The Journal of Antibiotics (2017) 70, 136–141

www.nature.com/ja

ORIGINAL ARTICLE

Polyols, not sugars, determine the structural diversity of anti-streptococcal liamocins produced by *Aureobasidium pullulans* strain NRRL 50380

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Liamocins are polyol lipids produced by the fungus *Aureobasidium pullulans*, and have selective antibacterial activity against *Streptococcus* species. Liamocins produced by *A. pullulans* strain NRRL 50380 on sucrose medium have a D-mannitol head group ester-linked to 3,5-dihydroxydecanoate acyl chains, three or four of which are joined together by 1,5-polyester bonds (liamocins Man-A1 and Man-B1), and similar 3′-*O*-acetylated analogs (Man-A2 and Man-B2). However, other types of liamocins are produced depending on the choice of strain and growth conditions. In the current study, growth on different polyols, but not sugars, resulted in considerable structural variation, including liamocins with D-galactitol (dulcitol), D-sorbitol (glucitol), D- and L-arabitol, D-xylitol, L-threitol and glycerol head groups. The head groups of liamocins produced on arabitol were shown to be entirely composed of D-arabitol. These liamocin variants were structurally characterized by NMR and MS, and tested for antibacterial activity. The new liamocin variants also had selective activity against *Streptococcus*. Liamocin structural variants are novel antibacterials against *Streptococcus* sp. that merit further investigation.
The Journal of Antibiotics (2017) **70,** 136–141; doi:10.1038/ja.2016.92; published online 20 July 2016

INTRODUCTION

The growth in antibiotic resistance has led to an increased interest in narrow-spectrum antibiotics that target specific groups of bacteria.[1] These have the advantage of minimizing the occurrence of resistance arising, and also reducing adverse side effects against commensal host bacteria. We have recently shown that liamocins produced by the fungus *Aureobasidium pullulans* are novel and selective agents against *Streptococcus* species.[2] *A. pullulans* is well-known as the source of the commercial polysaccharide pullulan, used as a water-soluble film in food and pharmaceutical applications,[3,4] as well as certain other valuable bioproducts.[5–8] The liamocins described to date consist of a single polyol head group partially *O*-acylated with polyester tails composed of three or four 3,5-dihydroxydecanoic ester groups, some of which were also 3-*O*-acetylated (Figure 1).[9] The polyester tail groups are structurally related to exophilins, described as having antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus*, but not against *Streptococcus epidermis*.[10] Thus, the spectrum of activity of liamocins is very different from that of exophilins. *Streptococcus* spp. are the source of a number of veterinary and clinical diseases, and it would be desirable to have a novel antibacterial agent with a narrow spectrum of action, particularly in prophylactic applications.

When grown on a simple medium containing sucrose, *A. pullulans* strain NRRL 50380 produced four mannitol-type liamocins (Man-A1, Man-A2, Man-B1 and Man-B2) that each contain a single D-mannitol head group.[9] More recently, it was shown that the choice of basal growth medium could affect the structure of liamocins.[11] In the current study, the effect of diverse sugar and polyol carbon sources on the production of liamocins was tested. Surprisingly, it was determined that polyols, but not sugars, could affect the nature of the polyol head group component of liamocins.

MATERIALS AND METHODS

Culture maintenance and growth and liamocin production

A. pullulans strain NRRL 50380 was obtained from the ARS Culture Collection, Peoria, IL, USA. Cultures were maintained on yeast malt extract agar. For liamocin production, 50 ml cultures in 250 ml flasks were grown for 7 days at 28 °C with shaking at 150 r.p.m. Liamocins were isolated as previously described.[12] Whole cultures were extracted with at least 50 ml methyl ethyl ketone (2-butanone). Solvent, aqueous and biomass layers were then isolated in a separatory funnel. Liamocins were extracted into the solvent layer. Solvent was evaporated by rotovap and then under a stream of air. Liamocin yields are reported as the means of dry weights with s.e.

Analytical procedures and bioassays

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were recorded on a Bruker-Daltonic Microflex (Bruker-Daltonics, Billerica, MA, USA) instrument operating in reflectron mode as previously

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Received 5 March 2016; revised 31 May 2016; accepted 10 June 2016; published online 20 July 2016

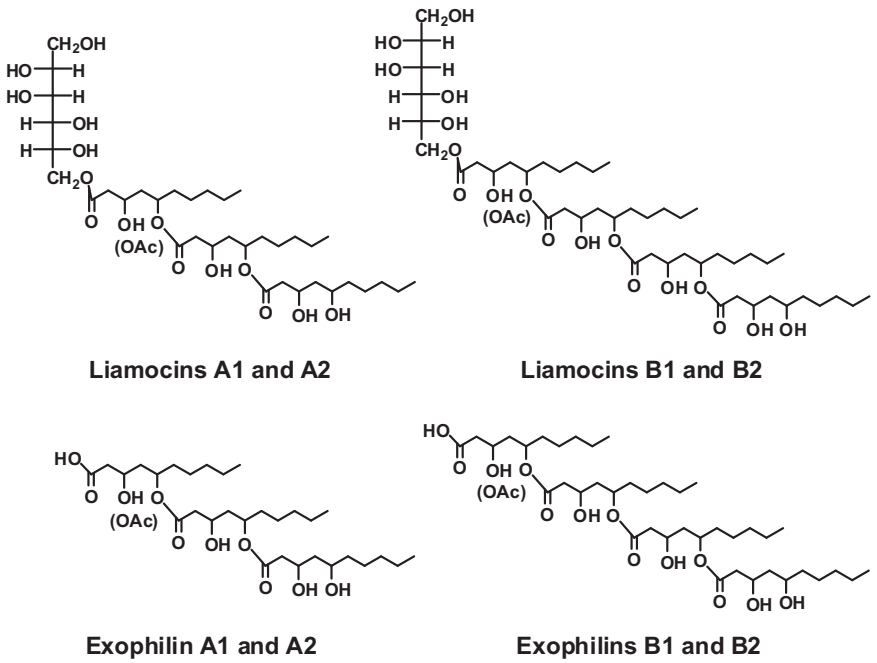

Figure 1 Structures of the mannitol liamocins and related exophilins.[9]

described.[9] NMR data were acquired on a Bruker Avance 500 instrument (Bruker Biospin Corp, Billerica, MA, USA). MICs of liamocins were determined by a modification of the broth microdilution method.[13] Serial two-fold dilutions of liamocins in methyl ethyl ketone:DMSO (1:1) were added to tryptic soy broth (or brain heart infusion broth for *Enterococcus 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 ml^{-1}), 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 AND DISCUSSION

Production of liamocins on sugars and polyols

A. pullulans strain NRRL 50380 was grown on a variety of sugars and polyols for production of liamocins. Cultures grown on myo-inositol, methyl-1-amino-1deoxy-sorbitol, *N*-acetyl-D-glucosaminitol, ethanolamine or 3-amino-1-propanol showed very little growth or liamocin production (data not shown). Other substrates tested supported growth and liamocin production, although liamocin yields were limited on lactose, D-mannitol and D-sorbitol (Table 1). Yields of liamocins were positively but not strongly correlated with growth yields ($r^2 = 0.54$, data not shown). In all cases, liamocins produced by strain NRRL 50380 were yellow and fluorescent, as previously described.[9,12]

Various sugar carbon sources did not affect the nature of the liamocin head group produced, and in each case only mannitol-type liamocins are produced (Table 1). However, some of the polyol carbon sources did affect the structure of the liamocins produced. Cultures grown on D-mannitol produced 100% mannitol-type liamocin head groups. Meso-erythritol produced mainly mannitol head groups, with only a slight amount of erythritol head groups. However, cultures grown on D-sorbitol, D-galactitol or D-glycerol produced a mixture of liamocins with head groups primarily containing either mannitol or the substrate polyol. Growth on D-xylitol or D-ribitol produced a mixture or mannitol, arabitol and substrate head groups. Interestingly, growth on D-arabitol produced liamocins with head groups composed almost entirely of arabitol, whereas growth on L-arabitol produced liamocins with a mixture of arabitol and mannitol head groups. Similarly, growth on D-threitol produced almost entirely mannitol-type head groups, whereas growth on L-threitol produced a mixture of mannitol and threitol types. Notably, strain NRRL 50380 grew and produced liamocins well on either isomer of arabitol or threitol.

The structures of liamocins produced on D-mannitol, D-arabitol and L-threitol were further examined by MALDI-TOF MS (Figure 2). Results confirmed the analyses of polyol head groups obtained by GC/MS (Table 1, Supplementary Figure S1), and further showed that liamocins contained the expected polyester tails groups containing three or four 3,5-dihydroxydecanoic ester groups, some of which are acetylated (A1, A2, B1 and B2). These results suggest that growth on various polyols primarily affects only the polyol head groups of liamocins, and not the tail groups.

It was of further interest to determine whether arabitol head groups were composed of D-arabitol, L-arabitol or an enantiomeric mixture of these isomers. Chiral GC/MS analysis was performed on hydrolyzed arabitol-type liamocins produced on D-arabitol (Figure 3). Results indicated that head groups were entirely composed of D-arabitol, suggesting that this polyol was directly incorporated into liamocins.

Further structural characterizations of the major liamocins (Man-liamocin, Ara-liamocin and Thr-liamocin) were made from NMR assignments (Table 2). The point of attachment of the fatty acid chains to the polyol head group was determined from long-range HMBC correlations from the FA1′ acyl ^{13}C carbonyl signals, across the ester oxygen, to the methylene protons on the polyol backbone. COSY ^{1}H-^{1}H correlations were then utilized for the assignment of the other polyol protons. We also note from the COSY data (Supplementary Figure S2) that the Man-2 and Man-5 signals of Man-liamocin were previously incorrectly assigned, and have corrected this in Table 2. Evidently, the point of attachment of the acyl chains is to the terminal primary hydroxyl group at one end of the polyol head group. This is apparent from the ~3 p.p.m. downfield chemical shift for the acylated carbon signals relative to C1, and from the corresponding downfield

Table 1 Liamocin production by *A. pullulans* strain NRRL 50380 grown on various sugars and polyols

			% Polyol head groups detected from acid-hydrolyzed liamocins[b]								
Carbon source[a]	*Growth (OD_{600})*	*Oil yield ($g\ l^{-1}$)*	*Glycerol (3.5)*	*Threitol (6.1)*	*Erythritol (6.7)*	*Ribitol (10.8)*	*Arabitol (11.1)*	*Xylitol (11.4)*	*Mannitol (15.2)*	*Sorbitol (15.25)*	*Galactitol (15.28)*
Sucrose	7.9±0.6	4.4±0.5	—	—	—	—	—	—	100	—	—
Lactose	2.5±0.1	0.2±<0.1	—	—	—	—	—	—	100	—	—
D-Fructose	6.7±0.3	4.1±0.2	—	—	—	—	—	—	100	—	—
D-Glucose	6.0±0.2	2.5±0.2	—	—	—	—	—	—	100	—	—
D-Mannose	7.3±0.2	3.0±0.2	—	—	—	—	—	—	100	—	—
D-Galactose	7.6±0.1	1.3±<0.1	—	—	—	—	—	—	100	—	—
D-Arabinose	3.6±0.4	1.5±<0.1	—	—	—	—	—	—	100	—	—
L-Arabinose	7.4±0.1	2.6±0.1	—	—	—	—	—	—	100	—	—
D-Xylose	9.3±0.4	3.6±0.1	—	—	—	—	—	—	100	—	—
D-Mannitol	3.7±0.1	0.6±<0.1	—	—	—	—	—	—	100	—	—
D-Sorbitol	3.2±0.1	0.7±<0.1	—	—	—	—	—	—	65	35	—
D-Galactitol	7.9±0.1	1.7±0.4	—	—	—	—	—	—	73	8	19
D-Arabitol	6.0±0.2	1.1±0.2	—	—	—	—	>98	—	<2	—	—
L-Arabitol	6.0±0.5	1.2±0.1	—	—	—	—	62	—	38	—	—
D-Xylitol	6.6±0.2	2.7±0.2	—	—	—	—	27	45	28	—	—
D-Ribitol	5.2±0.1	2.0±0.2	—	—	—	18	63	—	19	—	—
L-Threitol	5.3±0.1	2.5±0.2	—	~75	—	—	—	—	~25	—	—
D-Threitol	7.1±0.1	1.9±0.1	—	5	—	—	—	—	95	—	—
Meso-erythritol	9.5±0.1	4.0±0.5	—	—	5	—	—	—	95	—	—
D-Glycerol	6.0±0.2	1.8±0.3	75	—	—	—	8	—	17	—	—

[a]Sole carbon source at 5% in production medium (PM).[12]
[b]Analysis by GC/MS. Retention times (min) given in parentheses.

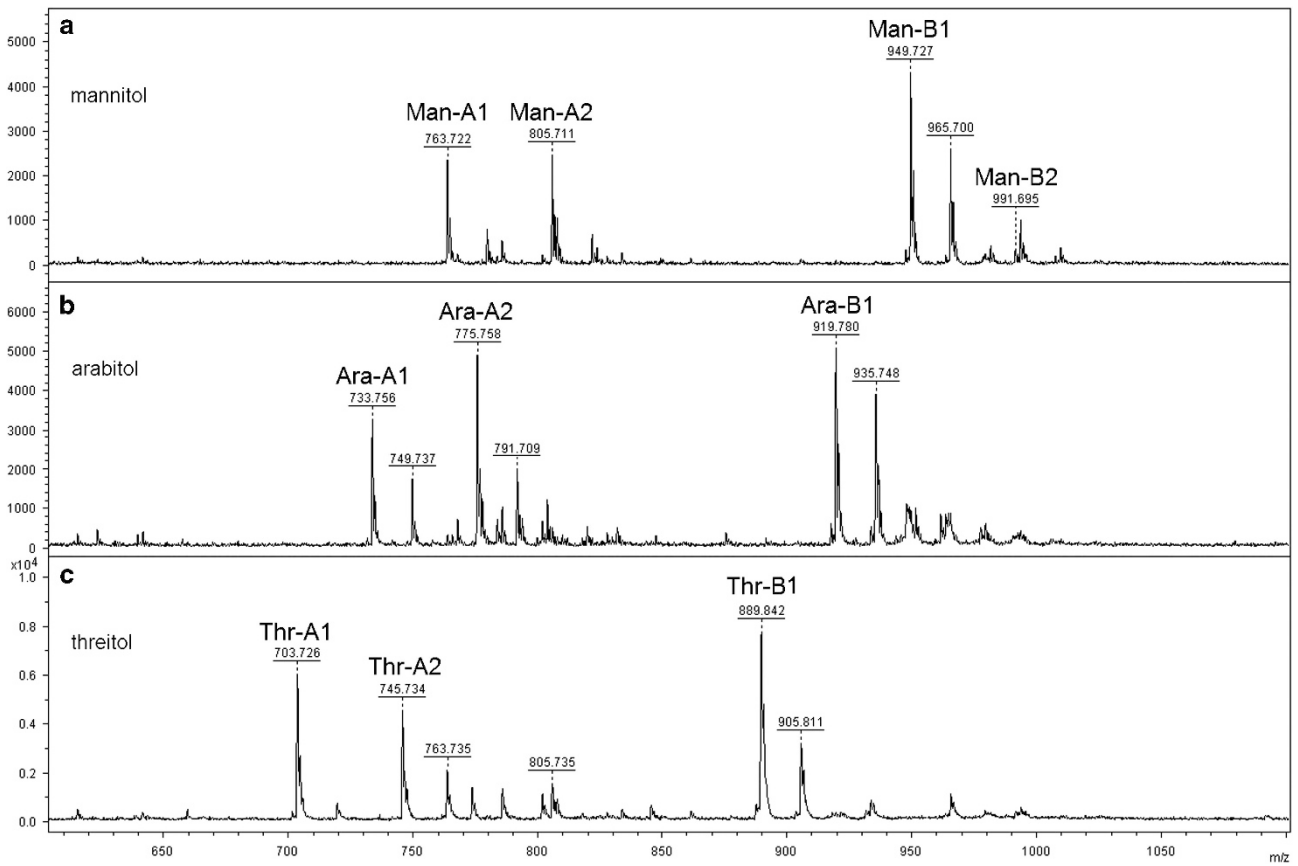

Figure 2 MALDI-TOF MS spectra of liamocins produced by *A. pullulans* strain NRRL 50380 grown on (**a**) D-mannitol; (**b**) D-arabitol; (**c**) L-threitol.

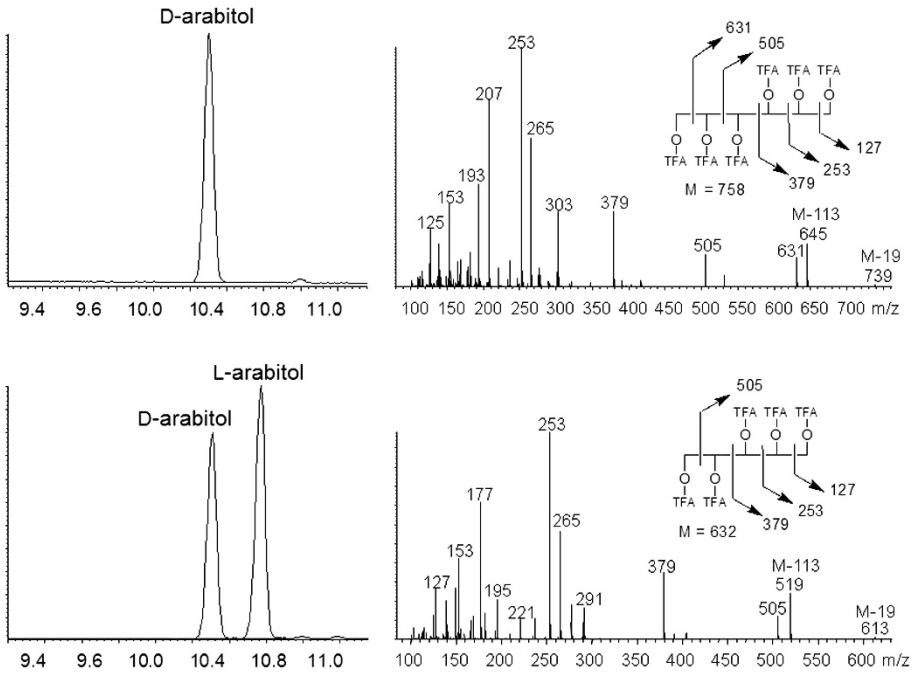

Figure 3 Chiral GC/MS analysis of hydrolyzed arabitol head groups. Liamocins produced on D-arabitol were acid hydrolyzed, pertrifluoroacetylated and separated on a Supelco Beta-Dex-120 chiral GC column. Retention times: D-arabitol (10.1 min); L-arabitol (10.4 min).

Table 2 NMR assignments for the polyol head groups of mannitol-, arabitol- and threitol-type liamocins

Structural assignment[a]	*Man-liamocin*		*Ara-liamocin*		*Thr-liamocin*	
	^{13}C *shift*	^{1}H *shift*	^{13}C *shift*	^{1}H *shift*	^{13}C *shift*	^{1}H *shift*
1	63.7	3.80, 3.65	63.0	3.59, 3.53	63.8	3.83, 3.66
2	71.5	3.71	69.7	3.76	69.5	3.80
3	69.7	3.80	69.7	3.76	68.9	3.88
4	69.7	3.80	69.1	3.90	66.5	4.42, 4.22
5	68.8	3.89	66.3	4.40, 4.21	—	—
6	66.5	4.38, 4.20	—	—	—	—

[a]Position 1 is the non-acylated end; the highest number is the point of attachment to the fatty acid. Chemical shifts reported in p.p.m. relative to acetone internal standard.

shift of the acylated CH_2OH methylene protons (Table 2). Hence, for the mannitol-, arabitol- and threitol-type liamocins, the polyol is similarly selectively ester-linked to the fatty acid motif at one end.

In our previous study we were able to separate mannitol liamocin species A1, A2, B1 and B2 using an RP18 HPLC column with a linear gradient of acetonitrile in water (Bischoff *et al.*[2]). This study showed that fraction Man-B1 had the greatest activity against *S. agalactiae*. Thus far we have not been able to separate liamocin species differing only in the polyol head group, and we anticipate that this separation will be very difficult. An alternative approach is to identify strains or culture conditions that mainly produce liamocins with various polyol head groups. In the current study, conditions have been identified under which the liamocins primarily have polyol head groups of D-mannitol, D-arabitol, L-arabitol, D-ribitol or D-glycerol (Table 1). The primary conclusion of this study is that liamocins with various polyol head groups retain antibacterial specificity toward *Streptococcus* sp. (Table 3).

Although little is understood about the biosynthetic pathway to liamocins, results suggest that the stereospecificity of polyol substrates has a role in determining how carbon sources are utilized for liamocin synthesis. Sugars and certain polyols may be metabolized and utilized for production of mannitol, possibly the preferred head group for liamocin production. Other polyols appear to be partially or completely incorporated directly into liamocins, possibly depending on how easily these substrates or metabolized.

Antibacterial activity of liamocins with different polyol head groups

Liamocins produced on various sugars and polyols were compared for inhibition of *S. agalactiae, S. aureus, E. faecalis and B. subtilis* (Table 3). Mannitol-type liamocin produced on sucrose showed an MIC of 20 μg ml^{-1} for *S. agalactiae* with strong specificity toward this strain, consistent with our previous report.[11] Liamocins produced on other sugars and polyols varied only slightly and showed similar specificity toward *S. agalactiae*. This includes liamocins produced on D-arabitol, which have head groups composed almost entirely of arabitol, and liamocins produced on D-xylitol, D-ribitol and L-threitol, all of which contain a mixture of head group types in which mannitol head groups are not predominant. Thus, the structural variability of the liamocin polyol head group appears to have little or no effect on the specificity of the antibacterial activity.

However, we note that the efficacy of the liamocins toward *Streptococcus* is determined in part by the nature of the polyol head group, and that the polyol is required for activity. Moreover, it has also been shown that the tetrameric liamocins, with four 3,5-dihydrodecanoate chains, are more active than the trimers, whereas dimeric and monomeric forms are inactive against *S. agalactiae*.[2] We have also noted previously that neither massoialactone nor exophilin have an anti-streptococcal activity.[2] Structurally similar compounds called halymectins F and G have been isolated from an antagonistic fungus *Simplicillium lamellicola* BCP. These compounds do not contain a polyol group, but rather are mannose-containing lipids structurally related to (3R,5R)-3-*O*-β-D-mannosyl-3,5-dihydrodecanoic acid.[14] These compounds have potent activity (IC_{50} values ranging from 1.6 to 24.8 μg ml^{-1}) against several Gram negative plant pathogenic bacteria,[14] and therefore quite different from the observed specificity of the liamocins. We conclude that the liamocins have potential therapeutic value as selective antibiotics against the important class of *Streptococcus* pathogens.

Table 3 Antibacterial activities of liamocins produced by *A. pullulans* strain NRRL 50380 grown on various sugars and polyols

	MIC (ug ml^{-1})[a]			
Growth substrate	*S. agalactiae* strain *NRRL B-1815*	*S. aureus* strain *ATCC 29212*	*E. faecalis* strain *ATCC 29212*	*B. subtilis* strain *BGSC 1A751*
Sucrose	20	>625	625	625
Lactose	20	>625	ND	>625
D-Galactose	39	>625	625	>625
D-Arabinose	78	>625	>625	625
L-Arabinose	39	>625	625	>625
D-Arabitol	39	>625	625	>625
D-Xylitol	39	>625	625	>625
D-Ribitol	20	625	625	625
Meso-erythritol	39	>625	625	>625
D-Threitol	39	>625	625	>625
L-Threitol	78	>625	625	625

Abbreviation: ND, not determined.
[a]Determined by the broth dilution method.

Streptococcus infections are treated with a variety of antibiotics, including erythromycin, clindamycin, cephalosporin, vancomycin, and particularly penicillin.[15,16] Many of these antibiotics have relatively broad spectra.[16] While broad-spectrum antibiotics are useful, they would be undesirable for prophylactic applications, such as in cattle dips to prevent mastitis. In previous studies, liamocins were shown to have antibacterial activity against all *Streptococcus* species tested, including *S. agalactiae*, *S. uberis*, *S. mutans*, *S. mitis*, *S. infantarius* and *S. salivarius*.[2] Liamocins had only weak antibacterial activity against *E. faecalis*, and no activity against *S. aureus*, *Lactobacillus fermentum*, *Escherichia coli* or *Pseudomonas aeruginosa*.[2,17] Thus, liamocins offer a novel antibacterial agent with specificity against *Streptococcus* sp.

While most *Streptococcus* sp. are susceptible to penicillin, antibiotic resistance is not uncommon among clinical isolates.[16] The U.S. Centers for Disease Control and Prevention has issued a "concerning" threat level for erythromycin-resistant group A *Streptococcus*, the

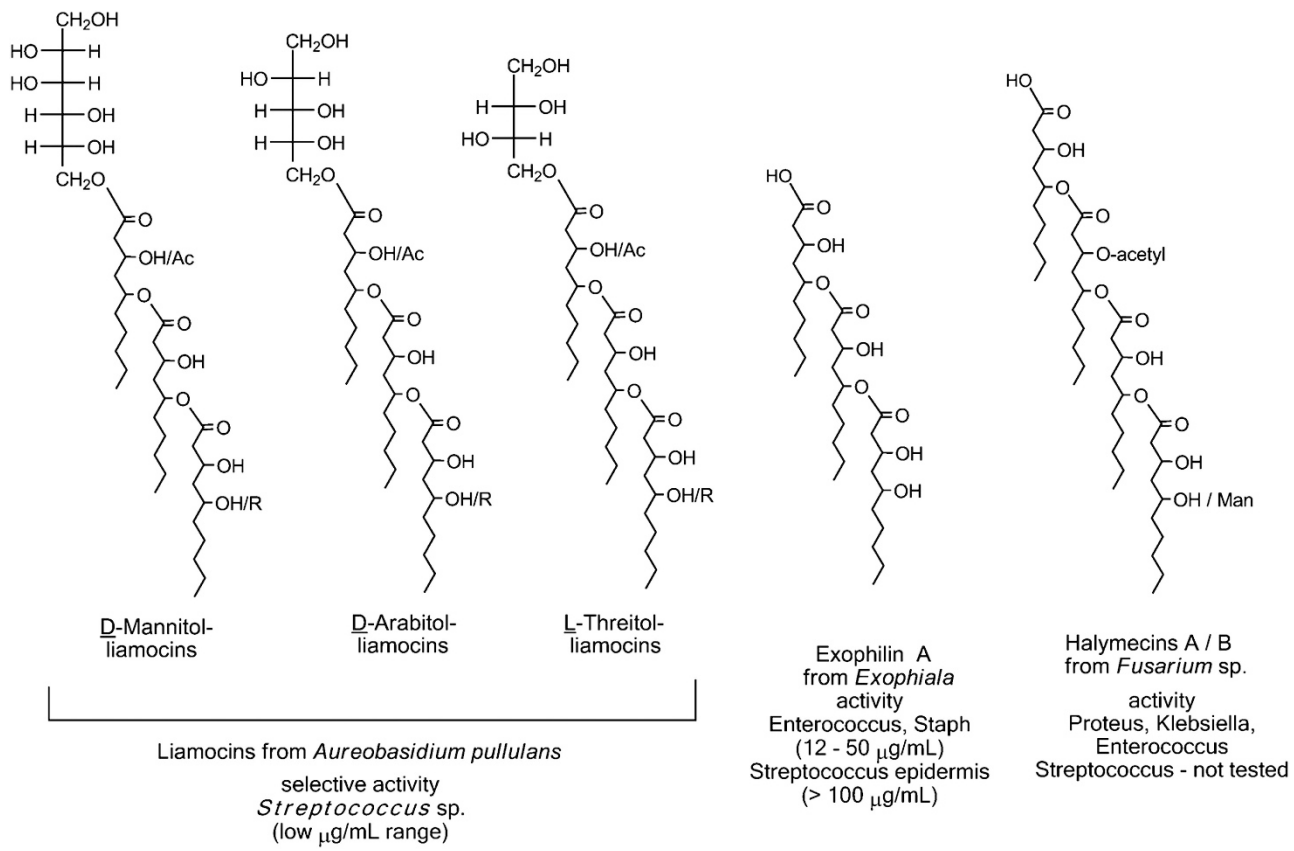

Figure 4 Comparison of liamocins, exophilins and halymecins.

leading cause of necrotizing fasciitis.[18] Antibiotic resistance also has been reported in up to 85% of pathogenic *S. suis* strains.[19] Since liamocins are structurally distinct from any class of broad-spectrum antibiotic, cross resistance to clinically important drugs seems unlikely.

Further studies are needed on the cytotoxicity of liamocins against mammalian cells. Several studies have observed that liamocins have antiproliferative effects against certain cancer cell lines.[12,17,20,21] However, liamocins from several strains of *A. pullulans* were not toxic to African green monkey kidney epithelium Vero cells.[12,17]

In conclusion, it was unexpectedly discovered that growth on various polyols as sole carbon sources affected the polyol head group structure of liamocins, whereas growth on various sugars did not. Mannitol-type liamocins were previously shown to have antibacterial activity with specificity for species of *Streptococcus*.[2] In the current study, evidence was obtained that liamocins with other types of polyol head groups are also active against *Streptococcus* spp. By comparison, exophilins lack polyol head groups, and are active against *Enterococcus* spp. and *Staphylococcus* spp. rather than *Streptococcus* spp. (Figure 4).[10] Halymecins produced by *Fusarium* and *Simplicillium* spp. appear to have a different spectrum of activity, although they have not to date been tested against *Streptococcus* spp. (Figure 4).[14,22]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

Expert technical assistance was provided by Trina Hartman, Eric Hoecker and Melinda S. Nunnally. Mention of any trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. USDA is an equal opportunity provider and employer.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)