

ORIGINAL ARTICLE

Production of novel lipopeptide antibiotics related to A54145 by *Streptomyces fradiae* mutants blocked in biosynthesis of modified amino acids and assignment of *lptJ*, *lptK* and *lptL* gene functions

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A54145 is a complex of lipopeptide antibiotics produced by *Streptomyces fradiae*. A54145 factors are structurally related to daptomycin, with four modified amino acids, only one of which is present in daptomycin. We generated three mutants defective in *lptJ*, *lptK* or *lptL*, whose gene products are involved in the formation of hydroxy-Asn₃ (hAsn₃) and methoxy-Asp₉ (moAsp₉). Each of the mutants produced novel lipopeptides related to A54145 and the profiles allowed assignment of functions for those genes. We constructed strains carrying different combinations of these genes coupled with a mutation in the *lptI* gene involved in the biosynthesis of 3-methyl-Glu₁₂ (3mGlu₁₂), and all recombinants produced novel lipopeptides. One of the compounds displayed very good antibacterial activity in the presence of bovine surfactant, which interacts with daptomycin or A54145E to inhibit their antibacterial activities.

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Keywords: A54145; antibiotic; combinatorial biosynthesis; lipopeptide; *Streptomyces fradiae*

INTRODUCTION

A54145 (Figure 1) is a complex of calcium-dependent lipodepsipeptide antibiotics produced by *Streptomyces fradiae* NRRL 18160.^{1,2} A54145 has a cyclic depsipeptide ring containing 10 amino acids and an exocyclic tail of three amino acids, and it is similar in overall structure to daptomycin. Like A21987C and daptomycin,^{3,4} the N-terminal Trp₁ of the exocyclic peptide is coupled to long-chain length fatty acids, the most common being *iso*-decanoyl, *n*-decanoyl and *anteiso*-undecanoyl in A54145 factors.^{1,2} A54145 factors also vary at positions 12 and 13, in which different factors have different combinations of Glu₁₂, L-3-methyl-Glu₁₂ (3mGlu₁₂), Ile₁₃, or Val₁₃. The most prevalent factors produced during the *S. fradiae* fermentation are shown in Figure 1. Typically, A54145 factors containing Glu₁₂ accumulate early in fermentation and those containing 3mGlu₁₂ accumulate later, with final yields of about 60% of factors containing Glu₁₂.¹ The study by Boeck *et al.*⁵ demonstrated that the distribution of A54145 factors can be manipulated by adding certain amino acids or lipids to the fermentation. For instance, feeding L-Ile, enriched for factors containing Ile₁₃ from 89 to 98%, whereas feeding L-Val (which inhibited overall lipopeptide production by about 70%) enriched for compounds containing Val₁₃ from 11 to 56%, almost exclusively in

factor F (containing Glu₁₂). Feeding either of these amino acids also dramatically shifted the distribution of lipid side chains attached to the core.

The fatty-acid side chains of the A54145 factors can be removed by a deacylase produced by *Actinoplanes utahensis*,⁶ thus providing a means to generate the peptide core as a starting material for chemical acylations with different fatty acids.⁷ Several semisynthetic derivatives were tested for antibacterial properties and acute toxicity in mice.⁸ The most potent natural factors (B, B₁ and E), contain 3mGlu₁₂ and Ile₁₃, but the LD₅₀ for A54145B is low at only 28 mg kg⁻¹. The natural factors containing Glu₁₂ and Ile₁₃ had about twofold lower antibacterial activities, but were substantially less toxic (for example, LD₅₀ > 500 mg kg⁻¹ for A54145A). A semisynthetic derivative of the A54145A core peptide containing an undecanoyl side chain had improved antibacterial activities *in vitro* (MICs ranging from 0.5 to 4 µg ml⁻¹ for different *Staphylococcal* and *Streptococcal* strains), *in vivo* efficacy against *Streptococcus pyogenes* in a mouse protection assay (ED₅₀ = 1.6 mg kg⁻¹), and low toxicity (LD₅₀ > 500 mg kg⁻¹).⁸

Daptomycin has been approved for the treatment of complicated skin and skin structure infections caused by Gram-positive bacteria,⁹ and for the treatment of bacteremia and right-sided endocarditis

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Dedicated to late Dr C Richard 'Dick'/Hutch' Hutchinson for his exceptional contributions to natural product biosynthesis, engineering and drug discovery.

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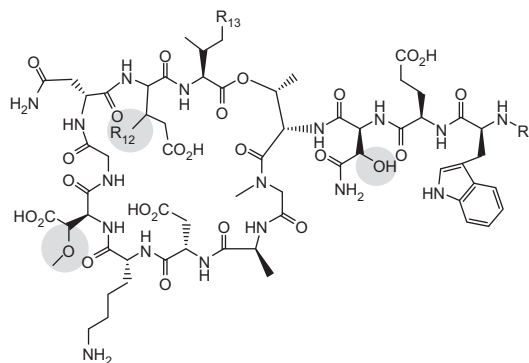


Figure 1 The structure of the A54145 factors normally produced by *S. fradiae*. Amino acid modifications to the lipopeptide that are of interest to this study are highlighted in gray.

caused by *Staphylococcus aureus*, including strains resistant to methicillin (MRSA).¹⁰ One shortcoming of daptomycin is its lack of efficacy in community-acquired pneumonia.¹¹ The poor efficacy in lung tissue appears to be caused by the sequestration of daptomycin in the lung surfactant.¹² To address this issue, derivatives of daptomycin were generated by modifying the lipid side chain or the δ -amino group of ornithine by medicinal chemistry,^{13,14} and by modifying the core peptide by combinatorial biosynthesis.^{15,16} Although improvements in antibacterial activity in the presence of surfactant relative to daptomycin were observed, none of the compounds were active enough to carry forward into clinical studies.

As part of the combinatorial biosynthesis program, the *lpt* gene cluster dedicated to the biosynthesis of A54145 was cloned and sequenced,¹⁷ the complete pathway was localized on a bacterial artificial chromosome (BAC) vector and expressed in heterologous streptomycete hosts.¹⁸ In addition, an ectopic transcomplementation system was developed to engineer the A54145 biosynthetic gene cluster in *Escherichia coli* using λ -Red-mediated recombination, conjugation from *E. coli* mediated by *oriT* from plasmid RP4, and site-specific integration of engineered plasmids into bacteriophage ϕ C31 and ϕ BT1 *attB* sites.¹⁸ Using this system, the A54145 nonribosomal peptide synthetase (NRPS) genes were genetically engineered to generate hybrid lipopeptides by module exchange.¹⁹ A mutant, defective in the *lptI* gene was engineered and confirmed that its gene product is the methyltransferase involved in the biosynthesis of 3mGlu₁₂, resulting in the production of only the Glu₁₂-containing factors.¹⁸ It is believed that LptI uses a similar mechanism, as the homologous GlmT methyltransferase from *Streptomyces coelicolor* converting α -ketoglutarate to the (2*S*,3*R*)-3-methylglutamate intermediate that is further converted to 3mGlu by a transaminase from primary metabolism.²⁰ When fermented in DSF medium with isoleucine, the strain produced $>0.5 \text{ g l}^{-1}$ of A54145D.¹⁸

In addition to 3mGlu₁₂, the A54145 factors have three other modified amino acids, L-hydroxy-Asn (hAsn₃), sarcosine (Sar₅) and L-methoxy-Asp (moAsp₉). The biosynthesis of Sar is encoded in the *lptA* NRPS gene as a methyltransferase (M) domain that converts Gly to Sar, but the other amino acid modifications are believed to be catalyzed by individual oxygenases and an O-methyltransferase.¹⁷ In the present study, we constructed strains containing combinations of deletions of *lptJ*, *lptK* and *lptL* genes encoding the three enzymes predicted to be involved in the biosynthesis of hAsn and moAsp, and analyzed the lipopeptides produced during fermentation to confirm the gene functions. We combined these deletions with the *lptI* deletion, and with plasmids containing combinations of the *lpt* genes to generate a series of novel lipopeptides. The antibacterial activities of

Factor	R	R ₁₂	R ₁₃	MW
A54145A	<i>iso</i> -decanoyl	H	CH ₃	1643
A54145A ₁	<i>n</i> -decanoyl	H	CH ₃	1643
A54145B	<i>n</i> -decanoyl	CH ₃	CH ₃	1657
A54145B ₁	<i>iso</i> -decanoyl	CH ₃	CH ₃	1657
A54145C	<i>anteiso</i> -undecanoyl	CH ₃	H	1657
A54145D	<i>anteiso</i> -undecanoyl	H	CH ₃	1657
A54145E	<i>anteiso</i> -undecanoyl	CH ₃	CH ₃	1671
A54145F	<i>iso</i> -decanoyl	H	H	1629

the A54145 derivatives were determined in the presence and absence of bovine pulmonary surfactant, of which, one compound displayed very good antibacterial activity.

MATERIALS AND METHODS

Strains, plasmids, media, growth conditions and conjugation

The strains and plasmids used in this study are shown in Table 1. Luria-Bertani broth or Luria-Bertani agar²¹ was used for the growth of *E. coli* strains at 37°C, except when the temperature-sensitive plasmid pKD119²² was present. When appropriate, media were supplemented with ampicillin (Amp; 100 $\mu\text{g ml}^{-1}$), apramycin (Am; 100 $\mu\text{g ml}^{-1}$), chloramphenicol (Cm; 25 $\mu\text{g ml}^{-1}$), tetracycline (Tc; 12.5 $\mu\text{g ml}^{-1}$), neomycin (Nm; 50 $\mu\text{g ml}^{-1}$), spectinomycin (Sp; 50 $\mu\text{g ml}^{-1}$) or hygromycin B (Hm; 50 $\mu\text{g ml}^{-1}$). The media and growth conditions for *S. fradiae* and the method for conjugation of plasmid DNA from *E. coli* to *S. fradiae* were as described.¹⁸ *S. fradiae* strains were grown in CSM broth or on mR2YE agar, and when appropriate supplemented with trimethoprim (Tmp; 50 $\mu\text{g ml}^{-1}$), neomycin (10 $\mu\text{g ml}^{-1}$), apramycin (10 $\mu\text{g ml}^{-1}$) or hygromycin B (150 $\mu\text{g ml}^{-1}$).

Truncation of the *lpt* cluster with t₀ terminator-Amp^R cassette

The t₀ terminator-Amp^R cassette was PCR amplified from pDA1836 and the λ -Red-mediated recombinations were carried out as described.^{18,22} The PCR product was made using primers P1 (5'-GGGTAACGCCAGGGTTTCCCAGTACAGCAGTGTGAAAACGACGGCCATGTGTAGGCTGGAGCTGCTTC-3', italicized letters indicate primer binding sites for pDA1836) and P2 (5'-CGCCCGCACCAGCAACTCCTGGCCGCCACAAAGTGCTGTTCCTCAGAACGGATAACAATTTACACAGGA-3') to delete *lptJ* and downstream DNA by recombination from the integrative *lpt* cluster BAC pDA2002 to generate pDA2060. Additional PCR templates made in this manner were used to delete *lptK* (P1 and P3 (5'-TCGGGGCCAACATCGGCATGTTACCCTCTTCGTACGCTCGGAACGATATATTAAGCGGATAACAATTTACACAGGA-3')) and *lptL* (P1 and P4 (5'-GGTCCCCACCTCCCGGAGAAACAGCATGGAACCCGAGAACACCTTACCCTGGATAACAATTTACACAGGA-3')), as well as downstream DNA, to generate pDA2074 and pDA2076, respectively, from pDA2002.

Construction of integrative, *ermE**p containing BACs expressing multiple *lpt* genes

Derivatives of pCB01 with the ϕ BT1 cassette and strong constitutive *ermE** promoter inserted immediately upstream of the start codon of *lptK* or *lptL* were constructed by λ -Red-mediated recombination as described previously.¹⁸ The initial 0.3 kb of the *lptK* gene was PCR amplified from pCB01 using the primers P5 (5'-CTAGAATTCACATATGACCATCGCCCTCGC-3', underlined letters indicate restriction sites) and P6 (5'-CAATCTAGACCGGATAGTGGGTGAACCTCG-3') and cloned adjacent to the ϕ BT1 cassette-*ermE** promoter to generate pDA1860. The $\sim 6 \text{ kb}$ *NheI* fragment containing the BAC flanking region- ϕ BT1 cassette-*ermE**p::*lptK* was excised from the plasmid and used for λ -Red-mediated recombination into pCB01 to generate the BAC pJR2012.

Table 1 Key *S. fradiae* strains and plasmids

Designation	Relevant characteristic(s)	Source or reference
Strains^a		
XH25	NRRL 18160 Sm ^R (<i>rpsL</i> K88R), A54145 high producer	18
DA613	XH25 Δ <i>lptI::tsr</i>	18
DA1187	XH25 Δ <i>lptEFABCDGHJKLMNPI::tsr</i> (" Δ <i>lptEF-I::tsr</i> ")	18
DA1243	DA1187 + <i>lptEF-P</i> (pDA2054), Δ <i>lptI</i>	This study
DA1327	DA1187 + <i>lptEF-H</i> (pDA2060), Δ <i>lptJKLMNPI</i>	This study
DA1333	DA1187 + <i>lptEF-J</i> (pDA2074), Δ <i>lptJKLMNPI</i>	This study
DA1336	DA1187 + <i>lptEF-K</i> (pDA2076), Δ <i>lptLMNPI</i>	This study
DA1449	DA1333 + <i>ermE</i> *p:: <i>lptK-I</i> (pJR2012), <i>wild type</i> ^b	This study
DA1453	DA1333 + <i>ermE</i> *p:: <i>lptL</i> (pDA2117), Δ <i>lptKMNPI</i>	This study
DA1459	DA1333 + <i>ermE</i> *p:: <i>lptI</i> (pDA2129), Δ <i>lptJKLMNP</i>	This study
DA1467	DA1336 + <i>ermE</i> *p:: <i>lptI</i> (pDA2129), Δ <i>lptLMNP</i>	This study
DA1470	DA1336 + <i>ermE</i> *p:: <i>lptL</i> (pDA2117), Δ <i>lptMNP</i> ^c	This study
DA1484	DA1327 + <i>ermE</i> *p:: <i>lptL</i> (pDA2117), Δ <i>lptJKMNPI</i>	This study
DA1489	DA1327 + <i>ermE</i> *p:: <i>lptI</i> (pDA2129), Δ <i>lptJKLMNP</i>	This study
DA1491	DA613 + <i>ermE</i> *p:: <i>lptI</i> (pDA2129), <i>wild type</i> ^b	This study
DA1553	DA1327 + <i>ermE</i> *p:: <i>lptK-I</i> (pJR2012), Δ <i>lptJ</i>	This study
DA1621	DA1336 + <i>ermE</i> *p:: <i>lptL-I</i> (pJR2015), <i>wild type</i> ^b	This study
DA1627	DA1333 + <i>ermE</i> *p:: <i>lptL-I</i> (pJR2015), Δ <i>lptK</i>	This study
Plasmids^c		
pCB01	pECBAC1:: <i>lpt</i> gene cluster	18
pRT802	ϕ BT1 integration cassette, Nm ^R	23
pDA1652	ϕ C31 <i>ermE</i> *p expression vector, Am ^R	18
pDA1834	pBR322::pECBAC1 + ϕ BT1 cassette + <i>ermE</i> *p:: <i>lptD</i> , Hm ^R	18
pDA1836	pOJ260 + t ₀ terminator-Amp ^R cassette	18
pDA1842	pOJ260 + t ₀ terminator-Cm ^R cassette	This study
pDA1860	pBR322::pECBAC1 + ϕ BT1 cassette + <i>ermE</i> *p:: <i>lptK</i>	This study
pDA1862	pBR322::pECBAC1 + ϕ BT1 cassette + <i>ermE</i> *p:: <i>lptL</i>	This study
pDA2002	pCB01:: ϕ C31 cassette at <i>orf21</i> , <i>lptEF-I</i>	18
pDA2054	pDA2002:: t ₀ terminator-Amp ^R cassette in <i>lptI</i> , <i>lptEF-P</i>	18
pDA2060	pDA2002:: t ₀ terminator-Amp ^R cassette in <i>lptJ</i> , <i>lptEF-H</i>	This study
pDA2074	pDA2002:: t ₀ terminator-Amp ^R cassette in <i>lptK</i> , <i>lptEF-J</i>	This study
pDA2076	pDA2002:: t ₀ terminator-Amp ^R cassette in <i>lptL</i> , <i>lptEF-K</i>	This study
pDA2112	pDA1652 <i>ermE</i> *p::Spc ^R	This study
pDA2113	pRT802 + <i>ermE</i> *p::Spc ^R	This study
pDA2117	pDA2113 + <i>ermE</i> *p:: <i>lptL</i> , <i>lptL</i>	This study
pDA2129	pDA2113 + <i>ermE</i> *p:: <i>lptI</i> , <i>lptI</i>	This study
pJR2012	pCB01:: ϕ BT1 cassette + <i>ermE</i> *p:: <i>lptK</i> , <i>lptK-I</i>	This study
pJR2015	pCB01:: ϕ BT1 cassette + <i>ermE</i> *p:: <i>lptL</i> , <i>lptL-I</i>	This study

^aFinal *lpt* genotype for plasmid containing *S. fradiae* strains is in bold.

^bControl strains generated while validating plasmid function.

^c*lpt* genes present on plasmids are in bold. Genes in the *lpt* pathway are not sequential so *lptEF-I* is actually *lptEFABCDGHJKLMNPI* and is an example of the shorthand used for this lengthy set of genes.

The resulting *lptKLI* expression plasmid was functionally validated by complementation of the DA1333 strain (Δ *lptKLI*) to generate DA1449.

Similarly, 0.3 kb from the start of the *lptL* gene was PCR amplified from pCB01 using the primers P7 (5'-AACCATATGGAACCCGAGAACACCTTCAC-3') and P8 (5'-TGTTCTAGACGGGAAGGTTGCGCAGGAGC-3') and cloned adjacent to the ϕ BT1 cassette-*ermE** promoter to generate pDA1862. The ~6 kb fragment containing the flanking region- ϕ BT1 cassette-*ermE**p::*lptL* was excised from the plasmid with *NheI* digestion and used for λ -Red-mediated recombination into pCB01 to generate the BAC pJR2015. The *lptLI* expression plasmid was functionally validated by complementation of the DA1336 strain (Δ *lptLI*) to generate DA1621.

Construction of single *lpt* gene complementation plasmids

A DNA fragment containing the Spc^R coding region was PCR amplified with the primers P9 (5'-CTAGAATTCACATATGAGGGGAAGCGGTGATCG-3') and P10 (5'-CAATCTAGAGTCTTCCCCAGCTCTCTAAC-3') and ligated into *NdeI* and *XbaI*-digested *ermE** promoter expression vector pDA1652,¹⁸ to generate pDA2112. The *ermE**p::Spc^R expression cassette flanked by the t₀ and fd terminators was PCR amplified with primers P11 (5'-TCGGGTTAAC TGCGGGTCTGTC AAC-3') and P12 (5'-TGAGCGGCCGCAAGCTTACT GAGCAACGCGGAAGGC-3') and digested with *HpaI* and *NotI*. The digested PCR product was inserted into pRT802,²³ digested with *NotI* and *EcoRV* to create pDA2113.

The t₀ terminator-Cm^R cassette was generated by replacing the *Amp*^R gene in pDA1836.¹⁸ with the *Cm*^R gene to generate pDA1842. The *Cm*^R gene was PCR amplified from pCB01 using the primers P13 (5'-ATCGCGGCCGACGGAA GATCACTTC-3') and P14 (5'-CGCAAGCTTCGCGCGTGCACCAATTCTC ATG-3'), digested with *NotI* and *HindIII* and ligated with pDA1836 digested with *NotI* and *HindIII* to remove the *Amp*^R gene.

The *lptL* gene was amplified from pCB01 using primers P15 (5'-AACCA TATGGAACCCGAGAACACCTTCAC-3') and P16 (5'-CGTCTAGAGT CAGTCCCAGCCGCGGAG-3') and digested with *NdeI* and *XbaI*. The *lptL* gene was inserted into pDA2113 digested with *NdeI* and *HindIII* (removes Spc^R and t₀ terminator) as part of a three-piece ligation with the t₀ terminator-Cm^R cassette, which was removed from pDA1842 as a *HindIII* and *XbaI* fragment, to produce pDA2117. The introduction of the t₀ terminator-Cm^R cassette was required because the conjugation helper plasmid in ML22 is also Nm^R. The *lptL* expression plasmid was functionally validated by complementation of the DA1336 strain (Δ *lptLI*) to generate DA1470.

In a similar manner, pDA2129 was created by inserting the *lptI* gene amplified from pCB01, using primers P17 (5'-GAGGAATTCACATATG CACCGCGGGGAAC-3') and P18 (5'-CAATCTAGACTCTCTGCGGG CAGTC-3') and the Cm t₀ terminator cassette digested with the appropriate restriction enzymes into pDA2113. The *lptI* expression plasmid was functionally validated by complementation of the DA613 Δ *lptI* mutant to generate DA1491.

Fermentation, measurement of lipopeptide production and purification of lipopeptides

Engineered strains of *S. fradiae* were fermented in DSF production medium,¹ supplemented with 0.79% (w/v) of L-Ile (DSF-Ile).¹⁸ Addition of Ile to the optimized media drives production towards factors containing Ile₁₃ and *anteiso*-undecanoyl side chains derived from precursors generated by Ile catabolism. Production of A54145 factors and novel lipopeptides was monitored by HPLC and LC-MS as described.¹⁸ Production of A54145 factors in liquid culture or on agar plates can also be assessed by a bioactivity assay using *S. aureus* ATCC 29213 with 5 mM CaCl₂.¹⁸ Engineered strains producing novel lipopeptides were scaled up to 5 l of DSF-Ile in multiple shake flasks and centrifuged culture supernatant was purified by column chromatography using HP20 resin and semipreparative HPLC as described.²⁴ Only the major lipopeptide produced in DSF-Ile media, containing Ile₁₃ and *anteiso*-undecanoyl side chain was purified and characterized. LC-MS-MS analysis of select lipopeptides was carried out as described.²⁴

Antibacterial activity assay for lipopeptides

Minimum inhibitory concentrations were determined by broth microdilution using the Mueller-Hinton broth supplemented with 50 mg l⁻¹ Ca²⁺ (MHBC). Cultures were incubated at 37°C with rotation (200 r.p.m.). Susceptibility of *S. aureus* ATCC 29213 for purified lipopeptides was determined in MHBC media with or without supplemented 1% (v/v) bovine pulmonary surfactant (Survanta; Abbott Laboratories, Columbus, OH, USA).¹²

Bioinformatics analyses of putative Lpt proteins

BLASTP analysis²⁵ of translated putative protein sequences was carried out at the NCBI website (<http://www.ncbi.nlm.nih.gov/>).

RESULTS

Engineering and characterization of mutants blocked in the biosynthesis of hAsn₃ and moAsp₉, and assignment of *lptJ*, *lptK* and *lptL* gene functions

S. fradiae A54145 normally produces a mixture of lipopeptide factors containing Glu or 3mGlu at position 12, Ile or Val at position 13, coupled with any of three lipid side chains (Figure 1). In a previous study, it was shown that production of the factors containing 3mGlu₁₂ can be eliminated by deleting the *lptI* methyltransferase gene in the biosynthetic gene cluster.¹⁸ Furthermore, factors A54145D and A54145E (containing Ile₁₃ and with *anteiso*-undecanoyl side chains) can be greatly enriched by supplementing the fermentation medium with L-Ile. Bioinformatic analyses of the region downstream of the NRPS gene *lptD* (Figure 2) initially suggested that *lptK*, *lptL* and *lptI* genes are involved in amino acid modification, with the *lptJ* gene possibly involved in gene regulation based on an incorrect annotation of *syfP* (see below), and the *lptM*, *lptN* and *lptP* genes involved in resistance or transport.¹⁷ These analyses provided a strong rationale for the determination of *lptJ*, *lptK* and *lptL* gene functions by genetic modification, namely deletion and complementation.

Using the tools and techniques developed for *S. fradiae* and described in Alexander *et al.*,¹⁸ a set of plasmids deleted for *lptI*, *lptLI*, *lptKLI* and *lptJKLI* was constructed by λ -Red-mediated recombination. The downstream ends of the *lpt* gene cluster containing BACs were deleted and replaced with the t₀ terminator-Amp^R cassette (Figure 2). The recombinant BACs were introduced into the *lpt* cluster deletion mutant DA1187 (Δ *lptEF-I::tsr*) and the engineered strains were fermented in DSF-Ile medium to characterize the lipopeptide(s) produced, with a focus only on the major lipopeptides produced containing Ile₁₃ and *anteiso*-undecanoate side chains. Host strains, BACs, plasmids, their relevant genotype with respect to the *lptJKLI* genes for the engineered strains and the resulting lipopeptide products are summarized in Table 2.

A54145E, which contains hAsn₃, moAsp₉, 3mGlu₁₂ and an *anteiso*-undecanoyl side chain, and has a mass ion of 1672.7, was used as the fully modified lipopeptide reference standard. Strain DA1243 (Δ *lptI*) produced A54145D, which has a mass ion of 1658.7 differing from A54145E by the loss of the Me group at Glu₁₂ and consistent with previous work.¹⁸ Strain DA1336 (Δ *lptLI*) produced the major lipopeptide (CB-182,325), which had a mass ion of 1642.7, consistent with

the loss of an additional hydroxyl group, indicating that *lptL* encodes one of the two hydroxylases needed. As this strain did not produce a compound lacking the methoxy group, *lptL* likely encodes the Asn₃ hydroxylase. Strain DA1333 (Δ *lptKLI*) produced CB-182, 349, with a mass ion of 1628.7, consistent with the loss of an additional Me group. Therefore, *lptK* likely encodes the methyltransferase that converts hAsp₉ to moAsp₉. Strain DA1327 (Δ *lptJKLI*) produced CB-182,348 with a mass ion of 1612.7, consistent with the loss of an additional hydroxyl group, indicating that *lptJ* probably encodes the Asp₉ hydroxylase.

Complementation of *lpt* mutants to generate novel lipopeptides

By introducing specific genes or gene sets into the mutants, it was possible to generate an expanded set of novel lipopeptides and further confirm the gene function assignments described above. Plasmid pDA2117 expressing the *lptL* gene was introduced into the DA1327 background giving DA1484, which produced CB-182,333, with a mass ion of 1628.7 (Table 2). When introduced into the DA1333 background, it generated DA1453, which produced CB-182,350, with a mass ion of 1644.7. The increase of 16 mass units to the parental lipopeptide is consistent with *lptL* encoding the Asn₃ hydroxylase.

The plasmid pDA2129 expressing the *lptI* methyltransferase gene was introduced into the three deletion mutants described to give DA1489, DA1459 and DA1467, which produced three novel lipopeptides CB-182,390, CB-182,597 and CB-182,363, respectively. As expected, the mass of each of the three novel lipopeptides increased by 14 mass units from the parental lipopeptide consistent with its function as the Glu₁₂ methyltransferase.¹⁸

Introduction of the pJR2015 BAC vector, containing the *lptL* and *lptI* genes, into DA1333 generated strain DA1627, which produced a novel lipopeptide with a mass ion of 1658.7, a 30 mass unit increase compared with the parental lipopeptide. Introduction of pJR2012 BAC vector with the *lptK*, *lptL* and *lptI* genes into DA1327 generated strain DA1553 and resulted in production of CB-182,443 with a 30 mass unit increase over the parental lipopeptide. An identical lipopeptide was produced when pJR2015 was introduced into DA1327, consistent with *lptL* encoding the Asn₃ hydroxylase and *lptI* encoding the Glu₁₂ methyltransferase, but suggests a lack of functional activity from the *lptK* methyltransferase present on the pJR2012 BAC on the hAsn₃, Asp₉ and 3mGlu₁₂ lipopeptides produced by DA1553.

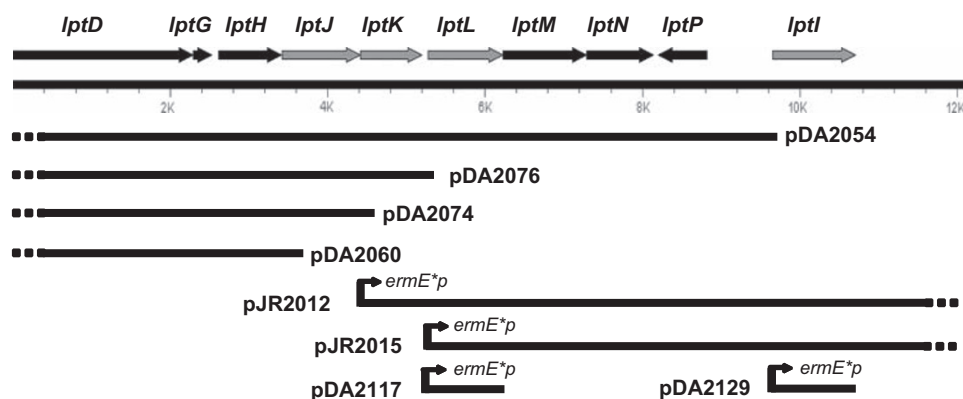


Figure 2 Genetic organization of a region of the *lpt* biosynthetic gene cluster, including the genes required for amino acid modification. Open reading frames are shown as arrows and the genes of interest are shown in gray and are located downstream from the NRPS (*lptD*) and NRPS-associated genes (*lptG* and *lptH*) in the gene cluster. The series of lines beneath the gene cluster show the extent of DNA present BACs or plasmids used in complementation experiments, with dotted lines representing *S. fradiae* DNA that continues beyond the region illustrated in the figure. For some of the plasmids gene expression is under the control of the *ermE** promoter. *lptI*, methyltransferase involved in formation of 3mGlu₁₂; *lptJ*, L-Asp₉ hydroxylase; *lptK*, L-hAsp₉ methyltransferase; *lptL*, L-Asn₃ hydroxylase.

Table 2 Novel lipopeptides produced by recombinant *S. fradiae* strains

Producing strain	Host strain	Insertion through ϕ C31	Insertion through ϕ BT1	Relevant genotype	Compound	Amino acid at position			Mass ions (m/z)	Yield ^a (mg l ⁻¹)
						3	9	12		
XH25	—	—	—	WT	A54145E	hAsn	moAsp	3mGlu	1672.7	152
DA1243	DA1187	pDA2054	—	Δ <i>lptI</i>	A54145D	hAsn	moAsp	Glu	1658.7	462
DA1327	DA1187	pDA2060	—	Δ <i>lptJKLI</i>	CB-182,348	Asn	Asp	Glu	1612.7	81
DA1333	DA1187	pDA2074	—	Δ <i>lptKLI</i>	CB-182,349	Asn	hAsp	Glu	1628.7	52
DA1336	DA1187	pDA2076	—	Δ <i>lptLI</i>	CB-182,325	Asn	moAsp	Glu	1642.7	78
DA1489	DA1327	pDA2060	pDA2129	Δ <i>lptJKL</i>	CB-182,390	Asn	Asp	3mGlu	1626.7	75
DA1459	DA1333	pDA2074	pDA2129	Δ <i>lptKL</i>	CB-182,597	Asn	hAsp	3mGlu	1642.7	32
DA1467	DA1336	pDA2076	pDA2129	Δ <i>lptL</i>	CB-182,363	Asn	moAsp	3mGlu	1656.7	41
DA1484	DA1327	pDA2060	pDA2117	Δ <i>lptJKI</i>	CB-182,333	hAsn	Asp	Glu	1628.7	118
DA1453	DA1333	pDA2074	pDA2117	Δ <i>lptKI</i>	CB-182,350	hAsn	hAsp	Glu	1644.7	360
DA1553	DA1327	pDA2060	pJR2012	Δ <i>lptJ</i>	CB-182,443	hAsn	Asp	3mGlu	1642.7	39
DA1627	DA1333	pDA2074	pJR2015	Δ <i>lptK</i>	NA	hAsn	hAsp	3mGlu	1658.7	76

Abbreviation: NA, not available.

^aYield of designated lipopeptide, which was the major lipopeptide produced during the fermentation in DSF-IIe. A54145E is an exception, as it was not the major lipopeptide produced by XH25; ~65% was A54145D.

The engineered strains produced novel lipopeptides at titers that ranged from 32 to 360 mg ml⁻¹ (Table 2). These titers were sufficient for compound isolation from the 5 l scale-up fermentations for further characterization.

LC-MS-MS characterization of select novel lipopeptides to confirm LptJ and LptL functions

Each of the engineered *S. fradiae* strains produced novel lipopeptides with mass ions consistent with the predicted gene functions for LptJ, LptK and LptL. Four of the compounds, A54145D (1), CB-182,333 (2), CB-182,348 (3) and CB-182,349 (4), were analyzed in detail to confirm the desired structural modifications. The structures of 1~4 were supported by their HR-MS data at *m/z* 1658.7921 (Δ 0.43 p.p.m.), 1628.7790 (Δ 1.11 p.p.m.), 1612.7892 (Δ 2.05 p.p.m.) and 1628.7807 (Δ 0.06 p.p.m.) [M+H]⁺, respectively. Amino acid sequences of 1~4 were determined by analysis of MS-MS data of both parent compounds (Figure 3a) and the linear hydrolysates 1a~4a (Figure 3b). As summarized in Figure 3a, MS-MS spectra of 1~4 provided limited but distinct product ions from γ_{10} to γ_{12} along with their corresponding water-loss peaks. The assigned three fragments γ_{12} , γ_{11} , γ_{10} confirmed the side-chain amino acid sequences of 1 and 2 as *anteiso*-undecanoyl-Trp-Glu-hAsn and 3 and 4 as *anteiso*-undecanoyl-Trp-Glu-Asn, respectively. Furthermore, the amino acid sequences of 1~4 were determined by the analyses of the MS-MS data from 1a~4a (*m/z* 1676.8, 1646.8, 1630.7, 1646.8 [M+H]⁺, respectively), which were produced by hydrolysis of 1~4 with lithium hydroxide. As shown in Figure 3b, experimental values of *yn* and *bn* agreed with their respective theoretical fragment ions. Therefore, the amino acid sequences of compound 1~4 were strongly supported by the linear hydrolysates 1a~4a, as *anteiso*-undecanoyl-Trp-Glu-hAsn-Thr-Sar-Ala-Asp-Lys-moAsp-Gly-Asn-Glu-Ile, *anteiso*-undecanoyl-Trp-Glu-hAsn-Thr-Sar-Ala-Asp-Lys-Asp-Gly-Asn-Glu-Ile, *anteiso*-undecanoyl-Trp-Glu-Asn-Thr-Sar-Ala-Asp-Lys-Asp-Gly-Asn-Glu-Ile and *anteiso*-undecanoyl-Trp-Glu-Asn-Thr-Sar-Ala-Asp-Lys-hAsp-Gly-Asn-Glu-Ile, respectively. The MS-MS data are consistent with our gene function predictions such that CB-182,333 produced by DA1484 (Δ *lptJKI*), which expresses LptL, hydroxylates Asn₃ and CB-182,349 produced by DA1333 (Δ *lptKLI*), which expresses LptJ, and hydroxylates Asp₉.

Antibacterial properties of A54145 derivatives

The antibacterial properties of A54145 derivatives against *S. aureus* in the presence and absence of 1% bovine surfactant are shown in Table 3. As controls, daptomycin and its close analog CB-181,220, which has an *anteiso*-undecanoyl rather than an *N*-decanoyl side chain, were compared with the A54145 derivatives. Both daptomycin and CB-181,220 had MICs of 0.5 μ g ml⁻¹ in the absence of surfactant and 64 μ g ml⁻¹ in the presence of 1% surfactant. Potency of CB-182,130, a Glu₁₂ analog of CB-181,220 was significantly higher (16-fold higher MIC or 8 μ g ml⁻¹) than daptomycin or CB-181,220 in the absence of surfactant but retained activity in the presence of 1% surfactant (16 μ g ml⁻¹). A54145E had an MIC of 1 μ g ml⁻¹ in the absence of surfactant, but the MIC in the presence of 1% surfactant was increased by 32-fold. Removal of the Me group from Glu₁₂ (A54145D) resulted in only a twofold increase in MIC (2 μ g ml⁻¹) in the absence of surfactant, and an eightfold reduction in MIC in the presence of surfactant (4 μ g ml⁻¹) relative to A54145E. A similar result was obtained by removing the methoxy group from Asp₉ (CB-182,443). Removal of the methoxy group from Asp₉ and the hydroxyl group at Asn₃ yielded CB-182,390, which had MICs of 2 μ g ml⁻¹ in the presence or absence surfactant. Other combinations of hypomodification of Asn₃, Asp₉ and Glu₁₂ gave antibiotics with less favorable MIC profiles (Table 3).

The results from inactivating the various amino acid modification enzymes indicate that the antibacterial properties of A54145 can be altered by simply changing the modified amino acid composition. Whereas the fully modified A54145E was the most active antibiotic, several compounds lacking one or two amino acid modifications were nearly as active as A54145E in the absence of surfactant and more active in the presence of surfactant. The best compound (CB-182,390) displayed only a twofold increase in MIC (2 μ g ml⁻¹) over A54145E in the absence of surfactant, with no change in potency in the presence of surfactant (2 μ g ml⁻¹).

Bioinformatic analysis of the *lptJKL* gene products

LptL showed highest sequence similarity (47%) to AsnO that hydroxylates Asn during the lipopeptide CDA biosynthesis.^{26,27} Furthermore, LptL has a binding pocket for the oxygenation of Asn identical to that of AsnO, EQNHENDHR (Table 4).²⁷ LptL and AsnO

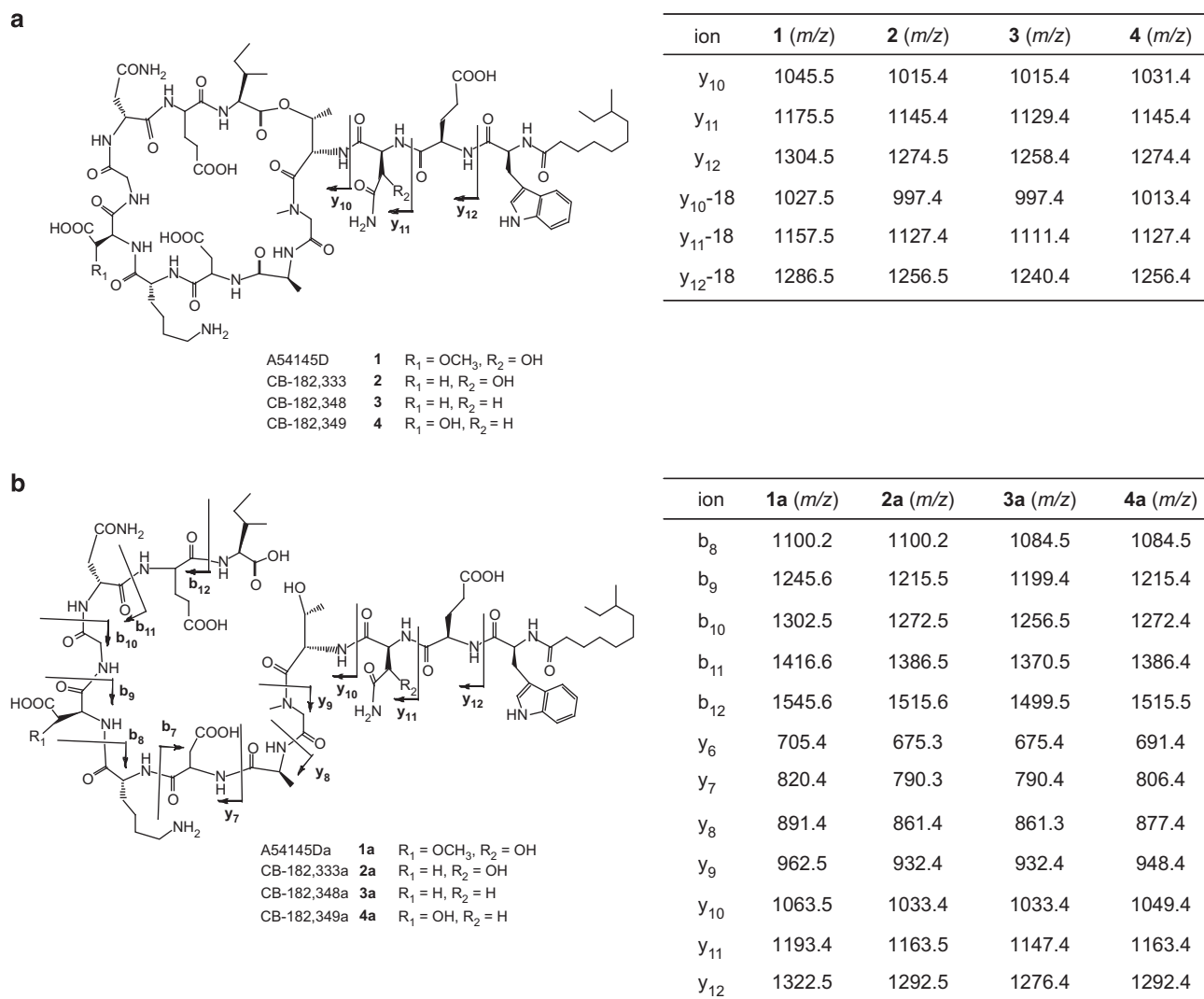


Figure 3 (a) Chemical structures of compounds **1-4** and product ions from y₁₀ to y₁₂ of their LC-MS-MS spectra. (b) Chemical structures of linear hydrolysates **1a-4a** with MS-MS fragmentation patterns and the corresponding product ions y_n and b_n.

Table 3 Antibacterial activities of lipopeptides

Compound ^a	Amino acid at position ^b			<i>S. aureus</i> MIC (μg ml ⁻¹)		
	3	9	12	- Surf	+Surf (1%)	Ratio (+/-)
Daptomycin	Asp	Asp	3mGlu	0.5	64	128
CB-181,220	Asp	Asp	3mGlu	0.5	64	128
CB-182,130	Asp	Asp	Glu	8	16	2
A54145E	hAsn	moAsp	3mGlu	1	32	32
A54145D	hAsn	moAsp	Glu	2	4	2
CB-182,443	hAsn	Asp	3mGlu	2	4	2
CB-182,363	Asn	moAsp	3mGlu	2	16	8
CB-182,350	hAsn	hAsp	Glu	8	16	2
CB-182,333	hAsn	Asp	Glu	32	64	2
CB-182,325	Asn	moAsp	Glu	32	32	1
CB-182,349	Asn	hAsp	Glu	32	64	2
CB-182,348	Asn	Asp	Glu	16	32	2
CB-182,597	Asn	hAsp	3mGlu	1	16	16
CB-182,390	Asn	Asp	3mGlu	2	2	1

Abbreviation: Surf, surfactant.

^aDaptomycin has an *N*-decanoyl side chain. All others compounds have *anteiso*-undecanoyl side chains.

^bAmino acids altered through genetic changes are in bold.

homologs with amino acid sequences ranging from 47 to 60% identities were observed in *Saccharopolyspora erythraea*, *Streptomyces scabies*, *Streptomyces* sp. SPB78, *Streptomyces* sp. SPB74 and *Streptomyces* sp. C, all of which have amino acid binding pockets identical to those of AsnO and LptL (Table 4). *S. coelicolor* and *Streptomyces lividans* encode AsnO proteins of identical sequence, and also encode AsnO/LptL homologs with binding pockets differing in one amino acid from the AsnO/LptL pocket (Table 4).

Although LptJ encodes a hydroxylase that converts Asp₉ to hAsp₉, BLASTP analysis indicated that it has no sequence similarity to LptL. The top BLASTP hits were to a conserved hypothetical protein from *Streptomyces clavuligerus* (ZP_050077403; 63% identity) and a SyrP-like protein from *Streptomyces avermitilis* (NP_824815; 63% identity). It also showed 33% identity with the SyrP_L-Asp hydroxylase from *Pseudomonas syringae*.²⁸ LptJ defines the first *L*-Asp hydroxylase encoded by a secondary metabolite biosynthetic gene cluster in an actinomycete, although it has been shown that *asnO* can be modified by site-specific mutagenesis to encode an enzyme that will hydroxylate Asp.²⁹

BLASTP analysis of LptK was consistent with it functioning as a methyltransferase. However, the only BLASTP hit above 31% identity

Table 4 Proteins related to asparagine oxygenases AsnO and LptL

Strain	Protein	Amino acid identity (%)		Amino acid binding pocket ^a
		AsnO	LptL	
<i>S. coelicolor</i> A3(2) ^b	AsnO	333/333 (100)	149/316 (47)	EQNHENDHR
<i>S. fradiae</i> A54145	LptL	149/316 (47)	319/319 (100)	EQNHENDHR
<i>S. erythraea</i> NRRL 2338	CAM03547	189/312 (60)	150/306 (49)	EQNHENDHR
<i>S. scabies</i> 87.22	CBG67543	178/303 (58)	144/288 (48)	EQNHENDHR
<i>Streptomyces</i> sp. SPB78	ZP_05486634	155/288 (53)	166/310 (53)	EQNHENDHR
<i>Streptomyces</i> sp. SPB74	ZP_04993663	155/317 (48)	170/318 (53)	EQNHENDHR
<i>Streptomyces</i> sp. C	ZP_05509616	149/311 (47)	167/316 (52)	EQNHENDHR
<i>S. coelicolor</i> A3(2) ^c	CAB92259	187/314 (59)	156/316 (49)	EENHENDHR

^aAmino acids at positions 125, 144, 146, 155, 157, 158, 241, 287 and 305 relative to the crystal structure of the amino acid binding pocket of AsnO.²⁷

^bSame amino acid sequence as *S. lividans* ZP_05525565.

^cSame amino acid sequence as *S. lividans* ZP_05526106.

was to a putative methyltransferase from *Streptomyces griseus* (BAG17399; 45% sequence identity).

DISCUSSION

The cyclic lipopeptide antibiotic A54145 complex of related factors is distantly related to the clinically important antibiotic daptomycin, and several of the factors demonstrate good antibacterial activities against Gram-positive pathogens.^{1,8} The most active compounds containing 3mGlu₁₂ were toxic to mice, whereas those containing Glu₁₂ were about 20-fold less toxic and displayed only twofold lower antibacterial activities.⁸ In a recent study, we generated strains deleted for the *lptI* gene, and the mutants produced >0.5 g l⁻¹ of the Glu₁₂-containing A54145D when fermentations were supplemented with L-Ile.¹⁸

In the present study, we generated a series of *S. fradiae* strains deleted for different combinations of the *lptJ*, *lptK*, *lptL* and *lptI* genes. By analyzing the products of the engineered strains, we were able to deduce the functions of the *lptJ*, *lptK* and *lptL* genes, as Asp₉ hydroxylase, hAsp₉ methyltransferase and Asn₃ hydroxylase, respectively, and further confirmed the function of the *lptI* gene as the Glu₁₂ methyltransferase. The genetic engineering tools developed for *S. fradiae* were well suited for a combinatorial biosynthesis approach to assign gene function and generate a series of novel lipopeptides. Fermentation of the engineered strains in DSF-Ile media shifted production predominantly to the factors containing Ile₁₃ and *anteiso*-undecanoyl side chains, which simplified HPLC and LC-MS analysis of the fermentation broths and purification of the novel A54145 factors. We chose to pursue the purification and characterization of only A54145D or A54145E analogs (Ile₁₃ and the *anteiso*-undecanoyl side chain) because of the concentration advantage afforded by the Ile supplementation and increased chromatographic separation of these lipopeptides resulting in higher-yielding purifications. Characterization of novel lipopeptides with the different lipid side chains would have increased the number of analogs generated, but the MIC data⁸ did not suggest significant differences among the different side chains to warrant purification of the remaining lipopeptides. Also in support of this decision to focus only on the major lipopeptide, we had confirmed the previous observation that Val₁₃-containing lipopeptides A54145F had inferior MICs in comparison with the Ile₁₃ counterpart (Counter *et al.*⁸; and data not shown). With all analogs having the same lipid side chain, direct antibacterial activity comparison among the hybrid lipopeptides was possible.

It is noteworthy that even the strain deleted for all four genes produced lipopeptide antibiotics (CB-182,348; Table 2). This means

that the NRPS enzymes can process Asn or hAsn at position 3, Asp, hAsp or moAsp at position 9 and Glu or 3mGlu at position 12. This astonishing flexibility was exploited to generate many novel compounds, discussed below. It is also noteworthy that the specific amino acid modifications were localized to the L-isomers of Asn, Asp and Glu at positions 3, 9 and 12, respectively, even though A54145E has a D-Asn, L-Asp and D-Glu residues at positions 11, 7 and 2, respectively. This site specificity of amino acid modifications is particularly relevant in light of the observation that the amino acid binding pockets for hAsn₃ and moAsp₉ are indistinguishable from those for Asn₁₁ and Asp₇, respectively.¹⁷ Further characterization of AsnO and its functional homolog LptL and their interactions with their respective NRPS proteins may shed light on the mechanism of relaxed amino acid binding specificity at positions 3, 9 and 12 for modified amino acids, coupled with the apparent stringent amino acid binding specificity at positions 2, 7 and 11.

Addition of pJR2012 (*lptKLI*) or pJR2015 (*lptLI*) to DA1327 (Δ *lptJKLI*) generated strains that produced the hAsn₃, Asp₉ and 3mGlu₁₂ lipopeptide CB-182,443. It is not a lack of function from the *lptK* methyltransferase present on the pJR2012, but rather *lptK* function requires the presence of *lptJ* to generate the hAsp₉ substrate necessary for *lptK* O-methylation. This sequential order was confirmed in an additional strain engineering experiment using plasmids expressing only *lptJ* or only *lptK* or *lptJ* and *lptK* together in the DA1327 background (data not shown). The strain containing the *lptK* plasmid alone produced only the parental lipopeptide, whereas *lptJ* alone and *lptJK* together produced lipopeptides with 16 and 30 mass unit increases to the parental lipopeptide, confirming *lptK* action on the *lptJ* catalyzed hAsp₉ substrate. These data further confirm that LptJ converts Asp₉ to hAsp₉, the obligatory substrate for the LptK O-methyltransferase action.

Titers of the novel lipopeptides ranged from 10 to 75% of the 462 mg ml⁻¹ of A54145D produced by DA1243. The majority of the novel lipopeptide producing strains lacked the LptM and LptN transporter pair, which are clearly not essential for antibiotic export, but may aid in optimal export of antibiotic. The *lptM* and *lptN* genes are only present in strains DA1243, DA1553 and DA1627. Strain DA1453 produces the hAsn₃, hAsp₉, Glu₁₂ (CB-182,350) at ~75% of the A54145D titers, whereas hAsn₃, Asp₉, Glu₁₂ (CB-182,333) is produced at only ~25% by strain DA1484 and Asn₃, moAsp₉, Glu₁₂ (CB-182,325) is produced at only ~17% by strain DA1336. This type of direct comparison would be more conclusive with individual in-frame deletion mutants for each gene, but it does suggest

less efficient production of Asn₃ or Asp₉-containing lipopeptides. Fermentation of *S. fradiae* XH25 typically generates a mixture of approximately 60% Glu₁₂ and 40% 3mGlu₁₂ factors, suggesting a shortage of 3mGlu or precursors or that there is an incorporation or processing preference for the Glu₁₂-containing peptides. Interestingly, in most cases the novel 3mGlu₁₂-containing lipopeptides generated in this study represented >75% over their Glu₁₂-containing counterpart produced during the fermentation.

MIC data demonstrated that modified amino acids are important for activity; this was especially true for the Glu₁₂ analogs. In all cases, the 3mGlu₁₂-containing analogs are more active than their Glu₁₂ counterparts. Similar to A54145D, novel Glu₁₂-containing lipopeptides displayed substantially lower MICs in the presence of bovine surfactant, but their potency is not sufficient for clinical consideration. It is noteworthy that the analogs CB-182,443 and CB-182,390 have 3mGlu₁₂, which results in good potency but uniquely displayed good activity in the presence of 1% surfactant. CB-182,390 (Asn₃, Asp₉ and 3mGlu₁₂) was targeted for further investigation, including extensive chemical analyses, including amino acid quantitation, tandem LC-MS-MS analysis and 2D-NMR to confirm the structure.²⁴

Potent antibacterial activity depends on the presence of modified amino acids, making total peptide synthesis or chemoenzymatic synthesis,³⁰ utilizing proteinogenic amino acids undesirable approaches to generate A54145 analogs. As chemical synthesis of the chiral modified amino acids at the necessary scale for SAR studies is not practical, combinatorial biosynthesis¹⁹ remains the most effective method to generate potent novel lipopeptides.

In a separate study, the M domain was deleted from the Sar₅ module to generate an A54145D analog (CB-182,391) containing Gly₅, which was eightfold less active than A54145D in the presence or absence of surfactant with MICs of 16 and 32 µg ml⁻¹, respectively (S Doekel and P Brian, unpublished). This indicates that Sar₅ is also important for overall activity of A54145 lipopeptides.

The study by Strieker *et al.*²⁷ have shown that AsnO converts the free L-Asn to L-hAsn before incorporation into the growing peptide. According to their model, L-hAsn binds to the adenylation (A)-domain of the CA_{hAsn9}TE module of CdaPS2, in which it is converted to D-hAsn and incorporated into the growing peptide chain. The AsnO protein was crystallized, and the catalytic binding pocket for L-Asn has been defined as a 10 letter code: EQNHENDHR at positions 125, 144, 146, 155, 157, 158, 241, 287 and 305.²⁷ The LptL protein has an identical L-Asn catalytic binding pocket displaced by eight amino acids relative to AsnO, and starting at position E117. Therefore, even though the *lptL* and *asnO* genes and their protein products have diverged considerably (47% identical amino acids), they have conserved the L-Asn binding pocket 100%. This supports the notion that although these enzymes have orthologous catalytic functions, they have likely diverged for a second non-orthologous function.³¹ One possible non-orthologous function is binding to different NRPS enzymes at specific modules to provide L-hAsn *in situ* on demand during peptide assembly.³¹ This model predicts amino acid sequence divergence in regions of the proteins involved in protein-protein interactions, and also provides a mechanism to sequester L-hAsn from the cytoplasmic amino acid pools that can be drawn on for protein biosynthesis. It also explains the absence of incorporation of hAsn at position 11.

There are currently over 20 streptomycete and many other actinomycete genome sequences in GenBank. There are homologs to LptL and AsnO showing >47% amino acid identities over nearly the entire proteins in seven streptomycetes and in *Saccharopolyspora erythraea*

(Table 4). Their high amino acid sequence similarities suggest that some or all may function as L-Asn oxygenases.

BLASTP analysis indicated that LptJ is unrelated to the LptL, but it displays high sequence similarities with only two proteins, a SyrP-like protein from *S. avermitilis* (62% identity) and a conserved hypothetical protein from *S. clavuligerus* (62% identity). LptJ shows 33% sequence identity with SyrP from *P. syringae*, an enzyme that has been shown to hydroxylate L-Asp tethered as the protein-bound S-pan-tetheinyl thioester on an NRPS module involved in syringomycin biosynthesis to produce L-threo-3-OH-Asp.²⁸ If LptJ hydroxylates L-Asp in a similar manner during A54145 biosynthesis, it might account for the specificity of hydroxylation at Asp₉ and not Asp₇. LptJ defines functionally the first of the SyrP-like L-Asp hydroxylases in actinomycetes, and the high sequence identities to the two related proteins from genome sequencing projects suggests that they may also function as L-Asp hydroxylases. Bioinformatics had originally predicted LptJ to have a role in gene regulation based on its initial annotation.¹⁵ Biochemical characterization of SyrP confirmed its actual role in syringomycin biosynthesis is as an Asp hydroxylase,²⁸ which is consistent with our prediction for the homologous LptJ in this study.

BLASTP analysis of LptK indicated that this protein class is even more obscure than LptJ. LptK showed 45% sequence identity to a putative methyltransferase from *S. clavuligerus* and 45% identity to a sequence (likely a methyltransferase) embedded in a NRPS from *Pseudomonas aeruginosa* strains. The uniqueness of LptK may not be surprising as the moAsp in A54145 represents the only known moAsp-containing natural product that we are aware of. The functional assignment of the *lpt* genes described herein should help in the annotation of cryptic secondary metabolite pathways that are being rapidly discovered by genome mining.

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