

# Formation and Attachment of the Deoxysugar Moiety and Assembly of the Gene Cluster for Caprazamycin Biosynthesis<sup>∇†</sup>

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Caprazamycins are antimycobacterials produced by *Streptomyces* sp. MK730-62F2. Previously, cosmid cpzLK09 was shown to direct the biosynthesis of caprazamycin aglycones, but not of intact caprazamycins. Sequence analysis of cpzLK09 identified 23 genes involved in the formation of the caprazamycin aglycones and the transfer and methylation of the sugar moiety, together with genes for resistance, transport, and regulation. In this study, coexpression of cpzLK09 in *Streptomyces coelicolor* M512 with pRHAM, containing all the required genes for dTDP-L-rhamnose biosynthesis, led to the production of intact caprazamycins. *In vitro* studies showed that Cpz31 is responsible for the attachment of the L-rhamnose to the caprazamycin aglycones, generating a rare acylated deoxyhexose. An L-rhamnose gene cluster was identified elsewhere on the *Streptomyces* sp. MK730-62F2 genome, and its involvement in caprazamycin formation was demonstrated by insertional inactivation of *cpzDIII*. The L-rhamnose subcluster was assembled with cpzLK09 using Red/ET-mediated recombination. Heterologous expression of the resulting cosmid, cpzEW07, led to the production of caprazamycins, demonstrating that both sets of genes are required for caprazamycin biosynthesis. Knockouts of *cpzDI* and *cpzDV* in the L-rhamnose subcluster confirmed that four genes, *cpzDII*, *cpzDIII*, *cpzDIV*, and *cpzDVI*, are sufficient for the biosynthesis of the deoxysugar moiety. The presented recombinering strategy may provide a useful tool for the assembly of biosynthetic building blocks for heterologous production of microbial compounds.

Caprazamycins are potent antimycobacterials isolated from *Streptomyces* sp. MK730F-62F2 (23). In a pulmonary tuberculosis mouse model, they showed a therapeutic effect but no significant toxicity (24). The caprazamycins are assigned to the translocase I inhibitors (27) due to their structural similarity to the liposidomycins (34, 43), which have been studied in more detail. Translocase I catalyzes the first step in the membrane-linked reaction cycle of bacterial cell wall formation (52): the transfer of phospho-N-acetylmuramic acid-L-Ala-γ-D-Glu-m-diaminopimelic acid-D-Ala-D-Ala from UMP to the lipid carrier undecaprenyl phosphate. Structurally unique in nature, the caprazamycins and liposidomycins share a 5'-β-O-amino-ribosyl-glycyluridine and a rare N-methylated diazepanone as their characteristic feature (Fig. 1) (25, 53). Attached at the 3'' position are β-hydroxylated fatty acid groups of different chain lengths, carrying a 3-methylglutarate. While the liposidomycins are sulfated, the caprazamycins lack this group. Instead, they are glycosylated with a 2,3,4-O-methyl-L-rhamnose and therefore belong to the large number of bioactive compounds containing 6-deoxyhexoses. Usually, these moieties contribute significantly to the compounds' properties, influencing, e.g., molecule-target interactions, cell import and export, pharma-

cokinetics, and solubility (56). The biosynthesis of deoxysugars has been studied in detail and generally starts from NDP-activated hexoses via 4-keto-6-deoxy intermediates (36). The formation of L-rhamnose involves four enzymes, a dTDP-D-glucose synthase, a dTDP-D-glucose 4,6-dehydratase, a dTDP-3,5-deoxyglucose epimerase, and a 4-ketoreductase. Recent advances in combinatorial biosynthesis have led to a variety of novel natural products with an engineered glycosylation pattern and altered bioactivity (37, 44). The key step in this approach is the attachment of different deoxysugars to the aglycones, which demands substrate-flexible glycosyltransferases.

We recently reported cloning and heterologous expression of cosmid cpzLK09 containing the first identified gene cluster of a translocase I inhibitor, the caprazamycins. However, genes for the formation of the dTDP-L-rhamnose could not be identified on this cosmid (Fig. 1) and therefore only caprazamycin aglycones were produced by the heterologous host, *Streptomyces coelicolor* M512/cpzLK09 (31). The absence of genes for the formation of the L-rhamnose moiety within the corresponding biosynthetic gene cluster has previously been reported for aranciamycin (38), steffimycin (16), spinosyn (55), and elloramycin (9). Since potential genes for O methylation (*cpz28* to *cpz30*) and a glycosyltransferase (*cpz31*) are encoded in the caprazamycin gene cluster, we speculated that only four genes are missing for successful heterologous production of intact caprazamycins.

Here we report the identification of the genes required for the biosynthesis of the caprazamycin deoxysugar moiety elsewhere on the genome of *Streptomyces* sp. MK730-62F2. A new

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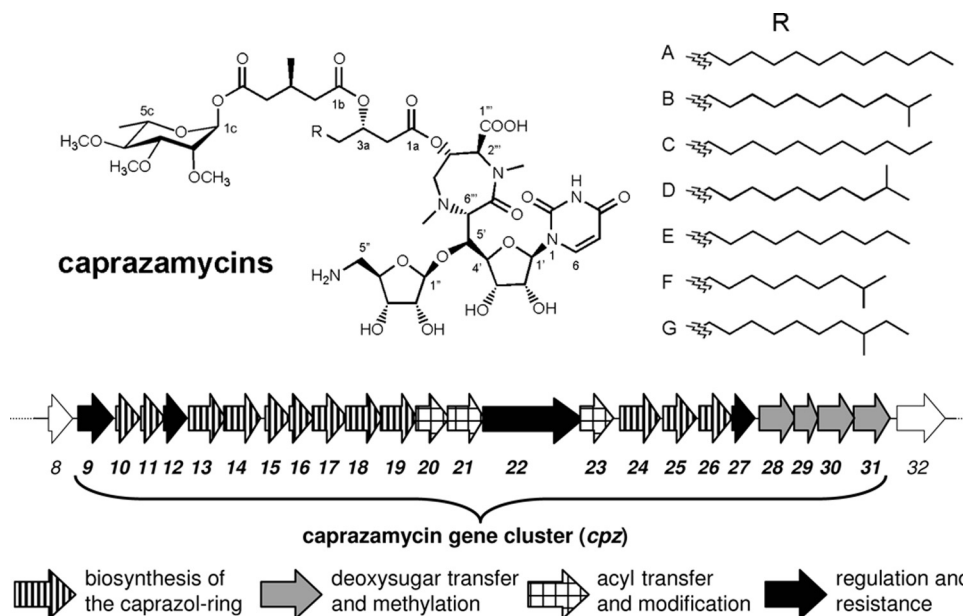


FIG. 1. Structure of caprazamycins and organization of the caprazamycin gene cluster (*cpz9* to *cpz31*) lacking the genes for deoxysugar formation. Assignments of genes to different steps in the biosynthesis are indicated.

strategy was developed, based on Red/ET-mediated recombination, to assemble the identified subcluster into cosmid cpzLK09. Expression of the assembled cluster readily resulted in the production of intact caprazamycins in the heterologous producer strain. Moreover, *in vitro* studies demonstrated that Cpz31 is the glycosyltransferase in caprazamycin biosynthesis.

#### MATERIALS AND METHODS

**Bacterial strains and general methods.** Chemicals and microbiological and molecular biological agents were purchased from standard commercial sources. *Streptomyces* sp. MK730-62F2 and *S. coelicolor* M512 (SCP1<sup>-</sup> SCP2<sup>-</sup>,  $\Delta$ *actIIorf4*  $\Delta$ *redD*) and their respective derivatives were maintained and grown on either MS agar (2% soy flour, 2% mannitol, 2% agar [components purchased from Carl Roth, Karlsruhe, Germany]) or tryptic soy broth (TSB) medium (Becton Dickinson, Heidelberg, Germany). *Escherichia coli* strains were cultivated in LB medium (components purchased from Carl Roth, Karlsruhe, Germany) supplemented with appropriate antibiotics. DNA isolation and manipulations were carried out according to standard methods for *E. coli* (51) and *Streptomyces* (33).

**Production, extraction, and detection of caprazamycin derivatives.** Fifty milliliters of TSB medium was inoculated with a spore suspension of *Streptomyces* sp. MK730-62F2, *S. coelicolor* M512, or a derivative thereof. The cultures were incubated for 2 days at 30°C and 200 rpm. For the production of caprazamycins, 1 ml of the precultures was inoculated into 100 ml of a medium containing 1% soytone, 1% soluble starch, and 2% D-maltose adjusted to pH 6.7 (components purchased from Becton Dickinson, Heidelberg, Germany). The cultures were incubated for 7 days at 30°C and 200 rpm. Partial purification of caprazamycins was achieved by extraction of the culture supernatant (adjusted to pH 4) with an equal volume of *n*-butanol. The organic phase was evaporated, and extracts were resolved in 500  $\mu$ l methanol. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed on a Surveyor high-performance LC (HPLC) system equipped with a Repronil-Pur Basic C<sub>18</sub> column (5  $\mu$ m; 250 by 2 mm; Maisch, Ammerbuch, Germany) coupled to a Thermo Finnigan TSQ Quantum triple-quadrupole mass spectrometer (heated capillary temperature, 320°C; sheath gas, nitrogen; collision gas, argon). Analysis of extracts from the coexpression of pRHAM experiments was performed with a linear gradient of 2% to 40% acetonitrile in aqueous formic acid (0.1%) over 5 min followed by a linear gradient of 40% to 100% acetonitrile in aqueous formic acid (0.1%) over 20 min. Otherwise, a linear gradient of 2% to 40% acetonitrile in aqueous formic acid (0.1%) over 4 min was used followed by a linear gradient of 40% to 100% acetonitrile in aqueous formic acid (0.1%) over 31 min. The flow rate was 0.2

liter min<sup>-1</sup>, and detection was at 262 nm. Positive electrospray ionization [(+)-ESI] was performed with an electrospray voltage of 3.8 kV, and collision-induced dissociation (CID) spectra were recorded with a collision energy of 35 eV.

**Coexpression of plasmid pRHAM.** Plasmid pRHAM (49), containing all required genes for the biosynthesis of dTDP-L-rhamnose *oleL*, *oleS*, *oleE*, and *oleU* from *Streptomyces antibioticus* ATCC 1891, was introduced into the nonmethylating strain *E. coli* ET12567 (40) and reisolated. The isolated plasmid DNA was used for polyethylene glycol (PEG)-mediated protoplast transformation as described by Kieser et al. (33) with either *Streptomyces* sp. MK730-62F2  $\Delta$ *cpzDIII* for complementation of the *cpzDIII* knockout or with *S. coelicolor*/cpzLK09 for the production of intact caprazamycins. For the preparation of protoplasts, the corresponding *Streptomyces* strain was cultivated in 100 ml S-YEME broth (34% sucrose, 1% glucose, 0.5% peptone, 0.3% yeast extract, 0.3% malt extract, 5 mM MgCl<sub>2</sub>; components purchased from Carl Roth, Karlsruhe, Germany) for 48 h at 30°C. Cells were harvested and washed with an aqueous solution of 10.3% sucrose. The degradation of the cell wall was performed at 30°C in P buffer [10.3% sucrose, 0.025% K<sub>2</sub>SO<sub>4</sub>, 0.202% MgCl<sub>2</sub> · 6H<sub>2</sub>O, 0.005% KH<sub>2</sub>PO<sub>4</sub>, 0.368% CaCl<sub>2</sub> · 2H<sub>2</sub>O, and 0.573% *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] supplemented with 4 mg/ml lysozyme (Serva, Heidelberg, Germany) and monitored by microscopy. Subsequently, PEG-mediated protoplast transformation was performed. Thiostrepton resistance mutants were selected and termed either *Streptomyces* sp. MK730-62F2/ $\Delta$ *cpzDIII*/pRHAM-(1-3) or *S. coelicolor*/cpzLK09/pRHAM-(1-3).

**Overexpression and purification of Cpz31.** *cpz31* was amplified from cosmid DNA using the primers cpzGTeco\_fw, GAATTCATGCGCGTGCTCTTC ACG, and cpzGTHind\_rv, AAGCTTCTAGGCCCTCCTCGCCAG (restriction sites for EcoRI and HindIII are underlined). A 1.2-kb PCR product was cloned into the pGEM-T cloning vector (Promega, Mannheim, Germany). The resulting plasmid, pLK01, was verified by sequencing. *cpz31* was subsequently cloned into the expression vector pHis8 (29), taking advantage of the EcoRI and HindIII restriction sites, and confirmed by sequencing. The resulting plasmid was named pLK02.

*E. coli* Rosetta2 (DE3)pLys (Novagen, Darmstadt, Germany) cells containing plasmid pLK02 were cultivated in 1 liter of TB broth (1.2% tryptone, 2.4% yeast extract, 0.4% glycerol, 0.23% KH<sub>2</sub>PO<sub>4</sub>, 1.25% K<sub>2</sub>HPO<sub>4</sub>) supplemented with 50  $\mu$ g ml<sup>-1</sup> kanamycin and 50  $\mu$ g ml<sup>-1</sup> chloramphenicol at 37°C. At an optical density at 600 nm of 0.7, the temperature was adjusted to 20°C, and isopropyl thiogalactoside (IPTG) was added to a 0.5 mM final concentration. After an additional 10-h cultivation at 20°C, the culture was harvested and 10 ml of buffer A (50 mM Tris-HCl [pH 8], 1 M NaCl, 10% glycerol, 20 mM imidazole, 10 mM  $\beta$ -mercaptoethanol) supplemented with 0.5 mg ml<sup>-1</sup> lysozyme and 0.5 mM phenylmethylsulfonyl fluoride was added to the pellet (12 g). Cells were dis-

rupted by sonication (Branson, Danbury, CT) at 4°C. The lysate was centrifuged (55,000 × g, 45 min), and the supernatant was applied to an affinity chromatography column with 4 ml of Ni-nitrilotriacetic acid (NTA)-agarose resin (Qiagen, Hilden, Germany) according to the manufacturer's instructions. His<sub>8</sub>-Cpz31 was eluted from the column with 250 mM imidazole in buffer A. Buffer exchange was carried out with PD10 columns (Amersham Biosciences, Freiburg, Germany) and buffer A without β-mercaptoethanol and imidazole. The low solubility of His<sub>8</sub>-Cpz31 resulted in a yield of 0.46 mg of partially purified protein per liter of culture. The protein was stored at -80°C in aliquots of 50 μl.

**Assay for glycosyltransferase activity.** Partially purified caprazamycin aglycones were obtained from a culture extract of *S. coelicolor* M512/cpzLK09. Five microliters of caprazamycin aglycones in methanolic solution was applied to a reaction tube, air dried, and dissolved in a 50-μl reaction mixture containing 50 mM Tris-HCl, pH 7.5, 5 mM MgCl<sub>2</sub>, 5 mM dTDP-L-rhamnose, and 9 μM protein. The assay was performed at 30°C for 2 h and stopped by the addition of 150 μl methanol. The activity of His<sub>8</sub>-Cpz31 was measured by LC-ESI-MS.

**Screening of the cosmid library.** The construction of the genomic cosmid library of *Streptomyces* sp. MK730-62F2 has been described previously (31). A 0.5-kb fragment of *cpzDIII* was amplified from genomic DNA by using a degenerated primer as described by Decker et al. (8). Perfect matching primers GDHsp<sub>f</sub>\_fw, CGTAGTTGTTGGAGCAGCGCG, and GDHsp<sub>r</sub>\_rv, GGTTCACCGCTCCCGCTACG, amplifying a 0.5-kb fragment of *cpzDIII*, were applied in a PCR screening of the cosmid library.

**DNA sequencing and computer-assisted sequence analysis.** Double-stranded sequencing of the entire cosmid clone 4H11 (39,442-bp insert) was performed by GenoTech (Baejeon, South Korea) by using a shotgun library with DNA fragments of approximately 0.5 to 1.0 kb in length. The DNASIS software package (Hitachi Software Engineering, Tokyo, Japan) and Artemis (Wellcome Trust Genome Campus, Cambridge, United Kingdom) were used for sequence analysis and annotation. Database comparisons were carried out in the GenBank database by using the BLAST program (1). Alignment and comparison of sequences were performed using the ClustalX algorithm (28) and GeneDoc alignment editor (<http://www.psc.edu/biomed/genedoc>).

**Inactivation of *cpzDIII* in *Streptomyces* sp. MK730-62F2.** An apramycin resistance cassette [*aac(3)IV*] was amplified from plasmid pIJ773 (18) using the primer pair *gdhKO\_fw*, GTGCTGGACAGCTCACCTACGCCGGCACCCCTC GACGACATCCGGGATCCGTCGACC, and *gdhKO\_rv*, GAGCTGGCAC GGGCCGTAGTTGTTGGAGCAGCGCGTACTGTAGGCTGGAGCTGC TTC. Italic letters represent 39-nucleotide (nt) homologous extensions for Red/ET-mediated recombination. The gene was replaced in *E. coli* BW25113/pIJ790/4H11 by using Red/ET-mediated recombination (18), and the resulting cosmid, cpzLK01, was confirmed by restriction analysis. cpzLK01 was transferred into the nonmethylating strain *E. coli* ET12567/pUZ8002 (46) and introduced into *Streptomyces* sp. MK730-62F2 by conjugation. Exconjugants, resistant to apramycin, were isolated and tested for the loss of their kanamycin resistance, indicating a successful double crossover. The mutants were further analyzed by PCR with chromosomal DNA as the template. Mutant strains were designated *Streptomyces* sp. MK730-62F2Δ*cpzDIII*-(1-3). Complementation of the mutants was performed by coexpression of plasmid pRHAM as described above.

**Assembly of the caprazamycin biosynthetic gene cluster and the L-rhamnose subcluster.** An apramycin resistance cassette [*aac(3)IV*] was amplified by PCR from plasmid pIJ774 (19) with primers 4H11\_774\_fw, GCCGAGCACCCGCA GCGGGTTCGCCCGGAGCCAGCAGCTAGCTTATGAGCAGCCAATC GAC, including a NheI restriction site (bold), and 4H11\_774\_rv, CAGAGGGT GCGCTCGGAAGACGGCGCGGAAGCCGCCGACTAGTATCCGGGG ATCCGTCGACC, including a SpeI restriction site (bold). Italic letters represent 39-nt homologous extensions to an internal region of *orf1* and to a region downstream of *orf9*, respectively. The resulting 1,310-bp PCR product contained the *aac(3)IV* gene and a single loxP site. The PCR product was inserted into cosmid 4H11 by Red/ET-mediated recombination (18), thereby replacing a 13,531-bp region (*orf1* to *orf9*) upstream of *cpzDI*, resulting in the apramycin- and kanamycin-resistant cosmid cpzEW01. A hygromycin resistance cassette (*hyg*) was amplified from pJ10700 by PCR with primers 4H11\_10700\_fw, TGACGGGGCGGGCGGGCTCGCAGCCCTAGGCTGAGGAGA TTTAAATTTGAGGCTGGAGCTGCTC, including a SmaI restriction site (bold), and 4H11\_10700\_rv, GGGGGCGGTCGCCGAAGTGGCGGGCCCGG GCGGGCACGGGTTTAAACATCCGGGGATCCGTCGACC, including a PmeI restriction site (bold). Italic letters represent 39-nt homologous extensions to sequences upstream of *orf10* and to an internal region of *orf25*, respectively. The resulting 1,678-bp PCR product contained the hygromycin resistance gene *hyg*, an origin of transfer, *oriT*, and was flanked by two FLP recombinase recognition sites (FRT). The PCR product was inserted into cosmid cpzEW01 by Red/ET-mediated recombination, thereby replacing a 16,144-bp region (*orf10* to

*orf25*) upstream of *cpzDVII* and resulting in the hygromycin-, apramycin-, and kanamycin-resistant cosmid cpzEW02. Restriction of cpzEW02 with NheI and PmeI generated the 9,950-bp L-rhamnose subcluster fragment (*cpzDI* to *cpzDVII*) flanked by the apramycin and the hygromycin resistance cassettes.

Cosmid cpzLK09, which already contained the ΦC31 integration cassette from pIJ787 (10), was prepared as the L-rhamnose subcluster acceptor cosmid. An apramycin resistance cassette [*aac(3)IV*] was amplified from pIJ774 (18) by PCR with primers 31C2\_774\_fw2, TGCCGTACCCCGGGTACCAGGTTCCGCTCGGCG GGTGCACTAGTTGTAGGCTGGAGCTGCTC, including a SpeI restriction site (bold), and 31C2\_774\_rv, CGTGCTGGTGTCCGCCAACCCCATATGCGGGTG GGCATGCTAGCAAATGCCGCTTGAATGG, including a NheI restriction site (bold). Italic letters represent 39-nt homologous extensions to sequences downstream of *cpz31* and to an internal region of *cpz33*, respectively. The resulting 1,079-bp PCR product contained the *aac(3)IV* gene and a single loxP site. The PCR product was inserted into cosmid cpzLK09 by Red/ET-mediated recombination, thereby replacing a 1,000-bp region downstream of *cpz31* and resulting in the apramycin- and kanamycin-resistant cosmid cpzEW03. A spectinomycin/streptomycin resistance cassette (*aadA*) was amplified from pIJ778 (18) by PCR with primers 31C2\_778\_fw, TCGTCAGATGCAGATGGACGTCGTGCCCGCGGTTCTA TTTAAATTTATTGCGGACTACTTGG, including a SmaI restriction site (bold), and 31C2\_778\_rv, CCGCGGTGGCGCACCCAGCAGAACTGGTCCGCG GGGCCGGTTTAAACATCCGGGGATCCGTCGACC, including a PmeI restriction site (bold). Italic letters represent 39-nt homologous extensions to an internal sequence of *cpz34* and to a region 351 bp upstream of *cpz34*, respectively. The resulting 1,406-bp PCR product contained the *aadA* gene, an *oriT*, and a single FRT site. The PCR product was inserted into cosmid cpzEW03, thereby replacing the 613-bp region between *aac(3)IV* and the beginning of the SuperCos1 backbone and resulting in the spectinomycin-streptomycin-, apramycin-, and kanamycin-resistant cosmid cpzEW05.

For the generation of cpzEW07, the cosmid containing the caprazamycin gene cluster, and the L-rhamnose subcluster, Red/ET-mediated recombination was used. A Red/ET-proficient *E. coli* strain harboring cpzEW05 was transformed with the 9,950-bp NheI/PmeI fragment from cpzEW02. Homologous recombination took place between a 924-bp region, represented by the apramycin resistance gene *aac(3)IV*, and a 523-bp region of identical sequence between the hygromycin and the spectinomycin-streptomycin resistance cassettes. Positive selection with hygromycin was possible for this recombination event due to the exchange of the resistance gene *aadA* with *hyg*. Note that after Red/ET-mediated recombination, the apramycin resistance cassette is now flanked by loxP sites and the hygromycin resistance cassette by FRT sites, allowing removal of the resistance cassettes by the use of Cre recombinase or FLP recombinase, respectively. The isolated cosmid DNA of cpzEW07 was verified by restriction analysis. cpzEW07 was introduced into *S. coelicolor* M512 by triparental intergeneric conjugation with the help of *E. coli* ET12567/pUB307 (14). Apramycin-, hygromycin-, and kanamycin-resistant clones were selected and termed *S. coelicolor* M512/cpzEW07-(1-3).

**Inactivation of *cpzDI* and *cpzDV* in *S. coelicolor* M512/cpzEW07.** The apramycin resistance cassette in cosmid cpzEW07 was eliminated by SpeI restriction and religation as described elsewhere (17). The resulting cosmid cpzEW08 was verified by restriction analysis. An apramycin resistance cassette [*aac(3)IV*] was amplified from plasmid pIJ774 (18) by PCR with primers containing an AflII site (bold), KOcpzDI\_fw, GTGGAAGCAGAAGGAAGATGATGAGTCGTGGAGT CCATCTTAAGATTCCGGGGATCCGTCGACC, and KOcpzDI\_rv, GCTCG GATCGTACCCCGCTGTCCACGAGGGAGGCGTCACTTAAGTGTAGGC TGGAGCTGCTC, for the inactivation of *cpzDI*, or KOcpzDV\_fw, GCATCC GCAAGGAAGGTCTTCTCGTGAACGCCATGAGCTTAAGATTCCGGG GATCCGTCGACC, and KOcpzDV\_rv, CGCCGGCCGCCCCGGGGCGGGT GCGGGCGCGGTCGTTCACTTAAGTGTAGGCTGGAGCTGCTtc, for the inactivation of *cpzDV*. Italic letters represent 39-nt homologous extensions to sequences up- and downstream of the target genes for Red/ET-mediated recombination. Resulting cosmids cpzEW09 [Δ*cpzDI::aac(3)IV*] and cpzEW11 [Δ*cpzDV::aac(3)IV*] were confirmed by restriction analysis. Excision of the cassette was performed by *in vitro* application of Cre recombinase (New England Biolabs, Frankfurt am Main, Germany) according to the manufacturer's instructions, taking advantage of the loxP recognition sites flanking the apramycin resistance cassette. The obtained cosmids cpzEW10 (Δ*cpzDI*) and cpzEW12 (Δ*cpzDV*) were screened for apramycin sensitivity and verified by restriction analysis. Cosmids cpzEW10 and cpzEW12 were transferred into *E. coli* ET12567 (40) and introduced into *S. coelicolor* M512 by triparental intergeneric conjugation with the help of *E. coli* ET12567/pUB307 (14). Kanamycin resistance exconjugants were selected and designated *S. coelicolor* M512/cpzEW10-(1-3) (Δ*cpzDI*) and *S. coelicolor* M512/cpzEW12-(1-3) (Δ*cpzDV*).



**Nucleotide sequence accession number.** The nucleotide sequence of the gene cluster for dTDP-L-rhamnose biosynthesis, *cpzDI* through *cpzDVII*, has been deposited in GenBank under the accession number HM051054.

## RESULTS

**Coexpression of pRHAM in *S. coelicolor* containing the caprazamycin gene cluster.** Previously, we reported that heterologous expression of cosmid cpzLK09, containing the caprazamycin gene cluster, resulted in the accumulation of caprazamycin aglycones in *S. coelicolor* M512 (31). The production of the nonglycosylated caprazamycin derivatives correlates with the absence of putative genes for the deoxysugar formation within the gene cluster. In order to investigate if all other genes required for the biosynthesis of the intact caprazamycins are functionally expressed on cpzLK09, we introduced plasmid pRHAM (49) into the heterologous host *S. coelicolor* M512/cpzLK09. pRHAM contains all genes required for dTDP-L-rhamnose biosynthesis from *S. antibioticus*: *oleS*, encoding a dTDP-glucose synthase, *oleE*, a dTDP-glucose 4,6-dehydratase, *oleL*, a dTDP-deoxyglucose 3,5-epimerase, and *oleU*, a 4-ketoreductase. Three individual clones termed *S. coelicolor* M512/cpzLK09/pRHAM-(1-3) were selected by their thiostrepton resistance and cultivated in production medium. Analysis of the culture extracts by LC-ESI-MS revealed that in comparison to *S. coelicolor* M512/cpzLK09, which only produced the caprazamycin aglycones (Fig. 2B; CPZ aglycones E/F with a retention time [Rt] of 18.2 min, CPZ aglycones C/D/G with Rt of 19.0 min, and CPZ aglycones A/B with Rt of 20.2 min), all mutant strains now accumulated additional metabolites (Fig. 2C). These new compounds correspond to the caprazamycins E/F (Rt, 20.0 min), caprazamycins C/D/G (Rt, 20.9 min), and caprazamycins A/B (Rt, 22.3 min), as found by ESI-MS product ion scans (see Fig. S1 in the supplemental material). MS/MS spectra from CID experiments (see Fig. S1) matched exactly the characteristic fragmentation profiles of the intact caprazamycins (31). This verified that the enzymes for methylation and attachment of the L-rhamnose moiety have to be encoded on the gene cluster. A set of genes, *cpz28* to *-31*, encoding three hypothetical sugar *O*-methyltransferases and a putative rhamnosyltransferase, are the most likely candidates for these reactions.

**In vitro investigation of Cpz31 with dTDP-L-rhamnose as substrate.** Cpz31 shows high sequence homology to known rhamnosyltransferases from other antibiotic gene clusters, e.g., ElmGT from *S. olivaceus* (38% identity, 50% similarity) (4). pLK02 containing *cpz31* was overexpressed in *E. coli*, and the resulting protein was purified for *in vitro* studies. His<sub>8</sub>-Cpz31 consists of 420 amino acids with a calculated molecular mass of 44.7 kDa. Expression of a corresponding protein could be observed after induction with IPTG (see Fig. S2 in the supplemental material). Solubility of the enzyme was low, and the yield could not be improved using different culture temperatures or IPTG concentrations for expression. However, His<sub>8</sub>-Cpz31 was partially purified by Ni-NTA chromatography, resulting in 0.85 mg of protein from a 1-liter culture (see Fig. S2). A butanolic extract from an *S. coelicolor* M512/cpzLK09 culture containing caprazamycin aglycones was incubated with His<sub>8</sub>-Cpz31 in the presence of dTDP-L-rhamnose. After 2 h, caprazamycin aglycones could not be observed in the assay

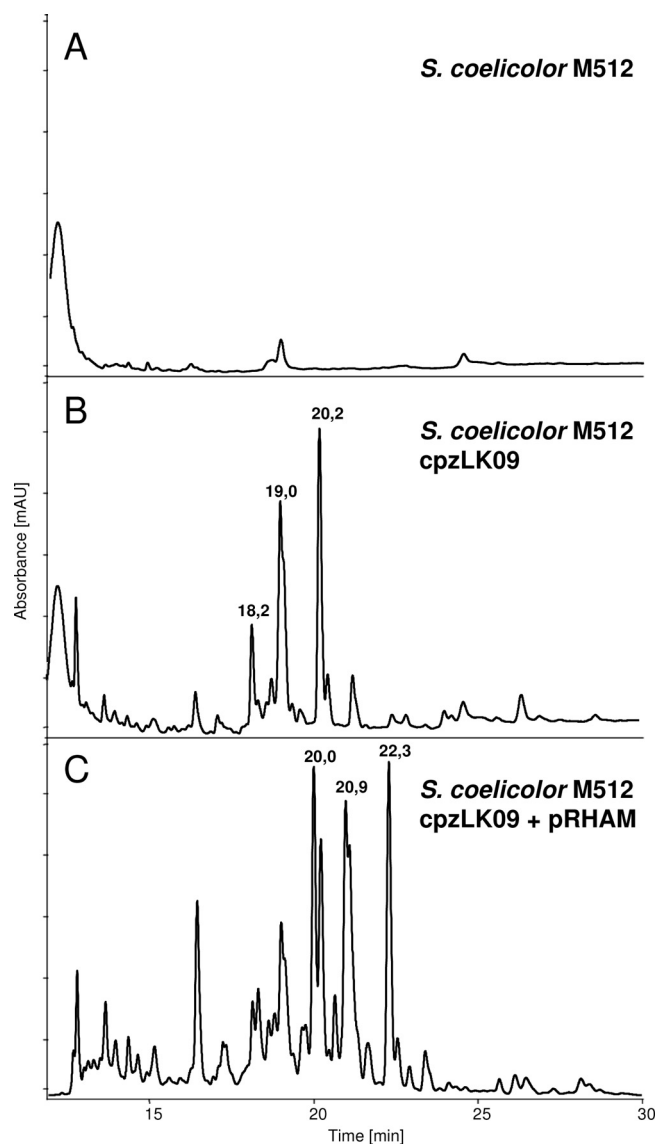


FIG. 2. HPLC chromatograms of *n*-butanolic extracts from *S. coelicolor* M512 (A), *S. coelicolor* M512 containing the caprazamycin gene cluster cpzLK09 (B) and *S. coelicolor* M512/cpzLK09 containing pRHAM coexpressing the biosynthetic genes for dTDP-L-rhamnose (C).

mixture anymore, and instead three new peaks appeared in the LC chromatograms (Fig. 3B; at Rts of 17.2 min, 18.2 min, and 19.7 min), indicating product formation. Precursor ions for the rhamnosylated caprazamycins E/F with  $m/z$  1,076.6 [M+H]<sup>+</sup>, C/D/G with  $m/z$  1,090.6 [M+H]<sup>+</sup>, and A/B with  $m/z$  1,104.6 [M+H]<sup>+</sup> were readily detected by ESI-MS product ion scans at Rts of 17.4 min, 18.5 min, and 19.9 min, respectively (Fig. 3C). The MS/MS spectrum obtained from the parent ion with  $m/z$  1,090.6 [M+H]<sup>+</sup> (Fig. 3D) was identical to the predicted fragmentation of the rhamnosylated caprazamycins C/D/G (Fig. 3A). Characteristic ions with  $m/z$  1,091,  $m/z$  960, and  $m/z$  848 displayed the presence of the rhamnosyl moiety. Similar spectra were produced by the parent ions with  $m/z$  1,076.6 [M+H]<sup>+</sup> and  $m/z$  1,104.6 [M+H]<sup>+</sup>, representing the rhamno-

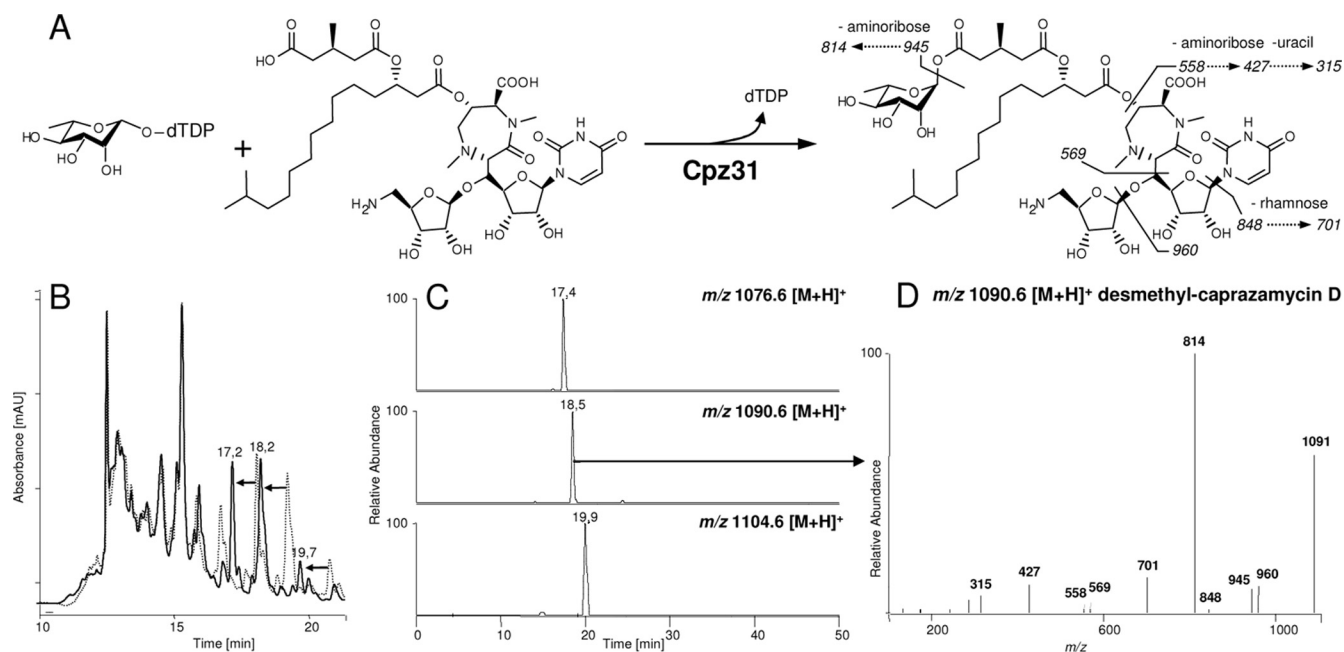


FIG. 3. Glycosylation of caprazamycins by Cpz31. (A) Reaction scheme of Cpz31 with dTDP-L-rhamnose and caprazamycin D aglycone as substrates. The suggested fragmentation of the reaction product 2c,3c,4c-desmethyl-caprazamycin D is depicted. (B) Overlay of HPLC chromatograms (262 nm) from activity assays with Cpz31 (black line) and without protein (dashed line). The observed shift in retention time by the complete conversion of caprazamycin aglycones to the rhamnosylated product is indicated by bold arrows. (C) LC-ESI-MS product ion scans in positive mode for desmethyl-caprazamycins E/F with  $m/z$  1,076.6  $[M+H]^+$ , for desmethyl-caprazamycins C/D/G with  $m/z$  1,090.6  $[M+H]^+$ , and for desmethyl-caprazamycins A/B with  $m/z$  1,104.6  $[M+H]^+$  in the activity assay with Cpz31. (D) Mass spectrometric fragmentation pattern in the CID experiment for 2c,3c,4c-desmethyl-caprazamycin C/D/G.

sylated caprazamycins with different fatty acid chain lengths (data not shown). These compounds were not detectable in assay mixtures without Cpz31 or dTDP-L-rhamnose. Therefore, we conclude that Cpz31 glycosylates the caprazamycin aglycones by using dTDP-L-rhamnose as a substrate (Fig. 3A).

**Identification of genes for biosynthesis of dTDP-L-rhamnose.** In bacteria, genes directing the biosynthesis of a certain secondary metabolite are generally clustered together. However, cosmid 31C2, containing the caprazamycin gene cluster, missed the genes for the formation of the L-rhamnose moiety (31). Because these genes are required for caprazamycin biosynthesis, they have to be located elsewhere on the chromosome of the original producer strain *Streptomyces* sp. MK730-62F2. Using degenerated primers for the amplification of NDP-glucose 4,6-dehydratases (8), we obtained a 500-bp partial sequence of *cpzDIII* from genomic DNA of the caprazamycin producer. This fragment was cloned and sequenced. Annotation revealed high homology to dTDP-glucose 4,6-dehydratases on the protein level. Perfect matching primers were constructed to screen a cosmid library of *Streptomyces* sp. MK730-62F2 (31). Sequencing and annotation of a positive cosmid, 4H11, revealed a stretch of 6.8 kb of DNA putatively involved in the biosynthesis of dTDP-L-rhamnose (Fig. 4). According to the gene function predictions (Table 1), *cpzDII* encodes a putative dTDP-glucose synthase, *cpzDIII* encodes a dTDP-glucose 4,6-dehydratase, *cpzDIV* encodes a 4-ketoreductase, and *cpzDVI* encodes a dTDP-deoxyglucose 3,5-epimerase. The identified gene cluster consists of two putative operons (Fig. 4). *cpzDII*, *cpzDIII*, and *cpzDIV* are translation-

ally coupled to *cpzDV*, as indicated by overlap of the stop and start codons. The deduced gene product of *cpzDV* shows homology to hypothetical proteins from various *Streptomyces* strains which are annotated as methyltransferases. In the opposite direction, *cpzDVI* is translationally coupled with *cpzDVII*, which encodes a putative glycosyltransferase. Homologues of CpzDVII have been assigned to bacterial cell wall biosynthesis. The putative gene *cpzDI*, 105 bp upstream of *cpzDII*, encodes a small protein of 57 amino acids with several homologues from *Streptomyces* genomes. Many of them, e.g., SvirD4\_37404 from *Streptomyces viridochromogenes* DSM40736 and SAMR0798 from *Streptomyces ambofaciens* ATCC 23877, are located next

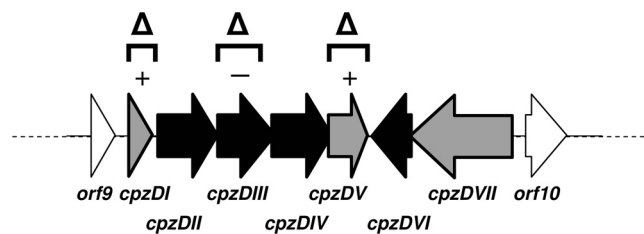


FIG. 4. Organization of the L-rhamnose gene cluster *cpzDI* to *cpzDVII* on cosmid 4H11. Black arrows indicate genes proposed to be essential in dTDP-L-rhamnose biosynthesis. Gray arrows indicate genes with unknown function. Squared brackets above the cluster mark the gene deletions performed in this study. The minus sign indicates that the deletion of the respective region led to an abolishment of caprazamycin production. Plus signs indicate that caprazamycin production was not influenced.

TABLE 1. Deduced functions of genes within the L-rhamnose subcluster

Gene <sup>a</sup>	No. of amino acids	Protein homologue (accession no.)	Overall homology <sup>b</sup>	Proposed function
<i>orf9</i>	140	SvirD4_010100037399 of <i>S. viridochromogenes</i> DSM 40736 (ZP_05536129)	87/93	Oxidoreductase
<b><i>cpzDI</i></b>	58	SAMR0798 of <i>S. ambofaciens</i> ATCC 23877 (CAJ88507)	64/75	Unknown
<b><i>cpzDII</i></b>	356	MtmD of <i>S. argillaceus</i> (CAK50774)	76/86	dTDP-glucose synthase
<b><i>cpzDIII</i></b>	327	NanG2 of <i>S. nanchangensis</i> (AAP42865)	77/83	dTDP-glucose dehydratase
<b><i>cpzDIV</i></b>	296	OleU of <i>S. antibioticus</i> (AAD55455)	63/71	4-Ketoreductase
<b><i>cpzDV</i></b>	268	SvirD4_010100037424 of <i>S. viridochromogenes</i> DSM 40736 (ZP_05536134)	96/99	Unknown
<b><i>cpzDVI</i></b>	202	StrM of <i>S. glaucescens</i> (CAA07389)	65/77	Sugar 3,5-epimerase
<b><i>cpzDVII</i></b>	647	SghaA1_010100002568 of <i>S. ghanaensis</i> ATCC 14672 (ZP_04684037)	76/82	Cell wall biosynthesis glycosyltransferase
<i>orf10</i>	170	SAMR0794 of <i>S. ambofaciens</i> ATCC 23877 (CAJ88503)	83/89	Phosphoesterase

<sup>a</sup> Genes shown in bold represent the proposed L-rhamnose subcluster.

<sup>b</sup> Homology is reported as percent identity/percent similarity.

to hypothetical NDP-glucose dehydratase genes. Since *orf9*, a putative oxidoreductase gene, and *orf10*, a possible phosphodiesterase gene, have no apparent role in deoxysugar metabolism, we proposed that the L-rhamnose biosynthetic gene cluster spans from *cpzDI* to *cpzDVII*.

#### Inactivation of *cpzDIII* and complementation with pRHAM.

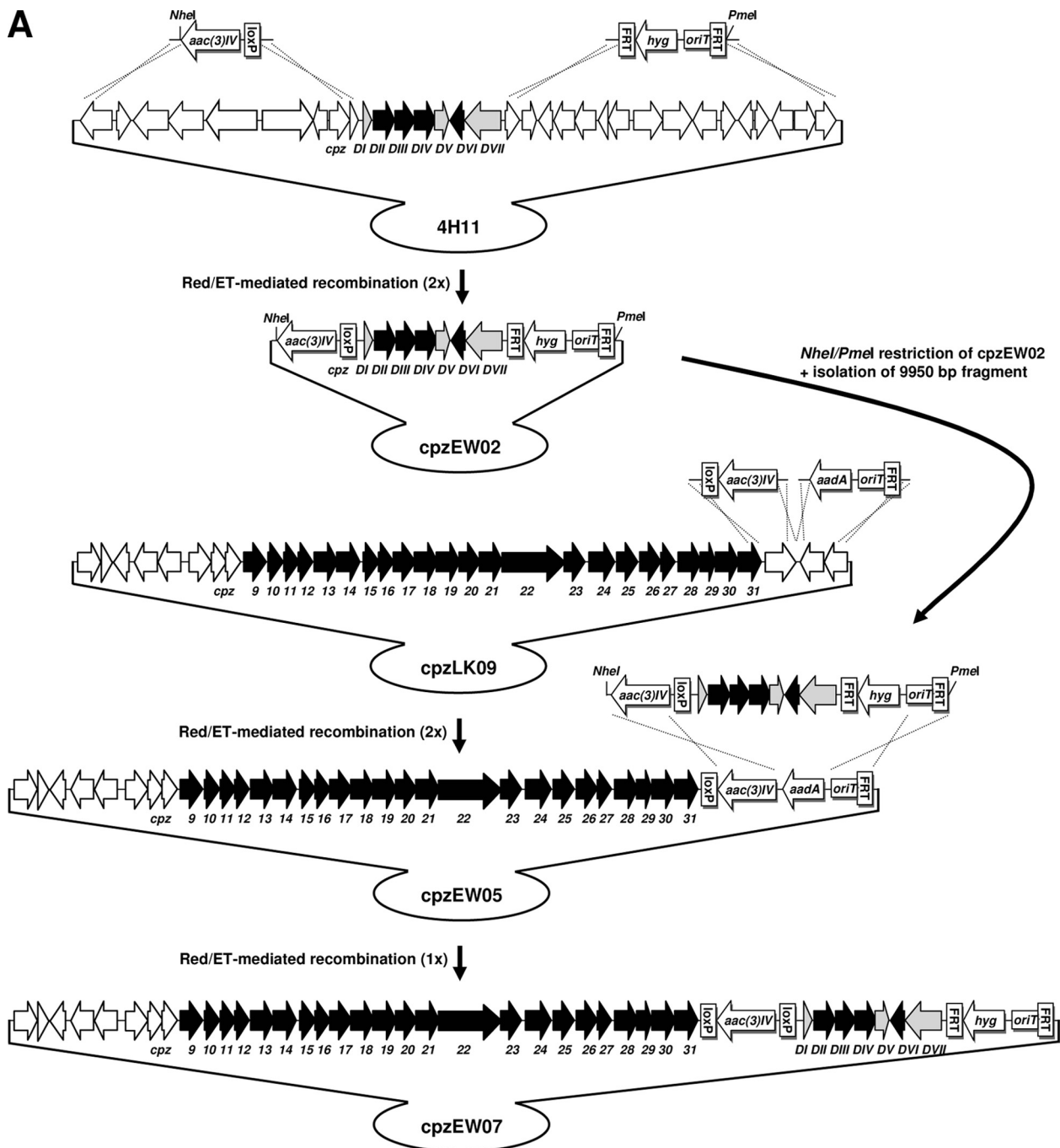
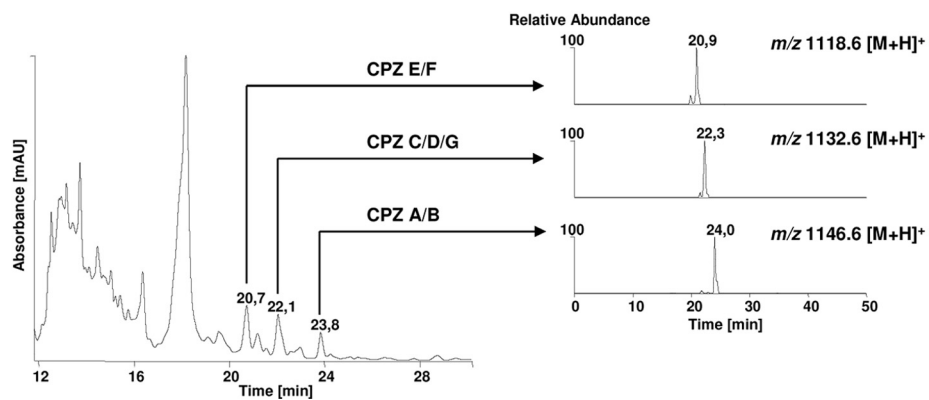
In order to confirm the involvement of the postulated L-rhamnose biosynthetic gene cluster in caprazamycin biosynthesis, the hypothetical dTDP-glucose 4,6-dehydratase gene *cpzDIII* was inactivated. The resulting cosmid, cpzLK01, was introduced into *Streptomyces* sp. MK730-62F2 by intergeneric conjugation. Four double-crossover mutants of *Streptomyces* sp. MK730-62F2  $\Delta$ *cpzDIII* were finally selected and confirmed by PCR. After cultivation, extracts of the mutant strains were applied to LC-ESI-MS analysis. In contrast to the wild-type strain, production of intact caprazamycins was completely abolished in the mutants (see Fig. S3 in the supplemental material). However, caprazamycin aglycones could clearly be detected, and extracts of the mutants still showed bioactivity in an agar diffusion assay against *Mycobacterium phlei* (data not shown). In order to validate that the disruption of *cpzDIII* is responsible for the abolishment of caprazamycin production, we complemented the  $\Delta$ *cpzDIII* mutant by introducing pRHAM. After cultivation, LC-ESI-MS analysis showed that caprazamycin production was restored (see Fig. S3). Analysis of the  $\Delta$ *cpzDIII* mutant and the complementation confirmed that the L-rhamnose biosynthetic gene cluster on cosmid 4H11 is required for the formation of the L-rhamnose moiety in caprazamycin biosynthesis.

**Assembly of the entire gene cluster for heterologous production of intact caprazamycins.** In order to prove that the identified genes, *cpzDI* to *-VII*, are sufficient for L-rhamnose biosynthesis, we assembled the subcluster to cosmid cpzLK09 containing the caprazamycin gene cluster (Fig. 5A). It was shown previously that the genes *cpz32* to *cpz34* are not involved in caprazamycin biosynthesis, based on inactivation experiments (31). Therefore, an apramycin resistance cassette and a spectinomycin-streptomycin resistance cassette were used to replace *cpz32* to *cpz34* by Red/ET-mediated recombination, generating cosmid cpzEW05. Within cosmid 4H11, containing the L-rhamnose subcluster, a 14-kb stretch of DNA upstream of *cpzDI* was replaced with an apramycin resistance cassette,

introducing a unique *NheI* restriction site. A 17-kb fragment upstream of *cpzDVII* was replaced by a hygromycin resistance cassette, introducing a unique *PmeI* restriction site. From the generated cosmid cpzEW02, a 10-kb *PmeI/NheI* restriction fragment, containing the *cpzDI-DVII* subcluster, was transferred into Red/ET-proficient *E. coli* cells harboring cpzEW05. Homologous recombination of the restriction fragment with cosmid cpzEW05 was possible between the apramycin resistance gene *aac(3)IV* (924 bp) and 523-bp identical sequences of the hygromycin and the spectinomycin-streptomycin resistance cassettes. These sequences include an *oriT* and an FRT site. Red/ET-mediated recombination would lead to an apramycin-, hygromycin-, and kanamycin-resistant transformant only if homologous recombination occurred within these regions.

The resulting cosmid, cpzEW07, was introduced into *S. coelicolor* M512 by intergeneric triparental conjugation. Positive clones were selected by their apramycin, hygromycin, and kanamycin resistance and termed *S. coelicolor* M512/cpzEW07. The mutant strain was cultivated, and extracts were analyzed by LC-ESI-MS. Three new peaks appeared in the UV chromatograms at retention times of 20.7 min, 22.1 min, and 23.8 min (Fig. 5B). These peaks were missing in extracts of *S. coelicolor* M512 and *S. coelicolor* M512/cpzLK09 (data not shown). Corresponding mass peaks were detected by product ion scanning for the caprazamycins E/F with  $m/z$  1,118.8 [M+H]<sup>+</sup> (Rt 20.9 min), caprazamycins C/D/G with  $m/z$  1,132.8 [M+H]<sup>+</sup> (Rt, 22.3 min), and the caprazamycins A/B with  $m/z$  1,146.8 [M+H]<sup>+</sup> (Rt, 24.0 min) (Fig. 5B). MS/MS fragmentation patterns matched exactly the intact caprazamycins (data not shown). The heterologous production of caprazamycins in *S. coelicolor* M512 containing the assembled gene cluster proves that the *cpzDI* to *-VII* subcluster is sufficient for the biosynthesis of dTDP-L-rhamnose.

**Deletion of nonessential genes in the L-rhamnose subcluster.** The production of intact caprazamycins by expression of the assembled gene cluster now enabled us to investigate other genes in caprazamycin biosynthesis in a heterologous background. However, to allow further gene deletion experiments, the existing apramycin resistance cassette needed to be eliminated from cosmid cpzEW07. This was achieved by a *SpeI* digestion and religation procedure, generating cosmid

**A****B**



cpzEW08. Corresponding SpeI sites were added to the primer sequence during the generation of cosmids cpzEW02 and cpzEW05 (see Materials and Methods).

Three of the hypothetical genes in the L-rhamnose subcluster have no obvious role in dTDP-L-rhamnose biosynthesis. The potential glycosyltransferase CpzDVII contains several conserved domains typical for glycan synthases and is most likely involved in cell wall biogenesis. *cpzDI* is similar to small hypothetical genes often located next to genes from deoxy-sugar metabolism, and *cpzDV* encodes a putative protein with unknown function. The genes *cpzDI* and *cpzDV* were individually deleted by replacement with an apramycin resistance cassette flanked by loxP sites. Subsequently, the resistance cassettes were eliminated *in vitro* using Cre recombinase. The resulting cosmids were termed cpzEW10 ( $\Delta$ cpzDI) and cpzEW12 ( $\Delta$ cpzDV). After introduction of the cosmids into *S. coelicolor* M512, hygromycin- and kanamycin-resistant exconjugants were selected and designated *S. coelicolor* M512/cpzEW10 and *S. coelicolor* M512/cpzEW12, respectively. After cultivation, culture extracts of the mutants showed no differences in LC-ESI-MS analysis in comparison to *S. coelicolor* M512/cpzEW07 containing the complete L-rhamnose cluster (see Fig. S4 in the supplemental material). Caprazamycins were accumulated by all of the strains. Therefore, we conclude that *cpzDI* and *cpzDV* have no role in caprazamycin biosynthesis and are probably involved in primary metabolism.

## DISCUSSION

Based on the identification of the L-rhamnose biosynthetic genes in this study, we propose two gene clusters, the caprazamycin and the L-rhamnose subcluster, to participate in caprazamycin biosynthesis. Coexpression of pRHAM, containing all genes required for the biosynthesis of dTDP-L-rhamnose, led to the accumulation of intact caprazamycins in a heterologous producer strain harboring cosmid cpzLK09. This clearly demonstrates that only the genes for the formation of dTDP-L-rhamnose are missing in the heterologous caprazamycin producer. Thus, the genes directing the attachment and methylation of the L-rhamnosyl moiety have to be carried on cosmid cpzLK09. Three putative methyltransferases, Cpz28, Cpz29, and Cpz30, show the highest sequence homology to known rhamnose O-methyltransferases from elloramycin (47) and spinosyn (54) biosynthesis. Most likely, these proteins have similar functions in caprazamycin formation. Notably, the recently identified gene cluster of liposidomycins, nonglycosylated caprazamycin derivatives, did not contain any *cpz28*, *cpz29*, or *cpz30* orthologues (32).

Translationally coupled to the genes *cpz28* to *cpz30* is the putative glycosyltransferase gene *cpz31*. An *in vitro* assay with His<sub>8</sub>-Cpz31 using dTDP-L-rhamnose and caprazamycin aglycones as substrates showed a complete conversion of the agly-

cones after a 2-h incubation. The products were unambiguously identified as 2c,3c,4c-desmethyl caprazamycins by LC-ESI-MS/MS analysis. This result implies that attachment of the deoxysugar occurs prior to its sequential methylation. A similar order of sugar transfer and subsequent tailoring reactions has been found in the biosynthesis of other glycosylated compounds, such as tylosin (2), oleandomycin (50), mycinamycin (26), and chromomycin (42). Cpz31, together with most glycosyltransferases involved in natural product biosynthesis, belongs to the GT-1 family of glycosyltransferases as classified by the CAZy system (6). Cpz31 exhibits significantly higher homology to, for example, the 4,6-dideoxy-4-hydroxylamino- $\alpha$ -D-glycosyltransferase CalG3 (36% identity, 50% similarity) from *Micromonospora echinospora* (59) than to the rhamnosyltransferases AraGT (29% identity, 38% similarity) from *S. echinatus* (38) and SpnG (29% identity, 39% similarity) from *Saccharopolyspora spinosa* (5). Luzhetskyy et al. (39) proposed that similarity-based relationships of glycosyltransferases are better understood with consideration of the aglycone substrate than of the sugar donor. In this aspect, Cpz31 is distinctive from other known glycosyltransferases involved in natural product biosynthesis. The rhamnosyl moiety of caprazamycins is linked to the methylglutaryl moiety by an ester bond and not by a glycosidic bond. While acylated deoxyhexoses are common in plants, they are rare in bacterial secondary metabolism. So far, only the phenazine derivatives aestivophoenins (35) and phenazoviridins (30) and the rhamnopyranosides (21) have been reported. Thus, Cpz31 may serve as a new enzymatic tool for the modification of bioactive compounds in the future.

Rhamnosyltransferases have been frequently exploited for the structural diversification of glycosylated antibiotics (4, 15, 38, 44), as they often exhibit high flexibility towards the donor substrate. However, heterologous expression of cosmid cpzLK09 in *S. fradiae* A0, which produces the deoxysugars D-olivose and L-rhodinose, and *Streptomyces* sp. T $\bar{U}$ 6071 $\Delta$ plaA6, producing L-amiketose, did not lead to the identification of new caprazamycin derivatives (data not shown). Since the caprazamycin aglycones were readily detected in the culture extracts, it can be speculated that Cpz31 is more specific for dTDP-L-rhamnose than related glycosyltransferases, e.g., ElmGT from *S. olivaceus* (4) or StfG from *S. steffisburgensis* (44). Further investigations will have to prove this assumption.

Screening of a cosmid library of the caprazamycin producer *Streptomyces* sp. MK730F-62F2 led to the identification of *cpzDII*, *cpzDIII*, *cpzDIV*, and *cpzDVI* elsewhere on the genome. The deduced gene products match the required enzymes for the biosynthesis of dTDP-L-rhamnose. Involvement in caprazamycin biosynthesis was demonstrated by inactivation of *cpzDIII*, resulting in the accumulation of caprazamycin aglycones. In contrast, inactivation of the collocated genes *cpzDI* and *cpzDV* had no influence on caprazamycin formation.

FIG. 5. (A) Strategy for assembly of the caprazamycin gene cluster and the L-rhamnose subcluster. *aac(3)IV*, apramycin resistance gene; *aad4*, spectinomycin-streptomycin resistance gene; *hyg*, hygromycin resistance gene; loxP, Cre recognition site; *oriT*, origin of transfer. The size of the resistance cassettes and their components are not drawn to scale. (B) LC-ESI-MS UV chromatogram and product ion chromatograms from mass scans for caprazamycins E/F with  $m/z$  1,118.6 [M+H]<sup>+</sup>, caprazamycins C/D/G with  $m/z$  1,132.6 [M+H]<sup>+</sup>, and caprazamycins A/B with  $m/z$  1,146.6 [M+H]<sup>+</sup> in butanolic extracts of *S. coelicolor* M512/cpzEW07.



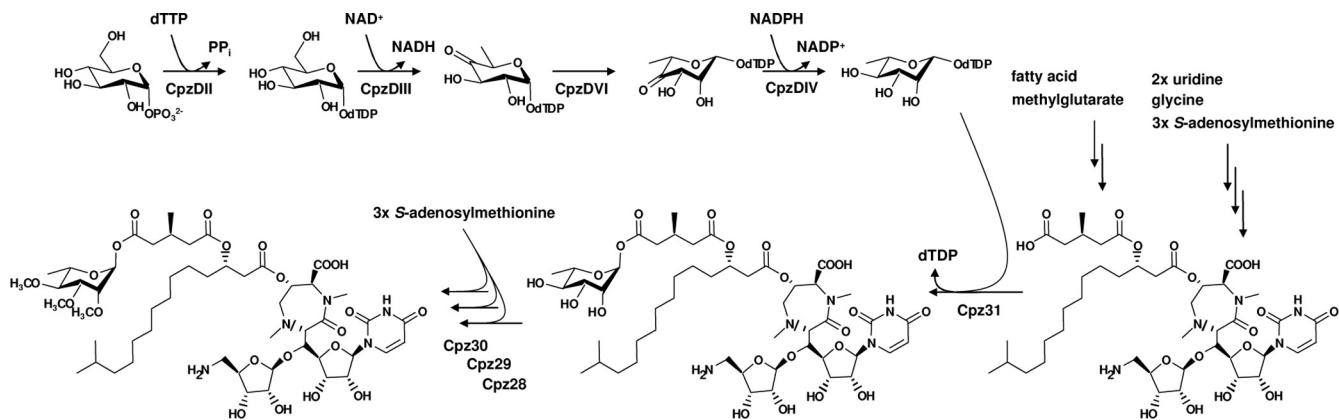


FIG. 6. Model for formation, attachment, and methylation of the L-rhamnosyl moiety in caprazamycin biosynthesis.

Based on the presented results, we propose the biosynthetic pathway for the latter steps in caprazamycin biosynthesis as follows (Fig. 6). Generation of the deoxysugar would start with the activation of glucose-1-phosphate by the addition of deoxythymidyldiphosphate (dTDP), catalyzed by the putative NDP-glucose synthase CpzDII. Subsequently, the glucose dehydratase CpzDIII would dehydrate dTDP-glucose to dTDP-4-keto-6-deoxyglucose. This product would serve as a substrate for the sugar 3,5-epimerase CpzDVI, resulting in dTDP-4-keto-L-rhamnose, which would be further reduced to dTDP-L-rhamnose by the 4-ketoreductase CpzDIV. Cpz31 would then attach the L-rhamnose to the caprazamycin aglycones, generating 2c,3c,4c,-demethyl caprazamycins. Sequential methylation of the deoxysugar moiety by the putative *O*-methyltransferases Cpz28, Cpz29, and Cpz30 would finally lead to the caprazamycins.

Usually, genes involved in the formation of a bacterial secondary metabolite are clustered together. However, biosynthetic gene clusters of rhamnosylated compounds often lack the genes for L-rhamnose formation (9, 16, 38, 54). In the case of elloramycin (48) and spinosyn (41), these genes were identified elsewhere in the genome. Inactivation experiments in *Saccharopolyspora spinosa* suggested that the *gt*, *gdh*, *epi*, and *kre* genes provide the rhamnosyl moiety for both spinosyn and cell wall biosynthesis. The presence of *cpzDVII*, a putative cell wall biosynthesis glycosyltransferase gene, may indicate a similar role for the L-rhamnose cluster in *Streptomyces* sp. MK730F-62F2. However, L-rhamnose seems not to be essential for the strain, as inactivation of the glucose dehydratase gene *cpzDIII* did result in a viable corresponding mutant.

In order to allow investigation of the entire biosynthetic pathway of caprazamycins, we assembled the L-rhamnose and caprazamycin gene cluster on cosmid cpzLK09, using a new strategy based on Red/ET-mediated recombination. This technology, designated recombineering, was initially established for DNA manipulations in *E. coli* (7, 60, 61) and has been adapted to *Streptomyces* (18). It has been successfully applied for functional studies and engineering of antibiotic biosynthetic machineries (17), e.g., heterologous expression (10) and combinatorial biosynthesis (11). More recently, recombineering was used for the reconstitution of large biosynthetic gene clusters from overlapping inserts (3, 22, 57). This “stitching”

overcomes the problem that genomic library clones rarely contain an entire gene cluster due to limitations of the average insert sizes. While “stitching” relies on the presence of overlapping, identical DNA sequences in both clones, we now show for the first time that assembly is possible without the need of overlapping DNA regions. For the presented strategy, Red/ET-mediated recombination is restricted to sequences in the form of resistance cassettes. These cassettes were introduced into the L-rhamnose donor cosmid and the recipient cosmid harboring the caprazamycin gene cluster. Final assembly was achieved by Red/ET-mediated recombination between a restriction fragment from the donor cosmid with the recipient cosmid. FRT and loxP recognition sites and SpeI restriction sites were introduced for the latter excision of the resistance cassettes. The *in vivo* usage of Cre (13) and the Flp recombinase (12) has now been successfully established in *Streptomyces* and allows the rapid generation of unmarked deletions. However, if Cre or Flp recombinase is used, a “scar” sequence comprising a loxP or an FRT site is maintained, which can cause undesirable recombination events in further recombineering experiments. Therefore, elimination of the apramycin resistance cassette was performed with SpeI digestion and religation of the cosmid DNA. In this study, *cpzDI* and *cpzDV* were successfully deleted on the assembled cosmid and the corresponding mutants were analyzed.

Since manipulation of natural producer strains can be difficult and time-consuming, heterologous expression of entire biosynthetic gene clusters in more-amenable and fully sequenced host strains is desirable. In addition, formation of a natural compound often relies on the supply of specific precursors provided by genes encoded outside the gene cluster, e.g., deoxysugars (48), isoprenoid derived moieties (20), or other small subunits (45, 58). Therefore, the new assembly approach described in this study may help to establish heterologous expression and genetic engineering of scattered gene clusters.

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