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# Genetic Characterization of the Chlorothricin Gene Cluster as a Model for Spirotetronate Antibiotic Biosynthesis

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## Summary

**The biosynthetic gene cluster for chlorothricin (CHL) was localized to a 122 kb contiguous DNA from *Streptomyces antibioticus* DSM 40725, and its involvement in CHL biosynthesis was confirmed by gene inactivation and complementation. Bioinformatic analysis of the sequenced 111.989 kb DNA region revealed 42 open reading frames, 35 of which were defined to constitute the CHL gene cluster. An assembly model for CHL biosynthesis from D-olivose, 2-methoxy-5-chloro-6-methylsalicyclic acid, and chlorothricolide building blocks was proposed. This work represents cloning of a gene cluster for spirotetronate antibiotic biosynthesis and sets the stage to investigate the unusual macrolide biosynthesis including tandem Diels-Alder cyclizations, Baeyer-Villiger oxidation, and incorporation of an enoylpyruvate unit.**

## Introduction

The spirotetronate antibiotics are a class of natural products that exhibit broad biological activities, including antibacteria, antitumor, antimalaria, and cholesterol biosynthesis inhibition [1–4]. They feature an unusual aglycone that contains a characteristic tetronic acid (spiro-linked to a cyclohexene ring) conjugated with a *trans*-decalin system either by a carboxylic ester, such as chlorothricin (CHL), or by a carbonyl group, such as kijanimicin (KIJ) or tetrocarcin A (TC-A) (Figure 1). Further decoration of the structurally related aglycones with a variety of deoxysugar and/or other peripheral moieties renders the diversity of structures and activities of members in this family.

Although most of the spirotetronate antibiotics possess moderate activity against gram-positive bacteria, CHL is the only member whose mechanism of action was demonstrated to inhibit pyruvate carboxylase, which catalyzes the anaplerotic $CO_2$ fixation on synthetic media [5]. Later screening of the microbial compounds indicated that CHL and its derivatives inhibit cholesterol biosynthesis via the mevalonate pathway [4]. KIJ is active in vitro against an unusual range of microorganisms (including some anaerobic bacteria), and studies in vivo have shown that it also has antimalarial activity [2]. TC-A exhibits remarkable activities against various experimental tumor models, but it has no significant myelosuppression and nephrotoxicity in mice [6]. Recently, studies of effects on T-acute lymphoblastic leukemia (T-ALL) and B-chronic lymphocytic leukemia (B-CLL) suggest that TC-A induces apoptosis by a novel signal transduction pathway and might represent an attractive lead for further antitumor drug development [7, 8]. The extensive investigation of the structure-activity relationships relied entirely on the semisynthesis from the available natural products [9], and alterations of the macrolide backbones have not yet been explored, probably in part because of the structural complexity.

CHL, isolated from *Streptomyces antibioticus* Tü99 (deposited in DSMZ with the accession number DSM 40725) in 1969 [1], was the first member of the spirotetronate family to be structurally elucidated. The structure of chlorothricolide (the aglycone of CHL) was originally elucidated by using spectroscopic methods and degradation experiments [10] and was ultimately confirmed by single-crystal X-ray analysis [11]. Thereafter, chlorothricolide, as a molecular model of the spirotetronate macrolides, has stimulated considerable efforts for its total synthesis [12, 13].

While great success has been achieved in the chemical synthesis of CHL and spirotetronates, the biosynthetic studies on spirotetronate natural products are still limited. Previous feeding experiments examining CHL biosynthesis showed that the backbones of 2-methoxy-5-chloro-6-methylsalicyclic acid and chlorothricolide (with the exception of C-22, -23, and -24; Figure 1) are likely of polyketide origin, and that the two deoxysugar (D-olivose) moieties derive from glucose [14–16]. Here, we report the cloning and sequencing of the CHL biosynthetic gene cluster from *S. antibioticus* DSM 40725, and the functions of the deduced gene products are proposed. Sequence analyses revealed a pathway for the biosynthesis of the characteristic macrolide aglycone and supported an assembly model for CHL biosynthesis from D-olivose, 2-methoxy-5-chloro-6-methylsalicyclic acid, and chlorothricolide building blocks. To our knowledge, this study represents the first cloning of a gene cluster for spirotetronate antibiotic biosynthesis. The findings set the stage to investigate the genetic and biochemical basis for CHL biosynthesis and make it possible to generate structural diversity for drug discovery and development by applying combinatorial biosynthesis to the CHL biosynthetic machinery.

## Results and Discussion

### Cloning and Identification of the CHL Gene Cluster from *S. antibioticus* DSM 40725

It has been well established that most deoxysugars derive from the common intermediate 4-keto-6-deoxyglucose

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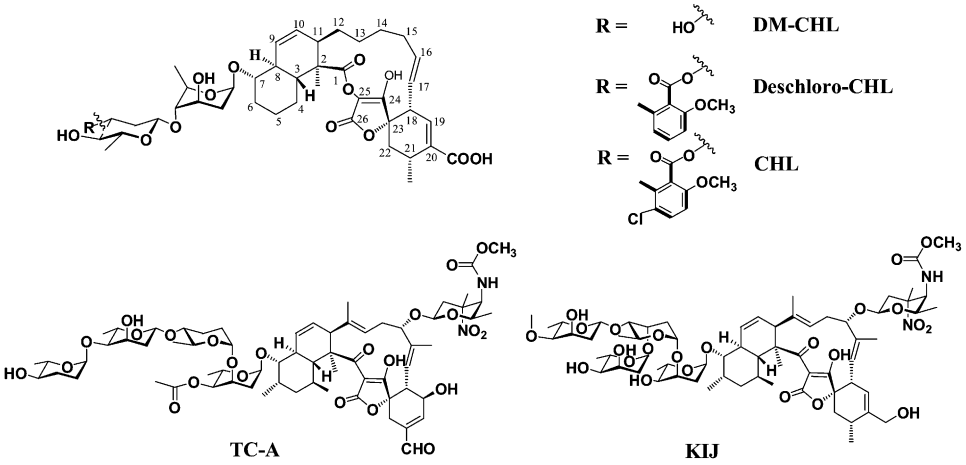


Figure 1. Structures of Chlorothricin, Deschloro-Chlorothricin, Desmethylsalicycloyl Chlorothricin, Kijanimicin, and Tetrocarcin A

nucleoside diphosphate, the biosynthesis of which originates from glucose nucleoside diphosphate and is catalyzed by a conserved $NAD^+$-dependent oxidoreductase (*dNDP*-D-glucose-4,6-dehydratase, NGDH) [17]. Using PCR and Southern hybridization approaches, Sohng and coworkers identified at least three putative NGDH genes from the genomic DNA of the CHL producer *S. antibioticus* Tü99 [18], and one of those, *oxiIII*, was suggested to be involved in the CHL deoxysugar biosynthesis due to the fact that *oxiIII* is adjacent to a partial type I PKS gene [19]. As a result, we utilized the *oxiIII* sequence and cloned a 550 bp internal fragment of *oxiIII* by PCR. However, inactivation of the cloned NGDH gene resulted in the mutant strain TL1001 that retains the ability of CHL production (Figure 2, II), excluding its involvement in CHL biosynthesis.

Since the feeding experiment by incorporation of isotope-labeled acetate and propionate into the CHL agylcone supported its polyketide origin [15], we adopted a strategy to clone the putative chlorothricolide PKS genes by PCR with degenerate primers designed according to the highly conserved motifs of type I PKSs (described in Experimental Procedures). A single band with the predicted size of 750 bp was amplified and cloned into pGEM-T easy vector (Table S1; see the Supplemental Data available with this article online). By digestion with various restriction enzymes, eight resultant clones were classified into two distinct groups (represented by pTL1023 and pTL1024, respectively), both of which were shown to be highly homologous to known type I PKS genes based on sequence analysis. To determine whether these putative PKS genes are required for

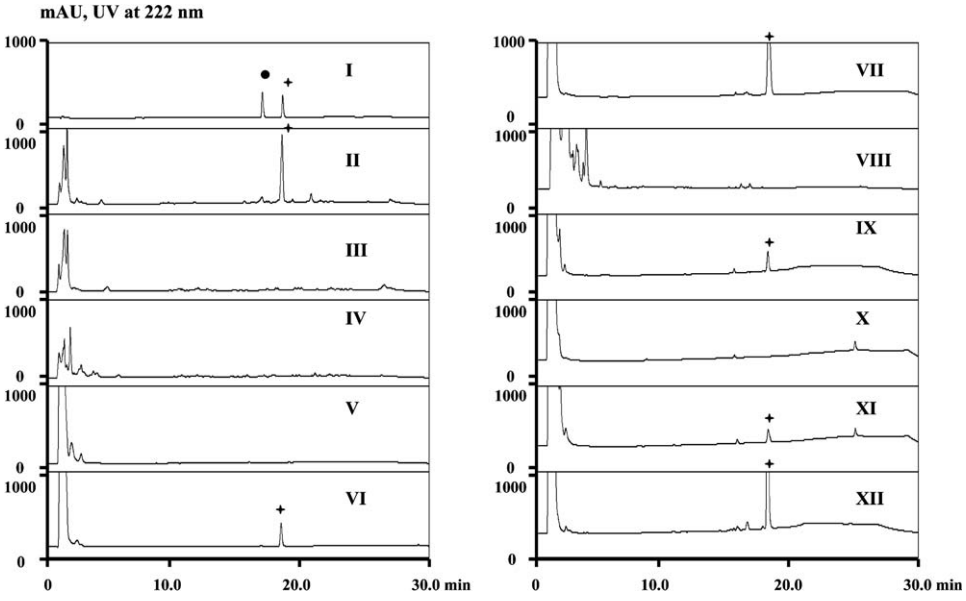


Figure 2. HPLC Analysis of CHL Production by *S. antibioticus* Wild-Type and Recombinant Strains

(I) An authentic standard, (II) TL1001 (*ΔNGDH*), (III) TL1002 (*ΔPKS-P1*), (IV) TL1003 (*ΔPKS-P2*), (V) TL1006 (*ΔchlD3*), (VI) TL1010 (*chlD3:ermE**), (VII) wild-type, (VIII) TL1005 (*ΔchlC7*), (IX) TL1009 (*chlC7:ermE**), (X) TL1007 (*ΔchlD4*), (XI) TL1011 (*chlD4:ermE**), and (XII) TL1004 (*Δorf(-1)*). The asterisk and dark dot indicate CHL and Deschloro-CHL, respectively.

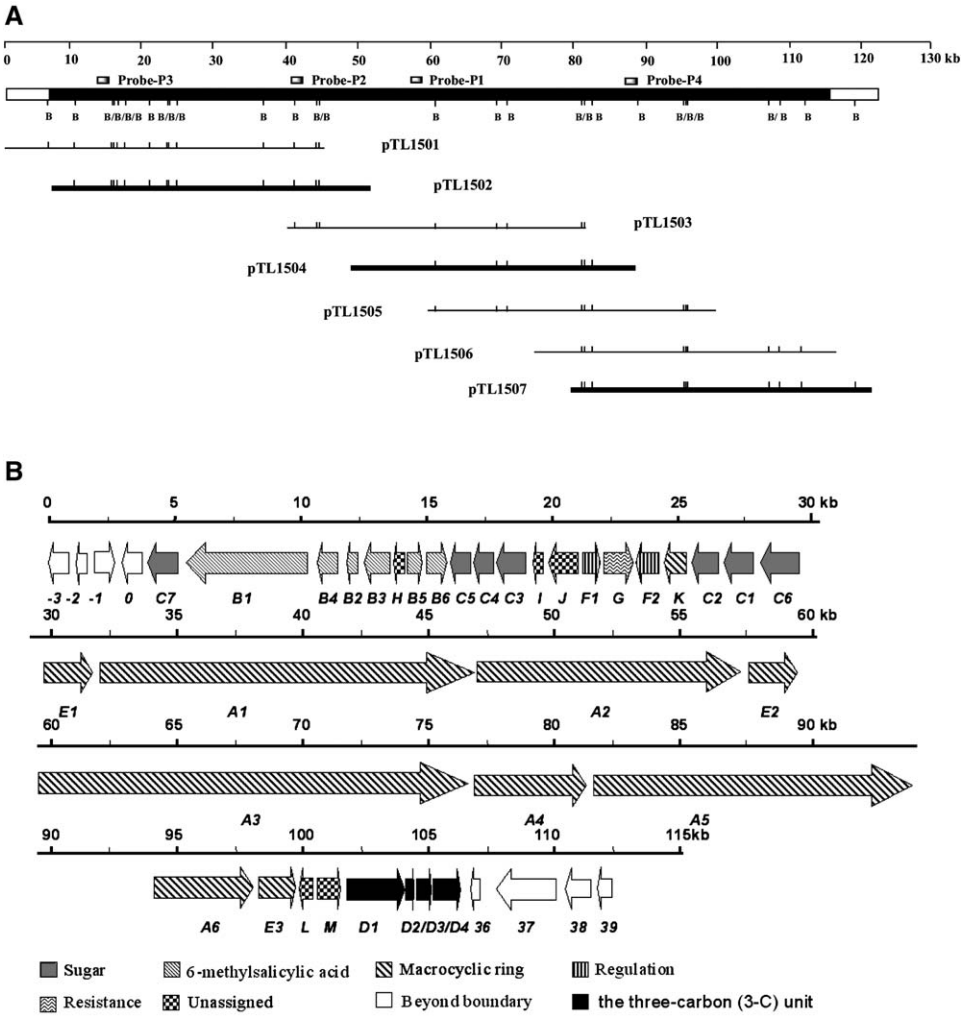


Figure 3. Restriction Map and Genetic Organization of the CHL Biosynthetic Gene Cluster

(A) Restriction map of the 122 kb DNA region from *S. antibioticus* DSM 40725 as represented by seven overlapping cosmid clones. "B," BamHI. Solid black bars indicate the sequenced DNA region; solid quadrates indicate the probe loci.

(B) Genetic organization of the CHL biosynthetic gene cluster. Proposed functions of individual *orfs* are shade coded and summarized in Table 1.

CHL biosynthesis, we set out to inactivate their target alleles and found that the resultant mutants, TL1002 and TL1003, completely lost the ability to produce CHL (Figure 2, III and IV), unambiguously confirming their involvement in CHL biosynthesis. Subsequently, the digoxigenin-(DIG)-labeled PCR product P1 from pTL1023 was utilized as a probe to screen approximately 6,000 clones of the genomic library, resulting in the isolation of a set of overlapping cosmids spanning 70 kb. Further Southern hybridization with the DIG-labeled PCR product P2 from pTL1024 indicated that both P1 and P2 are closely linked within a DNA region of approximately 15 kb (Figure 3A).

In addition, a novel iterative PKS gene, *chlB1*, for 6-methylsalicylic acid biosynthesis was cloned by PCR with the degenerate primer pair *C-D* designed according to the conserved motifs that are unique to iterative type I PKSs (L.S. et al., unpublished data). Consistent with the fact that genes for antibiotic production are commonly clustered in one region of the chromosome in *Actinomycetes*, the PCR product of *chlB1* was localized to a single 5.1 kb BamHI fragment that is ~25 kb upstream of the P2 locus. Taken together, these results provided strong support that the CHL gene cluster in *S. antibioticus* DSM 40725 was cloned. To ensure full coverage of the entire CHL cluster, we carried out additional chromosomal walking from the *chlB1* locus (P3, a 0.9 kb PCR product of *chlB1* amplified with the primer pair *C-D*) and the right end of pTL1504 (P4, a 0.5 kb PCR product), and we eventually identified a 122 kb contiguous DNA region of overlapping cosmids, as exemplified by pTL1501, pTL1502, pTL1503, pTL1504, pTL1505, pTL1506, and pTL1507 (Figure 3A).

## Sequence Analysis and Gene Organization of the CHL Cluster

The DNA region represented by three overlapping cosmids, pTL1502, pTL1504, and pTL1507, was selected for shotgun DNA sequencing, yielding 111,989 bp of contiguous sequence. The overall GC content of the sequenced region is 71.8%, characteristic of *Streptomyces* DNA. Bioinformatic analysis (described in Experimental

Table 1. Deduced Functions of *orfs* in the CHL Biosynthetic Gene Cluster

| Gene | Size[a] | Protein, Homolog,[b] and Origin | Similarity/ Identity, % | Proposed Function |
|---|---|---|---|---|
| *orf(-3)*[c] | 222 | YHJG (NP_266946); *Lactococcus lactis subsp.* | 47/29 | Acetyl transferase |
| *orf(-2)*[c] | 136 | — | — | Unknown protein |
| *orf(-1)*[c] | 206 | DVU2436 (YP_011649); *Desulfovibrio vulgaris subsp.* | 60/51 | Predicted hydrolases of the HD superfamily |
| *orf(0)*[c] | 246 | PF01526 (BAC68130); *S. avermitilis* MA-4680 | 74/65 | Transposase |
| *chlC7* | 411 | CmmGIII (CAE17545); *S. griseus subsp. griseus* | 54/42 | Glycosyltransferase |
| *chlB1* | 1756 | AviM (AAK83194); *S. viridochromogenes* | 70/58 | Type I PKS: KS-AT-KR-DH-ACP |
| *chlB4* | 449 | PltA (AAQ90172); *Pseudomonas sp.* M18 | 77/62 | Halogenase |
| *chlB2* | 88 | CouN5 (AAG29790); *S. rishiriensis* | 67/38 | Discrete ACP |
| *chlB3* | 347 | AviN (AAK83178); *S. viridochromogenes* | 64/49 | Ketoacyl-ACP synthase, condensing protein |
| *chlH* | 137 | — | — | Unknown protein |
| *chlB5* | 238 | COG4122 (ZP_00343262); *Desulfitobacterium hafniense* DCB-2 | 61/48 | O-methyltransferase |
| *chlB6* | 352 | CalO4 (AAM70354); *Micromonospora echinospora* | 51/37 | Ketoacyl-ACP synthase, condensing protein |
| *chlC5* | 247 | LanR (AAD13548); *S. cyanogenus* | 64/50 | 4-Ketoreductase |
| *chlC4* | 331 | AknQ (AAF73453); *S. galilaeus* | 63/53 | 3-Ketoreductase |
| *chlC3* | 438 | ORF18 (AAG52988); *Amycolatopsis mediterranei* | 63/51 | 2,3-Dehydratase |
| *chlI* | 70 | simX2 (AAK06794); *S. antibioticus* | 58/51 | Coupling protein of carboxyl transferase |
| *chlJ* | 535 | SCO5535 (CAA19983); *S. coelicolor* A3(2) | 95/91 | Carboxyl transferase |
| *chlF1* | 202 | TetR family (YP_103523); *Burkholderia pseudomallei* K96243 | 53/37 | Transcriptional regulator |
| *chlG* | 488 | SCO1194 (CAB61606); *S. coelicolor* A3(2) | 57/40 | Export protein |
| *chlF2* | 261 | MonRI (AAO65809); *S. cinnamonensis* | 84/75 | Response regulator of 2-component system |
| *chlK* | 266 | MonAX (AAO65810); *S. cinnamonensis* | 73/63 | Type II TE |
| *chlC2* | 327 | Med-ORF17 (BAC79030)*S. sp.* AM-7161 | 77/64 | dNDP-glucose-4,6-dehydratase |
| *chlC1* | 355 | CmmD (CAE17529); *S. griseus subsp. griseus* | 71/56 | dNDP-glucose synthase |
| *chlC6* | 404 | CmmGIII (CAE17545); *S. griseus subsp. griseus* | 53/40 | Glycosyltransferase |
| *chlE1* | 516 | CmmOIV (CAE17536); *S. griseus subsp. griseus* | 58/46 | FAD-dependent oxygenase |
| *chlA1* | 4699 | SCO6275 (CAD55506); *S. coelicolor* A3(2) | 62/52 | Type I PKS: $KS^{Q}$-AT-ACP-KS-AT-DH-KR-ACP-KS-AT-DH-KR-ACP |
| *chlA2* | 3955 | Orf16 (AAX98191); *S. aizunensis* | 66/54 | Type I PKS: KS-AT-DH-KR-ACP-KS-AT-DH-ER-KR-ACP |
| *chlE2* | 398 | Cyp234 (AAT45294); *S. tubercidicus* | 57/44 | Cytochrome P-450 hydroxylase |
| *chlA3* | 5718 | Orf16 (AAX98191); *S. aizunensis* | 65/53 | Type I PKS: KS-AT-DH-ER-KR-ACP-KS-AT-DH-KR-ACP-KS-AT-DH-KR-ACP |
| *chlA4* | 1853 | Orf13 (AAX98188); *S. aizunensis* | 63/52 | Type I PKS: KS-AT-DH[c]-KR-ACP |
| *chlA5* | 3936 | Orf16 (AAX98191); *S. aizunensis* | 66/54 | Type I PKS: KS-AT-DH-ER-KR-ACP KS-AT-DH-KR-ACP |
| *chlA6* | 1583 | Orf13 (AAX98188); *S. aizunensis* | 61/49 | Type I PKS: KS-AT-ACP |
| *chlE3* | 498 | CmmOIV (CAE17536); *S. griseus subsp. griseus* | 53/42 | FAD-dependent oxygenase |
| *chlL* | 191 | LSU (YP_190800); *Gluconobacter oxydans* 621H | 44/31 | Ribosomal protein L15P |
| *chlM* | 375 | COG0332 (ZP_00110099); *Nostoc punctiforme* PCC 73102 | 65/45 | 3-Oxoacyl-ACP synthase III |
| *chlD1* | 651 | FkbH (AAF86387); *S. hygroscopicus var. ascomyceticus* | 44/31 | Involved in glycerol-S-ACP biosynthesis |
| *chlD2* | 75 | COG0236 (ZP_00110101); *Nostoc punctiforme* PCC 73102 | 62/40 | Acyl carrier protein |
| *chlD3* | 271 | COG0508 (ZP_00110102); *Nostoc punctiforme* PCC 73102 | 65/44 | Pyruvate/2-oxoglutarate dehydrogenase/ dehydratase |
| *chlD4* | 362 | COG0596 (ZP_00110109); *Nostoc punctiforme* PCC 73102 | 59/38 | Predicted hydrolases or acyltransferases |
| *orf36*[c] | 131 | COG0624 (ZP_00134467); *Actinobacillus leuropneumoniae serovar 1 str.* 4074 | 46/36 | Unknown protein, similar to acetylornithine deacetylase |
| *orf37*[c] | 813 | SCP1.136 (CAC36657); *S. coelicolor* A3(2) | 44/31 | Putative helicase |
| *orf38*[c] | 374 | ANK (EAP00483); *Marinobacter aquaeolei* VT8 | 37/50 | Ankyrin repeat |
| *orf39*[c] | 168 | TAB182 (AAM15531); *Homo sapiens* | 29/40 | Tankyrase1-binding protein |

[a] Numbers are in amino acids.
[b] NCBI accession numbers are given in parentheses.
[c] *orfs* beyond the CHL gene cluster.

Procedures) revealed 42 open reading frames (*orfs*), and individual *orfs* were functionally assigned by comparison to proteins of known functions in the database (shown in Figure 3B; summarized in Table 1). The GenBank accession number for the CHL cluster is DQ116941.

### Determination of the CHL Gene Cluster Boundaries

To determine the boundaries of the CHL gene cluster, we inactivated a series of *orfs* by gene disruption or replacement within the sequenced region. Inactivation of *chlD3*, *chlD4*, and *chlC7* completely abolished CHL production (Figure 2, V, X, and VIII), confirming that they are

essential for CHL biosynthesis. In contrast, inactivation of *orf(-1)* had no effect on CHL production (Figure 2, XII), clearly indicating that its locus resides outside the *chl* cluster. Several attempts to inactivate *orf36* and *orf37* resulted in non-CHL-producing mutants whose genotypes did not match the predicted patterns based on Southern hybridization analysis (data not shown). Sequence analysis revealed that *orf37* encodes an 813 aa putative helicase, and a 1203 bp noncoding region between *orf36* and *orf37* exists, suggesting that *orf37* and its flanking noncoding region are outside of the *chl* cluster but may be indispensable for the replication of an extrachromosomal plasmid, and genetic manipulation at the adjacent *orf36* locus may affect its stability. This prediction is consistent with our recent evidence that the CHL gene cluster is carried on a linear plasmid (data not shown). We prefer the possibility that *orf36* is outside of the CHL gene cluster for two reasons: first, in addition to *orf37*, *orf38*, and *orf39*, *orf36* lacks significant similarity to genes involved in secondary metabolite biosynthesis; and second, the failure of generating an *orf36* mutant suggests that it may not be a "real" gene and that its putative coding region may function in *cis* for replication. These results, together with the functional assignment of deduced gene products within the sequenced region, support the conclusion that the CHL gene cluster is minimally contained within the region from *chlC7* to *chlD4*, which spans 101.8 kb and encompasses 35 *orfs*. Therefore, establishment of the boundaries of the CHL gene cluster provided a basis to predict the CHL biosynthetic pathway.

### Regulation and Self-Resistance of CHL Biosynthesis

Two putative regulatory genes, *chlF1* and *chlF2*, are identified, and the deduced functions would agree with their roles as pathway-specific regulators involved in CHL biosynthesis. While ChlF1 belongs to a TetR regulatory protein family, ChlF2 contains a putative effector domain of response regulators, suggesting that it is a member of a two-component system that could detect and respond to changes in the environment, and eventually control the CHL biosynthesis. Only one putative resistance gene, *chlG*, is found in the CHL cluster. The deduced protein of *chlG* is homologous to members of a general family of transporters. Since most of the CHL is extracted from the ultrasonic-fragmented mycelia, ChlG may not be efficient to transport the synthesized product out of the cells.

### Biosynthesis of the Deoxysugar Moiety

2,6-Dideoxysugars are frequently found to be the vital components in secondary metabolites [20]. In the past decades, a number of genes involved in their biosyntheses have been cloned and extensively investigated [21]. Seven genes, *chlC1–chlC7*, are identified in the CHL gene cluster and encode proteins that are homologous to enzymes for D-olivose biosynthesis, the pathway of which has been well established in mithramycin [22], chromomycin [23], urdamycin [24], and landomycin [25] producers, indicating that a similar strategy is utilized in the biosynthesis of the CHL deoxysugar moiety, as outlined in Figure 4A.

The deduced product of *chlC1* resembles a member of the family of *dNDP*-glucose synthases, consistent with the feeding experiment that established incorporation of isotope-labeled glucose into the CHL deoxysugar moiety. After *dNDP* activation of glucose, ChlC2, closely related to members of the NGDH family, assumedly catalyzes the formation of *dNDP*-4-keto-6-deoxyhexose (**1**). ChlC3, highly homologous to *dNDP*-hexose dehydratases that catalyze 2,3-dehydration to form $\alpha,\beta$-unsaturated 4-ketosugar, together with ChlC4, similar to 3-ketoreductases that render a hydroxyl group at C-3 with an equatorial configuration, is likely involved in the C-2 deoxygenation step, forming *dNDP*-4-keto-2,6-hexose (**2**). ChlC5, a putative 4-ketoreductase, may reduce the keto group at C-4, leading to the formation of *dNDP*-olivose (**3**). Both ChlC6 and ChlC7, resembling a family of glycosyl transferases, are presumably responsible for tandemly transferring two dexoysugar moieties to the CHL aglycone. The *chlC7* mutant strain TL1005 completely lost its ability to produce CHL (Figure 2, VIII), which was then restored by expressing *chlC7* in *trans* under the control of the *ermE** promoter (TL1009, Figure 2, IX), confirming its involvement in CHL biosynthesis.

### Biosynthesis of the 2-Methoxy-5-Chloro-6-Methylsalicyclic Acid Moiety

Six genes, *chlB1–chlB6*, are identified within the CHL cluster, and according to their deduced functions, the biosynthetic pathway of 2-methoxy-5-chloro-6-methylsalicyclic acid is established as outlined in Figure 4B. The deduced product of *chlB1* has the characteristic type I PKS domains, including a ketosynthase (KS), acyltransferase (AT), dehydratase (DH), ketoreductase (KR), and acyl carrier protein (ACP). Although it is well known that aromatic polyketide biosyntheses are catalyzed by iterative type I PKSs in fungi [26], a similar paradigm in bacteria in the biosyntheses of orsellinic acid and naphthoic acid has been undescribed until recently [27]. ChlB1 exhibits head-to-tail homology to NcsB that catalyzes the naphthoic acid biosynthesis for neocarzinostatin in *S. carzinostaticus* [28], and to AviM and CalO5 (excluding the KR domain), both of which catalyze orsellinic acid biosynthesis for avilamycin in *S. viridochromogenes* [29] and calicheamicin in *Micromonospora echinospora* [30]. In a mechanistic analogy, ChlB1 (as AviM or CalO5) could catalyze the assembly of a nascent linear tetraketide (**4**) from one acetyl-coenzyme A (CoA) and three malonyl-CoAs, and the additional KR domains of ChlB1 (the similar domain was found in NcsB) may offer a selective activity of keto reduction at the C-5 position. The resultant unsaturated intermediate undergoes an intramolecular aldol condensation to furnish the 6-methylsalicyclic acid (**5**) structure. Subsequently, ChlB5, a member of the SAM-dependent methyltransferase family, and ChlB4, a homolog of the halogenase family, are presumably responsible for C-2 *O*-methylation and C-5 chlorination to afford the fully modified methylsalicyclic acid moiety (**7**). This deduced biosynthetic pathway is consistent with the isotope-labeled feeding experiments that showed 2-methoxy-5-chloro-6-methylsalicyclic acid containing four head-to-tail acetates and an *O*-methyl group from S-adenosyl-L-methionine (SAM) [14, 15].

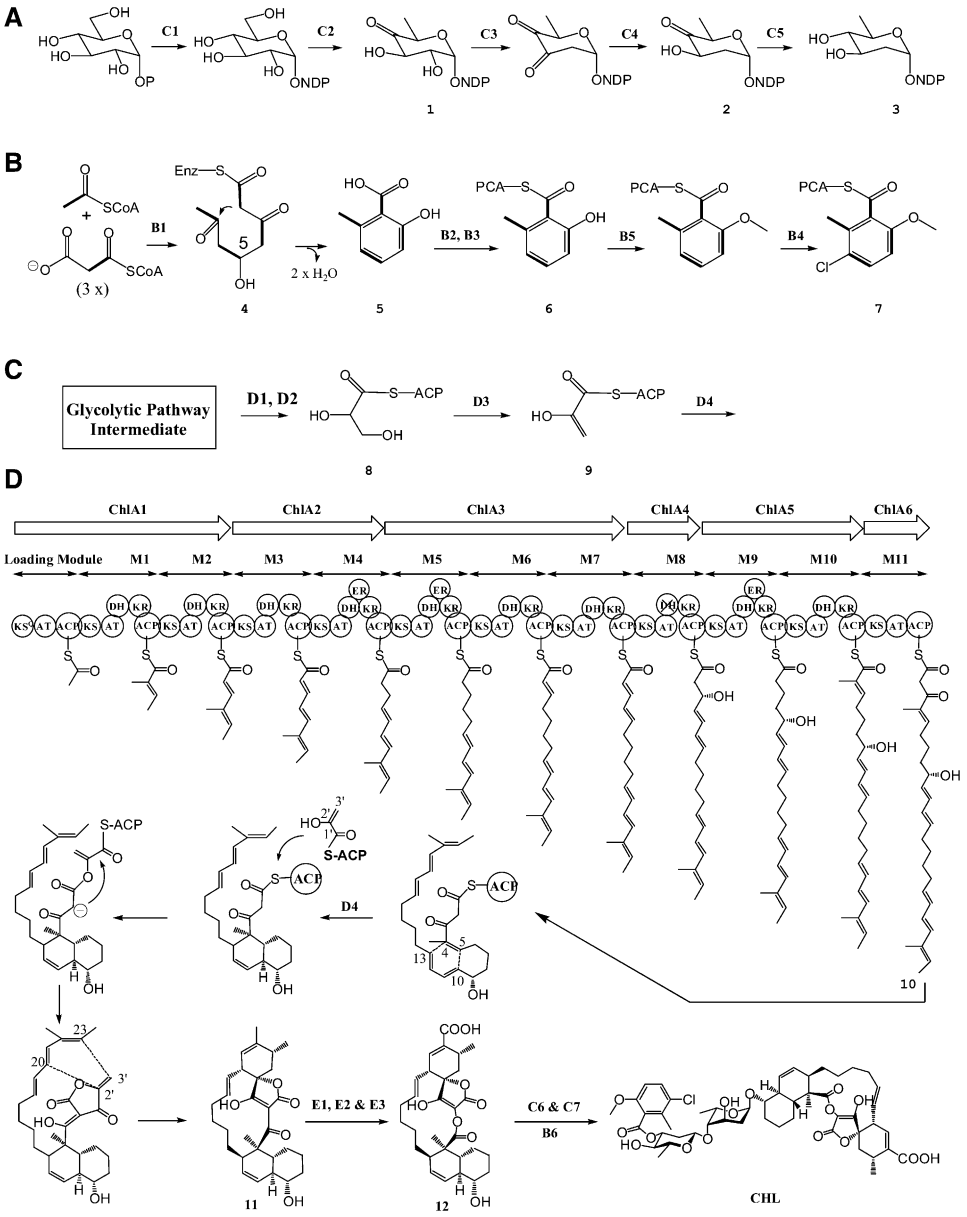


Figure 4. Proposed Biosynthetic Pathways

(A–D) Pathways for (A) deoxysugar olivose, (B) 2-methoxy-5-chloro-6 methylsalicyclic acid, (C) 3-carbon unit enoylpyruvate, and (D) chlorothricolide. A model for the assembly of CHL is also shown.

To confirm the role of ChlB1, we inactivated *chlB1* by replacing it with the apramycin resistance gene, *aac(3)IV*. As shown in Figures 5A and 5B (III), the resultant mutant strain TL1012 produces a compound that is biologically active but distinct from CHL upon HPLC analysis. The resulting compound was isolated and subjected to LC-MS analysis, exhibiting an $(M\text{-}H)^-$ ion at m/z = 771.3, consistent with the molecular formula $C_{41}H_{56}O_{14}$. By comparison with CHL, it was proposed to be demethylsalicycloyl CHL (DM-CHL) that lacks the entire modified methylsalicyclic acid moiety. The deduced conclusion was further confirmed by HR-MS and NMR spectra analyses (detailed data are summarized in Supplemental Data). Introduction of *chlB1* into the mutant strain TL1012 led to the recombinant strain TL1013 (Figure 5B, IV), in which the compound DM-CHL was converted into CHL (purified by HPLC and confirmed by LC-MS, data not shown). These results, together with the above-described functional assignments of ChlB4 as a halogenase and ChlB5 as an *O*-methyltransferase, respectively, support the conclusion that ChlB1 acts as a 6-methylsalicyclic acid synthase in the biosynthetic pathway of 2-methoxy-5-chloro-6-methylsalicyclic acid.

A pair of genes, *chlB2* and *chlB3*, that are just upstream of *chlB4*, encode a putative discrete ACP protein and β-ketoacyl-ACP synthase, respectively. Since the covalent tethering as the acyl-S-ACP (or PCP) is a common strategy to sequester the substrate and provide a platform for further decoration in many biosynthetic pathways of secondary metabolites, we propose that

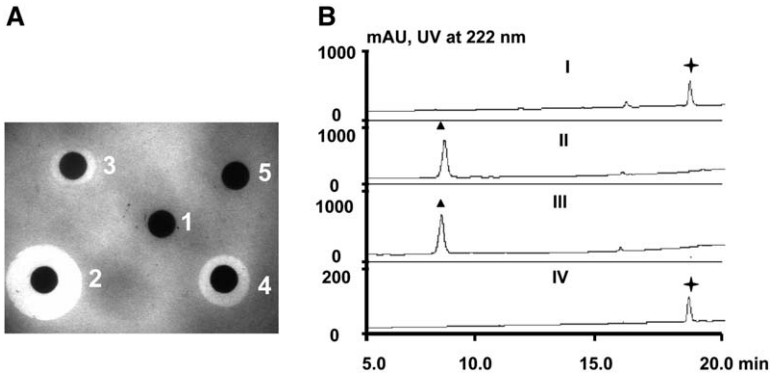


Figure 5. Bioassay and HPLC Analysis of CHL and DM-CHL

(A) Determination of antibacterial activities of CHL and DM-CHL against *B. subtilis*. Each sample was dissolved in 10 μl methanol and then added to the round filter paper. (1) 10 μl methanol (negative control), (2) 10 μg CHL, (3) 1 μg CHL, (4) 10 μg DM-CHL, and (5) 1 μg DM-CHL.

(B) HPLC analysis of CHL or DM-CHL production. (I) *S. antibioticus* DSM 40725, (II) TL1008 (*ΔchlB6*), (III) TL1012 (*ΔchlB1*), and (IV) TL1013 (*chlB1:ermE**). The asterisk and triangle indicate CHL and DM-CHL, respectively.

6-methysalicyclic acid could be activated by ChlB3 and then transferred to ChlB2. In other words, the methylsalicycloyl-S-ACP (**6**) is presumably formed before methylation and chlorination. ChlB6, a putative condensing protein, may catalyze the attachment of the methylsalicycloyl group to the glycosidically linked olivoses. It should be noted that ChlB3 and ChlB6 are highly homologous to AviN and CalO4 [29, 30], both of which could be required for transferring the orsellinic acid moiety to the deoxysugar. Although it remains to be established whether the free acid or acyl-S-ACP is the preferred substrate for AviN or CalO4, our functional assignments of ChlB3 and ChlB6 based on gene organization and sequence comparison indicate their differentiation of function evolution and suggest that formation of the activated substrates as acyl-S-ACP might be a common way in which the last attachment step occurs. To validate the hypothesis that *chlB6* is essential for CHL biosynthesis, we inactivated *chlB6* by gene disruption and constructed the mutant strain TL1008. Consequently, TL1008 produces the same compound, DM-CHL, as the *ΔchlB1* mutant strain TL1012 upon HPLC and LC-MS analyses (Figure 5B, II).These results support the functional assignment of ChlB6 and demonstrate the feasibility to genetically manipulate the CHL machinery for generation of the novel CHL analogs.

## Biosynthesis of the Three-Carbon Unit

Previous biosynthetic studies on the CHL aglycone demonstrated that the macrolide derives from a single polyketide chain that contains ten acetates and two propionates; however, they failed to account for the origin of the remaining three-carbon (3-C) atoms, C-22, -23, and -24. Later it was determined that intact isotope-labeled glycerol was incorporated into carbon atoms C-22, -23, and -24, clearly indicating that glycerol serves as the precursor of the 3-C unit [16]. Of the various metabolites that can derive from glycerol, a few, such as pyruvate and lactate, were ruled out as intermediate precursors of the 3-C unit by radioactive tracer experiments, leaving an enoylpyruvate derivative as the most attractive candidate mechanistically.

Four genes, *chlD1*, *chlD2*, *chlD3*, and *chlD4*, are subclustered within the CHL cluster, and the deduced functions support their involvement in the biosynthesis of the 3-C unit as outlined in Figure 4C. ChlD1, a FkbH-like protein, may act on the glycolytic pathway intermediate and transfer it to the discrete ACP protein ChlD2. ChlD3, the putative dehydratase, assumedly catalyzes 2,3-dehydration and generates enoylpyruvoyl-S-ACP (**9**). The *chlD3* mutant strain TL1006 lost its CHL productivity (Figure 2, V), which was then restored by expressing *chlD3* in *trans* (TL1010, Figure 2, VI), confirming that ChlD3 is essential for CHL biosynthesis. The final gene, *chlD4*, encodes a putative protein that shows homology to a predicted hydrolase or acyltransferase in *Nostoc punctofome*. Inactivation of *chlD4* resulted in the CHL-nonproducing mutant strain TL1007 (Figure 2, X), in which the CHL production was restored by expressing *chlD4* in *trans* (TL1011, Figure 2, XI), suggesting that ChlD4 may catalyze the incorporation of enoylpyruvoyl-S-ACP into the CHL aglycone (discussed below).

While we have proposed that the CHL 3-C unit formation starts with glyceroyl-S-ACP (**8**) that is catalyzed by the FkbH-like protein ChlD1, **8** could be alternatively synthesized by ChlM, which is similar to a ketoacyl-ACP synthase III family. However, inactivation of *chlM* retains its CHL productivity upon HPLC analysis, excluding its involvement in CHL biosynthesis (data not shown). FkbH homologs have been found in a few gene clusters of secondary metabolites, such as ansamitocin and FK520 [31, 32]. Although the exact substrate of FkbH-like protein remains to be determined, its function has been assigned to catalyze the formation of methoxymalonyl-S-ACP from a glycolytic pathway intermediate in association with other modification proteins [33]. Recently, an integrated FkbH domain identified in the loading module of PKS BryA, presumably responsible for the initial steps of bryostatin biosynthesis in the uncultivated marine bacterial symbiont *Bugula neritina*, was proposed to catalyze the glyceroyl-S-ACP formation in the 3-C starter unit biosynthesis [34]. Hence, evidence here supports that the FkbH-like protein ChlD1 serves as the preferred candidate to form **8**, which could represent a common intermediate for incorporation of the glycerol-derived metabolites into natural product biosynthesis.

## Biosynthesis of Chlorothricolide, 12, the Aglycone of CHL

Six genes in the CHL cluster, *chlA1–chlA6*, encode the multifunctional type I PKSs that consist of a loading module and 11 chain-elongation modules, supporting their roles in the biosynthesis of the chlorothricolide backbone as outlined in Figure 4D. The modules of ChlA1–A6, comprised of the predicted functional domains for chain

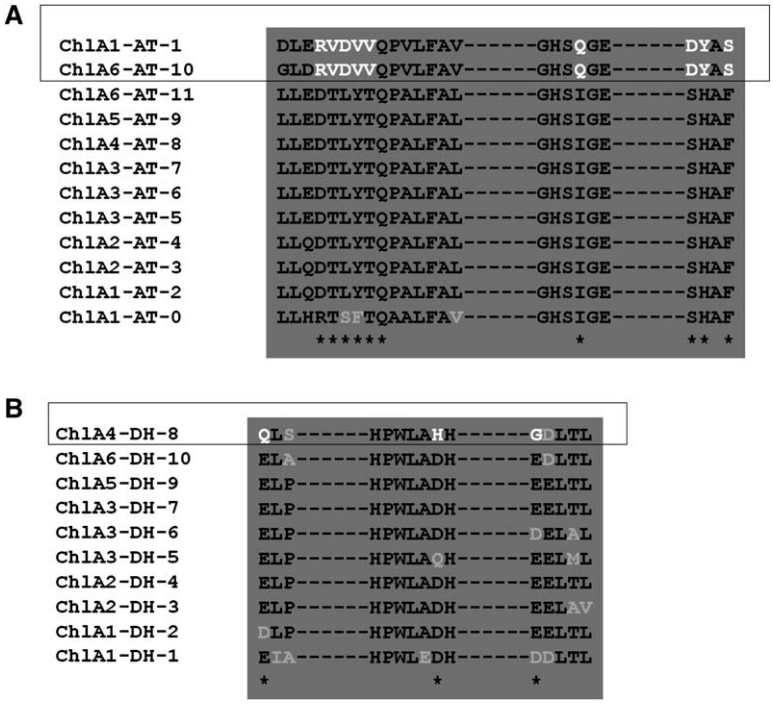


Figure 6. Sequence Analysis of the ChlA1–A6 PKSs

(A) Alignments of the conserved motifs of AT domains from CHL PKSs. Asterisks indicate the conserved amino acid residues that determine the substrate specificity, and ATs that activate methylmalonyl-CoA are boxed.

(B) Alignment of the conserved motifs of DH domains from CHL PKSs. Asterisks indicate the conserved acidic amino acid residues, and the putative mutant DH domain of module 8 is boxed.

extension and modification, are arranged collinear with their functions in the biosynthetic assembly process. Each of the modules contains at least three domains of KS, AT, and ACP that select, activate, and catalyze a decarboxylative condensation between the extender unit and the growing polyketide chain, generating a β-ketoacyl-S-ACP intermediate. Domains DH, enoyl-reductase (ER) and KR are found between AT and ACP, which carry out the variable set of reductive modifications of the β-keto group before the next round of chain extension [35]. On the basis of sequence analysis, the substrate specificities of these AT domains were predicted and fall into two groups (Figure 6A): methylmalonyl-CoA for ATs of module 1 and 10, and malonyl-CoA for ATs of modules 2–9 and 11 as well as the loading AT domain. An unusual DH domain is identified in module 8 of ChlA4, in which a few acidic amino acid residues within the conserved motifs shown in Figure 6B, such as E and D, are replaced by the neutral residues, such as Q and G, implying that this DH domain could be inactive and its function may not be required during the eighth round of condensation. The mutant KS domain ($KS^Q$), which has a glutamine in place of a conserved cysteine residue, is found in the loading module, suggesting that it catalyzes the decarboxylation of malonyl-S-ACP, which is activated by the loading AT domain, and forms the acetyl-S-ACP to initiate the elongation process. Consequently, we propose that ChlA1–A6, in a mechanistic analogy to a number of typical type I PKSs, catalyze the formation of the nascent tetracosanoyl-S-ACP intermediate (**10**) via 11 decarboxylation steps in a noniterative manner.

While **10** is being synthesized, the *trans*-decalin system is proposed to be enantioselectively formed via an intramolecular [4 + 2] Diels-Alder reaction by addition of the C-4–C-5 dienophile to the C-10–C-13 diene (Figure 4D). Catalyzed by the putative acyltransferase ChlD4, the hydroxyl group of enoylpyruvate may serve as the nucleophile to release the polyketide intermediate. After the resultant product undergoes a cyclization reaction to form the characteristic tetranoic acid moiety, an additional intramolecular [4 + 2] Diels-Alder reaction could occur by addition of the C-2′–C-3′dienophile of the 3-C unit to the C-20–C-23 diene of the polyketide chain, leading to the formation of the cyclohexene moiety, closing of the macrolide ring, and generation of pre-chlorothricolide (**11**, Figure 4D).

Three putative oxidoreductase genes, *chlE1*, *chlE2*, and *chlE3*, are identified for the postsynthetic modification of pre-chlorothricolide or pre-CHL. In contrast to the aglycone of KIJ or TC-A, which retains the acyltetronic acid structure, a Baeyer-Villiger oxidation is suggested to be responsible for converting the acyltetronic acid into a macrocyclic lactone in the CHL biosynthetic pathway. Both ChlE1 and ChlE3 resemble a family of FAD-dependent monooxygenases, implying that one of them serves as the candidate for catalyzing the Baeyer-Villiger oxidation reaction. The remaining one, functionally associated with ChlE2, which resembles a member of the P-450 family, may catalyze the oxidation of C-20 methyl branch to a carboxyl group, eventually resulting in chlorothricolide (**12**) or CHL (Figure 4D).

While it is proposed that two Diels-Alder reactions tandemly occurr in chlorothricolide biosynthesis, leading to the formation of the *trans*-decalin system and the cyclohexene moiety, respectively, it remains to be determined whether these two Diels-Alder reactions are performed enzymatically or nonenzymatically. The Diels-Alder reaction, which forms a six-membered ring from a dienophile and 1,3-diene, is widely applied for the chemical synthesis of cyclic products with a high region and stereoselectivity under mild conditions. Although it has been found to proceed in a number of biosyntheses of secondary metabolites [36, 37], only

three enzymes, solanapyrone synthase [38], lovastatin nonaketide synthase (LNKS) [39], and macrophomate synthase (MPS) [40], have been characterized as the natural Diels-Alderases [41], in which no sequence homology was found. Interestingly, they not only catalyze the Diels-Alder reaction, but they also have additional enzymatic activity, such as oxidation, polyketide chain formation, or decarboxylation. The enzyme converts the substrate into a reactive intermediate that is not released from the active site, forcing the intermediate into a conformation that readily undergoes the Diels-Alder reaction, catalyzing the [4 + 2] cycloaddition within an "entropy trap" [40]. Despite the fact that no obvious candidates catalyzing the two Diels-Alder reactions could be identified within the CHL cluster, we propose that the activities of Diels-Alder reactions might be associated with other enzymatic functions of certain proteins involved in chlorothricolide biosynthesis if it occurs enzymatically.

### Assembly of CHL from D-Olivose, 2-Methoxy-5-Chloro-6-Methylsalicyclic Acid, and Chlorothricolide Building Blocks

The CHL molecule could be synthesized by the assembly from the three building blocks: two D-olivoses, a modified methylsalicyclic acid, and a chlorothricolide as outlined in Figure 4D. Chlorthricolide may be first glycosylated by two dNDP-D-olivoses at the C-7 hydroxyl group, which is presumably catalyzed by the putative glycosyl transferases, ChlC6 and ChlC7. The modified methylsalicyclic acid could then be transferred to the C-3 hydroxyl group of the second olivose moiety, which is likely catalyzed by the putative condensing protein ChlB6. On the other hand, the assembly process could begin with pre-chlorothricolide and end with pre-CHL. If the latter strategy is correct, three modification proteins, ChlE1, ChlE2, and ChlE3, would act on pre-CHL, eventually converting it into CHL.

Interestingly, according to structural similarities of aglycones among the spirotetronate antibiotic family, such as CHL, KIJ, and TC-A, decoration of the aglycones with various peripheral moieties renders the versatile biological activities, underscoring nature's way of combinatorial biosynthesis in creating the diversity of structures and activities of complex natural products.

### Significance

**The spirotetronate antibiotics, with unique architectures and broad biological activities, have long been appreciated in the fields of chemistry, biology, and medical sciences. Decoration of structurally related aglycones with a variety of peripheral moieties leads to versatile biological activities, incarnating nature's wisdom to create the diversity of structures and activities of complex natural products via combinatorial biosynthesis. Here, we report the cloning, sequencing, and genetic characterization of the CHL biosynthetic gene cluster from *S. antibioticus* DSM 40725, and we provide an assembly model for CHL biosynthesis from D-olivose, 2-methoxy-5-chloro-6-methylsalicyclic acid, and chlorothricolide building blocks. To our knowledge, this work represents the first cloning of a gene cluster for spirotetronate antibiotic biosynthesis, and it sets the stage for investigating the unusual macrolide biosynthesis, including tandem Diels-Alder cyclizations, Baeyer-Villiger oxidation, and incorporation of an enoylpyruvate unit. Since many of these proposed enzymes and reactions are novel, mechanistic characterization of the CHL pathway will surely make a fundamental contribution to natural product chemistry and enzymology. The availability of the CHL biosynthetic gene cluster and its proposed pathway as a model for spirotetronate antibiotic biosynthesis also make it possible to apply combinatorial biosynthesis methods to the CHL biosynthetic machinery to generate structural diversity.**

### Experimental Procedures

#### Bacterial Strains, Plasmids, and Reagents

Bacterial strains and plasmids used in this study are summarized in Table S1. Biochemicals, chemicals, media, restriction enzymes, and other molecular biological reagents were obtained from standard commercial sources.

#### DNA Isolation, Manipulation, and Sequencing

DNA isolation and manipulation in *E. coli* and *Streptomyces* were carried out according to standard methods [42, 43]. PCR amplifications were carried out on an Authorized Thermal Cycler (Eppendorf AG 22331; Hamburg, Gemany) with either Taq DNA polymerase or PfuUltra High-Fidelity DNA polymerase. For the NGDH gene, a 0.55 kb internal fragment was obtained by two-stage PCR amplification: a distinct 1.0 kb fragment amplified from the genomic DNA of *S. antibioticus* DSM 40725 by using primers 5′-GATGAATTCATG AACCTCCTCGTCACC-3′ and 5′-CGGAAGCTTTCAGCCATCGCG-3′ [18] was purified and directly used as the template for the secondary amplification by using the degenerated pair of primers 5′-CSGGSGSSGCSGGSTTCATSGG-3′ and 5′-GGGWRCTGGYRSGGS CCGTAGTTG-3′ [44]. The identity of the PCR product as *oxiIII* was further confirmed by sequencing. For P1 and P2, a 0.75 kb fragment was amplified by PCR by using the following pair of degenerate primers: 5′-GGCCGGGCCTTCCAGGACSNSGGSNTSRACTC-3′ (designed according to the conserved motif of ACP domains of type I PKSs: GRASRDXGXD/NS) and 5′-CGCCAGGTGCATCGCCACSAR SGASGASGARCA-3′ (designed according to the conserved motif of KS domains of type I PKSs: CSSSLVAMHLA). For P4, a 500 bp fragment was amplified by PCR by using the following pair of primers: 5′-TCGTGGGTGCGCGCGATG-3′ and 5′-CGGAAACCGAG CAGTCCG-3′. The amplification of P3 was described (L.S. et al., unpublished data). Primer synthesis and DNA sequencing were performed at the Shanghai GeneCore Biotechnology, Inc. and the Chinese National Human Genome Center.

#### Genomic Library Construction and Screening

A genomic library of *S. antibioticus* DSM 40725 was constructed in Super-Cos1 according to standard protocols [43]. *Escherichia coli* XL1-Blue MRF and Gigapack III XL packaging extract (Stratagene, La Jolla, CA) were used for library construction according to the manufacture's instructions. The genomic library (6000 colonies) was screened by colony hybridization with the PCR-amplified products P1, P2, P3, or P4 as a probe, and resultant positive clones were further confirmed by Southern hybridization.

#### Sequence Analysis

The *orfs* were deduced from the sequence by using the FramePlot 3.0β program (http://watson.nih.go.jp/~jun/cgi-bin/frameplot-3.0b.pl). The corresponding deduced proteins were compared with other known proteins in the databases by using available BLAST methods (http://www.ncbi.nlm.nih.gov/blast/). Amino acid sequence alignments were performed by using the CLUSTALW method as well as by using the DRAWTREE and DRAWGRAM methods from BIOLOGYWORKBENCH 3.2 software (http://workbench.sdsc.edu).

### Gene Inactivation and Complementation

Introduction of plasmid DNA into *S. antibioticus* DSM 40725 was carried out by either polyethylene glycol (PEG)-mediated protoplast transformation or *E. coli-S. antibioticus* DSM 40725 conjugation, by following the procedure described previously [44].

The constructs for gene inactivation and complementation used in this study are summarized in Supplemental Data.

The constructs for gene inactivation were introduced into *S. antibioticus* DSM 40725. For gene disruption, colonies that were apramycin resistant at 37ºC were identified as the mutants. For gene replacement, colonies that were apramycin resistant and thiostrepton sensitive at 37ºC were identified as the mutants, each of which has also been complemented by expressing the target gene in *trans* under the control of the *ermE** promoter. The genotypes of the mutant strains were confirmed by Southern hybridization (Supplemental Data). Recombinant strains were cultured and analyzed for CHL production by HPLC with the *S. antibioticus* DSM 40725 wild-type strain as a control.

### Production, Isolation, and Analysis of CHL

*S. antibioticus* DSM 40725 wild-type and recombinant strains were grown on R2YE agar plates (with appropriate antibiotic for recombinant strains) at 30ºC for sporulation and CHL production on solid agar. For fermentation in liquid culture, 100 μl spore suspension (cfu $5.2 \times 10^6$ cells/ml) of the *S. antibioticus* DSM 40725 strain was inoculated into 50 ml seed medium (Soybean meal 2%, Mannitol 2%, and $CaCO_3$ 0.2%) in a 250 ml flask and incubated at 30ºC and 240 rpm for 2 days. A total of 2.5 ml seed culture was transferred into 100 ml of the same medium in a 500 ml flask and incubated at 30ºC and 240 rpm for 3 days.

For CHL or DM-CHL isolation from solid agar, each 20 ml of culture (R2YE agar plate) of *S. antibioticus* DSM 40725 strain was freeze dried, homogenized, and fragmented by ultrasound, and then extracted twice with 50 ml methanol. After the precipitate was removed, the combined extract was concentrated in a vacuum and resolved in 1 ml methanol. For CHL isolation from liquid culture, each 20 g mycelia collected by centrifuging was washed with water, lyophilized in a vacuum, and fragmented by ultrasound. A total of 10 g freeze-dried mycelia was extracted twice with 50 ml methanol, and the combined extract was concentrated and adjusted to a pH of 5.0 after addition of an equal volume of water. After it was extracted three times with ethyl acetate, the combined mixture was dried with $MgSO_4$, evaporated to dryness, and then resolved in 1 ml methanol.

HPLC analysis of CHL was carried out on a Nova-Pak C18 column (3.9 × 150 mm, Part No. WAT086344, Waters Corp., Ireland). The column was equilibrated with 50% solvents A ($H_2O$, 0.05% TFA) and B ($CH_3CN$, 0.05% TFA) and was developed with the following program: 0–5 min, 60% A/40% B; 5–20 min, a linear gradient from 60% A/40% B to 15% A/85% B; 20–25 min, constant 15% A/85% B; 25–30 min, a linear gradient from 15% A/85% B to 60% A/40% B. This was carried out at a flow rate of 1 ml/min and UV detection at 222 nm by using an Agilent 1100 HPLC system (Agilent Technologies. Palo Alto, CA). The identity of the compound was confirmed by liquid chromatography-mass spectrometry (LC-MS) analysis performed on LCMS-2010 A (Liquid Chromatograph Mass Spectrometer, SHIMADZU, JP). The CHL and deschloro-CHL showed an $(M\text{-}H)^-$ ion at m/z = 953.0 and m/z = 919.0, consistent with the molecular formula $C_{50}H_{63}ClO_{16}$ and $C_{50}H_{64}O_{16}$, respectively. NMR spectra were measured on a Varian Inova 600 (600 MHz) spectrometer (Supplemental Data).

### Bioassay of CHL and DM-CHL

To detect the biological activities of CHL and DM-CHL against *B. subtilis*, each 20 μl methanol extract from solid agar described above was added to the roundness filter paper that was placed on LB agar preseeded with an overnight *B. subtilis* culture. The plate was incubated at 37ºC for 24 hr, and the biological activity was estimated by measuring the sizes of the inhibition zones.

### Supplemental Data

Supplemental Data include bacterial strains and plasmids in this study, constructs for gene inactivation and complementation, Southern hybridization analysis of mutant strains, and chemical structural elucidation of Dm-CHL and are available at http://www.chembiol.com/cgi/content/full/13/6/575/DC1/.

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## Accession Numbers

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