹ reported that exophilin A, a substance produced by *Exophiala pisciphila*, had antimicrobial activity against *E. faecium*, *E. faecalis* and *S. aureus*, but it did not inhibit growth of *Streptococcus epidermidis* (it should be noted that *Exophiala pisciphila* has since been reclassified as a strain of

Figure 3 Viability of *S. agalactiae* following exposure to liamocins. Bacterial viability following exposure to the indicated concentration of liamocins from *A. pullulans* NRRL 50380 was determined by a two-color fluorescence assay and by enumeration of surviving cells. Controls ($0 \mu\text{g ml}^{-1}$ liamocins) include cells treated with saline and cells treated with 2-butanone:DMSO solvent (1.25% (v/v)). All assays were performed in triplicate. Bars indicate the percentage of live cells determined using the LIVE/DEAD BacLight Bacterial Viability Assay and using a standard curve plotting percent live cells versus the green:red fluorescence ratio (inset). The line indicates the bacterial density (log (CFU per ml)) of the surviving *Streptococcus* following 1 h exposure to the indicated concentration of liamocin. DMSO, dimethyl sulfoxide.

A. pullulans). In contrast, we observed that antibacterial activity of the liamocins shows specificity for *Streptococcus* over *Enterococcus* and *Staphylococcus*. Thus, despite sharing structural similarity (Figure 1), the liamocins appear to possess distinct and unique antibacterial activities compared with exophilin A. Halymecine F, a tetramer of DDA with a β -D-mannose situated at the C-3 position of the primary DDA group, was reported to have antimicrobial activity against several Gram-negative phytopathogens, including *Agrobacterium tumefaciens*, *Ralstonia solanacearum* and *Xanthomonas euvesicatoria*.¹⁰ Susceptibility testing against Gram-positive bacteria was not reported, however, and the available data do not allow for direct comparisons between halymecine F and liamocins.

Streptococcus is a genus of ubiquitous Gram-positive bacteria, some of which are etiologic agents of diseases in veterinary and clinical medicine. *S. agalactiae* and *S. uberis* are two commonly recognized species that cause mastitis in dairy cattle.^{11,12} *S. mutans* is associated with dental caries.¹³ *S. suis* is an emerging zoonotic pathogen associated with diseases like septicemia, pneumonia and endocarditis in pigs.¹⁴ In human clinical medicine, infections by species of *Streptococcus* cause numerous diseases including pharyngitis, impetigo, sepsis, toxic shock and necrotizing fasciitis.¹⁵

Some of these diseases may be controlled prophylactically with broad-spectrum antibiotics, but this is a controversial practice that may create reservoirs of antibiotic resistance and select for drug-resistant microorganisms even among the non-target population.¹⁶ An antimicrobial agent that selectively targets a pathogen would be a desired feature of new interventions to treat and prevent infectious disease. In this initial investigation, only species of *Streptococcus* were susceptible to the antibacterial activity of liamocins. Furthermore, as liamocins are chemically different from any class of broad-spectrum antibiotic, one would not expect cross-resistance to any clinically important drugs. Thus, liamocins merit further attention as antimicrobial agents that target streptococcal pathogens.

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