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Chalcomycin Biosynthesis Gene Cluster from *Streptomyces bikiniensis*: Novel Features of an Unusual Ketolide Produced through Expression of the *chm* Polyketide Synthase in *Streptomyces fradiae*

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Chalcomycin, a 16-membered macrolide antibiotic made by the bacterium *Streptomyces bikiniensis*, contains a 2,3-*trans* double bond and the neutral sugar D-chalcose in place of the amino sugar mycaminose found in most other 16-membered macrolides. Degenerate polyketide synthase (PKS)-specific primers were used to amplify DNA fragments from *S. bikiniensis* with very high identity to a unique ketosynthase domain of the tylosin PKS. The resulting amplimers were used to identify two overlapping cosmids encompassing the *chm* PKS. Sequencing revealed a contiguous segment of >60 kb carrying 25 putative genes for biosynthesis of the polyketide backbone, the two deoxysugars, and enzymes involved in modification of precursors of chalcomycin or resistance to it. The *chm* PKS lacks the ketoreductase and dehydratase domains in the seventh module expected to produce the 2,3-double bond in chalcomycin. Expression of PKS in the heterologous host *Streptomyces fradiae*, from which the *tyl* genes encoding the PKS had been removed, resulted in production of at least one novel compound, characterized as a 3-keto 16-membered macrolactone in equilibrium with its 3-*trans* enol tautomer and containing the sugar mycaminose at the C-5 position, in agreement with the structure predicted on the basis of the domain organization of the *chm* PKS. The production of a 3-keto macrolide from the *chm* PKS indicates that a discrete set of enzymes is responsible for the introduction of the 2,3-*trans* double bond in chalcomycin. From comparisons of the open reading frames to sequences in databases, a pathway for the synthesis of nucleoside diphosphate-D-chalcose was proposed.

Sixteen-membered macrolide antibiotics have important applications in human and veterinary medicine and are subdivided into three major groups on the basis of the structures of their macrolactone backbones. Chalcomycin (Fig. 1; compound 1), discovered in the late 1950s in a strain of *Streptomyces bikiniensis* (11), and mycinamicin I (compound 2) represent a group with 2,3-*trans* double bonds. Three congeners of chalcomycin have been recently identified (2, 13, 21). All contain the same polyketide backbone but differ in the degrees of oxidation or acylation. The other two subgroups are represented by tylosin (compound 3) and midecamycin A1 (compound 4), each of which carries a 3-OH group instead of the 2,3-double bond and which also differ in their C-12 and C-14 substituents. Chalcomycin was determined to have modest antibiotic activity against gram-positive organisms: the MIC at which 50% of 11 susceptible *Staphylococcus aureus* strains were inhibited was 0.19 μg/ml, with a range of 0.05 to 0.78 μg/ml; the MICs for two susceptible *Streptococcus pyogenes* strains were 0.19 and 0.78 μg/ml (11). Although its precise mechanism of action was not determined, chalcomycin was found to inhibit protein synthesis and to exhibit cross-resistance with a number of macrolides; hence, chalcomycin was thought to act in the same manner as tylosin (29). In addition, whereas macrolides are not known to inhibit tRNA synthetases, chalcomycin was shown to inhibit the incorporation of [^{14}C]glycine into glycyl-tRNA in *S. aureus* (18). Chalcomycin also exhibited very potent in vitro activity against a number of *Mycoplasma* species that were not susceptible to other macrolides, though the basis of this activity was not explored (25). Finally, chalcomycin was also found to inhibit protein synthesis in HeLa cells in culture (14), an activity not commonly associated with 16-membered macrolides. Despite these various activities, however, chalcomycin was not developed as a drug.

Recent physicochemical and X-ray crystallographic studies (none of which included chalcomycin) have provided detailed information on how macrolides interact with ribosomes and underscore the interesting structural differences between chalcomycin and the commercially important members of the 16-membered macrolide family, tylosin and midecamycin A1. In place of the amino sugar mycaminose in compounds 3 and 4 or desosamine in compound 2, the chalcomycins contain the neutral sugar chalcose substituted at C-5(*S*) of the macrolactone ring. It is thought that the positive charge of the 3′-substituted amino group together with the 2′-hydroxyl group contribute to the binding of amino sugar-macrolides to domain V of bacterial ribosomes (15, 31, 38). Chalcose lacks the 3′-amino group but has the 2′-hydroxyl group. The chalcomycins also contain a C-6(*S*) methyl side chain rather than the C-6(*R*) ethylaldehyde side chain found in compounds 3 and 4 and other clinically useful 16-membered macrolides. Reduction or removal of the

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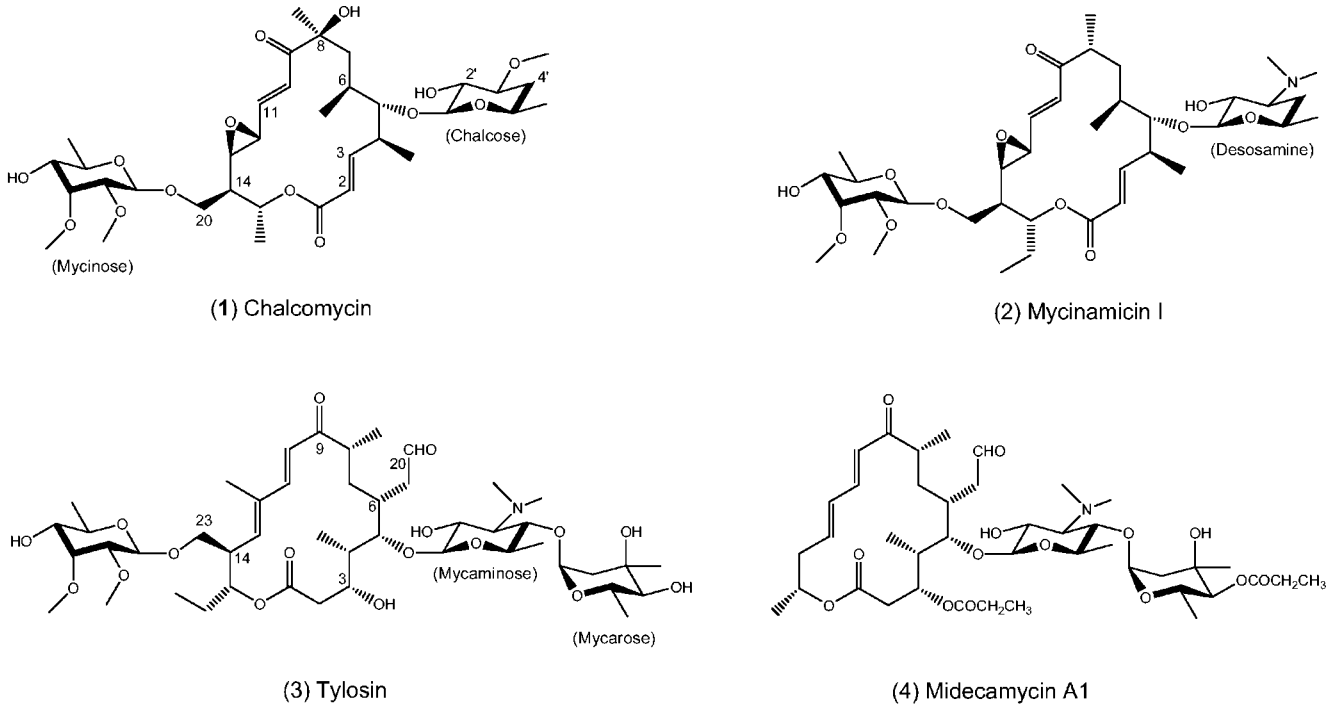

FIG. 1. Structures of selected 16-membered macrolides.

aldehyde in compound 3 and other 16-membered macrolides results in significant loss of ribosome binding and potency (26). X-ray analysis has shown a reversible covalent linkage between the aldehyde of compound 3 and the 6-amino group of A-2062 (*Escherichia coli* numbering) in domain V of the *Haloarcula marismortui* ribosome (15). In addition, chalcomycin contains an 8(*S*)-OH group not present in compound 2 or the clinically important 16-membered macrolides, but the contribution of this group to overall potency has not been explored. Finally, the 2,3-*trans* double bond of chalcomycin probably accounts for the large differences in the conformations of the macrocyclic backbones reported for the crystal and solution structures of chalcomycin and tylosin (44). Whether the differences in the backbones impact binding of the respective macrolides to the ribosome remains to be determined.

An important similarity between the chalcomycins and compound 3 is the presence of the sugar mycinose substituted at C-14(*R*) of the ring. Chemical footprinting of ribosomes from a number of bacteria and from X-ray data for *H. marismortui* ribosomes have indicated that the mycinose moiety of compound 3 makes contact with domain II of the ribosome and contributes to enhanced binding of the macrolide (4, 15).

To better understand the biosynthesis of chalcomycin and to determine how its components contribute to antibacterial potency, we isolated the chalcomycin biosynthesis cluster, determined its nucleotide sequence, and expressed some of the genes in heterologous hosts. The nucleotide sequence indicated that the 2,3-*trans* double bond is introduced by a reductase or dehydratase (DH) separate from the chalcomycin polyketide synthase (PKS), an unprecedented situation. Expression of the *chm* PKS in a mutant of *Streptomyces fradiae* from which the *tyl* genes encoding PKS had been removed confirmed the non-PKS origin of the 2,3-double bond and resulted in the production of compounds containing novel macrolactone-sugar combinations.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Routine DNA manipulations were performed with *E. coli* DH5α as a cloning host under standard culture conditions (36). *S. bikiniensis* NRRL2737, the wild-type producer of chalcomycin, was obtained from the ATCC strain collection and grown by a protocol described previously (28). For isolations of total DNA, *S. bikiniensis* was grown in tryptone soya broth (19). *S. fradiae* K342-45 was made by conjugal transfer of plasmid pKOS342-45 from *E. coli* DH5α/pUB307/pKOS342-45 to *S. fradiae* K159-1 as described previously (34). *S. fradiae* was maintained on AS-1 agar (8). For shake flask fermentations, seed cultures were grown in tryptone soya broth, and fermentations were performed in R medium [containing, per liter, 15 g of wheat flour, 10 g of corn gluten, 25 g of molasses, 2.5 g of fodder yeast, 1 g of $(NH_4)_2HPO_4$, 1 g of NaCl, 2 g of $CaCO_3$, and 34 ml of soybean oil] at 30°C for 5 to 7 days. When antibiotic selection of transformants was employed, carbenicillin (100 μg/ml), kanamycin (50 μg/ml), or apramycin (60 μg/ml) was used as required. *Micrococcus luteus* ATCC 9341 was used as an indicator strain in bioassays of zone of inhibition on medium 11 (Difco).

DNA manipulations. Total DNA isolation, plasmid DNA preparations, restriction endonuclease digestions, ligations, and other DNA manipulations were done using the standard protocols for *E. coli* (36) and *Streptomyces* (19). Digoxigenin labeling of DNA was performed according to protocols supplied by the distributor (Boehringer Mannheim).

DNA sequencing and analysis. DNA sequencing was performed with cosmids by standard shotgun cloning to obtain at least fourfold coverage. Primer walking was used to close the gaps. The sequence was assembled using the Sequencher software package (Gene Codes) and analyzed with MacVector (Accelerys) and the BLAST server of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST).

Isolation of the *chm* biosynthesis cluster. A genomic library of *S. bikiniensis* NRRL2737 was made by digestion of the total DNA with Sau3AI and ligation of 35- to 47-kb fragments into pSuperKos, a derivative of pSuperCos (Stratagene), which was digested with AfeI and self-ligated to eliminate the neomycin resistance gene. Degenerate primers designed based on conserved regions of keto-

TABLE 1. BLASTP analysis of unique KS amplimers from the genome of *S. bikiniensis*

KS amplimer	Best BLASTP match(es)[a]/organism(s)	% Identity/% Similarity	Accession no.
Sb1/5-60	MycAIV/*Micromonospora griseorubida* Tyl KS6/*Streptomyces fradiae*	82/91 77/86	BAC57031 AAB66507
Sb1/5-62	MycD/*Microcystis aeruginosa*	48/62	BAB12210
Sb1/5-67	Put. PKS ORF3/*Streptomyces* sp. strain GERI155 Tyl KS1/*Streptomyces fradiae*	85/89 83/90	AAM81586 AAB66504
Sb1/5–68	Put. PKS ORF1/*Streptomyces* sp. strain GERI155 Tyl KS2/*Streptomyces fradiae*	95/97 80/88	AAM81584 AAB66504
Sb1/5-72	Nid A1/*Streptomyces caelestis*	77/86	AAC46024
Sb1/5-75	Put. PKS ORF2/*Streptomyces* sp. strain GERI155 Ty1 KS3/*Streptomyces fradiae*	94/97 75/84	AAM81585 AAB66505
Sb1/5-76	Put. PKS/*Streptomyces avermitilis* MA4680	73/85	NP_822424
Sb1/5-78	NidA5/*Streptomyces caelestis* Ty1 KS7/*Streptomyces fradiae*	74/85 75/84	AAC46028 AAB66508
Sb1/5-80	Put. PKS ORF3/*Streptomyces* sp. strain GERI155 Ty1 KS5/*Streptomyces fradiae*	97/99 80/88	AAM81586
Sb1/5-81	GdmAI/*Streptomyces hygroscopicus*	75/86	AAO06916
Sb1/5-87	Put. PKS ORF3/*Streptomyces* sp. strain GERI155 Tyl KS4/*Streptomyces fradiae*	93/95 81/88	AAM81586 AAB66506
Sb2/5-A16	Put. PKS/*Nostoc* sp. strain PCC7120	45/59	NP_485688
Sb2/5-A17	NidA1/*Streptomyces caelestis*	76/86	AAC46024
Sb3/7-31	mycA ORF 1/*Micromonospora griseorubida* Ty1 KS^O/*Streptomyces fradiae*	72/80 75/84	BAA76543 AAB66504

[a] Put., putative.

synthase (KS) domains for the type I PKS cluster with a codon bias for high-G+C-content organisms are described elsewhere (33). DNA fragments (termed KS amplimers) obtained after PCR amplification were cloned into pLitmus28 (New England Biolabs) cut with EcoRV; 100 were sequenced. Three of the amplimers, Sb3/7-31 (KS^O), Sb1/5-75 (KS3), and Sb1/5-78 (KS7), believed to constitute segments of the *chm* PKS (Table 1), were used to probe the cosmid library under high-stringency conditions. To isolate both ends of the proposed >35-kb *chm* PKS cluster, plasmids from the colonies that showed strong hybridization signals were isolated and subjected to Southern blotting with the putative *chm* KS^O or KS7 amplimers as probes. End sequencing of the plasmids that hybridized to one or both probes revealed two cosmids, pKOS146-185.1 and pKOS146-185.10, that possessed high homology at one end with a segment of the PKS from the tylosin biosynthesis cluster. Specific primer pairs, designed for the putative *chm* KS^O (Sb3/7-31), KS3 (Sb1/5-75), and KS7 (Sb1/5-78) amplimers, were synthesized and used in PCRs with cosmids pKOS146-185.1 and pKOS146-185.10. pKOS146-185.1 produced correctly sized amplimers with the KS^O- and KS3-specific primers but not with KS7-specific primers, and pKOS146-185.10 produced a correctly sized amplimer with the KS7-specific primers but not with the KS^O- and KS3-specific primers, indicating that pKOS146-185.1 and pKOS146-185.10 contained the 5′ and 3′ regions, respectively, of the *chm* PKS genes. End sequencing and preliminary mapping suggested that the inserts of the two cosmids overlapped by ca. 4 kb. The complete nucleotide sequences of the inserts in the two cosmids were determined.

Assembly of the PKS genes on plasmid pKOS342-45. The *chm* PKS was pieced together for expression from cosmids pKOS146-185.1 and pKOS146-185.10 as follows. Restriction sites in the *chm* genes encoding PKS employed are shown in Fig. 2. PCR was used to obtain a 942-bp fragment encompassing the 3′ end of *chmGV* by use of the following oligonucleotides, with pKOS146-185.10 as the template: 5′-GACACGGCCGGTGAGAGCAGC-3′ and 5′-CTTCTAGATGT CGCGGTGTACGG-3′ (the restriction site is underlined). The PCR product was digested with NcoI and XbaI, and the 309-bp subfragment was ligated into pLitmus29 digested with NcoI plus XbaI to generate pKOS342-33. pKOS342-33 was digested with NcoI and XhoI and ligated with a ca. 2.4-kb fragment from similarly digested pKOS146-185.10 to produce pKOS342-35. This plasmid was digested with BglII and XhoI and ligated with a ca. 6.4-kb BglII- plus XhoI-generated fragment from pKOS146-185.10 to create pKOS342-36. The *chmGIII* gene was isolated by digesting pKOS146-185.1 with HindIII and PstI to obtain a ca. 5.4-kb fragment, and pKOS146-185.10 was digested with PstI and BglII to obtain a ca. 6.3-kb fragment. These two fragments were ligated into HindIII- plus BglII-digested pLitmus28 to produce pKOS342-38. pKOS342-36 was then cut with BglII and SpeI to produce a ca. 9-kb piece, which was introduced into the corresponding sites of pKOS342-38 to generate pKOS342-39. The first two open reading frames (ORFs) of the *chm* PKS were isolated from the cosmid pKOS146-185.1 as EcoRI/XhoI and XhoI/BspHI fragments. These two pieces were ligated into pKOS232-165, a pLitmus28 plasmid that had a linker to introduce PacI and NdeI sites at the start of the translation and was cut with EcoRI and NcoI. The plasmid that resulted from this three-piece ligation was designated pKOS232-172. To make the final expression plasmid, pKOS232-172 was cut with NdeI and HindIII to generate a ca. 19-kb fragment, and pKOS342-39 was digested with HindIII and SpeI to produce a ca. 20-kb fragment. These two fragments were ligated into NdeI- plus SpeI-digested pKOS244-20, a pSET152-based vector (5) that was modified to contain the λ *cos* site and *tylGI*p, and plasmid pKOS342-45, containing the ca. 40-kb segment of *chm* encoding PKS, was obtained by use of a λ phage packaging kit (Stratagene).

Detection of macrolides from fermentation broths. After 7 days of growth at 30°C, the culture broths (10 to 50 ml) were analyzed for 16-membered macrolide production by high-pressure liquid chromatography (HPLC) (Metachem Metasil Basic column; 4.6 by 150 mm; 5-μm-diameter particle) using a linear gradient of 15 to 100% organic phase (56% methanol, 5 mM ammonium acetate) at 1

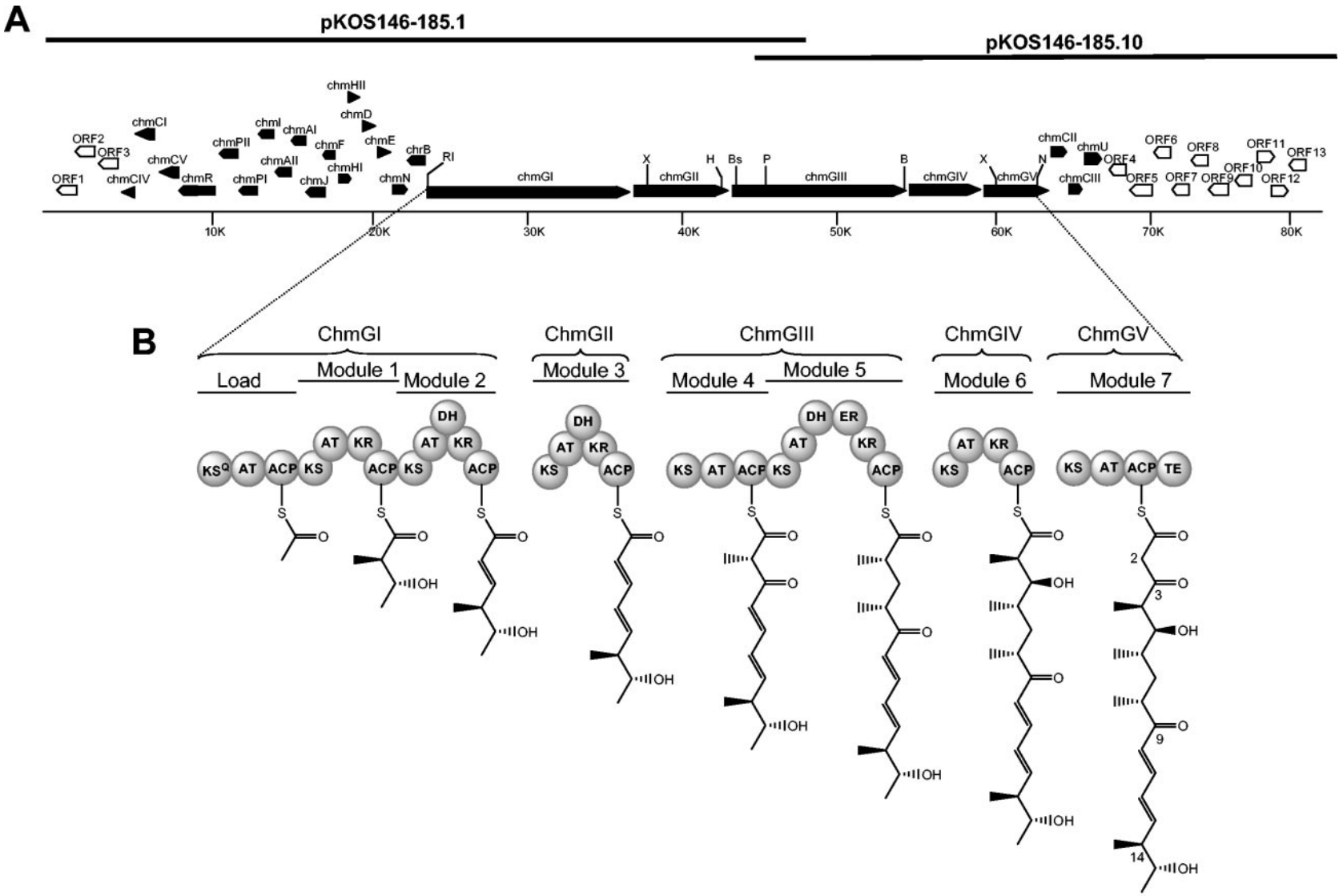

FIG. 2. (A) Map of the *chm* biosynthetic gene cluster on two overlapping cosmids. The *chm* biosynthesis or resistance genes are shown as closed polygons, and non-*chm* genes are shown as open polygons. Restriction site abbreviations: B, BglII; Bs, BspHI; H, HindIII; N, NcoI; Nd, NdeI; P, PstI; RI, EcoRI; X, XhoI; Xb, XbaI. (B) Domain organization of the *chm* PKS and proposed structures of the thioester intermediates at the end of each cycle of elongation. ACP, acyl carrier protein; AT; acyltransferase; ER, enoylreductase; KR, β-ketoreductase; KS, β-ketoacyl ACP synthase; KS^{Q}, KS domain with active site cys replaced by gln; TE, thioesterase.

ml/min over 7 min. The HPLC used simultaneous detection by electrospray mass spectrometry (Turbo Ionspray) and UV absorption at 282 nm.

Isolation and characterization of 5-*O*-mycaminosylchalcolactone. Approximately 500 ml of cell-free supernatant of fermentation broth from a culture of *S. fradiae* K342-45 was adjusted to pH 7.8 with solid $NaHCO_3$. The solution was filtered, and the filtrate was extracted with $CHCl_3$ (500 ml used for each of four extractions). The organic extracts were combined and dried with vigorous stirring over Na_2SO_4, filtered, and concentrated in vacuo into an amber oil. The crude material was partially purified by flash silica gel chromatography using a gradient of 0 to 35% acetone (plus 2% [vol/vol] triethylolamine [Net_3]) in hexane. The crude product (12 mg) was eluted in the 30% fractions. This material was further purified by preparatory HPLC with a 150- by 21.5-mm Polaris C_{18} column (MetaChem), and elution was monitored at 280 nm. Purification was performed at a flow rate of 10 ml/min, employing a linear 50 to 100% gradient of solvent A (CH_3CN-methanol [80/20] buffered with 5 mM NH_4 acetate). The compound eluted in 60 to 70% solvent A. The solvent was removed in vacuo, and the residual NH_4 acetate was removed by application of the sample to a silica gel plug. The plug was eluted with 30% acetone–2% (vol/vol) NEt_3 in hexane, providing purified 3-hydroxy-5-*O*-mycaminosyl-chalcolactone (5.6 mg) as a white solid. High-resolution mass spectra for $C_{28}H_{45}NO_8$ were calculated to be 523.3140 and observed to be (M + H) 524.3760. Nuclear magnetic resonance (NMR) spectra were collected in $CDCl_3$ with a 400-MHz spectrometer. In $CDCl_3$, the sample was present as a mixture of the C_3 enol and keto tautomers (enol/keto ratio of 3:1). NMR results were as follows: for ^1H NMR ($CDCl_3$, 400 MHz, data for enol tautomer), δ 0.98 (d, 3H, J = 6.8 Hz), 1.08 (d, 3H, J = 6.8 Hz), 1.15 (d, 3H, J = 6.8 Hz), 1.27 (m, 3H), 1.29 (m, 3H), 1.33 (d, 3H, J = 7.2 Hz), 1.38 (m, 1H), 1.50 (m, 1H), 1.57 (m, 1H), 2.22 (m, 1H), 2.36 (t, 1H, J = 10.0 Hz), 2.49 (s, 6H), 2.51 (m, 1H), 2.54 (m, 1H), 3.09 (t, 1H, J = 9.4 Hz), 3.27 (m, 1H), 3.52 (dd, 1H, J = 7.2, 10.0 Hz), 3.69 (d, 1H, J = 10.4 Hz), 4.28 (d, 1H, J = 7.2 Hz), 4.79 (m, 1H), 4.86 (s, 1H), 5.68 (dd, 1H, J = 15.2, 9.2 Hz), 6.11 (dd, 1H, J = 11.2, 15.2 Hz), 6.23 (d, 1H, J = 14.8 Hz), and 7.10 (dd, 1H, J = 14.8, 11.2 Hz); and for ^{13}C NMR (400 MHz, $CDCl_3$), δ 15.73, 17.16, 17.62, 17.81, 18.06, 18.25, 33.37, 41.67, 43.69, 44.74, 45.17, 70.13, 70.91, 71.05, 72.15, 73.19, 84.74, 89.82, 104.39, 123.39, 132.41, 141.64, 144.16, 171.92, 180.09, and 203.71. High-resolution mass spectra for $C_{28}H_{45}NO_8$ were calculated to be 523.3140 and found to be 524.3760 (M + H).

Nucleotide sequence accession number. The sequence reported in this paper has been deposited into GenBank under accession no. AY509120.

RESULTS

Identification of *chm* PKS probes by use of phylogenetic relationships. Comparisons of previously sequenced 16-membered macrolide PKS clusters suggested that the *chm* PKS genes are most homologous to the niddamycin or tylosin (*tyl*) PKS genes. Hence, five degenerate oligonucleotides, designed on conserved regions of KS domains of type I PKS genes from high-G+C-content organisms, were used in three specific combinations in PCR with total DNA from *S. bikiniensis* as the template. Amplimers of the expected sizes were obtained and sequenced. Fourteen different sequences were identified from 81 amplimers sequenced. Eight amplimers exhibited 71 to 81% identity at the protein level to a corresponding sequence in a KS domain of the *tyl* PKS (Table 1). Two additional amplimers exhibited very high similarity to segments of the PKS involved in the synthesis of the 16-membered macrolide niddamycin, but these sequences were much less similar to the corresponding tylosin PKS segments. Hence, three of the KS amplimers, which showed the highest degrees of homology to the tylosin KS domains from the loading module or from modules 3 or 7, were used as "perfect probes" (37) to probe a cosmid library of *S. bikiniensis* DNA, leading ultimately to isolation of the *chm* biosynthesis cluster on two overlapping cosmids (Fig. 2A).

Analysis of the *chm* biosynthesis cluster. Thirty-five ORFs were identified within the ca. 80-kb segment of *S. bikiniensis* DNA from cosmids pKOS146-185.1 and pKOS146-185.10 (Fig. 2A; Table 2). The *chm* cluster most likely starts with the gene designated *chmCIV*, similar to a gene involved in desosamine biosynthesis in the oleandomycin biosynthesis pathway (41), and ends with the gene designated *chmU*. ORFs 1 to 3 and 4 to 12 appear to have orthologs in the genomes of *Streptomyces coelicolor* and *Streptomyces avermitilis*, which are not known to be associated with secondary metabolism, and their proposed functions do not correspond to roles found in the synthesis of macrolides. ORF 13, at the far right end of the sequenced segment, appears to encode a type II thioesterase, homologs of which have been found in many other modular PKS systems and which are thought to play an editing role in the biosynthesis of the polyketide (8, 20). However, the protein encoded by *chmI* is more similar to the family of type II thioesterases associated with polyketide biosynthesis (57% amino acid identity) than ORF 13 (45% amino acid identity). Therefore, we propose that ChmI probably provides the required editing role in chalcomycin biosynthesis and that ORF 13 likely does not play a role in chalcomycin biosynthesis.

Resistance genes. Twenty-five of the ORFs showed similarities to genes in other macrolide pathways; all but one are designated *chm* (Table 2). The remaining gene, located immediately upstream of the *chm* PKS genes and designated *chrB*, strongly resembles *tlrB* and *myrA* in the tylosin and mycinamicin biosynthesis clusters, respectively. TlrB methylates nucleotide G748 (in domain II) of 23S rRNA to prevent tylosin from binding to and inhibiting the host's ribosomes (22). It is very likely, therefore, that ChrB catalyzes methylation of G748 in *S. bikiniensis* ribosomes, resulting in self-resistance. An *erm* gene responsible for methylation of A2058 in domain V of the 23S rRNA, a major factor in self-resistance in strains that produce most of the clinically important macrolides (all of which contain either desosamine or mycaminose), was not found in the *chm* cluster, but whether one is present elsewhere in the genome was not determined. A second putative resistance gene, *chmR*, strongly resembles *oleR* in the oleandomycin biosynthesis cluster of *Streptomyces antibioticus*. OleR is an extracellular β-glucosidase that removes the glucose moiety that is attached to the 2′OH of desosamine by the action of OleI (32). OleI-mediated glucosylation of oleandomycin confers self-resistance; removal of the glucose residue by OleR, after the glucosylated macrolide is excreted from the cell, restores activity to the compound. A counterpart to *oleI*, however, was not found within the *chm* cluster.

The chalcomycin PKS. The chalcomycin PKS is encoded by five genes (*chmGI* to *chmGV*) that together contain eight modules, including a loading module (Fig. 2A), and resemble, in both gene and module organization, the PKS genes that encode the macrolactone backbones of other 16-membered macrolides (17). The loading module contains a KS^Q domain; biosynthesis of the macrolactone is thus believed to require malonyl CoA as the starter, which is subsequently decarboxylated after attachment to the loading module (6, 43). The scheme representing the progression of the nascent polyketide through the modules of the *chm* PKS is shown in Fig. 2B. Examination of the sequence revealed that module 7, which determines the composition of carbons 2 and 3 of the macrolactone produced by the *chm* PKS, does not contain a ketoreductase or a DH domain. How and at what step during the synthesis of the completed molecule the 2,3-double bond in

TABLE 2. ORFs and their proposed functions

ORF designation	Size of encoded protein[a]	Proposed function	Closest homolog	% Identity
ORF 1	343	Membrane protein	SCO0450	56
ORF 2	280	Hypothetical protein	SCO0451	70
ORF 3	293	Oxidoreductase	DR1890	55
chmCIV	405	3,4-DH; D-chalcose pathway	OleN1	66
chmCI	259	*O*-Methyltransferase; D-chalcose pathway	SpnH 250	59
chmCV	485	D-Chalcose pathway	EryCV	76
chmR	836	β-Glucosidase, extracellular reactivator of chalcomycin	DesR	58
chmPII	401	P450; 12,13-epoxidase	LnmZ	51
chmPI	407	P450; C-8 hydroxylase	OleP	51
chmI	282	Thioesterase (TEII family)	TylO	57
chmAII	323	TDP-glucose 4,6-DH	AveBII	68
chmAI	305	TDP-glucose synthase	DesIII	69
chmJ	196	3-Epimerase; 6-deoxy-D-allose pathway	TylJ	64
chmF	255	*3-O*-Methyltransferase; D-mycinose pathway	TylF	69
chmHI	420	P450; C-20 hydroxylase	TylHI	60
chmHII	73	Ferredoxin	TylHII	53
chmD	326	4-Ketoreductase; 6-deoxy-D-allose pathway	TylD	58
chmE	403	2-*O*-Methyltransferase; D-mycinose pathway	TylE	71
chmN	418	6-Deoxy-D-allosyltransferase	TylN	67
chrB	280	23S-rRNA G748-methyltransferase	TlrB	58
chmGI	4,441	PKS, modules 0 to 2		
chmGII	1,974	PKS, module 3		
chmGIII	3,788	PKS, modules 4 and 5		
chmGIV	1,612	PKS, module 6		
chmGV	1,350	PKS, module 7 and thioesterase		
chmCII	412	NDP-hexose 3,4-isomerase; D-chalcose pathway	TylMIII	41
chmCIII	425	Chalcosyltransferase	TylMII	62
chmU	248	3-Ketoreductase	PP2783	50
ORF 4	382	Permease	SC0513	79
ORF 5	487	Membrane protein	SC2255	53
ORF 6	231	D-Alanyl-D-alanine carboxypeptidase	SAV3781	49
ORF 7	386	Sensory histidine kinase	SC5304	47
ORF 8	228	Response regulator	SAV4704	60
ORF 9	476	Permease	SC6214	71
ORF 10		Hypothetical protein	SC5427	35
ORF 11	612	Permease	SC6214	52
ORF 12	223	MerR family transcriptional regulator	SCO7698	34
ORF 13	251	Thioesterase (TEII family)	RifR	45

[a] Number of amino acids.

chalcomycin is produced are not immediately apparent from examination of the cluster and are discussed below.

Expression of the *chm* PKS in *S. fradiae* results in a 3-ketomacrolactone. The >40-kb segment of *chm* encoding the PKS was assembled as a single contiguous DNA fragment from cosmids pKOS146-185.1 and pKOS146-185.10 and then subcloned into a derivative of plasmid pSET152 that placed the PKS genes under the control of the promoter that drives the *tyl* PKS (*tylG*p). This plasmid, designated pKOS342-45, was transferred via conjugation from its *E. coli* cloning host to the streptomycete expression host, *S. fradiae* K159-1, from which the *tylGI* to *tylGV* (PKS) genes had been deleted (34). pSET152 does not carry genes enabling autonomous replication in *Streptomyces* but carries the *attP-int* segment of the *Streptomyces* phage ϕC31 and integrates, through site-specific recombination, into a unique site (*attB*) on the chromosome (5). *S. fradiae* K159-1/pKOS342-45 transconjugants were found to be stable for the maintenance of the *aacCIV* marker without the continued inclusion of apramycin in the fermentation medium. Ethyl acetate extracts of whole fermentation broths from four transconjugants exhibited antibiotic activity, whereas the extract of the fermentation broth of the parent was not bioactive. One of these strains was chosen for further analysis and designated *S. fradiae* K342-45.

Liquid chromatography-mass spectrometry analysis of extracted whole broths from a small-scale fermentation of K342-45 revealed four prominent compounds not present in the parent, with the following *m/z* values [$(M + H)^+$]: 525, 669, 843, and 845. Only the compound corresponding to *m/z* 525 was purified in sufficient amount from a large-scale fermentation to enable a determination of its structure. From the NMR data, it can be seen that this compound, named 5-*O*-mycaminosylchalcolactone (compound 5), contains a 3-keto group in the macrolactone backbone (present mainly as the 3-enol tautomer in the NMR solvent, $CDCl_3$) as predicted from the domain structure of the *chm* PKS, along with the sugar mycaminose attached at C-5 (Fig. 3).

The finding of the expected macrolactone is consistent with the hypothesis that the genes expressed in *S. fradiae* correspond to the chalcomycin PKS and that the cluster shown in Fig. 2A is indeed the chalcomycin biosynthesis cluster. Direct evidence that these genes are involved in chalcomycin biosynthesis was obtained from a loss of chalcomycin biosynthesis in *S. bikiniensis* after insertion of an antibiotic resistance marker

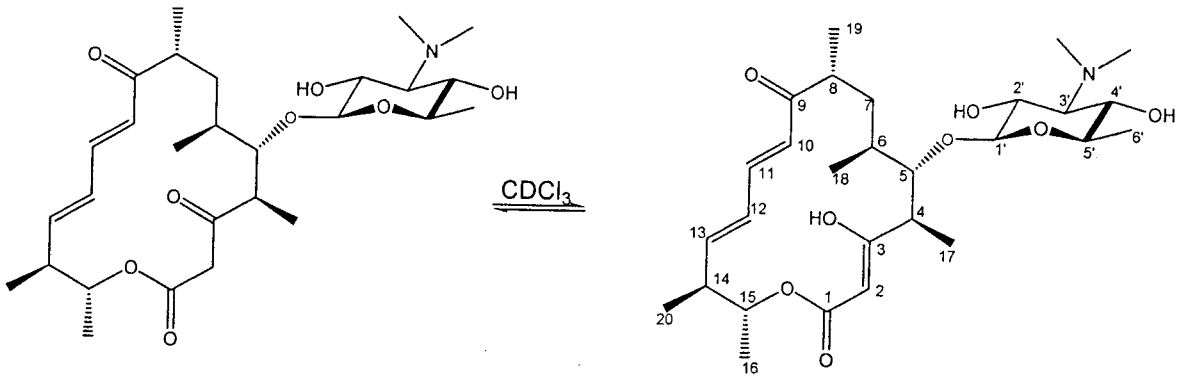

FIG. 3. 5-*O*-Mycaminosylchalcolactone.

into the *chm* PKS. This insertion was accomplished employing an in vivo cosmid packaging system to produce an *S. bikiniensis* integration vector and is described elsewhere (P. Revill et al., unpublished results).

Compound 5 was assayed for bioactivity and found to be virtually inactive (MICs, >12.5 μg/ml) against a number of *S. aureus* and *Streptococcus pneumoniae* strains. Since the original unpurified extracts of the transconjugants exhibited at least modest antibiotic activity, these results suggested the presence of at least one other more-potent chalcomycin-tylosin hybrid compound. Although structures of additional compounds were not determined, fragmentation patterns of the *m/z* 669 (M + H) compound are consistent with a structure consisting of the 3-keto macrolactone attached to the disaccharide of tylosin. Similarly, the fragmentation patterns of the *m/z* 843 (M + H) and *m/z* 845 (M + H) compounds are consistent with structures comprised of a 16-membered backbone and the three sugars of tylosin. It is conceivable that the *m/z* 845 (M + H) compounds may explain the antibacterial activity observed in the crude extract.

Deoxysugar genes. Genes for the synthesis of the two deoxysugar moieties of chalcomycin, D-chalcose and D-mycinose, are found in the cluster. Each is presumed to be derived from the common intermediate nucleoside diphosphate (NDP)-4-keto-6-deoxyglucose, which itself is produced from glucose-1-phosphate through the action of the gene products of *chmAI* and *chmAII* (Fig. 4).

Chalcose. As in the case of desosamine, synthesis of NDP-chalcose involves deoxygenation at C-4, which requires reduction of the radical formed after removal of the oxygen atom.

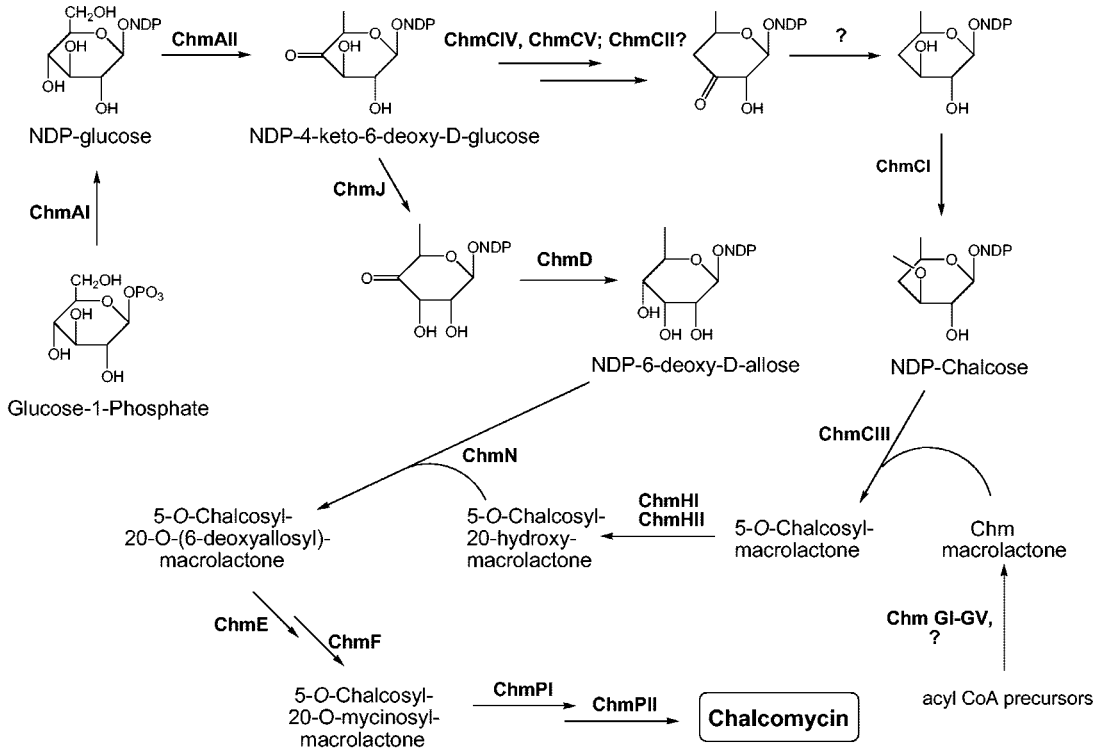

FIG. 4. Proposed pathways of deoxysugar biosynthesis and post-PKS modifications in *S. bikiniensis* showing roles for the various enzymes encoded by genes in the *chm* cluster.

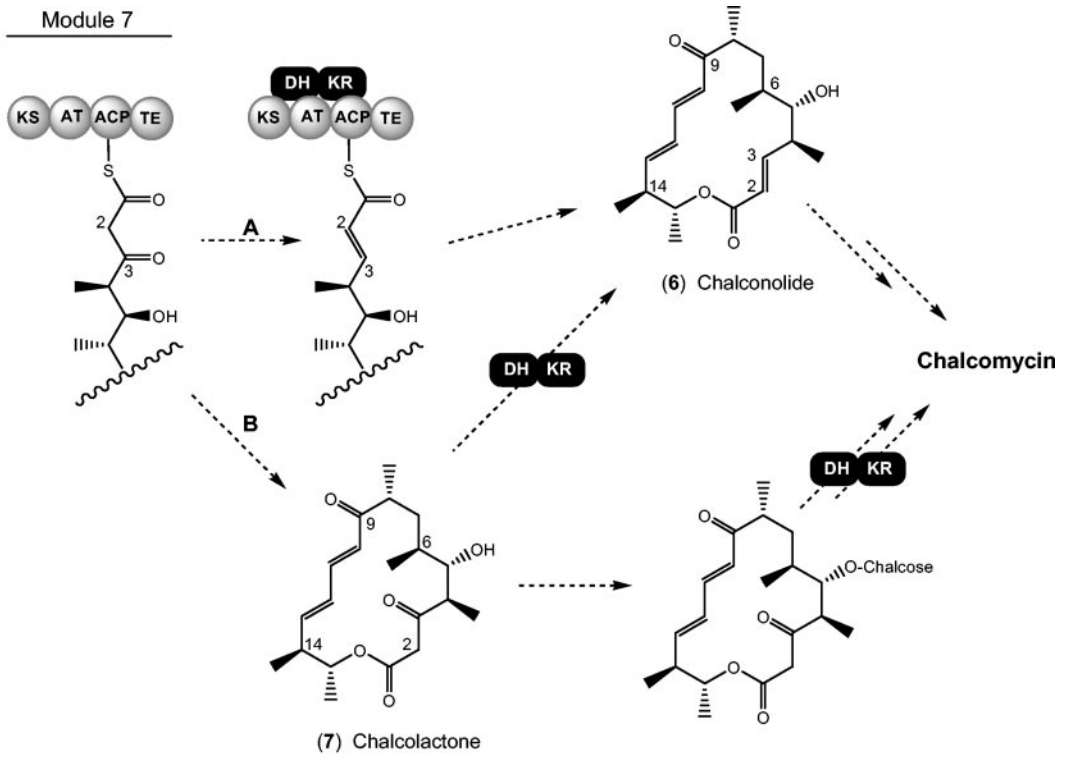

FIG. 5. Possible schemes for formation of the 2,3-*trans* double bond of the macrolactone produced from the *chm* PKS. (A) Two-step reduction and dehydration of the full-length polyketide chain prior to release from the PKS enzyme and cyclization; (B) post-PKS reduction and dehydration of the macrolactone showing two different points at which these reactions may occur.

We propose that these reactions are carried out by ChmCIV and ChmCV (Fig. 4), homologs of the desosamine biosynthesis enzymes EryCIV and EryCV, respectively, in the erythromycin pathway and of DesI and DesII, respectively, in the pikromycin pathway (12, 35, 40, 45). It has been proposed that deoxygenation takes place through the formation of a pyridoxamine-5′-phosphate (PMP)-sugar adduct at either C-3 (12, 40) or C-4 (16, 47). Formation of the PMP-sugar adduct at C-3 would require isomerization of the keto group from C-4 to C-3, a role proposed for EryCII in the desosamine pathway in erythromycin biosynthesis (12, 40). ChmCII is highly homologous to EryCII and could catalyze the analogous isomerization in chalcose biosynthesis, if the pathway proceeds through PMP-sugar adduct formation at C-3. If the adduct is formed at C-4, a role for ChmCII is not apparent. The next step after deoxygenation is reduction of the 3-keto group of the pathway intermediate. SpnQ is believed to serve this role in the biosynthesis of the forosamine component of spinosad (23). A homolog of SpnQ was not found within the >80-kb sequenced segment from the chalcomycin producer. The gene *chmU*, described below, which is presumed on the basis of sequence matching to encode a ketoreductase, lies immediately downstream of *chmCIII* but is thought to play a role in the introduction of the 2,3-double bond into the macrolactone component of chalcomycin. Hence, we do not presently have a candidate for the required 3-keto reduction step in chalcose synthesis in *S. bikiniensis*. The penultimate step, 3-*O*-methylation of NDP-4,6-dideoxy-D-glucose to produce NDP-chalcose, is likely carried out by ChmCI, which is highly similar to SpnH, a probable methyltransferase involved in O methylation of the rhamnose precursor of spinosyn (23, 42). Attachment of chalcose to the aglycone moiety is likely carried out by ChmCIII, a proposed glycosyltransferase.

Lankamycin is a 14-membered macrolide that also contains chalcose. Homologs of ChmCII, ChmCIII, ChmCIV, and ChmCV were found in the lankamycin (*lkm*) cluster, but their putative roles were not described (24).

Mycinose. Synthesis of the mycinose precursor NDP-6-deoxy-D-allose from NDP-4-keto-6-deoxyglucose requires two enzymes, a 3-epimerase and a 4-ketoreductase, most likely encoded by *chmJ* and *chmD* (Fig. 4); both are highly similar to their counterparts in the tylosin pathway. Attachment of 6-deoxy-D-allose to the macrolactone requires a glycosyltransferase, likely encoded by *chmN*. Attachment also requires a primary hydroxyl group at C-20 of the macrolactone, hence the need for the P450 enzyme encoded by *chmHI* and its corresponding ferredoxin encoded by the *chmHII* gene, which are orthologs of the corresponding proteins, TylHI and TylHII, respectively, that hydroxylate the C-23 position of the tylosin macrolactone. In the biosynthesis of tylosin, attachment of 6-deoxy-D-allose to the macrolactone takes place after attachment of the 5-*O*-sugar (mycaminose) and cannot take place in mutants that do not carry out this step (for a review, see reference 3). Hence, we propose that attachment of 6-deoxy-D-allose to the chalcose macrolactone also takes place after addition of chalcose to the ring, as shown in Fig. 4. Because of the uncertainty of the step at which the 2,3-double bond is introduced into the ring, we do not present the structure for

the intermediates that carry one or both sugars. Conversion of the 6-deoxy-D-allose residue to D-mycinose, after attachment to the macrolactone, requires the action of two *O*-methyltransferases, likely encoded by *chmE* and *chmF*, each ca. 70% identical to their respective counterparts in the tylosin biosynthesis cluster, TylE and TylF, which are also proposed to act after the sugar has been attached to the backbone. As can be seen in Fig. 2A, the seven genes required for the synthesis and attachment of the mycinose unit of chalcomycin are clustered.

Late steps in the pathway. Two oxidation steps are required to convert the product of ChmF to chalcomycin, i.e., hydroxylation at C-8 and 12,13-epoxidation of the lactone ring, but the order of the reactions is not known. Each of these steps is proposed to be carried out by one of the cytochrome P450 enzymes, ChmPI or ChmPII.

DISCUSSION

The structure of compound 5 confirms the prediction that the *chm* PKS does not encode the activities required for introduction of the 2,3-*trans* double bond and suggests that it is introduced by a 3-ketoreductase and a 2,3-DH that are not components of the *chm* PKS. This is an unprecedented finding in macrolide biosynthesis. Mycinamicin also contains a 2,3-*trans* double bond, but the mycinamicin PKS contains the expected DH and β-ketoreductase domains in module 7 (1). As shown in Fig. 5, introduction of the double bond into chalcomycin could take place either during the seventh elongation step of nascent polyketide chain synthesis to release a Δ-2,3-aglycone (chalconolide [compound 6]), or after the chain has been completed and cyclized to produce the 3-keto-aglycone (chalcolactone [compound 7]). Chalconolide would be produced if the required β-ketoreductase and DH enzymes interacted with the nascent polyketide chain while it is attached to the acyl carrier protein domain of module 7 (Fig. 5, scheme A). Interactions between type I PKS systems and discrete enzymes have been reported for acyltransferases (9, 30) but not for ketoreductases or DHs. The gene *chmU*, which lies ca. 3 kb downstream of the PKS-encoding *chm*, encodes a protein that appears to belong to the short-chain dehydrogenase/reductase family, which is composed of many ketoreductases, including those that participate in the synthesis of polyketides and fatty acids (as components of type II enzymes) and might fulfill the role of the 3-ketoreductase. A gene encoding a candidate 2,3-DH was not observed in the cluster, however.

The alternative pathway for introduction of the 2,3-*trans* double bond in chalcomycin biosynthesis, after the 3-keto acyl chain is released from the PKS and cyclized, is depicted in Fig. 5, scheme B. Because there are no reported examples of post-PKS 3-reduction or 2,3-dehydration in 16- (or 14-) membered macrolides, it is not possible to pinpoint the precise step at which these events would take place along the pathway of synthesis of chalcomycin after the formation of chalcolactone. A number of 16-membered macrolides, including spiramycin, have been identified in which the 9-keto group is reduced to its 9-hydroxy counterpart. The spiramycin PKS does not contain an active β-ketoreductase domain in the fourth module (7), hence reduction is believed to take place through the action of a discrete 9-ketoreductase following release of the 9-keto-macrolactone platenolide from the PKS. The seventh module of the type I PKS of *Streptomyces* strain HK-803, which produces phoslactomycin B, an antitumor polyketide, contains the required KR domain but lacks the DH domain for 2,3-dehydration of the nascent acyl chain; hence, post-PKS 2,3-dehydration is thought to occur. Again, however, a candidate 2,3-DH was not observed in the phoslactomycin biosynthesis cluster (27). A DH domain required for the biosynthesis of myxalamid is also absent from the corresponding PKS in the producing myxobacterial host (39).

Compound 5 represents the first 3-keto 16-membered macrolide isolated from a fermentation, in this case an unnatural natural product. The 14-membered macrolide pikromycin, produced from *Streptomyces venezuelae*, contains a 3-keto group which does not exist in the enol form to a significant degree, and a 3-keto derivative of the 14-membered macrolide erythromycin, termed "ketolides," has been successfully developed as a drug useful against many gram-positive hosts that are resistant to macrolides (for a review, see reference 46). 3-Keto derivatives of tylosin synthesized chemically were also found to adopt the 2,3-*trans* enol configuration and were shown to have significantly diminished antimicrobial activity compared to their 3-hydroxy counterparts (10). Whether the loss of antibiotic potency is due to the enolization of the 3-keto group remains to be determined.

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