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ORIGINAL PAPER

**Julia Penn · Xiang Li · Andrew Whiting
Mohammed Latif · Trevor Gibson · Christopher J. Silva
Paul Brian · Julian Davies · Vivian Miao
Stephen K. Wrigley · Richard H. Baltz**

Heterologous production of daptomycin in *Streptomyces lividans*

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Abstract Daptomycin and the A21978C antibiotic complex are lipopeptides produced by *Streptomyces roseosporus* and also in recombinant *Streptomyces lividans* TK23 and TK64 strains, when a 128 kbp region of cloned *S. roseosporus* DNA containing the daptomycin gene cluster is inserted site-specifically in the ϕC31 *attB* site. A21978C fermentation yields were initially much lower in *S. lividans* than in *S. roseosporus*, and detection was complicated by the production of host metabolites. However A21978C production in *S. lividans* was improved by deletion of genes encoding the production of actinorhodin and by medium optimization to control the chemical form of the calcium dependent antibiotic (CDA). This latter compound has not previously been chemically characterized as a *S. lividans* product. Adding phosphate to a defined fermentation medium resulted in formation of only the phosphorylated forms of CDA, which were well separated from A21978C on chromatographic analysis. Adjusting the level of phosphate in the medium led to an improvement in A21978C yield from 20 to 55 mg/l.

Keywords Daptomycin · A21978C · Calcium-dependent antibiotic (CDA) · Actinorhodin · *Streptomyces lividans*

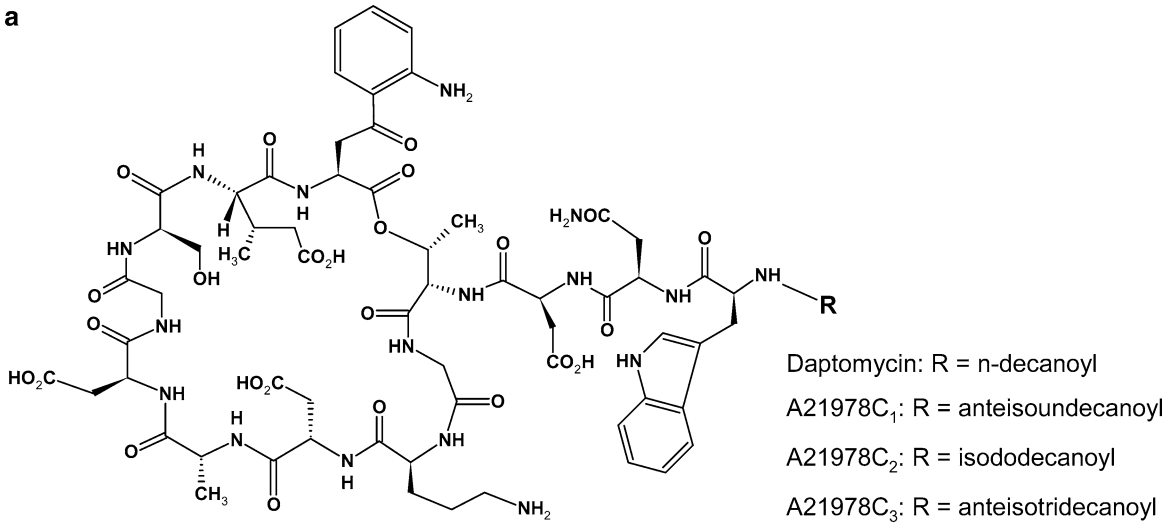
J. Penn · A. Whiting · M. Latif · T. Gibson · S. K. Wrigley
Cubist Pharmaceuticals (UK) Ltd, 545 Ipswich Road, Slough, Berkshire SL14EP, UK

X. Li · C. J. Silva · P. Brian · J. Davies · V. Miao
R. H. Baltz (✉)
Cubist Pharmaceuticals, Inc, 65 Hayden Avenue, Lexington, MA 02421, USA
E-mail: rbaltz@cubist.com
Tel.: +1-781-8608444
Fax: +1-781-8611164

J. Davies
Dept of Microbiology and Immunology, University of British Columbia, 6174 University Blvd, Vancouver BC V67 1Z3, Canada

Introduction

Daptomycin is a cyclic lipopeptide antibiotic with potent in vitro activity against Gram-positive pathogens including drug-resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *S. aureus* and vancomycin-resistant enterococci [6, 14]. Daptomycin has a novel, calcium-dependent mechanism of action involving binding to bacterial cell membranes; this is not yet fully understood [11, 20] but may account for its efficacy against strains resistant to other antibiotics in clinical use. Cubicin® (daptomycin-for-injection) has recently been approved for the treatment of Gram-positive infections of the skin and skin structures [18]. The structure of daptomycin (Fig. 1a) consists of a cyclic depsipeptide portion comprising 13 amino acids, cyclized through an ester bond between the threonine hydroxyl and the *C*-terminal kynurenine to form a 10 amino acid ring, and an *n*-decanoyl acyl tail attached to the amino group of the *N*-terminal tryptophan. Daptomycin is produced by precursor-directed fermentation of *Streptomyces roseosporus*. In the absence of a lipid feed, *S. roseosporus* produces the A21978C family of lipopeptides with the same tridecapeptide nucleus and different fatty acyl tails, formed predominantly from branched chain C_{11}, C_{12} and C_{13} fatty acids [4]. Daptomycin was developed from A21978C, initially through a semi-synthetic chemical route involving deacylation and reacylation of the tridecapeptide nucleus with a variety of lipid and other tail groups [5]. Methods to produce daptomycin directly by feeding *n*-decanoic acid to fermentations of *S. roseosporus* were subsequently devised [10]. A 128 kbp region of *S. roseosporus* DNA was cloned and shown by heterologous expression in *Streptomyces lividans* to contain the complete daptomycin gene cluster (*dpt*) [17]. However, total yields of the three main lipopeptides produced by recombinant *S. lividans* were much lower than those produced by *S. roseosporus*, in which yields range from 150 mg/l up to 1 g/l, the latter for a production strain.



Compound	R_9	R_{10}	R_{11}
CDA1b	OPO_3H_2	H	H, H
CDA2a	OPO_3H_2	CH_3	π-bond
CDA2b	OPO_3H_2	CH_3	H, H
CDA3a	OH	H	π-bond
CDA3b	OH	H	H, H
CDA4a	OH	CH_3	π-bond
CDA4b	OH	CH_3	H, H

Fig. 1 Structures of: **a** daptomycin and members of the A21978C complex; **b** actinorhodin; **c** members of the CDA complex

Table 1 Strains and plasmids

Organism	Relevant characteristics	Reference
Streptomyces lividans 66		
TK64	*str-6* (K88E)	[12]
TK23		[12]
TK23-521	Δ*act*	This study
CBUK136736	TK64 (pStreptoBac V)	This study
CBUK136742	TK64 (pCV1)	[17]
CBUK136744	TK64 (pCV2)	This study
CBUK137028	TK23 (pStreptoBac V)	This study
CBUK137027	TK23 (pCV1)	This study
CBUK137026	TK23-521 (pStreptoBac V)	This study
CBUK137024	TK23-521 (pCV1)	This study
Plasmids		
pStreptoBac V	BAC vector with *oriT*, Am^r, *att*/*int* $^{\phi C31}$	[17]
pCV1	pStreptoBac V::128 kbp *dpt* region	[17]
pCV2	pStreptoBac V::90 kbp *dpt* region	[17]

The situation was further complicated by the existence of a complex background of host metabolites, including actinorhodin [7, 19, 21] and a series of metabolites that we characterized as belonging to the CDA complex, which consists of cyclic lipopeptides structurally similar to daptomycin [8, 13]. CDA has only previously been characterized chemically as a product of *Streptomyces coelicolor* A3(2). Although *S. lividans* and *S. coelicolor* are closely related, most strains of the former do not normally produce actinorhodin and evidence for production of CDA by *S. lividans* has been indirect [3, 9]. The structures of actinorhodin and the known CDA components are shown in Fig. 1b, 1c, respectively.

We describe improvement of the yield of the A21978C lipopeptides in *S. lividans* through minimizing expression of the host background metabolites. This was done using both genetic and medium optimization approaches. The first heterologous production experiments for the A21978C lipopeptide in *S. lividans* used TK64 and TK23 strains with an intact actinorhodin pathway [15–17]. The actinorhodins are colored polyketides produced in copious quantities by *S. coelicolor* and some strains of *S. lividans* under many fermentation conditions, and interfere with the detection and purification of other secondary metabolites from the fermentation. As a consequence, medium development to enhance expression of the heterologous NRPS pathway in *S. lividans* was restricted to formulations that minimize the production of actinorhodin. However, *S. lividans* is also capable of producing large amounts of other metabolites that, in comparison, dwarfed the A21978C lipopeptides produced. It was therefore desirable to minimize the production of host metabolites in order to achieve cleaner chromatographic profiles for easier detection and purification of new compounds produced by recombinant strains.

Materials and methods

Strains and plasmids

Key strains and plasmids are shown in Table 1. Plasmid pCV1 is comprised of a 128 kbp fragment of *S. roseosporus* chromosomal DNA cloned in the BAC shuttle vector, pStreptoBAC V (Fig. 2) [17]. Plasmid pCV2 comprised an 90 kbp fragment of *S. roseosporus* chromosomal DNA cloned in the same BAC shuttle vector. *S. lividans* strains were routinely grown on trypticase soy agar or broth at 28–30°C, and mycelial biomass was archived in 20% glycerol at −135°C for storage.

Transformation of *S. lividans*

Plasmids were introduced into *S. lividans* by protoplast transformation using methods previously described [12, 17].

Construction of Act$^-$ *S. lividans*

In order to eliminate actinorhodin production from *S. lividans*, a cassette was constructed to genetically inactivate the pathway by replacing a portion of the actinorhodin biosynthetic gene cluster (*act*) with a resistance gene (Fig. 3). A 6.3 kbp PCR product containing part of the actinorhodin polyketide synthase genes (*act*I-ORF1, *act*I-ORF2, *act*I-ORF3, *act*VII, *act*IV) was amplified using *S. lividans* TK23 as template DNA and cloned into a pGEM–T derived vector containing an apramycin resistance (Am^R) gene from pOJ436 [2]; a 1 kbp *Bam*HI fragment, including the 3′ end of *act*I-ORF2, all of *act*I–ORF3 and the 5′ end of *act*VII, was deleted from the center of the fragment and replaced by a 1.5 kbp fragment containing the *ermE* gene [1] amplified from *S. erythraea* genomic DNA, using primers P1 (5′-GCAGATCTTTGCGCCCGATGCTAGTC-3′) and P2 (5′-GAAGATCTGGGCCGACATCAACCTCTG-3′; *Bgl*II sites in the primers (underlined) allow for cloning into the gap made by *Bam*HI.

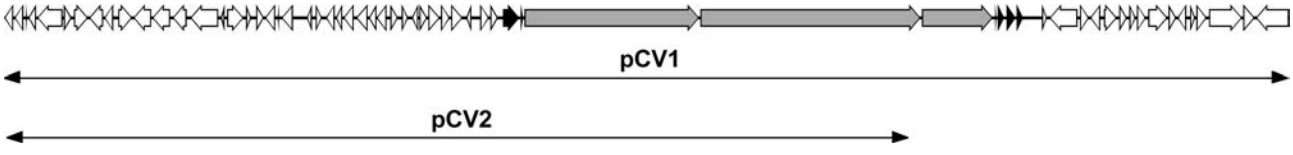


Fig. 2 The *S. roseosporus dpt* gene region. The 128 kbp *dpt* region includes the three daptomycin NRPS genes (*gray*), accessory biosynthetic genes (*black*), as well as flanking ORFs [17]

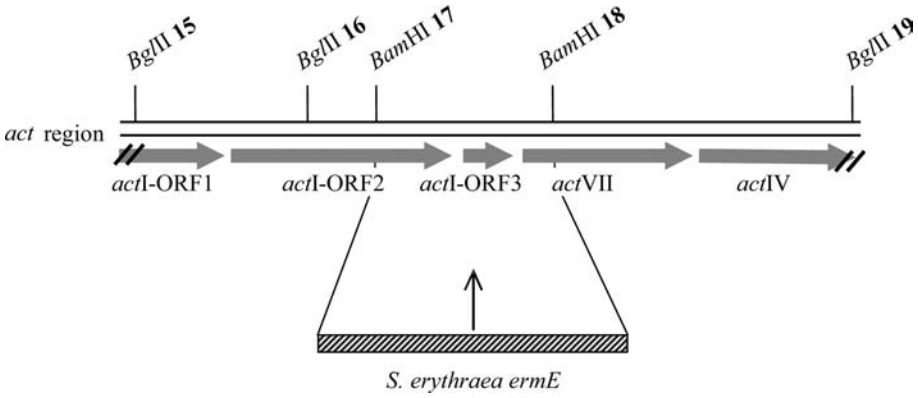


Fig. 3 Diagram of *act* deletion construction. The deleted *act* region (*Bam*HI 17 to *Bam*HI 18, numbering follows [16]) is replaced by *ermE* [1] to inactivate the pathway and abolish actinorhodin production.

The resulting suicide plasmid was introduced into *S. lividans* TK23 by protoplast transformation [12]. Cells were plated on trypticase soy agar (TSA) containing apramycin (100 μg/ml) to recover single crossover recombinants. After 6 days, colonies were replicated onto TSA plates with erythromycin to identify colonies that were resistant to erythromycin but sensitive to apramycin. These were analyzed by Southern hybridization and sequencing for evidence of a double crossover in which marker exchange had occurred between the suicide plasmid and the genome, leading to replacement of part of the native *act* locus by *ermE*. Strain TK23-521 exhibited the desired *act* replacement and was used for further experiments.

Culture media and growth conditions

Although a number of different media were initially explored, two were examined in more detail for their ability to support production of the A21978C lipopeptides in *S. lividans*. Both media also support good production of the A21978C lipopeptides in *S. roseosporus*. Medium A was a complex medium consisting of 1% glucose (BDH), 2% soluble starch (Sigma), 0.5% yeast extract (Difco), 0.5% casein (Sigma) and 4.6% MOPS (Sigma), adjusted to pH 7 and autoclaved. Medium B was a defined medium consisting of 2% glycerol, 0.25% sucrose, 1.2% L-proline, 1.5% MOPS, 0.056% K_2HPO_4, 0.05% NaCl, 1% Tween 80, 0.5% trace salts solution (per liter: 1 ml 1 M H_2SO_4, 1.722 g $ZnSO_47H_2O$, 1.112 g $FeSO_47H_2O$, 0.223 g $MnSO_44H_2O$, 0.062 g H_3BO_3, 0.125 g $CuSO_45H_2O$, 0.048 g $Na_2MoO_42H_2O$, 0.048 g $CoCl_26H_2O$, 0.083 g KI), 0.2% vitamin mixture solution (per liter: 0.025 g thiamine, 0.025 g riboflavin, 0.025 g pantothenate, 0.025 g nicotinic acid, 0.025 g pyridoxine, 0.025 g thioctic acid, 0.0025 g folic acid, 0.0025 g cyanocobalamin, 0.0025 g *p*-aminobenzoic acid, 0.05 ml vitamin K1, 2 ml Tween 80), 0.02% 10 mg/l $CaCl_22H_20$, 0.02% 10 mg/l $MgSO_47H_2O$, 0.00055% $FeSO_47H_2O$, adjusted to pH 7 and filter sterilised.

Fermentations were initiated by inoculation of an enriched oatmeal slant (medium A9 [17]) containing 100 mg/l apramycin with approximately 0.25 ml of biomass from a cryovial stored at −135°C. After 7–10 days incubation at 28°C, a mixed mycelial and spore suspension was generated by the addition of 4 ml 0.1% Tween 80, and 2 ml were inoculated into 40 ml of seed medium A345 [17] containing 25 mg/l apramycin in a baffled flask to initiate the seed stage. Seed flasks were shaken at 240 rpm and 30°C for 24–28 h before a 5% transfer to production flasks containing 50 ml of medium A or B without apramycin (to facilitate detection of A21978C production through its antibacterial activity; data not reported here). Replicate flasks were sampled from day 2 until day 6 of the production fermentation by aseptically removing approximately 1 ml broth and centrifuging it for 10 min at 10,000 rpm.

Analysis by HPLC, HPLC-MS and NMR

Broth supernatants were analyzed by HPLC at ambient temperature using a Waters Alliance 2690 HPLC system and 996 PDA detector with a 4.6×50 mm Symmetry C8 3.5 μm column and a Phenomenex Security Guard C8 cartridge. The mobile phase, buffered with 0.10% trifluoracetic acid, was initially held at 10% acetonitrile(aq) for 2.5 min, followed by a linear gradient over 6 min to 100% acetonitrile and re-equilibration after a further 3.5 min; the flow rate was 1.5 ml/min. Up to 50 μl of the supernatant was injected to monitor the production of the native A21987C lipopeptides; their concentrations were determined by comparison to reference daptomycin purified from *S. roseosporus* fermentations and provided by Cubist Pharmaceuticals, Inc., Manufacturing Dept. Preparative HPLC was performed on a radially compressed cartridge column consisting of two 40×100 mm Waters Nova-Pak C18 60 Å 6 μm units and a 40×10 mm Guard-Pak with identical packing.

Mass spectrometric (MS) data were obtained by LC-MS analysis on a Finnigan SSQ710C system using electrospray ionization in positive ion mode, with a scan range of 200–2,000 daltons and 2 s scans. The LC method was run at ambient temperature on a Waters Symmetry C8 column (2.1×50 mm 3.5 μm particle size).

The initial conditions of 90% water, 10% acetonitrile and 0.01% formic acid were maintained for 0.5 min, followed by a linear gradient to 100% acetonitrile and 0.01% formic acid over 6 min, and this composition was held for 3.5 min before re-equilibration; the flow rate was 0.35 ml/min. The electrospray capillary voltage was 21.2 V with a collisional induced dissociation offset of 0 or −10 V. The capillary temperature was maintained at 250°C. ^{1}H and ^{13}C spectra were recorded in DMSO-d_6 at 308 K using a Bruker ACF400 spectrometer at 400 and 100 MHz, respectively.

Results and discussion

An HPLC chromatogram illustrating heterologous expression of the A21978C lipopeptide series in *S. lividans* TK64 (CBUK136742) in medium A is shown in Fig. 4. Production of three of the A21978C lipopeptides with characteristic UV/visible spectra was evident, with retention times of 5.62, 5.77 and 5.90 min (λ_{max} 223, 261 and 364 nm) under the analytical conditions applied. On LC-MS analysis, these three A21978C lipopeptides yielded molecular ions $(M+H)^+$ at m/z of 1634.7, 1648.7 and 1662.7, which are in agreement with the masses reported for the major A21978C lipopeptide metabolites (C_1, C_2 and C_3 respectively) produced by *S. roseosporus* [4]. Production of these lipopeptides was accompanied by high levels of pigments with characteristics of actinorhodins [19].

Streptomyces lividans TK64 harbors *str-6*, a Lys88 to Glu mutation in ribosomal protein S12 (encoded by *rpsL*) that not only confers resistance to streptomycin, but also has been implicated in enhancing the production of actinorhodin in *S. lividans* TK24 [19]. As the high level of actinorhodin production might be related to the *rpsL* mutation present in the host, pCV1 was also introduced into *S. lividans* TK23, which lacks the *str-6* marker [12] but the same result was obtained, production of both A21978C and high levels of actinorhodin. Furthermore, similarly high levels of actinorhodin were also produced by control strains of both TK64 and TK23 carrying only the BAC vector even though neither strain could produce the A21978C lipopeptides.

It was difficult to accurately quantify the A21978C lipopeptides produced in crude broth by these strains due to co-chromotography with host peaks. A total maximum yield of the three main A21978C factors was estimated at around 20 mg/l. These were produced early in the fermentation along with numerous other host metabolites. One series of host metabolites caused particular problems for A21978C analysis. These had UV/visible spectra with λ_{max} at 222, 281 and 345 nm. LC-MS analysis suggested molecular weights of 1,494, 1,500 and 1,514. These are similar, but not identical to, the non-phosphorylated members of the CDA complex

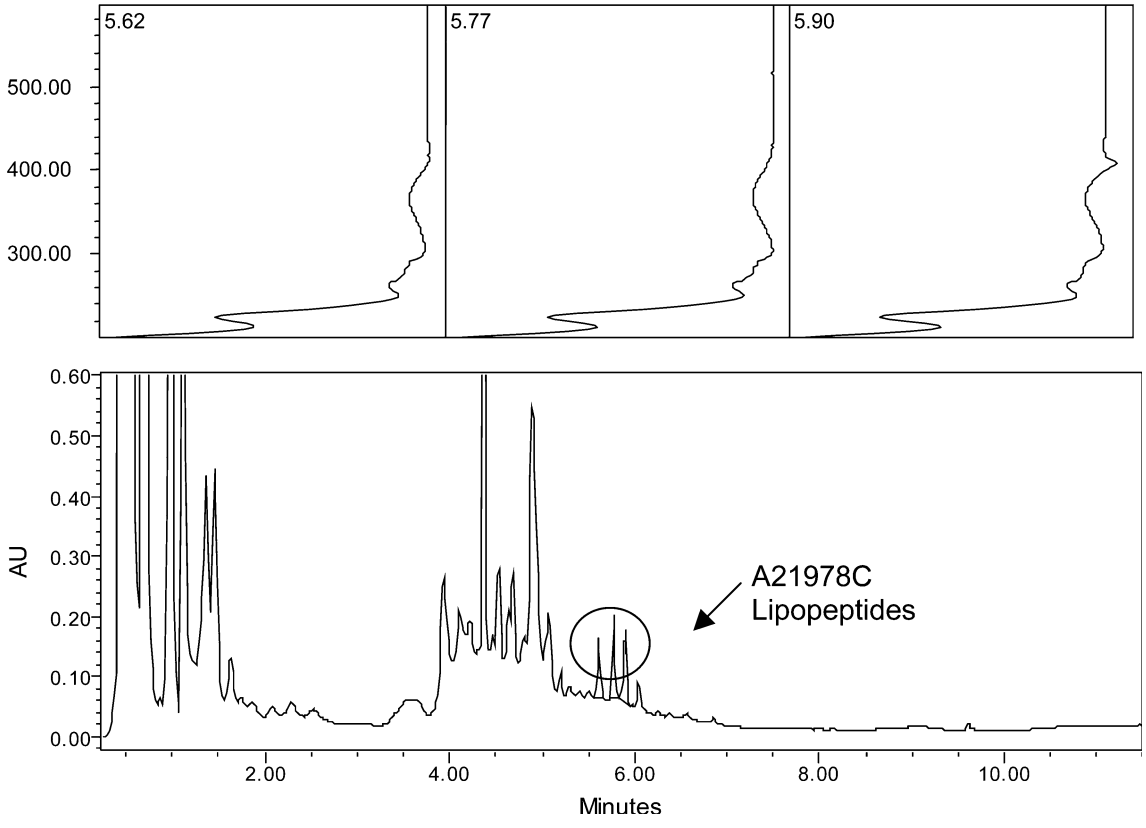


Fig. 4 HPLC chromatogram of the broth of the *S. lividans* TK64 clone containing the *dpt* cluster. The three major A21978C lipopeptides at retention times 5.62, 5.77 and 5.90 min were identified by their characteristic UV-visible spectra.

(1,494 corresponds to CDA4a; 1,500 and 1,514 have not previously been reported; the UV-visible maxima correspond to members of the complex containing a *Z*-2,3-dehydrotryptophan residue) [8]. For further characterization of these compounds, a number of other *S. lividans* strains containing smaller DNA fragments cloned from *S. roseosporus* that were known not to produce A21978C [17] were examined. One of these, CBUK136744, containing an 90 kbp DNA insert, was an effective producer of the putative CDA factors and the absence of A21978C production yielded a simpler chromatographic profile, facilitating purification. A 5l fermentation was conducted in multiple shake flasks and the component produced in the highest abundance (that with putative molecular weight 1,514) was purified by preparative HPLC. NMR spectroscopic analysis clearly indicated the presence of two tryptophan residues, and supported the presence of one hydroxyphenylglycine residue, one serine and at least two aspartate residues and methyl groups consistent with the presence of 3-methyl-glutamic acid and a short alkyl chain. These features are as expected for a member of the CDA complex. The compound was not characterized further.

The actinorhodin biosynthetic pathway (*act*) in TK23 was inactivated by replacement of a portion of the gene cluster with a resistance gene (Fig. 3). The profile of production of the A21978C lipopeptides remained unaffected in fermentations of the *S. lividans* CBUK137024 (act^-) in medium A. The absence of an intact *act* pathway in this strain allowed application of the defined medium B, in which normally high levels of actinorhodin are supported. Variations of the defined medium were evaluated and a 2–4 g/l level of K_2HPO_4 was found to be advantageous for both production of the A21978C lipopeptides and suppression of some of the host metabolites. Figure 5 shows the production of the A21978C lipopeptides by *S. lividans* CBUK137024 over time in medium B with and without the phosphate supplementation. Although antibiotic biosynthesis is generally suppressed by such levels of inorganic phosphate, the decrease in the complexity of the chromatographic profiles during the early stages of the fermentation was striking. It is possible that, in this case, the production of other metabolites by pathways more sensitive to phosphate than the daptomycin biosynthetic pathway was repressed. If these pathways had previously competed with the daptomycin pathway for precursors, this may have led to a greater availability of precursors for daptomycin biosynthesis. Figure 6a shows the much simpler HPLC profile obtained from broths of this strain grown in a phosphate supplemented medium at 50 h. As the fermentation progressed, the level and diversity of host metabolites increased, although never to the level previously observed in medium A. Although the production of many host metabolites was suppressed early in the fermentation, the production of the CDA lipopeptides was not. CDA can exist in both non-phosphorylated and phosphorylated forms [8, 13]. Under the chromatographic conditions used, the non-phosphorylated forms of CDA co-chromatographed in the same region as the A21978C lipopeptides and complicated detection and quantification. Incorporation of phosphate into the fermentation medium biased production, at least initially, to the phosphorylated forms of CDA, which were well resolved from the three A21978C lipopeptides by HPLC. These compounds had slightly different UV/visible characteristics compared to the non-phosphorylated forms of CDA observed previously, with maxima at 222 and 281 nm only (indicating the absence of *Z*-2,3-dehydrotryptophan). LC-MS analysis yielded data suggesting putative molecular weights of 1,562, 1,576, 1,578 and 1,592. As for the non-phosphorylated forms observed in earlier fermentations, these molecular weights are not identical but overlap with those of the known members of the CDA complex. The compounds with putative masses 1,562 and 1,576

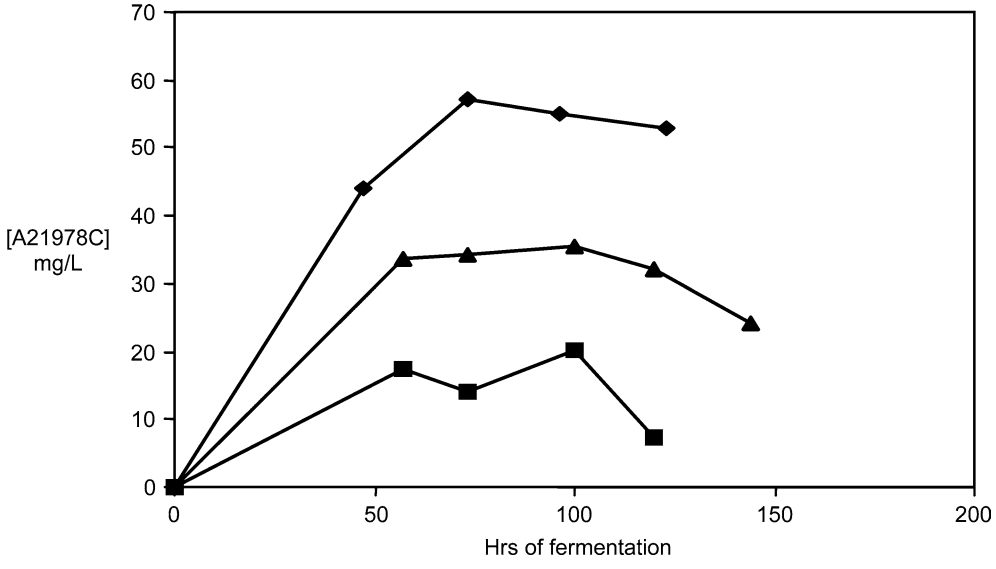


Fig. 5 Production of A21978C lipopeptides over time by the *S. lividans* CBUK 137024 in medium B supplemented with phosphate. (*filled square*) Medium B; (*filled triangle*) medium B plus 2 g/l K_2HPO_4; (*filled diamond*) medium B plus 3 g/l K_2HPO_4

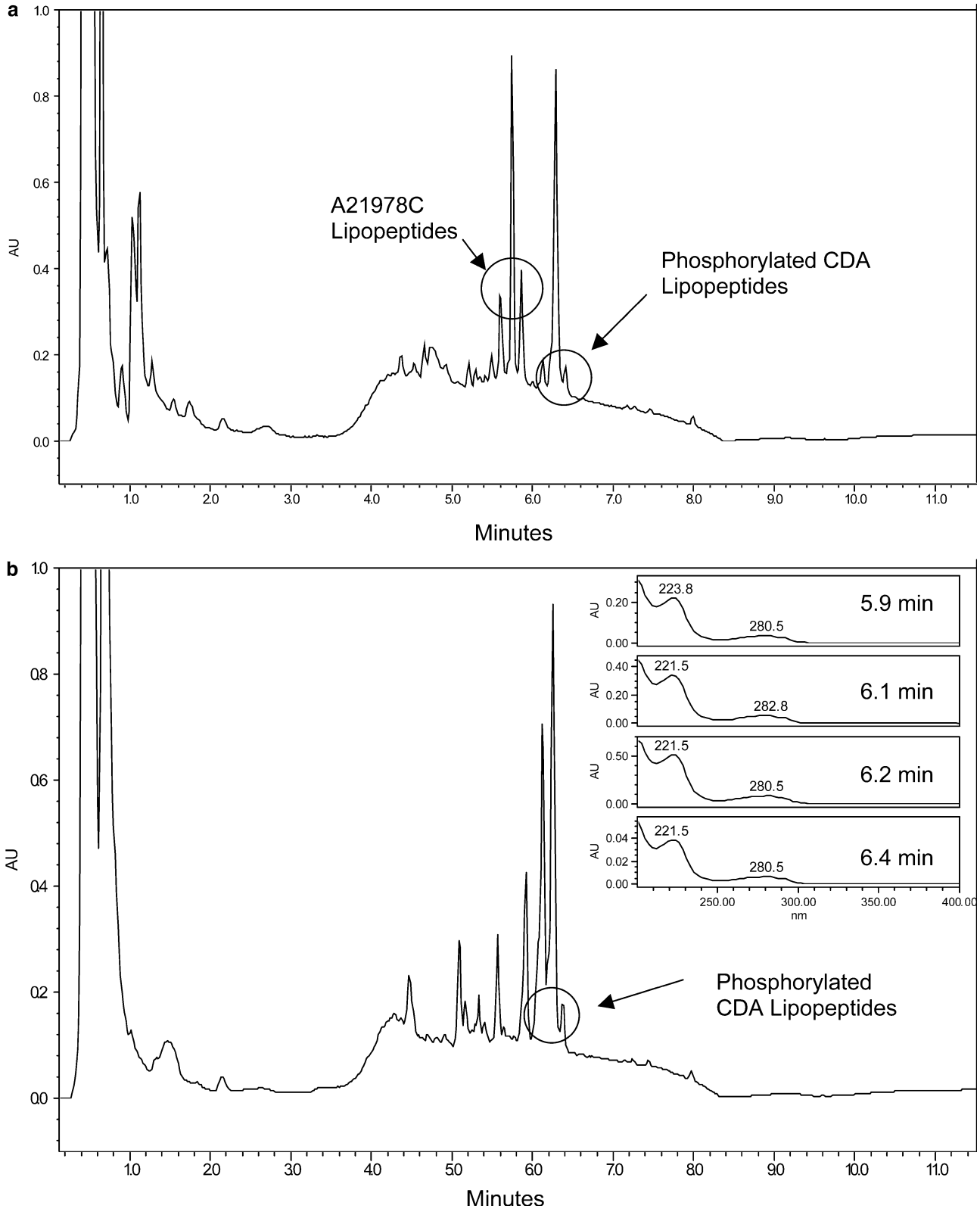


Fig. 6 **a** HPLC profile of A21978C lipopeptides produced in medium B plus 2 g/l K_2HPO_4 by *S. lividans* CBUK 137024. **b** HPLC profile of CDA lipopeptides produced in medium B plus 2 g/l K_2HPO_4 by *S. lividans* CBUK 137026. CDA lipopeptides were identified by their characteristic UV-visible spectra

may tentatively be identified as CDA1b and CDA2b respectively while those with putative masses 1,578 and 1,592 do not correspond to any compounds reported previously. These compounds have not been characterized further. This effect on CDA production in high phosphate supplemented medium B was also clearly evident from fermentation of the control *S. lividans* CBUK137026 containing pStreptoBAC V only (Fig. 6b).

In conclusion, we demonstrated that daptomycin can be produced in *S. lividans* when the daptomycin gene cluster is inserted site-specifically in the chromosomal ϕC31 *attB* site. The yields were improved to 55 mg/l, about one-third the amount produced by wild type *S. roseosporus*, by adjusting the level of phosphate in the medium, and compound isolation was facilitated by deleting the *act* gene cluster. Surprisingly, the conditions that favored the expression of the heterologous daptomycin gene cluster also enhanced the expression of the otherwise cryptic CDA pathway in all the modified TK23 and TK64 *S. lividans* strains studied. Since both lipopeptide pathways compete for some of the same precursors, deletion of the CDA pathway should facilitate further improvement in daptomycin yields, and product isolation. The cloning of complete antibiotic or other secondary metabolite pathways on BAC vectors, coupled with the stable insertion into the chromosome of robust streptomycetes, such as *S. lividans* in the present work, should serve as a particularly useful approach to express biosynthetic gene clusters from slow-growing actinomycetes not amenable to facile scale-up in large scale fermentation.

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