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The Leptomycin Gene Cluster and Its Heterologous Expression in *Streptomyces lividans*

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Abstract Leptomycin exerts its antifungal and antitumoral activity *via* inhibiting nucleo-cytoplasmic translocations in eukaryotic cells. To learn more about the biosynthesis of leptomycin and in an effort to generate leptomycin analogues through genetic engineering, 90 kb segment of DNA containing the putative leptomycin (*lep*) biosynthesis cluster from *Streptomyces* sp. ATCC 39366 was cloned and sequenced. The *lep* cluster consist of 12 polyketide synthase (PKS) modules distributed in four genes (*lepA*, *B*, *C* and *D*) and a P450 encoding gene. The *lep* gene cluster was confirmed by its successful expression in *Streptomyces lividans*, where it directed the production of the two natural congeners-leptomycins A and B. The production of leptomycin B showed that the host has the capability to synthesize ethylmalonyl-CoA.

Keywords leptomycin, heterologous expression, nuclear export inhibitor

Introduction

Streptomycetes produce a family of structurally similar natural products which have in common an unsaturated lactone moiety and two diene systems. This family of compounds, which includes leptomycin [1, 2], ratjadone [3], kazusamycin [4～6], anguinomycin [7, 8], leptofuranin [9, 10], leptolstatin [11, 12], and callystatin A [13] (Fig. 1), selectively block transport of proteins containing leucine-rich nuclear export signal domains from the cell nucleus to cytoplasm. Compounds in this family are usually extraordinarily potent, typically displaying IC_{50} values of 10 to 100 nM. Leptomycin B, a representative of the family, has been studied extensively and became an important tool for studying nuclear localization and molecules trafficking in cells [14～18]. It is known that leptomycin B (LMB) blocks the nucleus-cytoplasm export of proteins by targeting and covalently binding exportin 1/CRM-1, which was original identified as a protein essential for maintaining chromosome structure of the yeast [19～21]. The high potency and novel mechanism of action implicated potential therapeutic uses of LMB as an antiviral or antitumor agent, especially when used in combination with other drugs. For example, fusion of the *bcr-abl* genes and expression as a fusion protein is the cause for chronic myelogenous leukemia. Treatment with either LMB or STI-571 can only inhibit, but not kill, cells expressing the Bcr-Abl oncoprotein. However, the combined treatment of both LMB and STI-517 can achieve the goal to kill these tumor cells effectively and completely [22].

Here we report the cloning of the gene cluster for leptomycin biosynthesis from *Streptomyces* sp. ATCC 39366 and its confirmation through heterologous expression of the entire gene cluster in *Streptomyces lividans*.

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Materials and Methods

Bacterial Strains and Culture Conditions

The leptomycin-producer strain *Streptomyces* sp. ATCC 39366 was obtained from the American Type Culture Collection. *Streptomyces lividans* K4-114 [23] was used as a surrogate host for the expression of the putative *lep* gene cluster. Both *Streptomyces* sp. ATCC 39366 and *S. lividans* K4-114, or their recombinant strains, were maintained in R5 medium [24] supplied with 50 mg/liter of thiostrepton and/or 60 mg/liter of apramycin, when needed. FKA [25]

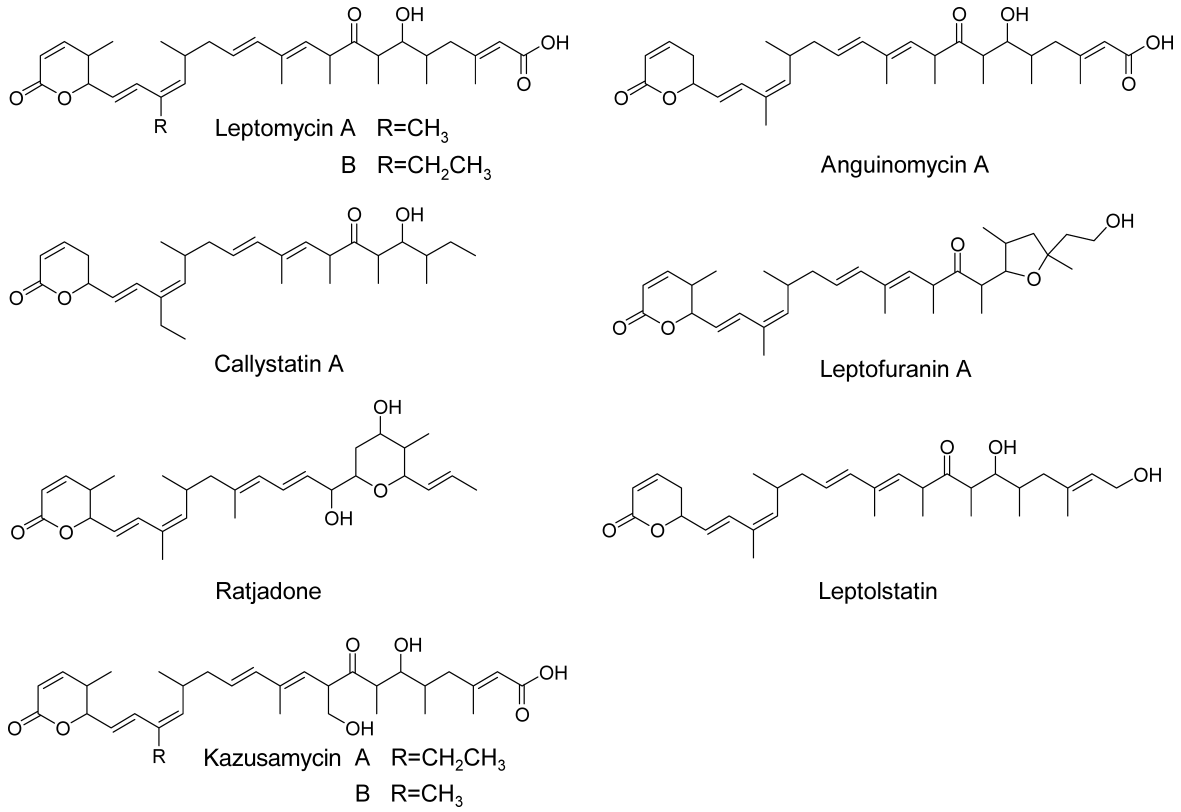

Fig. 1 The representative members of the leptomycin family.

was utilized as a fermentation medium for the production of leptomycins in *S. lividans* K4-114 derivative strains.

Construction of Plasmids for Heterologous Gene Expression

Two plasmids, pKOS279-166B and pKOS279-180, were constructed to accommodate the entire *lep* gene cluster. The former plasmid, having a SCP2* replicon and carrying the *lepA* gene under the control of *act*I promoter was constructed through a couple of subcloning steps. First, a 1 kb fragment called *lepAFR*, starting from the putative start codon of *lepA* to the nearest *Nsi*I site was amplified with the forward primer lepAF (5′-AAGCTT*TTAATTA-A*GGAGGACA*CATATG*CAGGTCATGGAGCGCGGAAT GACG) and the reverse primer lepAR (5′-*ATGCAT*-GTGCAGTTCGACGTATTGGACGGCGG). The primers introduced a *Pac*I and a *Nde*I at the 5′ end and a *Nsi*I site at 3′ end of *lepAFR* (the *Pac*I, *Nde*I and *Nsi*I sites are italicized in the primers sequences). After verification of the sequence, the *Pac*I-*Nsi*I fragment of *lepAFR* was used to replace the *Pac*I-*Nsi*I fragment of pCK7 [26], to yield pKOS279-163A. Then, the *Eco*RI-*Pst*I fragment carrying the rest of *lepA* gene from pKOS279-PF21 (see Fig. 2 for pKOS279-PF21) was inserted into the *Eco*RI and *Nsi*I sites of pKOS279-63A to give pKOS279-166B.

The second expression plasmid, pKOS279-180, carrying *lepBCDEF* genes plus the integrase gene and the corresponding *attP* site from ϕC31 phage [27～29] , was also made through a series of subcloning experiments. The 5′ end of *lepB* was amplified as a 721 bp *lepBFRC* fragment from pKOS279-2L78 and with the primers lepBFC (5′-*AAGCTTTTAATTAA*GGAGGACACATATGG-CTGAGTCGGAAGAG) and lepBR (5′-*ACTAGT*G*AATTC*-GGA*AGATCTC*CGGCGTGCACATCACCGTCG) (*Hin*dIII, *Pac*I, *Eco*RI and *Spe*I sites are italicized). The *Hin*dIII-*Pac*I *lepBFRC* fragment was then ligated with the *Hin*dIII-*Pac*I fragment of pKOS146-103A [30] and then together inserted into *Hin*dIII site of pLitmus 28 (New England Biolabs) to yield pKOS279-166A. The newly made plasmid was then cut with *Xba*I and self-ligated to produce pKOS279-166S in order to get rid of one unwanted *Eco*RI site. After that, the 14.7 kb *Bgl*II-*Eco*RI fragment carrying part of *lepB* gene from pKOS279-PFA42 was inserted into *Bgl*II-*Eco*RI sites of pKOS279-166S and resulted in pKOS279-167A. The *Avr*II-*Eco*RI fragment, containing *act*I promoter and 5′ end of lepB, of pKOS279-167A was used to replace the *Avr*II-*Eco*RI fragment of pKOS279-PFA42. The resulting plasmid, pKOS279-173

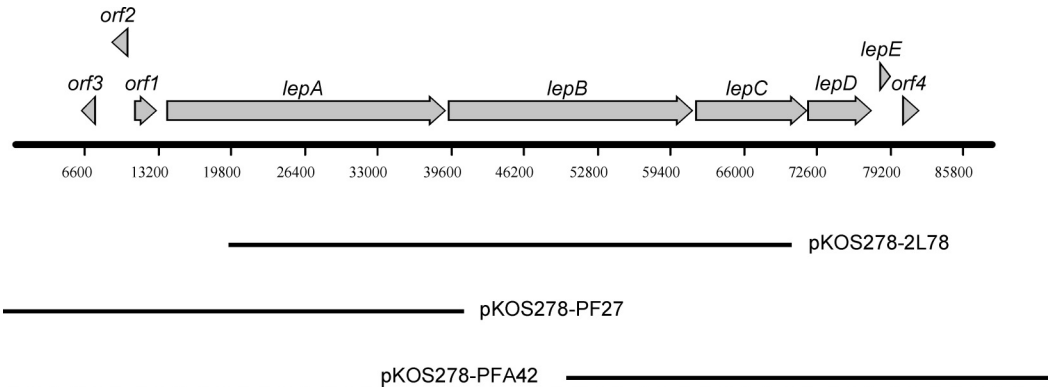

Fig. 2 A series of three overlapping cosmids containing the putative *lep* gene cluster and genes around it. The *lep* gene cluster consists of four pks genes (*lep*A, B, C, D) and a P450 gene (*lep*E).

was then equipped with the integrase gene and the corresponding *attB* site of ϕC31 and a *cos* site from HU152′ [30] by ligating the larger *Nhe*I-*Eco*RI fragment of pSET152 [31] and the small *Xba*I-*Eco*RI fragment of Hu152′ into *Xba*I linearized pKOS279-173. The *Avr*II-*Stu*I fragment of the newly made plasmid -pKOS279-174 carried *lepBCDEF* gene with *act*I promoter, the integrase gene, *attB* and *cos* site and was used to replace the *Xba*I-*Eco*RV fragment of Hu152′ so that the apramycin resistance gene was introduced into the final expression plasmid, pKOS279-180.

DNA Sequencing and Analysis

Sequencing was done on an ABI 3730 using Big Dye-terminator chemistry (Applied Biosystems). DNA sequencing of the 5′ and 3′ ends of the 475 cosmids picked randomly from the cosmid library was obtained by using the primers T7cos (5′-CATAATACGACTCACTATAGGG) and T3cos-1 (5′-TTCCCCGAAAAGTGCCAC). Individual cosmids were sequenced to completion using shotgun-cloning procedures by cloning 2.5∼3.5 kb of fragments resulted from partial *Sau*3AI digested cosmids into *Bam*HI site of PCR®-Blunt (Invitrogen). The DNA was assembled with Sequencher 4.1 (Gene Codes Corporation) and the corresponding deduced protein sequences were analyzed with Mac Vector 6.5.3 (Accelrys) software and compared with sequences in the public databases using CLUSTAL W and BLAST [32].

LC-MS Analysis of Leptomycins

Leptomycin A and B were analyzed by LC-MS on a system consisting of an Applied Biosystems Mariner time of flight mass spectrometer equipped with a Turbo Ion Spray source (spray chamber temperature 400°C; nozzle potential 110V) and an Agilent 1100 HPLC. In general, samples were injected on to a Zorbax Eclipse XDB-C8 column (3.5 μm; 2.1×150 mm) and eluted with a linear gradient from 50% to 100% CH_3CN (0.1% acetic acid) over 8 minutes at 0.250 ml/minute. The eluate was monitored by MS and DAD-UV. Under these conditions, the retention time of leptomycin A was 7.6 minutes, and leptomycin B was 8.1 minutes.

Results and Discussion

Cloning of the Leptomycin Biosynthesis Genes

The identification and isolation of a PKS (or NRPS) gene cluster are required for heterologous expression of the polyketide and for genetic engineering of the PKS to produce novel analogs. Over the past decade several methods have been developed for identification and isolation of modular (Type I) PKS genes from various organisms [33]. One such method involves sequencing small fragments of a random genomic DNA library, containing one or more modular PKS gene clusters, and then identifying which clones contain PKS genes. The PKS fragments obtained from the sequencing effort can then be used as “perfect probes” in experiments aimed at isolating one or all modular PKS gene clusters in an organism [34]. A modification of this method was developed in this study for the cloning of the *lep* gene cluster. For this, a cosmid library of the *Streptomyces* sp. ATCC 39366 genome was constructed on the Supercos-1 vector (Stratagene) and the end sequences (approximately 700 bp) of 475 cosmids were obtained and used for BLAST analysis. Type I PKS genes were found at one end of 16 cosmids and at both ends of four cosmids. Restriction analysis of the four cosmids containing PKS sequence at both ends, showed that three contained overlapping inserts while the fourth cosmid

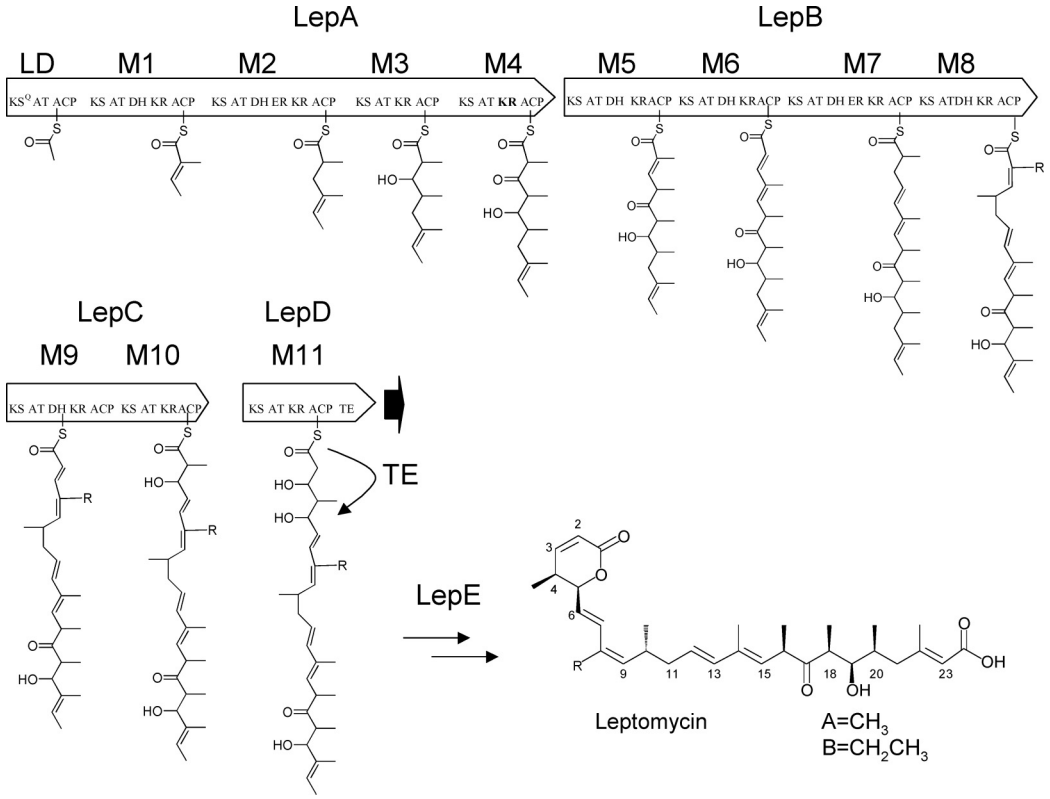

Fig. 3 The lep biosynthesis genes are shown as closed polygons. ACP, acyl carrier protein; AT, acyltransferase; ER, enoylreducase; KR, β-ketoreductase; KS, β-ketoacyl ACP synthase; KS^Q, KS domain with active site cysteine replaced by glutamine; TE, thioesterase.

(pKOS279-2L78) contained unique sequence. pKOS279-2L78 and one of the three overlapping cosmids were then completely sequenced by shotgun cloning. The sequence obtained for pKOS279-2L78 revealed six complete modules, and two incomplete ones, of which the organization and domain structure was consistent with the hypothetical biosynthesis of leptomycin (Fig. 2). In order to obtain the complete sequence of the cluster we searched for cosmids containing the missing part of the *lep* biosynthetic genes by using as a probe 1 kb segment from each end of the DNA fragment cloned in of pKOS279-2L78. A total of 89 positive cosmids were found and their ends sequenced. Three cosmids (pKOS279-PF27, pKOS279-PF21 and pKOS279-PFA42) (see Fig. 2) contained overlapping sequences with the right or the left hand ends of the insert present in pKOS279-2L78 and their sequences provided the complete DNA sequence of the *lep* gene cluster. The advantage of this method over the "perfect probe" method is that only one genome library, instead of two, has to be screened for the successful cloning of a gene cluster of interest.

Analysis of the Leptomycin Gene Cluster

A DNA fragment spanning aproximately 90 kb contained all the putative biosynthetic genes required for leptomycin biosynthesis (Fig. 2). The *lep* gene cluster was defined upstream by the orf1 and downstream by the orf4. The ORF1 is homologous (72% identity) to a carbon starvation protein from *Frankia* sp. CcI3 (CstA, Accession number ZP_00546194.11); ORF4 is a homolog (43% identity) of *N*-acyl-D-amino-acid deacylase from *Sphingopoyxis alaskensis* RB2256 (Accession number ZP_00577851.1). These two proteins are unlikely to be related to leptomycin biosynthesis. Analysis of the predicted coding regions present in the *lep* gene cluster revealed five relevant orfs. The leptomycin PKS is encoded by four genes (*lepA*, *B*, *C* and *D*) that together contain a total of 12 modules.

The amino acid sequence analysis of the putative loading module predicts the presence of a KS^Q type domain [35, 36], whose role is to provide, through decarboxylation of malonyl- or methylmalonyl-CoA, the starter substrate for the first condensation reaction catalyzed by the adjacent fully competent module [35, 36]. This suggests that malonyl-CoA, which is subsequently decarboxylated to acetate by the KS^Q activity, is utilized as the starter unit in

A.

ROSSMANN FOLD REGION

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KR4x-lep                          [no homology]
KR8x?-lep                        GTVLVTVATDPTDPTDGTDPV
KR9_1-lep                        GTTLVTGGTGALGALVARHLV
KR7_1-lep                        GTVLITGGTGTLGGLLARHLV
KR6_1-lep                        GTVLITGGTGALGRQVARHLV
KR5_1-lep                        GTVLITGGTGTLGTLLARHLV
KR3_1-lep                        GTVLVTGATGGLGALVARHLV
KR2_1-lep                        GTVLITGGTGTLGRSLARHLV
KR1_1-lep                        GTALITGGTGTLGALIARRLA
KR11_2-lep                       EAALITGGTGVLGAHAARWLV
KR10_2-lep                       GTALVTGGTGALGGRVARWLV
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B.

PREDICTED ACTIVE SITE REGION

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KR4x-lep        DGHDPGVLAALVTEHRPAGVVDASGESDAAWALHELTADVD-PAFFVLFSSAASLLGSSAHAATAGVD
KR8x?-lep       EHSPLTAVVHTAGLGRSHTEAMLRARVDAAVHLHELTRDAD-LSAFVLCTALDGVLADPGRGEHAAGD
KR9_1-lep       HVAGVVDDGVIGALTPERVDRVLRPKVDAALHLHELTRDAD-LTAFVLFSSVAGVIGSLGQANYAAGN
KR7_1-lep       HAAGVIDDAALTALTPERLDRVLRPKLHAAWNLHELTRDLD-LAEFVLFSSMAGTFGGAGQANYAAAN
KR6_1-lep       HLAGVTDDGLVGTLTPERLAAVLRPKIDAALHLDELTADAD-LSAFVLFSSAAGPVGNPGQANYAAAN
KR5_1-lep       HAAGVVDDGVVQSLTADRLDAVLRPKVDAAWNLHEATRHLD-LTAFVLFSSAAGVLGNPGQGNYAAAN
KR3_1-lep       HTAGVVDDATIANLTDAHMEHALRPKADAAFHLDELTRDVN-PAAFVLFSSGATTFGGPGQGNYAAAN
KR2_1-lep       HAAGTLDDAPIEALTPERVDHVLRPKVDAALVLDELTRDAD-LAAFVLFSSVAGVLGVAGQGGYAAGN
KR1_1-lep       HAAGVLDDATLLSLTPDRLDAVLRPKVDAAWHLHELTRAAN-PAAFVLFSSITAITGNAGQGAYTAAN
KR11_2-lep      HAAGVPGS-PTATGADAVAD-TVTAKVAGALALDTLFGADRALDAFVLYSSGAGVWGGAGQGAYAAAN
KR10_2-lep      HAAGAVVVGPLADSTVADLADASAAKVGGALLLDELLRADE-PDTVVLFSSAAGVWGGAGQGAYAAAN
                      #D                *K                      **SS          *Y
*: predicted catalytic residues
#: residue usually diagnostic of stereospecificity
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Fig. 4 Two conserved regions from leptomycin PKS ketoreductase domains (KR domains). A) A region containing a Rossmann fold motif, near the *N*-terminus of the region homologous to eubacterial independent FAS ketoreductases. The ketoreductase domain homolog in module 4, expected to be inactive, lacks this motif. The ketoreductase domain homolog in module 8, expected to be active, is very altered, and lacks four conserved glycines. B) A region containing a predicted active site triad (K, SS and Y). In most active ketoreductases, the tyrosine is expected to be the proton donor, and at least one of the two serine residues is expected to be present. In addition, this region contains a site predicted to be highly diagnostic of the stereospecificity of active ketoreductases; ketoreductases whose products have the stereostructure seen in the first module product of DEBS are normally seen to have aspartyl residues (here, modules 1, 2, 3, 5, 6, 7 and 9 are predicted to be of this type); those whose products have the opposite stereostructure do not (here, modules 10 and 11; for module 8, see discussion).

the first biosynthetic step of leptomycin . The organization of the rest of the modules and their domain composition is consistent with the hypothetical biosynthesis of leptomycin (Fig. 3), with certain exceptions described below. The ketoreductase (KR) domain set has certain unusual features. Each KR domain in a PKS falls into one of two stereospecificity classes. In one class (type 1) the stereospecificity of hydride attack is the same as that used by the homologous KR of vertebrate FAS [37, 38], as seen in the *lep* module 3. The opposite stereospecificity of attack (type 2) is that used here by the *lep* module 10. In many PKS modules, the KR mechanism is masked by subsequent dehydration (sometimes followed by enoyl reduction). We have proposed [39] that in modules producing *trans* double bonds using DH domains, or double bonds followed by enoyl reduction, that the stereospecificity on the KR is of type 1 and that ketoreduction of type 2 followed by dehydration would produce a *cis* double bond. These proposals imply that leptomycin ketoreductases of the *lep* modules 1, 2, 5, 6 and 9 should be of type 1, as is KR3, and that ketoreductases of type 2 would be consistent with the structures of the products of modules 8, 10 and 11. Sequence analysis of the KR domains support these predictions (Fig. 4), but suggests that some modules require activities external to themselves to produce their final products. The product of module 11, which contains a *cis* double bond, is consistent with a type 2 ketoreductase activity followed by dehydratation by a DH domain, however no such DH domain is found in this module; a similar case was reported in phoslactomycin biosynthesis [40]. Module 8 is even more unusual; although it contains an expected DH, and its product has structure consistent

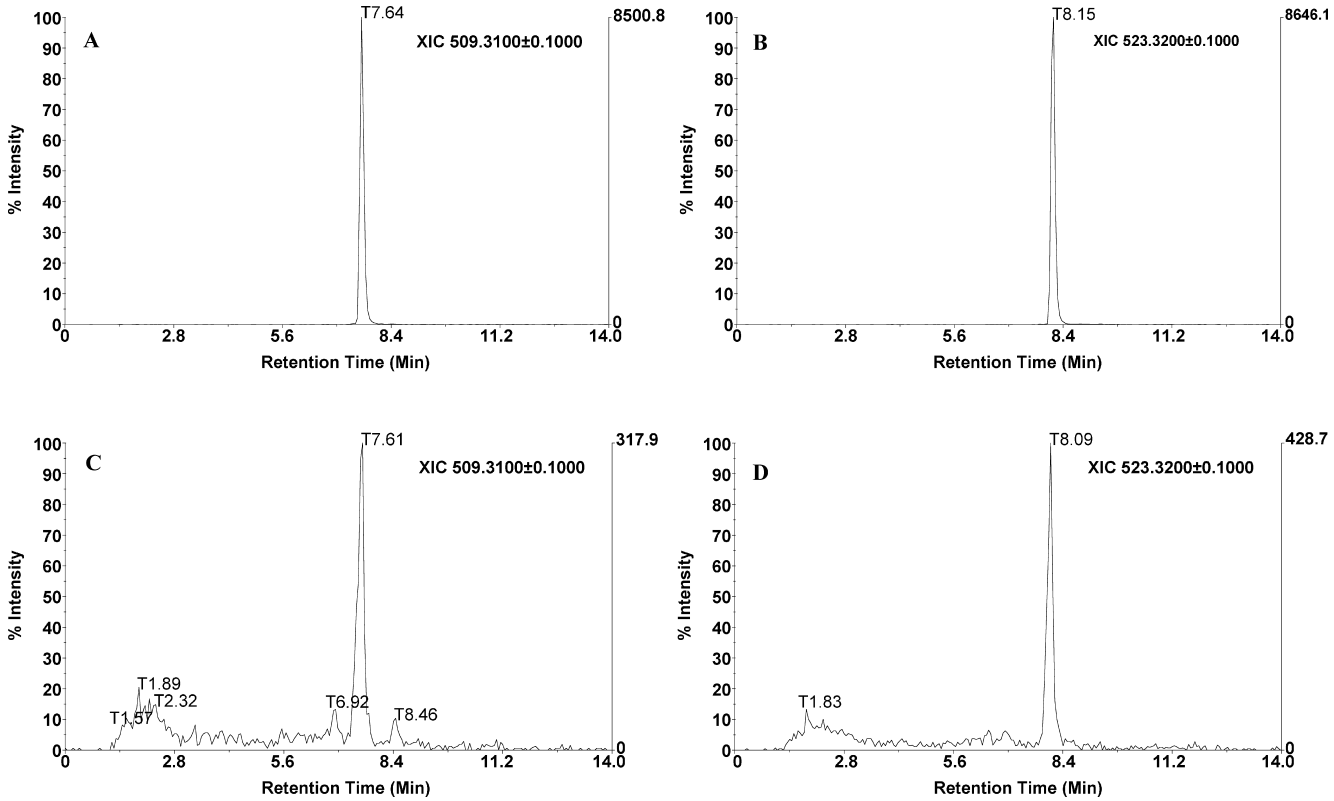

Fig. 5 Extracted ion chromatograms of A. leptomycin A standard; B. leptomycin B standard; C and D. leptomycin A and B from the crude extract of fermented *S. lividans* K4-414/pKOS279-180/pKOS279-166B.

with a type 2 KR plus a DH, its KR is likely to be inactive. In this module the predicted catalytically conserved triad K.S.Y, in which Y is expected to be the proton donor, appears to be replaced by R.T.H. Although suggestively similar in chemical types (*e.g.*, H could be the proton donor), such a dramatic change is unprecedented among the active KRs. Moreover, the alteration found at the glycine-rich region of the Rossmann fold, where four conserved glycines are absent (Fig. 4A), strongly suggests that this KR might be inactive. One possibility is that ketoreduction is performed by a domain from another module; alternatively, although less plausible, the ketoreduction could be catalyzed by an independent ketoreductase not found in the gene cluster.

Modules 6 and 11 of the LepB and LepD proteins, respectively, have acyltransferase (AT) domains highly conserved with respect to the other ATs domains that utilize malonyl-CoA as a substrate [41~43]. All the other ATs, except for AT 8, strongly resemble to those AT motifs that are specific for the utilization of 2-methylmalonyl-CoA [41~43]. Instead, the AT8 in LepD protein, like AT1 in Cfa 6 [44] and AT5 in TylGIII [45] is capable of utilizing either 2-methylmalonyl-CoA to produce leptomycin A or ethylmalonyl-CoA and lead to the production of leptomycin B. At a site known to be diagnostic for specificity, all the malonyl type ATs have the motif (A/S)HAFH and all the methylmalonyl type ATs present the motif DYASH. The F of HAFH is expected to fill a pocket left open by the S of DYASH for the methyl residue. In AT8, with overall higher homology to others methylmalonyl typeATs, the motif becomes AYSSH, with the D→A change perhaps allowing space for an extended ethyl side chain.

The remaining ORF, *lepE*, is also believed to be involved in leptomycin biosynthesis. LepE is homologous to several cytochrome P450 hydroxylases and shows 40% identity at the amino acid level with PikC, which is involved in methymycin/pikromycin biosynthesis in *S. venezuelae* [46]. We propose that LepE is likely involved in the generation of C_{24}–COOH.

Production of Leptomycin in the Heterologous Host *S. lividans*

Genetic engineering of PKS gene clusters is a powerful technique to generate analogs of natural products with improved pharmacokinetic properties, or to produce modified scaffolds used later as substrates for chemobiosynthesis or for chemical modifications. In our hands, *Streptomyces* sp. ATCC 39366 was intractable from

a genetic stand point, limiting our possibilities of generating analogs *via* genetic engineering. As an alternative we examined the ability to express the *lep* gene cluster in a heterologous host with well established genetic techniques. In order to produce leptomycin in a surrogate host like *S. lividans*, several requirements had to be met: 1) the *lep* genes had to be properly expressed, 2) the translation and post-translational modification of the PKSs had to occur at reasonable levels, and 3) the substrates needed for the biosynthesis of the final product had to be synthesized. To obtain adequate expression levels of the *lep* genes, all the ORFs of the cluster were cloned under the *act*I promoter, which has been successfully used in this microorganism for the expression of several heterologous PKS genes [26, 47, 48].

Transformants of *S. lividans* K4-114 containing plasmids, pKOS279-180 and pKOS279-166B were fermented in FKA media [25] for 7 days. HPLC/MS analysis of ethyl acetone extracts of the fermentation broth revealed the production of leptomycin A (~18 μg/liter) and B (~33 μg/liter). Although the production levels of leptomycin in the recombinant *S. lividans* strain was low, the result led us to conclude that all the genetic information needed for leptomycin biosynthesis was contained in the two expression plasmids. At the same time, the production of leptomycin B showed that *S. lividans* does produce ethylmalonyl-CoA, which is used as extender units in biosynthesis of several polyketides [43, 45, 49, 50]. Finally, the successful expression of this gene cluster in a genetically well characterized organism, opens the possibility of constructing genetically modified version of the cluster in order to produce new analogs of leptomycin. Although the level of production is low, there are many ways now to rationally improve the titers, for example using the pBOOST system recently used for increasing the levels of 6-dEB production in *Streptomyces coelicolor* [51, 52].

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