Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 345 (2006) 133–139
BBRC
www.elsevier.com/locate/ybbrc

Cloning and characterization of a bacterial iterative type I polyketide synthase gene encoding the 6-methylsalicyclic acid synthase

Lei Shao [a,b,1], Xu-Dong Qu [b,1], Xin-Ying Jia [b,1], Qun-Fei Zhao [b], Zhen-Hua Tian [b], Min Wang [a], Gong-Li Tang [b,*], Wen Liu [b,*]

[a] School of Life Science and Technology, Chinese Pharmaceutical University, 24 Tongjiaxiang, Nanjing 210009, China
[b] State Key Laboratory of Bio-Organic and Natural Product Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, 354 Fenglin Rd., Shanghai 200032, China

Received 3 April 2006
Available online 25 April 2006

Abstract

Unusual polyketide synthases (PKSs), that are structurally type I but act in an iterative manner for aromatic polyketide biosynthesis, are a new family found in bacteria. Here we report the cloning of the iterative type I PKS gene *chlB1* from the chlorothricin (CHL) producer *Streptomyces antibioticus* DSM 40725 by a rapid PCR approach, and characterization of the function of the gene product as a 6-methylsalicyclic acid synthase (6-MSAS). Sequence analysis of various iterative type I PKSs suggests that the resulting aromatic or aliphatic structure of the products might be intrinsically determined by a catalytic feature of the paired KR–DH domains in the control of the double bond geometry. The finding of ChlB1 as a 6-MSAS not only enriches the current knowledge of aromatic polyketide biosynthesis in bacteria, but will also contribute to the generation of novel polyketide analogs via combinatorial biosynthesis with engineered PKSs.

Keywords: Aromatic polyketide; Polyketide synthase; Chlorothricin; 6-Methylsalicyclic acid; Biosynthesis

Polyketide metabolites, widely found in bacteria, fungi, and plants, are one of the largest families of natural products. They are structurally classified into four major groups: aromatics, macrolides, polyenes, and polyethers, many of which are clinically valuable drugs. Despite their apparent structural diversity, polyketides are biosynthesized by a common mechanism in a manner resembling fatty acid biosynthesis: the carbon backbone of a polyketide is assembled from sequential condensation of short chain acyl Co-enzyme A (CoA) precursors, and the process is catalyzed by polyketide synthases (PKSs) [1,2]. Over the past decades, intensive attempts to establish a predictable relationship between the polyketide structures and functions of PKSs have been facilitated by an exponential growth of genetic and biochemical information regarding polyketide biosynthesis. In bacteria, reduced polyketide (i.e., macrolide, polyene, and polyether) biosynthesis usually requires type I PKSs, which are giant multifunctional enzymes that are organized into modules. Each type I PKS module contains a set of distinct domains responsible for the catalysis of one cycle of polyketide chain elongation in a non-iterative process [3]. In contrast, bacterial type II and III PKSs are heterologous or homologous multienzyme complexes that carry a single set of iteratively acting activities that are generally responsible for aromatic polyketide biosynthesis [4,5].

Current studies on polyketide biosynthesis have been providing the molecular basis to explain the vast structural diversity of polyketide natural products, however, the emergence of many novel PKS systems is becoming apparent in recent publications, revealing a rich variety of

* Corresponding authors. Fax: +86 21 64166128 (W. Liu).
E-mail addresses: gltang@mail.sioc.ac.cn (G.-L. Tang), wliu@mail.sioc.ac.cn (W. Liu).
[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.04.069

chemistry and architecture of PKSs beyond our previous understanding of bacterial type I, II, and III paradigms [1,2]. PKSs could be structurally type I, but function in an iterative manner for aromatic polyketide biosynthesis. Three such iterative type I PKSs have been found in bacteria to date. AviM [6] and CalO5 [7] catalyze the biosynthesis of an orsellinic acid moiety (monocyclic polyketide) for avilamycin (AVI) in *Streptomyces viridochromogenes* and calicheamicin (CAL) in *Micromonospora echinospora*, respectively (Fig. 3A). The third PKS is NcsB [8,9] that catalyzes the biosynthesis of a naphthoic acid moiety (bicyclic polyketide) for neocarzinostatin (NCS) in *Streptomyces carzinostaticus* (Fig. 3C). Although aromatic polyketide biosynthesis in fungi is catalyzed by iterative type I PKSs, as exemplified by the 6-methylsalicyclic acid synthases (6-MSASs), the general paradigm in bacteria had been thought to only consist of type II and III PKSs until recently [1,4]. Indeed, this raises the question if the new emerging paradigm of iterative type I PKSs for aromatic polyketide biosynthesis is far more prevalent in bacteria.

Chlorothricin (CHL), produced by *Streptomyces antibioticus* DSM 40725, is the first member of the spirotetronate antibiotic family to be discovered [10]. The CHL aglycone contains a *trans*-decarlin system and a characteristic tetronic acid that is spiro-linked to a cyclohexene ring. The macrolide is further decorated by two deoxysugars and a specific peripheral moiety, 2-methoxy-5-chloro-6-methylsalicyclic acid (Fig. 1). With unique architectures and broad biological activities, the spirotetronate antibiotics are attractive leads for novel drug development and have stimulated considerable effort on their total synthesis and structural modifications [11–13]. Recently, advances in biotechnology have provided a promising alternative to make complex microbial metabolites and their analogs by genetic manipulation of the biosynthetic pathways [14–16]. The success of this approach on spirotetronate antibiotics critically depends on characterization of their biosynthetic machineries that could be utilized for combinatorial biosynthesis, including the biosynthetic pathways of macrolides, deoxysugars, and other peripheral moieties.

Previous feeding experiments with ^{13}C-labeled precursors on CHL biosynthesis in *S. antibioticus* showed that 2-methoxy-5-chloro-6-methylsalicyclic acid contains four head-to-tail acetates, suggesting this substituted moiety of CHL is of polyketide origin [17,18]. According to the similarity in both aromatic structure and polyketide origin to orsellinic and naphthoic acids, we hypothesized that a bacterial iterative type I PKS catalyzes the biosynthesis of 6-methylsalicyclic acid (6-MSA), the precursor of 2-methoxy-5-chloro-6-methylsalicyclic acid. To address this fundamental issue, we have utilized a rapid PCR method to specifically clone *chlB1* from *S. antibioticus*, characterized the function of the gene product as a bacterial 6-MSAS, and analyzed the intrinsic activities of various iterative type I PKSs in the control of the double bond formation.

Materials and methods

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 from standard commercial sources.

PCR amplification. To clone the iterative type I PKS gene from *S. antibioticus*, the following two pairs of degenerate primers were used: primers 5′-TCC TAC GCG CTG GAC CTG CRS GGS CCS AG-3′

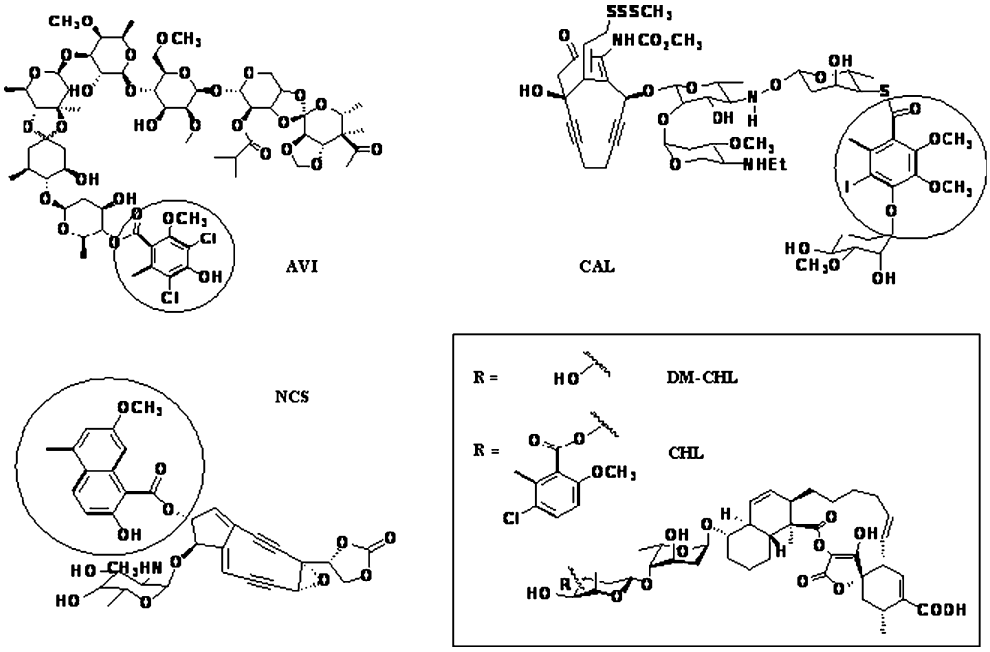

Fig. 1. Structures of avilamycin (AVI), calicheamicin (CAL), neocarzinostatin (NCS), chlorothricin (CHL), and desmethylsalicycloyl chlorothricin (DM-CHL).

(*A*, forward) and 5′-GTC GAG GGT CTC CCG CAC MGA GTG SGC SAC-3′ (*B*, reverse), and primers 5′-AAA GAA TTC GCG GAC GGC TAC GGS MGN GGN GAR GG-3′ (*C*, forward, *Eco*RI site underlined) and 5′-A AAA AAG CTT CGA GCC GTG GCC SGA RAA NAC CCA NAC-3′ (*D*, reverse, *Hin*dIII site underlined).

Random library construction. The genomic DNA of *S. antibioticus* was completely digested with *Bgl*II. The fragments of approximate 10.0 kb were recovered and ligated into vector pSP72 that was prepared by digestion with *Bam*HI and treatment with shrimp alkaline phosphatase. The resulting ligation mixture was transformed into *Escherichia coli* DH5α. The transformed cells were spread onto LB plates containing ampicillin (100 μg/ml), and the plates were incubated at 37 °C overnight.

Heterologous expression of chlB1 in Streptomyces lividans TK24. To make the*chlB1* expression construct, a 10.0 kb *Bgl*II fragment that contains intact *chlB1–orf4–orf3* and 0.5 kb *Eco*RI/*Bam*HI fragment that harbors the *ermE** promoter were cloned into the *Eco*RI/*Bam*HI site of pBS3031 to yield pTL1039. Expression construct pTL1039 was introduced into *S. lividans* TK24, resulting in the recombinant strain TL1015.

Production, isolation, and analysis of 6-MSA. Summarized in the section of Supplementary Material.

Sequence and phylogenetic analysis. DNA sequencing was performed at Chinese National Human Genome Center. The *orfs* were deduced from the sequence by performing FramePlot 3.0beta program (http://watson.nih.go.jp/~jun/cgi-bin/frameplot-3.0b.pl). The deduced proteins were compared with other known proteins in the databases by available BLAST methods. Amino acid sequence alignment and phylogenetic analysis were performed by the CLUSTALW method, and the DRAWTREE and DRAWGRAM methods, respectively, from BIOLOGY-WORKBENCH 3.2 software (http://workbench.sdsc.edu).

Results and discussion

Primer design and PCR amplification of the iterative type I PKS gene

From the CHL producer *S. antibioticus*, Sohng and co-workers [19] previously cloned a partial gene cluster containing a set of sugar biosynthetic genes and a typical type I PKS gene, which were originally assumed to be involved in CHL biosynthesis. However, we inactivated the *dNDP*-D-glucose-4,6-dehydratase gene within this partial cluster and found that the resulting mutant retains the ability to produce CHL, excluding its involvement in CHL biosynthesis (unpublished data). On the basis of sequence comparison with published data, Floss and co-workers [20] suggested that these genes may represent part of the oleandomycin biosynthetic cluster that is cryptic in *S. antibioticus*.

With the assumption that the modified 6-MSA moiety of CHL is of polyketide origin and shares a biosynthetic pathway similar to those of orsellinic and naphthoic acids, we altered cloning strategy to specifically probe for iterative type I PKS gene by PCR. Sequence analysis of the three cloned iterative type I PKSs in bacteria, AviM, CalO5, and NcsB, reveals high homology in both amino acid sequence and domain organization to the modules of well-known non-iterative type I PKSs. To avoid the amplification of genes encoding the latter, particularly those involved in oleandomycin biosynthesis in the CHL producer *S. antibioticus*, we designed two sets of degenerate primers ($A - B$ and $C - D$) according to the conserved motifs within the ketosynthase (KS) to acyltransferase (AT) regions of AviM, CalO5, and NcsB. The motifs for primer designation are distinct from those of typical modular type I PKSs encoding the oleandomycin biosynthesis (Fig. S1).

Genomic DNA from *S. antibioticus* was used as the template in this study. While no product was amplified by PCR under various conditions with primer pair $A - B$, a distinct product with the expected size of 0.9 kb was readily amplified with primer pair $C - D$ (as illustrated in Fig. S2). The PCR product was subsequently cloned into pGEM®-T easy vector for sequencing. Intriguingly, sequence analysis of six randomly selected clones confirmed an identical product (indicative of a specific PCR amplification), the deduced amino acid sequence of which is highly homologous to the iterative type I PKSs. To confirm that the obtained putative gene resulted from *S. antibioticus*, Southern analysis of the genomic DNA was performed with the 0.9 kb PCR product as a probe. Distinct bands were detected in all genomic DNA samples digested with various restriction enzymes (as shown in Fig. S3). Clearly, the PCR method presented above provides a rapid route to specifically access the iterative type I PKS gene in *S. antibioticus*.

Cloning and sequence analysis of the putative gene encoding the iterative type I PKS

Southern hybridization led to identify the largest 10.0 kb *Bgl*II fragment (as shown in Fig. S3), which likely contains the intact gene encoding the iterative type I PKS from *S. antibioticus*. Consequently, a random library of the genomic DNA digested with *Bgl*II was constructed in cloning vector pSP72. With the 0.9 kb PCR product as a probe, three positive clones were identified from approximate 950 clones, and then confirmed to be identical by restriction enzyme digestion and PCR amplification with primer pair $C - D$ (data not shown). The inserted *Bgl*II fragment was sequenced, yielding a 9906 bp of contiguous sequence. Bioinformatic analysis revealed six open-reading frames (*orfs*) as shown in Fig. 2A. Within this sequenced region, the largest gene *orf5* (renamed as *chlB1*) was identified. The deduced protein of *chlB1* showed the highest homology to the known bacterial iterative type I PKSs for aromatic polyketide biosynthesis, such as AviM (58% identity, 70% similarity), CalO5 (58% identity, 69% similarity), and NcsB (47% identity, 61% similarity).

The deduced 1756 aa product of *chlB1* consists of characteristic domains for type I PKS, including a KS, AT, dehydratase (DH), ketoreductase (KR), and acyl carrier protein (ACP). ChlB1 exhibits head-to-tail homology in both organization and sequence to NcsB for naphthoic acid biosynthesis, and to AviM or CalO5 (with an exception of the KR domain) for orsellinic acid biosynthesis (Fig. 3). In a mechanistic analogy, ChlB1 could be envisaged to catalyze the assembly of a linear tetraketide intermediate from one acetyl CoA and three malonyl CoAs in an iterative process as AviM and CalO5. However, the additional KR domain of ChlB1, like that of NcsB, may

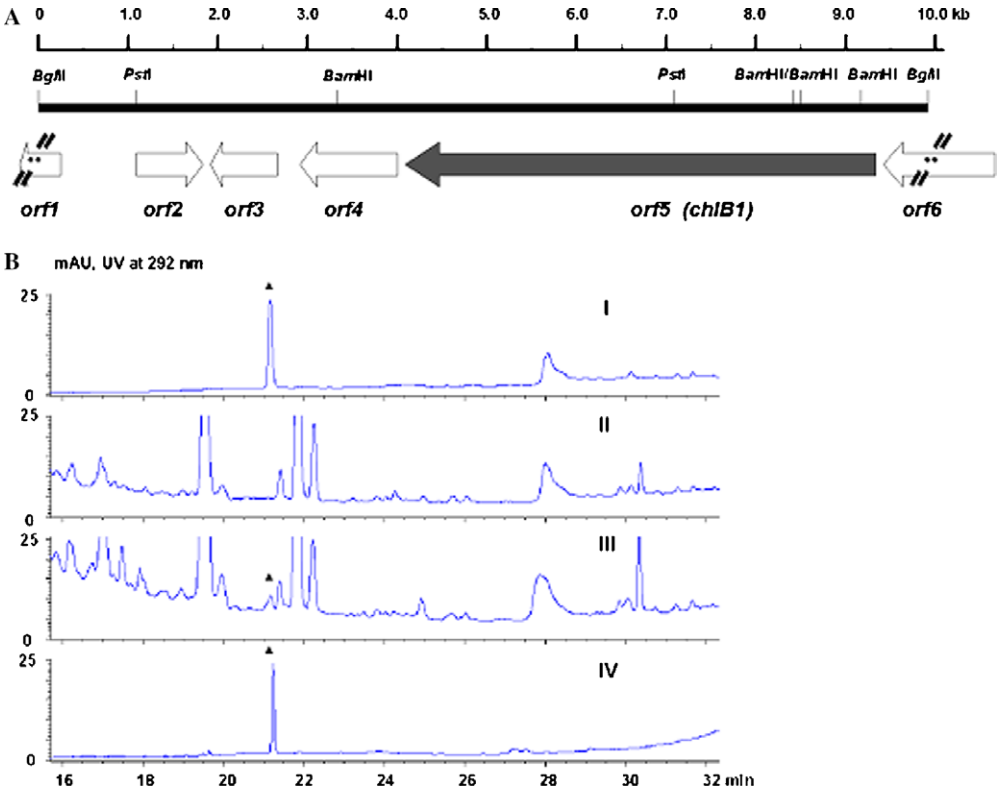

Fig. 2. Gene organization of *orf1*, *orf2*, *orf3*, *orf4*, *orf5* (*chlB1*), and *orf6* within the sequenced *Bgl*II fragment of the size of approximate10.0 kb (A), and HPLC analysis of 6-MSA production in *S. lividans* TK24 (B): authentic standard (I), *S. lividans* TK24 wild-type (II), TL1015 (*S. lividans* TK24 recombinant strain, *ermE*:chlB1–orf4–orf3*) (III), and 6-MSA purified from the fermentation culture of TL1015 (IV).

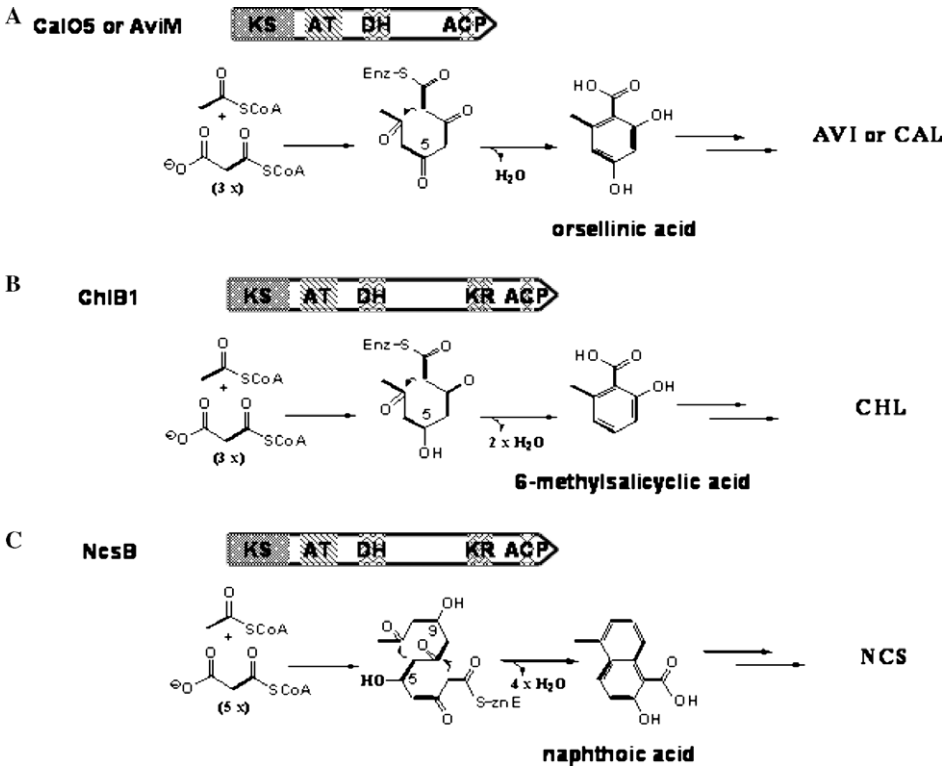

Fig. 3. Domain organizations and mechanisms of iterative type I PKSs for aromatic polyketide biosynthesis in bacteria: CalO5 or AviM for orsellinic acid biosynthesis (A), ChlB1 for 6-methylsalicyclic acid biosynthesis (B), and NcsB for naphthoic acid biosynthesis (C).

offer a selective activity of keto-reduction at C-5 position of the nascent polyketide intermediate, which then undergoes an intramolecular aldol condensation to furnish the 6-MSA structure (Fig. 3B). Further decoration of O-methylation at C-2 position and chlorination at C-5 position afford 2-methoxy-5-chloro-6-methylsalicyclic acid, the peripheral moiety of CHL. Accordingly, the first finding of ChlB1 for 6-MSA biosynthesis not only adds a new example to the pool of bacterial iterative type I PKSs, but also provides a third mode of aromatic biosynthesis encoded by this family (as summarized in Fig. 3). With the available *chlB1* as a probe and combining the strategy of cloning the typical type I PKS genes, we cloned the entire CHL biosynthetic gene cluster by chromosomal walking (The sequence of the CHL gene cluster has been deposited into GenBank under Accession No. DQ116941. X.-Y. Jia, unpublished).

Heterologous expression of chlB1 in S. lividans TK24 and identification of the resulting product as 6-methylsalicyclic acid

The involvement of ChlB1 in the biosynthetic pathway of 2-methoxy-5-chloro-6-methylsalicyclic acid has been confirmed by gene inactivation and complementation (X.-Y. Jia, unpublished). The Δ*chlB1* mutant strain produces a novel compound, desmethylsalicycloyl CHL (DM-CHL), the structure of which lacks the modified 6-MSA moiety of CHL. In this study, the polar effect of *chlB1* inactivation was excluded by in trans expressing the downstream genes *orf4–orf3* in the Δ*chlB1* mutant strain (summarized in the section of Supplementary Material). To further characterize the function of ChlB1, a construct that carries the genes *chlB1–orf4–orf3* under the control of *ermE** was introduced into heterologous host *S. lividans* TK24, yielding the recombinant strain TL1015. With the *S. lividans* TK24 wild-type strain as a negative control (Fig. 2B, II), TL1015 was cultured and analyzed, revealing a new peak (Fig. 2B, III) that showed the same retention time as that of the authentic 6-MSA upon HPLC analysis. To elucidate the structure, the resulting compound was extracted and purified (Fig. 2B, IV). LC–MS analysis of the obtained powder exhibited an $(M-H)^-$ ion at $m/z = 150.9$, consistent with the molecular formula $C_8H_8O_3$. Indeed, ^1H NMR spectrum comparison with that of the authentic standard further supports the conclusion that this compound is 6-MSA (summarized in the section of Supplementary Material). Given the functional assignment of *orf4* (encoding a putative glycosyltransferase) and *orf3* (encoding a putative transposase), and the fact that experiments on gene complementation excluded their involvement in the biosynthesis of the modified 6-MSA moiety of CHL, these results clearly confirmed the function of ChlB1 as a 6-MSAS in *S. antibioticus* for the biosynthesis of 6-MSA, the first intermediate of 2-methoxy-5-chloro-6-methylsalicyclic acid.

Phylogenetic analysis

To our knowledge, ChlB1 is the first bacterial 6-MSAS identified for 6-MSA biosynthesis, the pathway of which has been widely found in fungi (reviewed in [4]). Despite the remarkable homology of ChlB1 in both organization and sequence to various fungi 6-MSASs, phylogenetic analysis revealed that ChlB1 is genetically closer to AviM, CalO5, and NcsB (Fig. S4A), consistent with its bacterial origin. This combined family now is mechanically comprised of three groups (Fig. 3): (1) AviM and CalO5 for orsellinic acid biosynthesis (no reduction step); (2) ChlB1 for 6-methylsalicyclic acid biosynthesis (selective reduction at C-5 position); and (3) NcsB for naphthoic acid biosynthesis (selective reduction at C-5 and C-9 positions). While the former two groups result in monocyclic polyketides that are assembled from one acetyl-CoA and three malonyl-CoAs, NcsB leads to bicyclic polyketide that is assembled from one acetyl-CoA and five malonyl-CoAs. It is likely that these iterative type I PKSs are diverged from the same ancestor to evolve different activities including selective reduction and chain length determination, leading to the structural diversity that is seen within these aromatic compounds. In addition, sequence comparison of KS to AT regions revealed that the iterative type I PKSs for aromatic polyketide biosynthesis are phylogenetically distinct from those of the selected non-iterative type I PKSs for reduced polyketide biosynthesis (Fig. S4B), such as oleadomycin (macrolide), candicidin (polyene), and monensin (polyether), in agreement with the specificity shown by PCR amplification of the iterative type I PKS gene from *S. antibioticus*.

Studies on biochemistry of the fungal 6-MSAS from *Penicillium patulum* revealed that it catalyzes the biosynthesis of 6-MSA from one acetyl CoA and three malonyl CoAs, requiring a NADPH-dependent reduction and a dehydration occurred on the triketide intermediate (Fig. S6). Although the origins of the hydrogen atoms at C-3, C-4, and C-5 positions of 6-MSA were well established by incorporation of the chiral substrates (reviewed in [4]), the stereochemistry of the cryptic β-hydroxyacyl intermediate from the KR domain action remains to be determined.

Recently, an alignment of over 200 KR domains of the typical type I PKSs led to the identification of a D residue (within a conserved L-D-D motif) that occurs in all KR domains that catalyze ketoreduction known to produce the D-hydroxy configuration [21,22]. Such a KR-paired DH domain was further characterized to catalyze the following *trans*-double bond formation [24]. In contrast, KR domains that generate an L-hydroxy group lack this conserved aspartate [23], and their associated DH domains could catalyze the dehydration step to form a *cis*-double bond. Our sequence analysis of the iterative type I PKSs, including various 6-MSASs both in bacteria and fungi as well as NcsB that catalyzes the naphthoic acid biosynthesis,

revealed that all of the KR domains lack the conserved aspartate residue or the characteristic L-D-D motif (Fig. S5). Extension of the configuration prediction to this iterative PKS family implicates that the KR domain may act on the β-keto group to form exclusively an L-hydroxy configuration. Consequently, a *cis*-double bond is formed with the dehydration activity of the paired DH domain, facilitating the folding of the resulting polyketide intermediate for undergoing an aldol condensation to furnish the aromatic structure (Fig. S6). The iterative type I PKS MchA, unlike those for bacterial aromatic compound biosynthesis, is responsible for the assembly of an aliphatic chain of myxochromides S [24]. The KR domain of MchA contains a characteristic L-D-D motif (Fig. S5) that is consistent with a resulting D-configuration of the cryptic hydroxyl group, and its functional association with the paired DH domain leads to formation of the conjugated *trans*-double bonds. Therefore, whether the resulting compounds of the iterative type I PKSs are aromatic or aliphatic might be intrinsically determined by the catalytic feature of their KR–DH domains in the control of double bond geometry.

In conclusion, we specifically cloned the iterative type I PKS *chlB1* from the CHL producer *S. antibioticus* with a rapid PCR approach, which should be applicable to other bacterial systems. Characterization of ChlB1 for the biosynthesis of 6-methylsalicyclic acid, a precursor of the peripheral moiety of CHL, provides a starting point to access the entire gene cluster of CHL and elucidate its biosynthetic machinery. The enrichment of modes of iterative type I PKS family for aromatic polyketide biosynthesis will undoubtedly contribute to the great potential of generating novel polyketide analogs via combinatorial biosynthesis with engineered PKSs in bacteria.

Acknowledgments

We thank Dr. Isao Fujii, Graduate School of Pharmaceutical Sciences, The University of Tokyo, for providing the authentic 6-MSA; Dr. Steven G. Van Lanen and Prof. Ben Shen, School of Pharmacy, University of Wisconsin-Madison, for reading of the manuscript and comments. This work was supported in part by Grants from the Chinese Academy of Sciences (Grant KGCX2-SW-209), the National Natural Science Foundation of China (Grants 20321202, 30425003, and 30525001), and the Science and Technology Commission of Shanghai Municipality (Grants 04DZ14901, 04JC14082, 05QMX1468, and 05PJ14112).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.04.069.

References

[1] B. Shen, Polyketide biosynthesis beyond the type I, II and III polyketide synthase paradigms, Curr. Opin. Chem. Biol. 7 (2003) 285–295.
[2] S.C. Wenzel, R. Muller, Formation of novel secondary metabolites by bacterial multimodular assembly lines: deviations from textbook biosynthetic logic, Curr. Opin. Chem. Biol. 9 (2005) 447–458.
[3] J. Staunton, K.J. Weissman, Polyketide biosynthesis: a millennium review, Nat. Prod. Rep. 18 (2001) 380–416.
[4] B. Shen, Biosynthesis of aromatic polyketides, Curr. Top. Chem. 209 (2000) 1–51.
[5] M.B. Austin, J.P. Noel, The chalcone synthase superfamily of type III polyketide synthases, Nat. Prod. Rep. 20 (2003) 79–110.
[6] S. Gaiser, A. Trefzer, S. Stockert, A. Kirschning, A. Bechthold, Cloning of an avilamycin biosynthetic gene cluster from *Streptomyces viridochromogenes* Tü57, J. Bacteriol. 179 (1997) 6271–6278.
[7] J. Aherlt, E. Shepard, N. Lomovskaya, E. Zazopoulos, A. Staffa, B.O. Bachmann, K. Huang, L. Fonstein, A. Czisny, R.E. Whitmam, C.M. Farnet, J.S. Thorson, The calicheamicin gene cluster and its iterative type I enediyne PKS, Science 297 (2002) 1173–1176.
[8] B. Sthapit, T.J. Oh, R. Lamichhane, K. Liou, H.C. Lee, C.G. Kim, J.K. Sohng, Neocarzinostatin naphthoate synthase: an unique iterative type I PKS from neocarzinostatin producer *Streptomyces carzinostaticus*, FEBS Lett. 566 (2004) 201–206.
[9] W. Liu, K. Nokana, L. Nie, J. Zhang, S.D. Christenson, J. Bae, S.G. Nan Lanen, C.F. Yang, B. Shen, The neocarzinostatin biosynthetic gene cluster from *Streptomyces carzinostaticus* ATCC 15944 involving two iterative type I polyketide synthases, Chem. Biol. 12 (2005) 293–302.
[10] W. Keller-Schierlein, R. Muntwyler, W. Pache, H. Zähner, Metabolic products of microorganisms. Chlorothricin and deschlorothricin, Helv. Chim. Acta 52 (1969) 127–142.
[11] W.R. Roush, R.J. Sciotti, Enantioselective total synthesis of (−)-chlorothricolide, J. Am. Chem. Soc. 116 (1994) 6457–6458.
[12] W.R. Roush, R.J. Sciotti, Enantioselective total synthesis of (−)-chlorothricolide via the tandem inter-and intramolecular Diels-Alder reaction of a hexaenoate intermediate, J. Am. Chem. Soc. 120 (1998) 7411–7419.
[13] M. Kaneko, T. Nakashima, Y. Uosaki, M. Hara, S. Ikeda, Y. Kanda, Synthesis of tetrocarcin derivatives with specific inhibitory activity towards Bcl-2 functions, Bioorg. Med. Chem. Lett. 11 (2001) 887–890.
[14] C. Khosla, J.D. Keasling, Metabolic engineering for drug discovery and development, Nature Rev. Drug Discov. 2 (2003) 1019–1025.
[15] H.B. Bode, R. Muller, The impact of bacterial genomics on natural product research, Angew. Chem. Int. Ed. Engl. 44 (2005) 6828–6846.
[16] K.J. Weissman, P.F. Leadlay, Combinatorial biosynthesis of reduced polyketide, Nature Rev. Microbiol. 3 (2005) 926–935.
[17] R. Holzbach, H. Page, D. Hook, E.F. Kreuzer, C. Chang, H.G. Floss, Biosynthesis of the macrolide antibiotic chlorothricin: basic building blocks, Biochemistry 17 (1978) 556–560.
[18] O.A. Mascaretti, C. Chang, D. Hook, H. Otsuka, E.F. Kreuzer, H.G. Floss, Biosynthesis of the macrolide antibiotic chlorothricin, Biochemistry 20 (1981) 919–924.
[19] J.C. Yoo, J.M. Han, J.K. Sohng, Expression of *orf7* (*oxi*III) as dNDP-glucose 4,6-dehydratse gene cloned from *Streptomyces antibioticus* Tü99 and biochemical characteristics of expressed protein, J. Microbiol. Biotechnol. 9 (1999) 206–212.
[20] G. Draeger, S. Park, H.G. Floss, Mechanism of the 2-deoxygenation step in the biosynthesis of the deoxyhexose moieties of the antibiotics granaticin and oleandomycin, J. Am. Chem. Soc. 121 (1999) 2611–2612.
[21] P. Caffrey, Conserved amino acid residcues correlating with ketoreductase stereospecificity in modular polyketide synthases, Chem. Bio. Chem. 4 (2003) 649–662.

[22] R. Reid, M. Piagentini, E. Rodriguez, G. Ashley, N. Viswanathan, J. Carney, D.V. Santi, C.R. Hutchinson, R. McDaniel, A model of structure and catalysis for ketoreductase domains in modular polyketide synthases, Biochemistry 42 (2003) 72–79.
[23] J. Wu, T.J. Zaleski, C. Valenzano, C. Khosla, D.E. Cane, Polyketide double bond biosynthesis. Mechanistic analysis of the dehydratase-containing module 2 of the picromycin/methymycin polyketide synthase, J. Am. Chem. Soc. 127 (2005) 17393–17404.
[24] S.C. Wenzel, F. Gross, Y. Zhang, J. Fu, F.A. Stewart, R. Muller, Heterologous expression of a myxobacterial natural products assembly line in pseudomonads using red/ET recombineering, Chem. Biol. 12 (2005) 349–356.