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ORIGINAL PAPER

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Active-site residue, domain and module swaps in modular polyketide synthases

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Abstract Sequence comparisons of multiple acyltransferase (AT) domains from modular polyketide synthases (PKSs) have highlighted a correlation between a short sequence motif and the nature of the extender unit selected. When this motif was specifically altered in the bimodular model PKS DEBS1-TE of *Saccharopolyspora erythraea*, the products included triketide lactones in which acetate extension units had been incorporated instead of propionate units at the predicted positions. We also describe a cassette system for convenient construction of hybrid modular PKSs based on the tylosin PKS in *Streptomyces fradiae* and demonstrate its use in domain and module swaps.

Keywords Polyketide synthase · *Streptomyces fradiae* · Tylosin · *Saccharopolyspora erythraea* · Erythromycin · Acyltransferase · Docking domain

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Introduction

Hybrid modular polyketide synthases (PKSs), defined as PKSs whose genes are engineered from portions of two or more natural PKSs, in principle offer an unrivalled access to chemical diversity around polyketide natural product templates, and provide a powerful tool to gain greater understanding of key determinants of specificity and stereospecificity in these multienzymes. The demonstration in 1996 that hybrid PKSs were indeed functional [11, 15] was a breakthrough because it showed that the key to success was not some vague recipe for in vivo recombination, but the use of specific restriction sites to splice together the DNA encoding functional protein units, particularly concentrating on junctions at or near surface-associated linker regions [22]. Large numbers of engineered complex polyketides have now been produced in exactly this way [17, 23, 26]. Initial hybrids were made between erythromycin and avermectin or rapamycin, because those were the PKS multienzymes for which nucleotide sequences were available, but rapid advances in the cloning and characterisation of PKS-containing gene clusters for all major polyketide groups, including macrolides, polyenes and polyethers, have now provided additional ample supplies of genes for such hybrid construction.

The accumulation of sequence information for individual enzymatic domains has also highlighted subtle variations that clearly relate to the specificity and/or the stereospecificity of catalysis. For example, for ketoreductase (KR) domains, two research groups independently [19; P. Caffrey, personal communication] have recently noticed small but systematic sequence differences between KR enzymes that produce different stereochemical outcomes in their 3-hydroxyacylthioester products. The Caffrey motif is sufficiently precise that we have already found it invaluable in predicting the products of newly discovered modular PKS genes. We report here experimental tests of the significance of

another such short sequence motif found by careful analysis of multiple sequences of the acyltransferase (AT) domains of modular PKSs. It is distinct from a sequence motif we have previously described [8] and from a "hyper-variable" region described by others [12], but also appears to correlate with the known chemical specificity of individual AT domains for either malonyl- or methylmalonyl-CoA as substrates. From close inspection of three-dimensional structures of an AT domain using the crystal structure of the fatty acid synthase malonyl-CoA:ACP transacylase (MAT) of *Escherichia coli* [21], it was evident that the amino acids of the new sequence motif lay at the active site of the enzyme and therefore could make direct contact with the substrate. We describe here experiments to test the effect of targeted alterations of residues in this motif on the in vivo production of polyketides. Similar observations have been made independently by others [18].

Such mutagenesis experiments, together with the established methods of domain, multi-domain, module and multimodule swapping, create hybrid multienzymes that are not necessarily optimised for their new role. In particular, there are frequently negative effects of unfavourable interactions between the "incoming" residues and/or domains and their new neighbours. Gene-shuffling technologies [24] can be applied to such hybrid PKSs to improve their performance. We describe here the construction and validation of a cassette system for creating hybrid modular PKSs that will facilitate generation and screening of such combinatorial families of AT mutants. We also exemplify its use by showing that the specific inter-polypeptide domain:domain contacts between the C-termini and N-termini of adjacent PKS multienzymes are not significantly disrupted by adding two residues to the C-terminal partner. It appears that the extreme C-terminus is not involved in this protein: protein recognition.

Materials and methods

Strains, media and culture conditions

The growth and cultivation of *Saccharopolyspora erythraea* NRRL2338 (red variant) JC2 [20] from which almost all of the erythromycin PKS genes have been deleted, and of the tylosin-producing strain *Streptomyces fradiae* S66 (ATCC 19609) were as previously described [4,10, 20].

Mutagenesis of the AT1 domain of DEBS1-TE

Plasmid pHP41 is a pCJR24-based plasmid [20] containing the truncated model PKS DEBS1-TE gene, which comprises the loading module, the first and second extension modules of DEBS and the chain-terminating thioesterase (TE). In addition, the DNA encoding the amino acid sequence YASH in the AT domain of the first extension module has been specifically altered to encode the sequence HAFH. Plasmid pHP41 was constructed via several intermediate plasmids as follows. Plasmid pCJR26 [20] was digested with *Msc* I and *Avr* II to remove the AT1 domain, and the larger PKS-containing fragment was isolated by gel electrophoresis. The modified AT domains were produced by PCR, incorporating the desired mutations into the design of oligonucleotide primers. A DNA segment of the *eryAI* gene from *S. erythraea* was amplified by PCR using the primers 5′-TTTTTTTGGCCA GGGTTGGCAGTGGGCGGGCA-3′ and 5′-TTTTTACGGCCAGCCGCTTGGCGCG GAT-3′. One of the primers introduced an *Msc* I site and the second primed across a *Bst* XI site. The PCR product was cloned into *Sma* I-cut, phosphatase-treated pUC18, and demethylated plasmid DNA was produced by transformation of *E. coli* strain ET12567 [14]. The resulting DNA was digested with *Msc* I and *Bst* XI to release the insert. A second DNA segment of the *eryAI* gene from *S. erythraea* was amplified by PCR using the following primers; 5′-CGGTGCCTAGGTGCACCGACTCCCAGTCC-3′ and 5′-TTTTTCCAAGCGGCTGGCCGTGGACCACGCGTTCCAC TCCTCGCACGTCGAGACGAT-3′. The primers respectively introduced an *Avr* II site and primed across a *Bst* XI site, and mutated the amino acid sequence of the AT from YASH to HAFH. The PCR product was cloned into *Sma* I-cut pUC18 and the insert released by digestion with *Avr* II and *Bst* XI. The two cloned PCR fragments were ligated together with the ~13-kb fragment from pCJR26 to give pHP41. Sequence analysis confirmed that pHP41 contained the altered amino acid sequence HAFH.

Plasmid pHP048 is an analogous pCJR24-based plasmid in which the amino acid sequence YASH of the AT domain of the first DEBS extension module has been altered to HASH. A DNA segment of the *eryAI* gene from *S. erythraea* was amplified by PCR using oligonucleotide primers; 5′-CGGTGCCTAGGTGCACC-GACTCCCAGTCC-3′ and 5′-TTTTTCCAAGCGGCTGGCCGT GGACCACGCGTCGCACTCCTCGCACGTCGAGACGAT-3′. The primers respectively introduced an *Avr* II site and extended to a *Bst* XI site and also provided the mutated amino acid sequence HASH. The PCR product was cloned into *Sma* I-cut pUC18 and the insert released by digestion with *Avr* II and *Bst* XI. This fragment was ligated with the *Msc* I-*Bst*XI PCR fragment already described together with the ~13-kb fragment from pCJR26, and the resulting plasmid was designated pHP048. Sequence analysis confirmed that the clone contained the altered amino acid sequence HASH.

Production and analysis of triketides

Plasmids pHP41 and pHP048 were used separately to transform *S. erythraea* NRRL2338 JC2 protoplasts. Thiostrepton-resistant colonies, in which the plasmids had integrated by virtue of their sequence homology with the TE region of *eryAIII*, were selected on R2T20 agar containing 40 μg thiostrepton/ml . For analysis, strains were plated onto SM3 agar containing 40 μg thiostrepton/ml and allowed to grow for 11 days at 30 °C. Approximately 1 cm^2 of the agar was homogenised and extracted with a mixture of 1.2 ml ethyl acetate and 20 μl formic acid. The solvent was decanted and removed by evaporation and the residue dissolved in methanol and analysed by GC/MS. Alternatively, strains were used to inoculate 5 ml TSB containing 5 μg thiostrepton/ml . After 3 days growth, 1.5-ml of this culture was used to inoculate 25 ml of SM3 medium containing 5 μg thiostrepton/ml in a 250-ml flask. The flask was incubated at 30 °C, 250 rpm for 6 days, and then the supernatant was adjusted to pH 3.0 with formic acid and extracted twice with an equal volume of ethyl acetate. The solvent was removed by evaporation and the residue was analysed by GC/MS as previously described [15].

Deletion of the *tylGIV* gene encoding module 6 of the tylosin PKS of *S. fradiae*

A 2.0-kb fragment identical to the 3′ end of *tylGIII*, which lies immediately upstream of the target gene, was amplified by PCR using the oligonucleotide primers 5′-TCTAGACTACGCGCTG ACCCGGCTGGCCCGGGCCCGCA-3′ and 5′-TCTAGACG CGATCTCGCCGACGCTGTGCCCGCACA-3′. Likewise a 2.0-kb fragment identical to the 5′ end of *tylGV*, which lies

immediately downstream of the target gene, was amplified by PCR using the primers 5′-CATATGTCCGCCGAGAGGCTGACGAG GCGCTGCGGA-3′ and 5′-TCTAGACCCCAGCAGCAGGT CGGGGCGCATCCCCCA-3′. Each PCR product was initially cloned into pUC18 and then ligated together via the *Nde* I site. The 4-kb insert was subsequently excised with *Xba* I and cloned into the conjugation vector pCK1132 [2]. Plasmid pFdV14, containing the 4-kb insert in the correct orientation, was used for conjugation into *S. fradiae* S66. Selection for resistance to apramycin, followed by repeated culture of selected exconjugants in non-selective liquid medium, led to the production of strain S66, specifically deleted for the *tylGIV* gene. The nature of the deletion was confirmed by PCR analysis.

Complementation of *S. fradiae* S66 (DtylGIV)

The wild-type *tylGIV* gene was cloned as an *NdeI –Xba* I fragment and placed under the control of the *ermEp** promoter [1] in the integrative plasmid vector pIB139, which contains the attachment site for bacteriophage FC31. The plasmid was constructed by Dr. I.U. Böhm (Department of Biochemistry, University of Cambridge) by cloning the *ermEp** promoter as a *Spe* I–*Xba* I fragment into the unique *Xba* I site of pSET152 [2]. An altered copy of *tylGIV* containing the C-terminal sequence PGENS (wild type sequence: PGE) was cloned in the same vector. To facilitate AT domain swaps into *tylGIV*, an *Msc* I restriction site was introduced at the 5′ margin of the DNA encoding the AT6 domain, where the amino acid sequence GQG is highly conserved among extender AT domains, and a natural *Msc* I site near the 5′ end of the *tylGIV* gene was removed, by PCR mutagenesis. Advantage was taken of a naturally occurring *Stu* I site at the 3′ margin of the DNA encoding the AT6 domain. Donor AT domains (*tyl* AT6, *tyl* AT1, *ery* AT1) were accordingly amplified by PCR and cloned as *Msc* I–*Stu* I fragments into this engineered version of *tylGIV*, housed in the pUC18-derived plasmid pFdV42. The recombinant *tylGIV* genes were then subcloned as *Nde* I–*Xba* I fragments into pIB139 and used for conjugation into *S. fradiae* S66 (DtylGIV) (Fig. 3). Growth of exconjugants, selection for resistance to apramycin, and subsequent fermentation for tylosin production were carried out using established protocols [2,4]. After 7 days in fermentation medium at 30 °C and at 250 rpm, isopropanol was used to extract polyketide products from the culture broths and these extracts were checked using HPLC/MS analysis for restoration of tylosin production. At least ten independent exconjugants of each type were examined.

Results

Multiple sequence alignments of AT domains from modular PKSs were used to identify the amino acid sequence YASH, typically lying about 90–100 amino acid residues C-terminal of the active-site serine residue, as one highly conserved among the majority of extender AT domains specifying the incorporation of propionate units from methylmalonyl-CoA. In contrast, AT domains specifying the incorporation of acetate units from malonyl-CoA were found to have a highly conserved sequence, HAFH, at this position (Fig. 1). In AT domains responsible for incorporation of units from ethylmalonyl-CoA or other bulky CoA esters the sequence motif is less conserved but generally has the sequence XAGH (where X is most frequently F, T, V or H). In a module from the epothilone cluster known to incorporate either malonyl- or methylmalonyl-CoA this motif appeared to be a hybrid form of the two motifs, HASH and HAFH. Intriguingly, the size of the third residue in the motif was inversely proportional to the size of the side-chain in the substituted malonyl thioester substrate. Although we noted that additional individual residues in AT domains do also vary systematically, depending upon the substrate normally recruited, we selected this motif for an initial test of the possibility of rationally influencing product ratios by site-specific mutagenesis of AT domains.

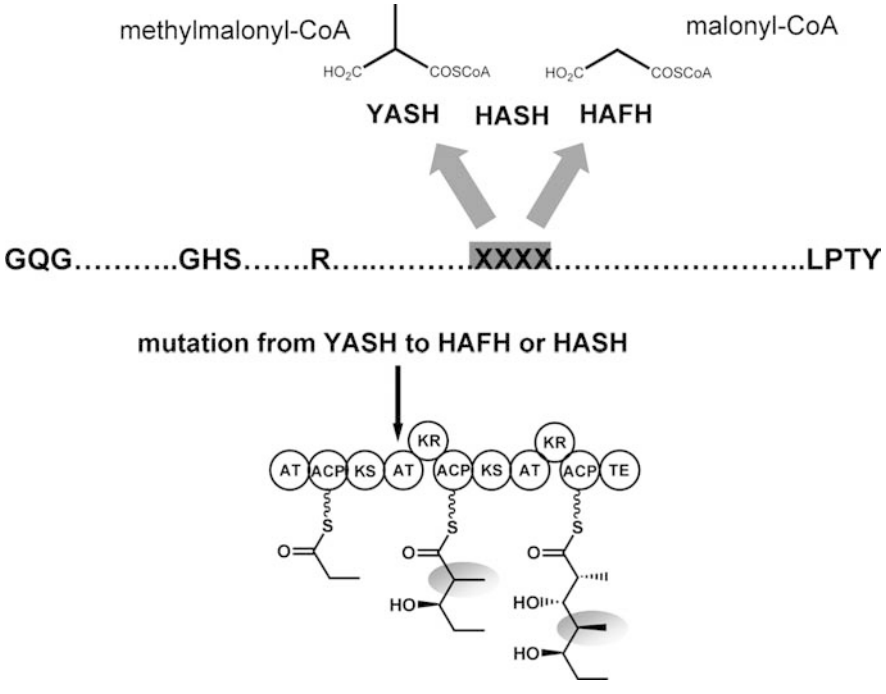
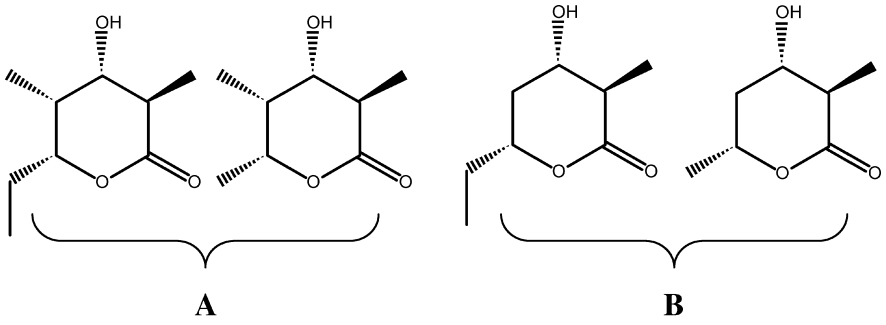


Fig. 1 Mutation of the proposed specificity motif in the acyltransferase (AT) domains of a modular PKS. The position of the YASH/HAFH motif in the primary sequence is shown relative to the N-terminus (GQG), the C-terminus (LPTY), the active-site residues GHS and R. The domain mutagenised in DEBS1-TE is highlighted



Strain	Ratio of A/B products
DEBS1-TE (YASH) (control)	100:0
DEBS1-TE (HAFH)	40:60
DEBS1-TE (HASH)	60:40

Fig. 2 Triketide lactone ratios obtained from strains of *Streptomyces erythraea* housing DEBS1-TE, mutated in the active site of the AT1 domain. The sequence of the targeted specificity motif is shown in *parentheses* for each mutant

As outlined in Materials and methods, versions of the DEBS1-TE gene were constructed in which the DEBS AT1 domain, flanked by unique restriction enzymes *Msc* I and *Avr* II [15], was replaced by mutant versions in which the sequence YASH was specifically replaced either by HASH (expected to favour a mixture) or by HAFH (expected to favour incorporation of acetate units rather than propionate). Recombinant *S. erythraea* clones containing the mutant genes were cultured along with controls (in which the wild-type sequence was present) and analysed, using GC/MS, for production of triketide lactones (Fig. 2) [15]. As expected [3], the product mixture included triketides derived from a propionate starter and also ones derived from an acetate starter. The wild-type strain produced triketides derived exclusively from propionate extender units. In contrast, both mutants, carrying respectively the sequence HASH or HAFH, produced an approximately equal mixture of lactones arising from incorporation of acetate units and propionate units during the first cycle of polyketide chain elongation (Fig. 2). There was significant variability in the propionate:acetate ratio in individual experiments. For example, fermentation of three independent clones housing the HAFH mutant resulted in two that produced A/B ratios of 40:60 and 43:57, while the third clone, which grew less well, had an A/B ratio of 78:22. Furthermore, the separation of certain individual triketide lactones was not complete in the GC/MS system used. The overall yield of triketide lactones was also diminished in the mutant strains, to 10% (HAFH-containing) or 20% (HASH-containing) of the levels of triketide produced by the control strain (which contained DEBS1-TE). Nevertheless, these results provide proof of the principle that site-specific mutagenesis can be used in these PKS systems to effect the specificity of an individual AT domain.

When the *tylGIV* gene of *S. fradiae*, which houses module 6 of the tylosin PKS, was wholly deleted, as described in the Materials and methods, the resulting mutant was, as expected, incapable of producing tylosin. When this mutant was complemented by the cloned *tylGIV*, placed under the *ermE** promoter [1] of *S. erythraea* and integrated at the ΦC31 attachment site in *S. fradiae*, production of tylosin was restored to 80 mg/l (average of ten independent exconjugants), about 40% of the levels produced by wild-type *S. fradiae* under these culture conditions. When two mutant versions of *tylGIV*, bearing alternative propionate-specifying AT extender domains in place of *tyl* AT6, were used for complementation, the percentage yields of tylosin compared to when *tylGIV* was used were as follows: control (*tyl AT6*), 100%; erythromycin AT1, 40%; tylosin AT1, 0%. Complementation of the *tylGIV* deletion strain with a version of the cloned *tylGIV* gene in which the C-terminus of the multienzyme was extended by two residues (see Materials and methods) led to restoration of tylosin production to essentially the same level (80 mg/l) as seen when *tylGIV* itself was used for complementation.

Discussion

The results of mutagenesis of the YASH/HAFH motif in a typical extender AT domain of a modular polyketide synthase are fully consistent with the idea that these residues are located in the active site and involved in selection of the substrate for elongation. Separate replacement of single amino acid residues within the YASH sequence led in each case to an apparent relaxation of the substrate specificity so that acetate units as well as propionate units were introduced (Fig. 2). Similar observations have been made independently by others [18]. A complete switch of specificity was not observed, although it was clear to us from the outset that it might be necessary to change additional residues of the active site to further modulate the specificity to favour one substrate over another. It must also be kept in mind

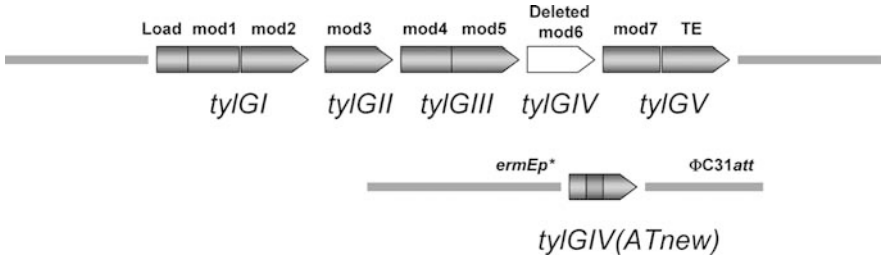


Fig. 3 Complementation *in trans* of *Streptomyces fradiae* (ΔtylGIV) by *tylGIV* cloned under the *ermEp** promoter in the ΦC31 *attB* site restores tylosin production. Unique restriction sites, flanking the acyltransferase (AT) domain of module 6 of the cloned TylGIV, were used for AT domain replacement

that in type I modular systems, several factors other than intrinsic selectivity of the AT domain help determine the product ratios in vivo, most notably the availability of competing substrates. In any engineering experiment it is therefore important to specify exactly the host strain and the conditions of growth, and to analyse a number of independent clones.

The likely functional importance of the YASH/HAFH motif, originally identified solely by sequence comparisons but subsequently analysed using the 1.5Å crystal structure of the *E. coli* MAT, has been borne out by the recently solved crystal structure (at 2.0Å resolution) of the *S. coelicolor* MAT [9], which contains acetate ions at the active site. The phenylalanine residue of the motif described here (Phe-200 in *S. coelicolor* MAT numbering) [9] apparently acts sterically, to make less favourable the binding of α-branched CoA ester substrates. McDaniel and colleagues [18] have questioned the robustness of AT domain swapping technology because they were unable to achieve the successful replacement of a malonyl-CoA-specific AT domain for the AT4 domain of the erythromycin PKS. However, this swap has been achieved by others (H. Petkovic, personal communication) by the utilisation of an alternative set of splice sites. It is therefore premature to propose mutagenesis as a superior alternative to domain swaps—although making additional mutations in or around the active site of the acyltransferase could improve the overall yields and observed specificity achieved by this approach. Based on present evidence it would appear that the best outcome in switching the specificity of AT domains should be achieved by a combination of domain swapping and standard methods of site-specific mutagenesis, or combinatorial mutagenesis coupled to an activity screen.

We also introduce here a cassette system in which such domain swapping and mutagenesis may be conveniently carried out. The deletion of an entire module from the tylosin PKS, and its substitution *in trans* by the same module (or an altered version) expressed from a gene cloned in the remote *attB* chromosomal attachment site for ΦC31 (Figure 3), was suggested by the elegant work of Kuhstoss and colleagues [11], who used this approach to substitute ORF1 of the *tyl* PKS for the counterpart ORF of the closely related spiramycin PKS. In our case, the *tylGIV* gene housing extension module 6 of the PKS, when expressed from the *ermE** promoter of *S. erythraea*, was able to restore levels of tylosin production to 40% of wild-type levels. There are several reasons why the levels of production after complementation might fall short of those of wild-type, including the known depressive effect on tylosin production of cloning into the ΦC31 *attB* site, possible disruption of PKS gene transcription and possible disruption of the regulatory network that governs tylosin biosynthesis in S. *fradiae* [6]. Still, this level of tylosin production is sufficiently high to permit use of the system in module and domain swaps.

Our results showed that of two heterologous, propionate-specifying AT domains, swapped in place of the *tyl* AT6 domain, only *ery* AT1 served to restore *tyl* production, and that to levels some 40% of the control (*tyl* AT6 itself). In contrast, the AT1 domain of tylosin was wholly inactive. This again illustrates the importance of utilising more than one donor domain in such experiments. Since the chemistry of polyketide chain extension remains exactly the same, the introduced AT domain probably makes unfavourable protein contacts with its new neighbours that lower or abolish its activity (while bearing in mind that there may also be secondary effects on the level of gene expression or protein folding). Using this cassette system, it should now be possible to undertake combinatorial and/or structure-assisted mutagenesis of each of the heterologous AT domains to "evolve" its ability to function in this new context, using tylosin production as a convenient assay.

As an additional illustration of the potential of the cassette system, a modified form of the TylGIV PKS multienzyme was expressed in the *S. fradiae* ΔtylGIV mutant, in which the C-terminus of the protein was modified by the addition of two residues (asparagine and serine). (This construct was the kind gift of Dr A Schneider, who had constructed this mutant for other purposes.) Analysis showed that the complemented strain produced exactly the same amount of tylosin as the control containing unmodified TylGIV. A current model for modular PKS structure [5, 22] proposes that non-covalent interactions between successive modules are limited to the N-terminal regions (including the KS domains) of the downstream module and the C-terminal regions (including the ACP domains) of the upstream module. In the case of modules interacting between different PKS proteins, there is considerable interest in

understanding how the correct partnerships are formed, including the role of ''linker regions'' [7] lying at the extreme N- and C- termini of the proteins. Our finding that the C-terminus of the upstream protein can be modified without prejudicing tylosin production adds to previous indications [13] that the C-terminus itself is not involved in any critical interaction. Again, the cassette system offers a convenient way to target these ''linker regions'' for combinatorial mutagenesis and to study the effects of such changes on polyketide production.

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