Organization of the biosynthetic gene cluster in *Streptomyces* sp. DSM 4137 for the novel neuroprotectant polyketide meridamycin

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Meridamycin is a non-immunosuppressant, FKBP-binding macrocyclic polyketide, which has major potential as a neuroprotectant in a range of neurodegenerative disorders including dementia, Parkinson's disease and ischaemic stroke. A biosynthetic cluster predicted to encode biosynthesis of meridamycin was cloned from the prolific secondary-metabolite-producing strain *Streptomyces* sp. DSM 4137, not previously known to produce this compound, and specific gene deletion was used to confirm the role of this cluster in the biosynthesis of meridamycin. The meridamycin modular polyketide synthase consists of 14 extension modules distributed between three giant multienzyme proteins. The terminal module is flanked by a highly unusual cytochrome P450-like domain. The characterization of the meridamycin biosynthetic locus in this readily manipulated streptomycete species opens the way to the engineering of new, altered meridamycins of potential therapeutic importance.

INTRODUCTION

Polyketide macrolides constitute a large family of bioactive natural products and include many compounds with important clinical applications in both human and veterinary medicine. Meridamycin is a 27-membered macrocyclic polyketide, isolated independently from two different streptomycete strains (Fehr *et al.*, 1994; Salituro *et al.*, 1995). Although it lacks immunosuppressant activity, it shares the structural motif common to the macrocyclic polyketide immunosuppressants rapamycin (Schwecke *et al.*, 1995), FK506 (Motamedi & Shafiee, 1998) and FK520 (Wu *et al.*, 2000), which is known to interact with the hydrophobic cavity on the surface of FK506-binding protein-12 (FKBP-12) (Van Duyne *et al.*, 1991, 1993). This structural moiety is proposed to mimic a natural peptide substrate and hence inhibit the peptidyl-prolyl isomerase activity of FKBP-12. Meridamycin, however, differs from rapamycin, FK520 and FK506 in the absence of the substituted cyclohexane ring characteristic of these molecules (Fig. 1). Meridamycin competitively inhibits the binding of FK506 and rapamycin to FKBP-12. It also inhibits the immunosuppressant action of FK506 in a

functional assay of interleukin (IL)-2 transcription; and that of rapamycin in an IL-6-dependent cell-proliferation assay.

Rapamycin (sirolimus), FK506 (tacrolimus) and more recently FK520 (pimecrolimus) have now become well established in clinical practice. In addition to immunosuppressant and anti-proliferative activity, they also possess neurotrophic activity through binding to neuroimmunophilin FKBP that are markedly enriched in the central nervous system and in peripheral nerves. These agents can stimulate nerve growth regeneration in a range of animal models. They are currently under investigation for the treatment of a number of important human neurological disorders, including multiple sclerosis (Lubetski *et al.*, 2005), traumatic spinal cord injury (Sosa *et al.*, 2005) and Parkinson's disease (Tanaka & Ogawa, 2004). It is clear that the immunosuppressant and neurotrophic properties of FKBP-binding macrolides represent separate and distinct pharmacological effects. Neuroregenerative activity, in the absence of immunosuppressant activity, is shown by compounds which bind to FKBP but do not interact with the effector proteins calcineurin or mTOR (Steiner *et al.*, 1997). It has recently been proposed that the neurotrophic activity may in fact be due to binding to targets other than FKBP-12. In FKBP-12 knockout mice, FKBP-12-binding ligands retain their neurotrophic activity on hippocampal neurones (Gold *et al.*, 1999) and FK506 is still able to protect dopaminergic neurones against MPP⁺-induced toxicity (Guo *et al.*, 2001). Both studies indicate that binding to

Abbreviations: AT, acyltransferase; FKBP, FK506-binding protein; LC-MS, liquid chromatography-mass spectrometry; NRPS, non-ribosomal peptide synthetase; PKS, polyketide synthase.

The GenBank/EMBL/DBJ accession number for the sequence data reported in this paper is DQ885223.

Fig. 1. FKBP-binding ligands meridamycin, rapamycin, FK506 and FK520 show common structural features.

FKBP-12 is not required for neurotrophic activity. Similarly, non-immunosuppressant derivatives of FK506 that lack FKBP-12 binding are found to stimulate neurite elongation in SH-SY5Y cells (Gold *et al.*, 2005). It is likely that binding to members of the FKBP family other than FKBP-12 underlies the neurotrophic activity of these agents. In particular, FKBP-52 has been proposed as the true target (Gold *et al.*, 1999). In keeping with these observations, meridamycin has recently been shown to possess similar neuroprotectant and neurotrophic activities. The absence of immunosuppressant activity is a considerable advantage as such activity results in a range of serious and unwanted side-effects. The production of modified meridamycin analogues by manipulation of the biosynthetic gene cluster would be an attractive approach to compounds of potential utility in a

range of neurodegenerative disorders with huge unmet need, causing enormous morbidity and mortality.

Our analysis of the biosynthesis of meridamycin was prompted by our investigation of the remarkable polyketide biosynthetic potential of *Streptomyces* sp. DSM 4137. This organism contains biosynthetic gene clusters for at least 11 complex modular polyketides as well as numerous aromatic polyketides, non-ribosomally synthesized peptides and other secondary metabolites. We have completed an analysis of the gene clusters encoding the novel macrodiolide elaiophyllin (Haydock *et al.*, 2004) from this organism, and of the cluster encoding biosynthesis of the related polyethers nigericin and abierixin (B. M. Harvey and others, unpublished). Our analysis of the remaining clusters has

now led to the identification of a 100 kbp locus whose organization is entirely consistent with the biosynthesis of meridamycin. We have confirmed that the strain produces meridamycin, and have used targeted gene deletion to confirm that the cloned genes govern this pathway. The genetic manipulation of the biosynthetic genes for meridamycin biosynthesis in *Streptomyces* sp. DSM 4137 represents a convenient system for the generation of a family of meridamycin metabolites with potential in the treatment of a range of debilitating neurological disorders.

While the present paper was in preparation, a chromosomal DNA sequence reported to encode meridamycin biosynthesis in *Streptomyces* sp. NRRL 30748 was deposited in GenBank, under accession number DQ351275, to accompany a brief description and a number of claims relating to the sequence, recently published in US Provisional Patent Application 60/664,483, originally filed on March 23, 2005. Comparison of our sequence with that of the sequence from *Streptomyces* sp. NRRL 30748 has helped to confirm the boundaries of the cluster but has also identified significant and unexpected differences in gene organization between the two clusters. An account of the meridamycin cluster in *Streptomyces* sp. NRRL 30748 has recently been published (He *et al.*, 2006).

METHODS

Bacterial strains and cloning vectors. *Streptomyces* sp. DSM 4137 was the kind gift of Dr Lake Ee Khaw, Department of Biochemistry, University of Cambridge. *Streptomyces* sp. DSM 7348 is a known meridamycin-producing strain and was the kind gift of Dr Rose Sheridan, Biotica Technology Ltd, Chesterford Research Park, Essex. The following *Escherichia coli* strains were used: DH10B (Gibco-BRL) for routine cloning; XL-1 Blue MR for cosmid library construction; and ET12567 (MacNeil *et al.*, 1992), into which the helper plasmid pUZ8002 (Paget *et al.*, 1999) was cloned, as a donor strain for conjugation into strain DSM 4137. Disruption of the meridamycin gene cluster was carried out using plasmid pYH24, derived from pJTU412 and pHZ1358 (Sun *et al.*, 2002). Routine subcloning was performed in pSHG397 (Hashimoto-Gotoh *et al.*, 1995).

Culture conditions and molecular genetic procedures. *E. coli* strains were grown on 2 × TY medium supplemented with appropriate antibiotics. *Streptomyces* sp. DSM 4137 and *Streptomyces* sp. DSM 7348 were maintained on SFM medium (Kieser *et al.*, 2000). Liquid cultures of DSM 4137 for isolation of genomic DNA were grown in tryptone soya broth (Difco). Cultures of strains DSM 4137, DSM 7348 and disruptants of DSM 4137 to be used in analysis of meridamycin production were grown in 50 ml SFM liquid medium in a 250 ml flask for 6 days at 30 °C. Standard genetic techniques for *E. coli* and for *in vitro* DNA manipulations were as described by Sambrook *et al.* (1989). Total DNA was isolated from DSM 4137 by 'procedure B' of Kieser *et al.* (2000). For the generation of the cosmid library, total DNA was partially digested with *Sau*3AI, dephosphorylated with shrimp alkaline phosphatase, ligated directly into pSuperCos and packaged with Gigapack Gold packaging extract (Stratagene) without size fractionation. All procedures were in accordance with the manufacturer's recommendations. Initial screening of the cosmid library of DSM 4137 was with a 9.5 kbp *Kpn*I fragment from the rapamycin PKS cluster (Schwecke *et al.*, 1995) encoding ketosynthase (KS), acyltransferase (AT), dehydratase (DH), ketoreductase (KR) and acyl carrier protein (ACP)

domains. Colony hybridization of the cosmid library was performed with a digoxigenin-labelled probe in accordance with the manufacturer's recommendations (Boehringer Mannheim). Cosmid DNA for complete sequencing was prepared using Qiagen midi-prep DNA purification kits from 50 ml liquid cultures in 2 × LB medium containing ampicillin and kanamycin. The complete sequence of each cosmid was obtained from overlapping *Sau*3AI fragments obtained after a partial digestion of the parent cosmid. The 2–5 kbp fraction of this digest was eluted from an agarose gel using the Gene Clean kit (Bio 101, Bio-Rad) and subcloned into pSHG397 (Hashimoto-Gotoh *et al.*, 1995).

For construction of the plasmid used for disruption of the *mer* gene cluster, two DNA fragments, a 4164 bp *Bcl*I–*Bam*HI fragment and a 4434 bp *Bcl*II fragment, were cloned from cosmids FB4 and H3G7 covering the *mer* gene cluster, respectively, then inserted into the unique *Bam*HI restriction site of the delivery vector pYH7 with its natural orientation to give the plasmid pYH24. The plasmid pYH24 contains an origin of replication and can replicate in *Streptomyces* sp. DSM 4137.

The following primers were used for screening double crossover mutants in this study: mer-3, 5'-AAAAGTATGGGTTGACAGCG-3'; mer-4, 5'-CGGCACGATGCGACA-3'. Genomic PCR with the mer-3 and mer-4 primers was performed in a final volume of 30 µl for 30 cycles of amplification using a programmer (94 °C, 5 min; 94 °C, 30 s; 55 °C, 40 s; 72 °C, 50 s; 72 °C, 5 min). A PCR product (894 bp) was recovered from gel for sequencing. Southern hybridization was carried out with digoxigenin-labelled probes. Sequencing of DNA fragments generated by PCR amplification was performed directly using automated DNA sequencing as described below.

Extraction, isolation and characterization of meridamycin.

Broths derived from 50 ml cultures of DSM 7348 (an authentic meridamycin producer), DSM 4137 and disruptants were extracted with equal volumes of ethyl acetate. The residue, after evaporation of the solvent, was dissolved in 0.8 ml methanol and subjected to liquid chromatography-mass spectrometry (LC-MS) analysis. On-line LC-MS and LC-MS/MS analysis were carried out on a LCQ mass spectrometer (Thermo Finnigan) using positive electrospray ionization. The LCQ was coupled with an HP 100 LC (Agilent) fitted with a Prodigy C18 column (5 µ, 4.6 × 250 mm) (Phenomenex). Samples were eluted with a 40 min gradient from 25 to 100% acetonitrile in water. High-resolution mass analysis was performed on a BioApex II (4.7 Tesla) FTICR mass spectrometer (Bruker Daltonics).

DNA sequencing analysis. Automated DNA sequencing was carried out on double-stranded DNA templates by the dideoxynucleotide chain-termination method in the University of Cambridge, Department of Biochemistry DNA sequencing facility. An Applied Biosystems 800 molecular biology CATALYST robot was used to apply *Taq* dideoxy terminator sequencing reactions (Big Dye Terminator kit, ABI) to an ABI 373A sequencer according to the manufacturer's protocols. Cosmid DNA for initial end sequencing was prepared from overnight growth of 1 ml cultures in 96-well plates in 2 × LB medium containing kanamycin and ampicillin. The complete sequence of the cosmid was obtained from analysis of overlapping *Sau*3AI fragments obtained via a partial digestion of the parent cosmid. The 2–5 kbp size fraction of this digest was eluted from an agarose gel using the Gene Clean kit (Bio 101, Bio-Rad) and subcloned into the *Bam*HI site of dephosphorylated pSHG397 (Hashimoto-Gotoh *et al.*, 1995). These random subclones were sequenced using template DNA obtained from 1 ml cultures grown overnight at 37 °C in 96-well plates, using the universal forward and reverse sequencing primers. SeqEd version 1.03 was used for sequence editing. Database searches used the BLAST algorithm. Sequence assembly employed the GAP (Genome Assembly Program) version 4.2 (Bonfield *et al.*, 1995). To fill remaining contig gaps and

resolve any remaining ambiguities, custom-designed oligonucleotide primers were used to sequence from selected subclones or from the parent cosmid. Both strands were completely sequenced with a mean eightfold coverage per base.

RESULTS AND DISCUSSION

Cloning and sequencing of the meridamycin biosynthetic cluster

A genomic library of strain DSM 4137 was constructed in pSuperCos vector (Stratagene). This was screened by hybridization with a 9.5 kbp *Kpn*I DNA fragment of *rapA* of the rapamycin PKS of *Streptomyces hygroscopicus* as described previously (Haydock *et al.*, 2004). It was concluded on the basis of stringent hybridization conditions that 8 % of the genome of this organism encoded type I PKS genes. To date, we have identified 11 type I PKS gene clusters in the genome of this organism. We are in the process of characterizing all of these loci. We have already reported the complete sequence of the cluster responsible for biosynthesis of the macrodiolide elaiophylin from this prolific organism (Haydock *et al.*, 2004). Analysis of the DNA sequence of four overlapping cosmid clones that comprised another of these loci led to the identification of a cluster predicted to encode the biosynthesis of the macrolide meridamycin. This locus spans approximately 95 kbp. Extensive sequencing of flanking regions revealed the likely boundaries of the gene cluster.

Confirmation of the identity of the meridamycin biosynthetic locus by gene disruption

LC-MS analysis of the extracts of DSM 4137 showed a peak at 27.04 min with $[M + Na]^+$ at 844.5 *m/z* (Fig. 2a). High-

Fig. 2. LC-MS analysis of meridamycin ($[M + Na]^+$: 844.5) production from cultural broth extracts. (a) Ion trace of *m/z*: 844.5 from DSM 4137 (wild-type); (b) ion trace of *m/z*: 844.5 from the meridamycin-producing strain DSM 7348; (c) ion trace of *m/z* 844.5 from DSM 4137 Δ *mer* (mutant).

resolution mass analysis confirmed its formula as $C_{45}H_{75}NO_{12}Na^+$ (Obs. 844.5187, Cal. 844.5181). To further confirm the production of meridamycin from strain DSM 4137, the meridamycin-producing strain *Streptomyces* sp. DSM 7348 was grown, harvested and analysed under the same conditions as for DSM 4137. LC-MS analysis of this strain clearly showed that meridamycin with $[M + Na]^+$ at 844.5 *m/z* elutes at 26.98 min (Fig. 2b). LC-MS/MS analysis of both strains showed that the MS/MS spectrum of 844.5 from strain DSM 4137 was identical to that from strain DSM 7348 (data not shown). To identify the meridamycin biosynthetic gene cluster, the recombinant plasmid construct pYH24 was introduced into DSM 4137 by conjugation using ET12657/pUZ8002 as donor host strain on SFM plates (Fig. 3). After incubation at 30 °C for 18 h, exconjugants were selected with 1 ml water containing apramycin and nalidixic acid. Single colonies from this plate were transferred to an SFM plate containing apramycin for further confirmation of antibiotic resistance. Confirmed colonies were propagated on SFM plates without antibiotic selection to allow the double crossover to occur. To screen the potential double crossover mutants, single colonies from non-selective plates were replicated to an SFM plate containing 12.5 μ g apramycin ml^{-1} . The candidates with the correct phenotype (Apr^S) were chosen for further identification by PCR (Fig. 4a) and Southern blotting. Sequencing, hybridization (Fig. 4b) and subsequent LC-MS (Fig. 2c) results showed that disruption of the locus specifically abolished production of a metabolite with an LC-MS profile identical to that of authentic meridamycin from the known producer DSM 7348.

Organization of the meridamycin biosynthetic gene cluster

The meridamycin biosynthetic cluster is encoded by a 95 kbp locus and encodes only five ORFs (Fig. 5, Table 1). The biosynthesis of the polyketide backbone of meridamycin is encoded by three large ORFs designated *merA*, *merB* and *merC*, which are adjacent and transcribed in the same direction and which encode, respectively, the multienzyme polypeptides MerA, MerB and MerC. Examination of the encoded AT domains of MER for distinctive amino acid sequence motifs allows the chemical nature of the extender unit recruited by each AT domain (especially, malonate versus methylmalonate or ethylmalonate) to be inferred (Haydock *et al.*, 1995; Lau *et al.*, 1999; Reeves *et al.*, 2002; Del Vecchio *et al.*, 2003). Examination of the 'reductive loop' (Staunton & Weissman, 2001) domains [KR, DH, enoyl-reductase (ER)] in each extension module allows the level of reduction to be deduced for that extension. In the case of hydroxyacyl extension units, even the stereochemical configuration of the hydroxy group can be predicted (Caffrey, 2003). The predicted polyketide product of the *mer* PKS from our analysis is entirely consistent with assembly of the polyketide chain of meridamycin, the modules being encoded along the chromosome in the order they are used from *merA* to *merC* (Table 2). Inspection of

Fig. 3. Organization of the chromosomal *mer* genes before and after deletion. The numbers between *Scal* sites represent the expected size of the fragments hybridizing to the probe (1920 bp *Scal* fragment obtained from digestion of plasmid pYH24) after digestion of chromosomal DNA with *Scal*.

Fig. 4. Confirmation of gene disruption by PCR (a) and Southern blotting (b). A pair of primers, *mer-3* and *mer-4*, covering the deleted region in the chromosome were used for quick screening to identify double crossover mutants. Southern blot analysis was performed with chromosomal DNA isolated from wild-type and mutants digested with *Scal*. The hybridization probe was the 1920 bp *Scal* fragment obtained from digestion of plasmid pYH24, used for the *mer* cluster disruption. Plasmid pYH24 was digested with *Scal* and used as positive control. Extra bands hybridizing on the Southern blot are partial digestion products and cross-hybridization with other genomic fragments with PKS genes from the numerous other gene clusters in this organism. Lanes: 1, Hyper ladder I; 2, pYH24; 3, DSM 4137 (wild-type); 4, DSM 4137 $\Delta mer-a$ (mutant); 5, DSM 4137 $\Delta mer-b$ (mutant).

Fig. 5. Organization of the meridamycin biosynthetic locus in *Streptomyces* sp. DSM 4137. Genes proposed to be involved in meridamycin biosynthesis are coloured black. Hatched genes are not thought to be required for biosynthesis.

the structure of meridamycin suggested that, if it is synthesized by a canonical modular PKS, the PKS can be predicted to comprise either a loading module for an acetate starter unit and 14 PKS extension modules, or a loading module that recruits a C₅ starter unit followed by 13 extension modules. The analysis allows a clear choice in favour of the first of these possibilities: MerA (5721 aa, approx. 630 kDa) encodes the loading module (containing a KSQ-type decarboxylase) (Bisang *et al.*, 1999) and extension modules 1–3; MerB (7178 aa, approx. 790 kDa) encodes extension modules 4–7; and MerC (11 604 aa, approx. 1276 kDa) encodes modules for extensions 8–14. The organization of the PKS for meridamycin shows close similarity to that of the related cluster from *Streptomyces* sp. NRRL 30748, with 95 % identity at the protein level for most of the PKS translation.

There are two significant differences in the organization of the PKS genes in the two clusters. Module 14 in the *mer* cluster is unusual in that the expected ACP domain at the C-terminus of MerC is followed by a domain (about 400 aa)

with significant end-to-end sequence similarity to a cytochrome P450 monooxygenase. Such an arrangement is to our knowledge unprecedented in modular PKS biosynthetic gene clusters that have been previously studied. This terminal P450 monooxygenase domain is absent from the counterpart PKS of strain NRRL 30748. It contains the conserved cysteine residue that provides a ligand to the haem iron in these enzymes. However, it lacks the absolutely conserved ExxR sequence upstream of the cysteine residue. The function of this domain is obscure, given that it is not present in the meridamycin PKS of NRRL 30748. Screening of the databases with this sequence identifies several putative cytochrome P450 monooxygenases, the best match being to a putative P450 from *Nocardia farcinica* IFM 10152, with 45 % identity and 61 % similarity at the protein level (accession no. BAD59368). This and other closely matching proteins also lack the ExxR sequence of the functional P450 haem oxygenase.

In *Streptomyces* sp. DSM 4137, MerC encodes extensions 8–14, whilst in *Streptomyces* sp. NRRL 30748 these

Table 1. Identifications of ORFs of the meridamycin biosynthetic gene cluster and flanking genes

ORF	Amino acids	Best match, organism, (% identity), (% similarity)	Putative function
L6	Partial	ABC87052, <i>Streptomyces</i> NRRL 30748, (93), (96)	Mycodextranase
L5	532	ABC87503, <i>Streptomyces</i> NRRL 30748, (93), (98)	α-1,4-Glucosidase
L4	291	ABC87504, <i>Streptomyces</i> NRRL 30748, (95), (98)	Sugar transporter
L3	318	ABC87505, <i>Streptomyces</i> NRRL 30748, (97), (98)	Sugar transporter
L2	476	ABC87506, <i>Streptomyces</i> NRRL 30748, (93), (96)	Sugar transporter
L1	336	ABC87507, <i>Streptomyces</i> NRRL 30748, (95), (98)	LacI regulator
MerP	1 605	ABC87508, <i>Streptomyces</i> NRRL 30748, (93), (95)	Non-ribosomal peptide synthetase
MerA	5 721	ABC87509, <i>Streptomyces</i> NRRL 30748, (91), (93)	Polyketide synthase
MerB	7 178	ABC87509, <i>Streptomyces</i> NRRL 30748, (91), (92)	Polyketide synthase
MerC	11 604	ABC87509, <i>Streptomyces</i> NRRL 30748, (90), (92)	Polyketide synthase
MerD	408	BAD59369, <i>Nocardia farcinica</i> IFM 10152 (63), (76)	Cytochrome P450 monooxygenase
R1	687	BAC70101, <i>S. avermitilis</i> MA-4680, (86), (88)	Amino acid permease
R2	445	CAA16438, <i>S. coelicolor</i> A3(2), (79), (87)	RNA methyltransferase

Table 2. Organization of the meridamycin PKS

Mer protein	Module	Domain structure*
MerA	Loading	KSQ ATa ACP
	Extension 1	KS ATp KR ACP
	Extension 2	KS ATp DH KR ACP
	Extension 3	KS ATa KR ACP
MerB	Extension 4	KS ATe DH KR ACP
	Extension 5	KS ATp KR ACP
	Extension 6	KS ATp DH ER KR ACP
	Extension 7	KS ATp DH KR ACP
MerC	Extension 8	KS ATa KR ACP
	Extension 9	KS ATa KR ACP
	Extension 10	KS ATp KR ACP
	Extension 11	KS ATa KR ACP
	Extension 12	KS ATa KR ACP
	Extension 13	KS ATp DH ER KR ACP
	Extension 14	KS ATa ACP

*KS, ketosynthase; KSQ, decarboxylating ketosynthase; ATa, acyltransferase with malonyl specificity; ATp, acyltransferase with methylmalonyl specificity; ATe, acyltransferase with ethylmalonyl specificity; DH, dehydratase; ER, enoylreductase; KR, ketoreductase; ACP, acyl carrier protein.

extensions are reportedly split between two large ORFs designated MerC and MerD, in such a way that one module is split, with a C-terminal KS domain in MerC and an N-terminal AT domain in MerD. Given this unusual arrangement and the otherwise very similar organization to that of the *Streptomyces* sp. DSM 4137 sequence, we examined this region more closely. The *merC* and *merD* genes occupy positions 64760–88666 and 88050–98327, respectively, of the deposited sequence from *Streptomyces* sp. NRRL 30748, that is, two genes are apparently coded for on the same strand and overlap by 600 bp. A more likely explanation, from comparison of the sequences, is that there is a single base error in the previously deposited sequence, so that a run of five guanines between positions 88303 and 88308 was incorrectly assigned as a run of six guanines. This correction would restore the reading frame and result in a contiguous sequence encoding a single ORF for extensions 8–14, just as in strain NRRL 4317.

Immediately upstream of *merA* lies another large ORF designated *merP* (1607 aa with an approximate molecular mass of 177 kDa), corresponding to *merP* in the deposited sequence from strain NRRL 30748, and encoding the protein MerP. MerP and its homologue in NRRL 30748 exhibit very high end-to-end sequence similarity to many authentic non-ribosomal peptide synthetase (NRPS) enzymes (comprising the domains in the following order: condensation, adenylation, peptidyl carrier protein and a further condensation domain). In particular, it has a high degree of identity to the NRPS proteins incorporating pipecolate, including the pipecolate-incorporating enzyme (PIE) of the biosynthetic gene clusters for other

FKBP-binding ligands such as rapamycin and FK506. MerP is therefore proposed to be responsible for addition of the pipecolate unit to the polyketide chain and subsequent formation of the macrocycle.

Immediately downstream of *merC* lies a single ORF predicted to encode a protein of 408 aa. This predicted gene product, MerD, shows end-to-end homology to authentic cytochrome P450 hydroxylases commonly encountered in macrolide gene clusters. In particular, it contains both the active-site cysteine and the conserved ExxR sequence. The meridamycin cluster in strain NRRL 30748 also has a P450 hydroxylase immediately downstream of the PKS. Interestingly, the genes from strains DSM 4137 and NRRL 30746 do not show particular homology and are no more alike than any two P450 hydroxylase genes. It also does not show particular homology to other P450 hydroxylases from either the rapamycin or FK506 gene clusters. The DSM 4137 sequence most resembles an authentic P450 gene from *Nocardia farcinica* IFM 10152 (accession no. BAD59369). It shows little similarity to the terminal domain of MerC. There is no position on the ring of meridamycin other than at C-9 that requires hydroxylation. Further work will be required to establish whether MerD, acting alone or in concert with the terminal domain of MerC, performs the oxidation at C-9 to give the characteristic 1,2,3-triketo arrangement in the FKBP-binding pharmacophore.

The probable limits of the *mer* gene cluster were probed by DNA sequence analysis. Upstream of *merP* is an apparently truncated version of an ornithine or lysine cyclodeaminase gene. This pseudogene would have been an obvious candidate to catalyse the synthesis of the pipecolate extender unit, if it had been intact. We have identified (S. F. H. and T. M., unpublished data) an intact copy of a cyclodeaminase gene in another gene cluster within this organism, adjacent to PKS genes. Possibly, the product of this gene is sufficient to provide pipecolate for both pathways. Immediately upstream of the presumed pseudogene are genes involved in carbohydrate metabolism. The first of these encodes a 532 aa protein with 93 % identity to α -1,4-glucosidase of *Streptomyces* sp. NRRL 30748 (accession no. ABC87503). This appears to be transcriptionally coupled to a gene encoding a protein with 94 % identity to mycodextranase that also lies adjacent to the α -1,4-glucosidase above in the same organism (accession no. ABC87502). The organization of genes upstream of the PKS and NRPS components of the clusters in the two organisms is thus identical. The clearly identified function of these two genes appears to mark the upstream boundary of the cluster. To confirm this, we have sequenced a further 10 kbp upstream and have identified no other genes that could be implicated in meridamycin biosynthesis. The downstream limits of the cluster were also explored by sequence analysis. Downstream of ORF1 is a gene encoding a putative 687 aa protein with 86 % identity to a permease from *Streptomyces avermitilis* MA-4680. Adjacent to this is a gene

predicted to encode a 445 aa protein with 79 % identity to an RNA methyltransferase from *Streptomyces coelicolor*. Following this gene is a 5 kbp region of higher AT content DNA that is not predicted to contain any ORFs. We have sequenced a further 10 kbp beyond this and have again been unable to identify any genes that could be implicated in meridamycin biosynthesis. The different organization of genes downstream of ORF1 indicates that this gene marks the downstream limit of the gene cluster.

The *mer* cluster contains relatively few genes. In particular, there is an absence of regulation and export genes, and of genes for biosynthesis of the ethylmalonate extender unit. Such 'minimalist' clusters are a feature of *Streptomyces* sp. DSM 4137. Due to the extensive biosynthetic capacity, there appears to be some redundancy of function with genes shared between pathways. We have identified other functional clusters containing genes for ethylmalonate biosynthesis and pipecolate biosynthesis, and several that do not have associated export or regulatory genes (S. F. H. and T. M., unpublished data).

Evidence for convergent evolution of the FKBP-binding pharmacophore

Despite exhaustive screening for naturally occurring FKBP-binding macrocyclic polyketides, such metabolites are rare. Compared to the FK520, FK506 and rapamycin gene clusters, meridamycin shows some key differences in organization. Its final extension module encodes non-functional dehydratase and ketoreductase domains, rather than these being absent (Fig. 6), as well as bearing a pendant P450 domain. Also, although an ER domain is required for the complete reduction at C-12 in all four compounds, the *mer* ER domain sequence is highly divergent from the equivalent domains of rapamycin, FK506 and FK520, which show very high mutual homology (data not shown). The biosynthesis of the FKBP-binding pharmacophore of meridamycin may have arisen by a process of convergent evolution to the other compounds.

Concluding remarks

The analysis of the meridamycin biosynthetic locus from *Streptomyces* sp. DSM 4137 has identified a set of modular PKS genes clustered with an NRPS-like gene and a gene for a cytochrome P450. The organization is similar to, but shows distinct differences from, the analogous gene cluster recently reported from *Streptomyces* sp. NRRL 30748. The minimal PKS cluster of DSM 4137 apparently recruits genes from other clusters within this organism to provide biosynthesis of the ethylmalonate and pipecolate extender units. The characterization of the genes for this interesting non-immunosuppressant FKBP-binding macrolide, together with methods for the genetic manipulation of the producing strain, opens the way to genetic modification of the cluster to obtain novel structural variants of meridamycin with potentially improved bioactivity.

Fig. 6. Comparison among the last module in the FK506, FK520, rapamycin and meridamycin biosynthetic gene clusters. The arrows represent the PKS-encoded polypeptides. Domains within each module are represented by circles. Abbreviations: KS, ketoacyl synthase; AT, acyltransferase; DH, dehydratase; KR, ketoreductase; ACP, acyl carrier protein; M, cytochrome P450 monooxygenases.

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