Substrate specificity of the acyl transferase domains of EpoC from the epothilone polyketide synthase

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The production of epothilone mixtures is a direct consequence of the substrate tolerance of the module 3 acyltransferase (AT) domain of the epothilone polyketide synthase (PKS) which utilises both malonyl- and methylmalonyl-CoA extender units. Particular amino acid motifs in the active site of AT domains influence substrate selection for methylmalonyl-CoA (YASH) or malonyl-CoA (HAFH). This motif appears in hybrid form (HASH) in epoAT3 and may represent the molecular basis for the relaxed specificity of the domain. To investigate this possibility the AT domains from modules 2 and 3 of the epothilone PKS were examined in the heterologous DEBS1-TE model PKS. Substitution of AT1 of DEBS1-TE by epoAT2 and epoAT3 both resulted in functional PKSs, although lower yields of total products were observed when compared to DEBS1-TE (2% and 11.5% respectively). As expected, epoAT3 was significantly more promiscuous in keeping with its nature during epothilone biosynthesis. When the mixed motif (HASH) of epoAT3 within the hybrid PKS was mutated to HAFH (indicative of malonyl-CoA selection) it resulted in a non-productive PKS. When this mixed motif was converted to YASH (indicative of methylmalonyl-CoA selection) the selectivity of the hybrid PKS for methylmalonyl-CoA showed no statistically significant increase, and was associated with a loss of productivity.

Introduction

There has been considerable interest in the epothilones as potential anticancer therapeutics since they were identified as microtubule stabilising agents which bind to β-tubulin at sites comparable to Taxol and its derivatives.[1,2] Moreover, the demonstration that these compounds act against cells resistant to paclitaxel and other anticancer agents stimulated further excitement and a number of epothilones entered clinical trials.[3] Thus, a thorough understanding of epothilone biosynthesis and its enzymatic machinery provides an opportunity to generate new analogues, or to enhance the yield or purity of epothilone congeners, through biosynthetic engineering.

Hardt and colleagues have purified approximately forty minor epothilone congeners produced during large-scale fermentation of an improved isolate of the producing organism *Sorangium cellulosum* So ce90.[4] This provides an illustration of the range of compounds which are produced through the incorporation of alternative substrates by acyltransferase (AT) domains, through other aberrations in the polyketide synthase (PKS) machinery/post-PKS processing enzymes, or as a result of product degradation. However, it is well established that in their natural environment the majority of AT domains display tight substrate selectivity and, as noted by Hardt and co-workers, the production of minor metabolites represents less than 1% of the total epothilones produced by *Sorangium cellulosum*. A notable exception is the production of epothilones A and C *vs.* epothilones B and D (Fig. 1), in ratios reported to range from 8 : 2 to 1 : 1 depending upon fermentation conditions.[4] These pairs of mixtures arise due to the incorporation of either malonyl-CoA or methylmalonyl-CoA by the AT domain of the PKS module 3 during the polyketide chain elongation[5] (with another difference between the two pairs of compounds being the presence or not of a post-PKS introduced epoxide moiety as shown in Fig. 1). The production of mixtures of compounds as a consequence of poor substrate specificity of the biosynthetic machinery is often a significant problem for the industrial production of natural products. Notable examples are the production of avermectin and monensin which are synthesised as mixtures of at least two chemical species.[6,7] Techniques that push production ratios towards the desired chemical species have considerable commercial potential as these increase production yields and reduce the cost of both purification and the disposal of unwanted analogues and associated waste streams.

Cloning and sequencing of the biosynthetic gene cluster for the epothilones has been reported.[8,9] This was followed by its genetic manipulation and expression in heterologous hosts,[9,10] and has enabled the production of a range of epothilone analogues which possess structural modifications around the macrolide ring.[10]

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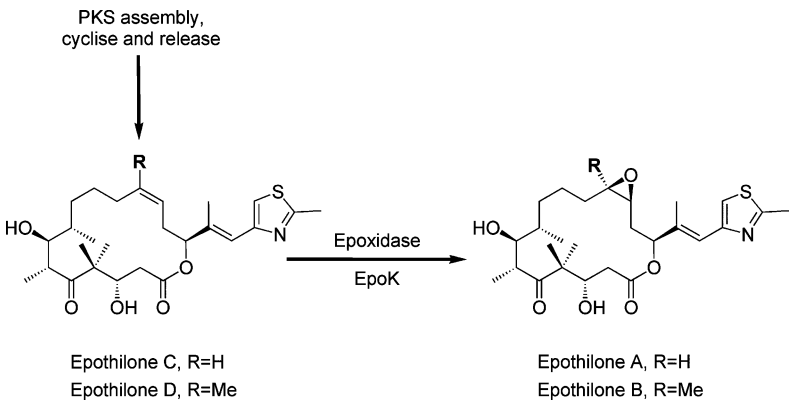

Fig. 1 Biosynthesis and structure of epothilones A–D. The position of the methyl or hydrogen moiety derived from the promiscuous EpoAT3 is highlighted in bold.

The availability of PKS sequence information may enable us to influence the relative yields or ratios of analogues by rational biosynthetic engineering.

The production of epothilone A/B (or C/D) (Fig. 1) is a direct consequence of the incorporation of both malonyl- and methylmalonyl-CoA by the epoAT3 housed in EpoC (utilising the Molnar[6] nomenclature rather than that of Tang[7]). Replacement of this promiscuous AT in its entirety with one specific for methylmalonyl-CoA might prevent formation of the epothilones A and C.[7] Several examples of such AT domain swap experiments have been described in other systems.[11–13] An alternative approach involving site directed mutagenesis of AT domains to alter their selectivity has also been reported.[14–16] This latter strategy, which involves the manipulation of particular amino acid motifs that define the specificity towards the substrate CoA esters, is particularly attractive in the epothilone case as we have observed that in the offending epoAT3, one of the motifs conferring a major determinant for selective incorporation of a particular CoA substrate appears to be a hybrid of the motifs defining malonyl- and methylmalonyl-CoA selectivity (Fig. 2). In addition, we found that the amino acid sequence of EpoAT2 (selecting malonyl-CoA) is almost identical to that of EpoAT3 (selecting malonyl-CoA plus methylmalonyl-CoA).[17] Therefore, we anticipated that modification of the hybrid motif to that observed for ATs capable of selecting specific substrates might result in the predominant production of a single (target) epothilone.

Here we report the results of a preliminary study in which the performance of the epothilone ATs was tested by transferring them into the well-characterised DEBS1-TE model system[18] in place of the native AT1 domain. We then sought to investigate the possibility of influencing the product ratios of these hybrid systems by single amino acid substitutions in the aforementioned hybrid selectivity motif of the inserted epoAT3 domain.

Results

We designed a number of plasmid constructs containing the truncated erythromycin PKS DEBS1-TE in which the AT domain of module 1 was replaced with the epothilone ATs from module 2 (epoAT2) and module 3 (epoAT3) as specified in Table 1. The epothilone AT2 utilises malonyl-CoA as predicted from its HAFH motif signature. The epothilone AT3 domain, which contains a mixed motif, utilises both malonyl- and methylmalonyl-CoA extender units. Furthermore, we have constructed two additional plasmids in which the hybrid motif sequence HASH of the promiscuous epothilone AT3 was modified to YASH (methylmalonyl-CoA selective) and HAFH (malonyl-CoA selective) motifs. The plasmid constructs pCJR26, in which the native methylmalonyl-CoA specific AT1 was replaced with rapamycin AT2 which utilises malonyl-CoA, and the native DEBS1-TE (pCJR65)[19] were used as controls. All plasmid constructs were transformed into the *S. erythraea* JC2 strain which lacks the entire erythromycin PKS. All the strains and plasmid constructs are listed in Table 1.

Analysis of TKL production by *S. erythraea* JC2 transformants expressing hybrid PKSs

When expressed in *S. erythraea* JC2[16] the hybrid PKSs produced the four triketide lactone (TKL) products **1–4** which are shown in Fig. 3. This was expected as the loading module of DEBS1-TE accepts both acetyl- and propionyl-CoA, to give the two products

Table 1 Strains and plasmid constructs used in this work

Polyketide synthase (PKS)	Plasmid	Description	Reference
DEBS1-TE	pCJR65	Native DEBS1-TE	19
DEBS1-TE/rapAT2	pCJR26	Module 1 AT from DEBS1-TE replaced with rapamycin AT from module 2	19
DEBS1-TE/epoAT2	pABS61	Module 1 AT from DEBS1-TE replaced with the AT from epothilone module 2	This paper
DEBS1-TE/epoAT3	pABS62	Module 1 AT from DEBS1-TE replaced with the AT from epothilone module 3	This paper
DEBS1-TE/epoAT3(HAFH)	pCJRHY.6	Module 1 AT from DEBS1-TE replaced with the AT from epothilone module 3 in which the mixed HASH motif was modified to HAFH	This paper
DEBS1-TE/epoAT3(YASH)	pCJRSF.4	Module 1 AT from DEBS1-TE replaced with the AT from epothilone module 3 in which the mixed HASH motif was modified to YASH	This paper

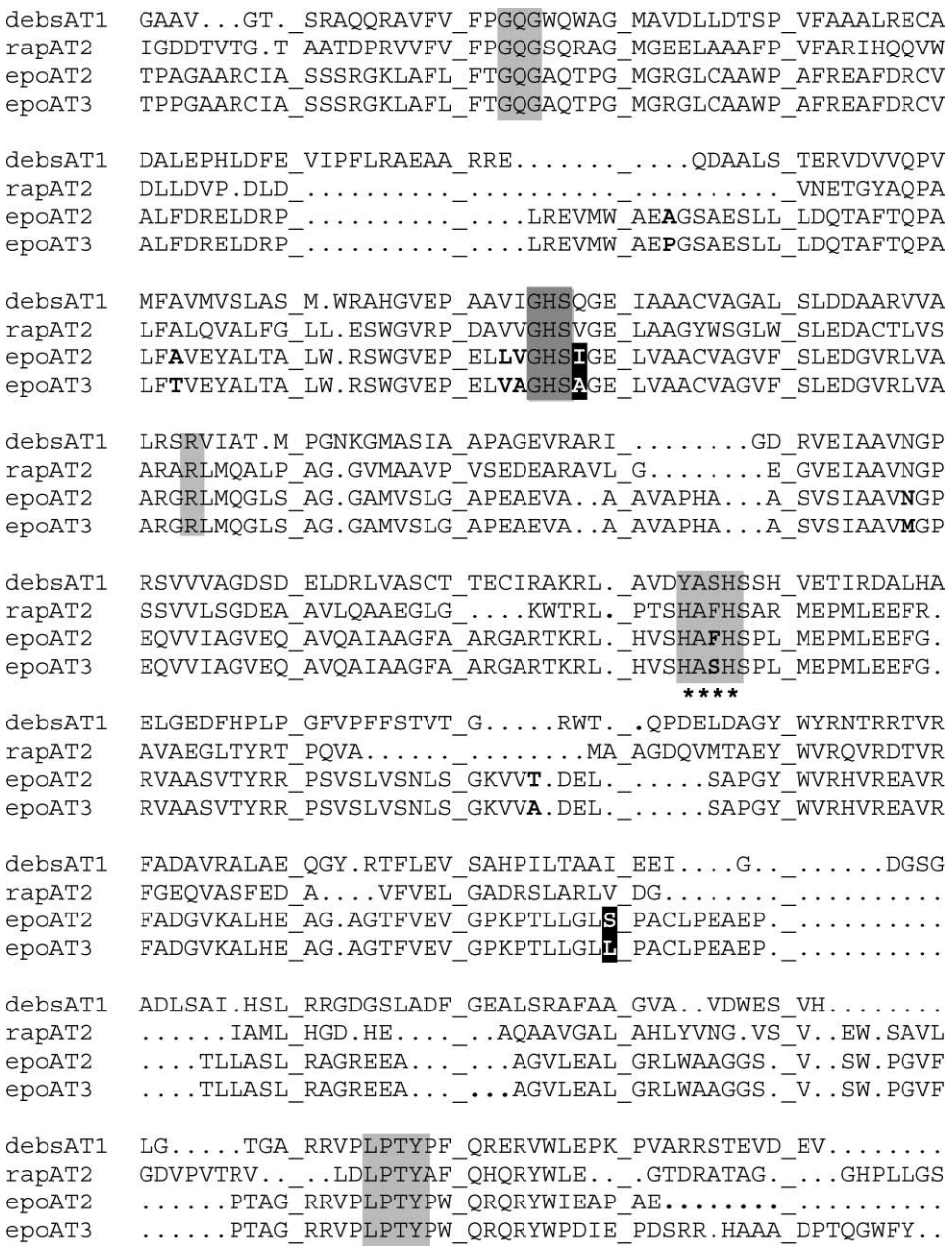

Fig. 2 Amino acid sequence comparison of AT domains from module 1 of the erythromycin PKS, module 2 of the rapamycin PKS, and from module 2 (epoAT2) and 3 (epoAT3) of the epothilone PKS. The motifs HAFH, YASH and HASH in the primary AT sequences are labelled by asterisk. The ascribed N-terminus (GQG), C-terminus (LPTY), and the active site residues GHS and R are shaded. The nine amino acid differences between epoAT2 and epoAT3 are marked as bold. Two key amino acid residues which may be readily identified from sequence protein alignments and previously reported to have important role in the substrate selectivity are marked as white on black background.

1 & **2** respectively. The extender units utilised are all methylmalonyl-CoA. Thus, two pairs of TKLs are produced by the hybrid PKSs, with each pair arising from recruitment of the two different PKS starter units (**1** & **3** *vs.* **2** & **4**), and the second difference being due to the selectivity of the inserted AT1 domain, and thus the first extension unit selected (**1** & **2** *vs.* **3** & **4**). The AT domain of DEBS1-TE module 2 always selects methylmalonyl-CoA in these experiments. Our analytical approach used LCMS/MS measurement and authentic standards. In Table 2 the total TKL production level is displayed for each experiment, both as an actual yield (mg L^{-1}) and as a percentage relative to the total TKL production for DEBS1-TE expressing strains. This provides a direct indication of the relative productivity of the various hybrid PKSs. The data for each of **1**–**4** is then shown for each strain. This data is also presented as a combined figure to allow direct analysis of the selectivity of the particular AT1 domain in question (*i.e.* the data for **1** & **2** *vs.* **3** & **4**). These ratios are normalised to aid clarity.

Strains expressing DEBS1-TE produced **1** and **2** demonstrating the high fidelity of both AT domains in their natural environment. The control strain *S. erythraea* containing the plasmid pCJR26 expresses a hybrid PKS in which the malonyl-CoA specific rapAT2 from the rapamycin PKS replaces the native AT1 of DEBS1-TE.

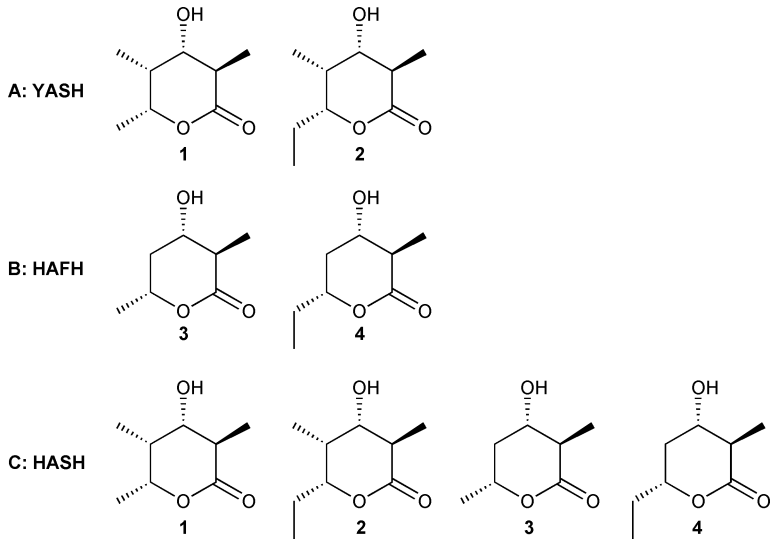

Fig. 3 AT domain active site motif sequences and their corresponding triketide lactone products: A. DEBS1-TE AT1; B. RapsAT2; C. EpoAT3 (mixed motif).

Table 2 PKS productivity and product (**1–4**) distributions; errors are given as a sample standard deviation (σ_{n-1})

	Total TKL yield		Relative titre of TKLs (%)				
PKS	mg L^{-1}	% of X	**3**	**1**	**4**	**2**	AT1 selectivity [**3** + **4**] : [**1** + **2**]
DEBS1-TE	844.1 ± 104.0	100	1.81 ± 0.85	52.47 ± 4.24	0.00 ± 0.00	45.72 ± 5.61	1 : 54.2
DEBS1-TE/rapAT2	173.9 ± 54.9	20.4	49.09 ± 6.32	0.19 ± 0.61	49.73 ± 6.76	0.99 ± 0.76	83.7 : 1
DEBS1-TE/epoAT2	17.1 ± 12.9	2.0	37.51 ± 8.52	3.72 ± 1.01	52.63 ± 6.25	6.14 ± 1.84	9.1 : 1
DEBS1-TE/epoAT3	97.0 ± 28.8	11.5	39.31 ± 8.95	12.27 ± 4.92	32.34 ± 4.46	16.08 ± 3.56	2.5 : 1
DEBS1-TE/epoAT3(YASH)	8.1 ± 2.4	1.0	27.10 ± 4.82	15.51 ± 2.52	34.16 ± 2.41	23.23 ± 4.60	1.6 : 1
DEBS1-TE/epoAT3(HAFH)	0.0 ± 0.0	0.0	—	—	—	—	—

This produced the TKLs **3** and **4** due to selection of malonyl-CoA by the inserted rapAT2. The total yield of TKLs from this construct was approximately 20% of that of DEBS1-TE. This result confirms earlier studies[20] showing that the introduction of rapAT2 converts the extender unit specificity of this module almost entirely.

Replacing the AT1 domain of DEBS1-TE with each of the ATs derived from the epothilone PKS was successful, resulting in the expression of functional hybrid PKSs which produced all four products **1–4**. These hybrid PKS-expressing strains were less productive than either DEBS1-TE or the control utilising the rapAT2 (see Table 2). In addition, in this system the epoATs were considerably less selective than either of the native AT1 of DEBS1-TE or the rapAT2 domains. Surprisingly, both epoATs were able to incorporate malonyl- and methylmalonyl-CoA. As expected from the performance of these systems in their natural context, the strains expressing a hybrid PKS containing epoAT2 were most selective and incorporated a greater proportion of malonyl-CoA; this selectivity is similar to that reported during epothilone production.[4] The strains expressing a hybrid PKS containing epoAT3 also behaved as anticipated, showing low selectivity and utilising malonyl-CoA preferentially, but only approximately 2.5 times more so than methylmalonyl-CoA. Once again, these data are similar to those for epothilone production in *S. cellulosum* (where the selectivity ratio is approx. 2 : 1).[4]

Analysis of hybrid PKSs containing mutated active site motifs

Site directed mutagenesis was used to convert the HASH mixed motif of epoAT3 to YASH (methylmalonyl-CoA selection) and HAFH (malonyl-CoA selection). The new YASH-containing epoAT3 construct produced less total TKL than the epoAT3 containing hybrid (~10%), and only 1% of the titre for DEBS1-TE-expressing strains.

However, it appears to produce a greater proportion of TKLs **1** and **2** than did the epoAT3 hybrid (1.6 : 1 *vs.* 2.5 : 1) when the mean values of the analysis are examined, indicating an increase in its ability to select methylmalonyl-CoA from the metabolite pool. However, when the individual data for rows 4 and 5 in Table 2 are directly compared, it is clear that the standard deviation for each of **1–4** overlaps, indicating that the differences in the ratios of mean data are not statistically significant. When the specificity motif of the hybrid epoAT3 containing PKS was altered to HAFH no production of **1–4** was observed (*i.e.*, it resulted in a non-functional PKS). None of 75 independent clones derived from this plasmid produced any TKL.

Discussion

Genetic manipulation of modular type I PKS multi-enzymes by the swapping of functional domains in order to produce

polyketides with an altered structure is now well established.[21,22] In particular, AT domains have been successfully swapped between modules in order to generate novel compounds including rationally designed erythromycin, FK520 and geldanamycin analogues.[12,13,23] However, the efficiency of these hybrid PKSs is highly variable, and some desired compounds are produced at very low levels. It has been demonstrated that by utilising a range of donor domains, as well as a range of splice sites at which the hybrid proteins are fused, it is possible to overcome at least some of the issues associated with the low productivity of hybrid PKSs.[11] Another factor leading to the observation of low productivity may involve the examination of an insufficiently large pool of clones after transformation. Extreme variation in colony-to-colony production of natural products after genetic engineering is a common problem with the actinomycetes.[24] In our present report the productivity of the engineered PKS containing clones was evaluated. We found that the productivity of clones varies significantly and it is therefore important to introduce a pre-selection procedure into experiments of the type described here. We screened in the region of 60 primary clones for each experiment, and each of the data-points presented in Table 2 represents an average of 21 fermentations (the 7 best clones, each grown in triplicate).

We then showed that DEBS1-TE (a truncated form of the erythromycin PKS) can be engineered by substitution of the native AT1 domain by ATs derived from myxobacterial as well as from streptomycete PKSs. The total yield of TKL products from each hybrid PKS ranged from approximately 20% of that for DEBS1-TE, to as little as 1%. This confirms that utilising a range of AT domains from alternative sources is important in order to generate functional and productive PKS. While epoAT2 and epoAT3 are extremely similar in protein sequence (only nine of around 340 amino acid residues are different (Fig. 2)),[17] these differences appear to have a significant effect both on the productivity of the resulting hybrid PKS, as well as on their selectivity for malonyl- or methylmalonyl-CoA substrates. This latter point is also true in their natural context. Interestingly, our results demonstrate that AT domains from different sources can have a range of fidelity with regard to their ability to incorporate alternative substrates. In the case of epoAT3 this was not surprising as this domain is known to incorporate both malonyl- and methylmalonyl-CoA substrates in its natural context. It was surprising however that epoAT2 incorporated ~10% of the incorrect substrate (methylmalonyl-CoA), especially since an epothilone structure containing a methylmalonyl-CoA derived unit at the corresponding position of the macrocycle was not identified among the forty minor congeners produced by *S. cellulosum.*[4] However, we cannot discount the possibility that the corresponding epothilone intermediate of such a compound cannot be further extended or cyclised by the epothilone PKS (although, clearly such a diketide intermediate can be extended by DEBS1-TE). Perhaps incorporation of an alternative extender unit may be the result of the AT being in a heterologous context. Another possibility is that the incorporation of an alternative substrate is due to differences in the supply levels of each substrate in different organism metabolic backgrounds. While *S. erythraea* has an abundant methylmalonyl-CoA supply (at least during erythromycin production), relative levels in *S. cellulosum* may be different, and therefore the selection pressure to evolve an ability to discriminate against alternative substrates (selectivity) is lower. Indeed, the differences in relative levels of malonyl-CoA and methylmalonyl-CoA are proposed to cause the increase in the proportion of epothilone B to epothilone A (1 : 10) when the compound is heterologously produced in *Myxococcus xanthus*, whereas generally the ratio is 2 : 1 in *Sorangium cellulosum.*[25] In addition, medium composition and fermentation conditions can have a significant influence on the final yield as well as on ratios of the epothilones in the broth at the end of the process.[4] We did not attempt to investigate the effect of medium composition during our experiments. It is likely that utilising alternative media and cultivation conditions might additionally influence product ratios.

Site directed mutagenesis has been used to influence the nature of the extender unit utilised by AT domains in their native context.[14–16] Herein we investigated the possibility of increasing the proportion of methylmalonyl-CoA incorporated by using such an approach for the epoAT3 when in the hybrid DEBS1-TE PKS. This would indicate whether subsequent engineering of the AT in the natural epothilone producer strain might increase the proportion of epothilone B *vs.* A (or D *vs.* C). This investigation was incited by the considerable similarity observed between the epoAT2 and epoAT3 domains and the key observation that, of the few observed sequence differences between the domains, one was in the active site sequence motif proposed to influence substrate specificity. Furthermore, this motif signature in the epoAT3 sequence is a hybrid (HASH) of the motifs (HAFH/YASH) found in malonyl-/methylmalonyl-CoA ATs respectively. It was thus reasonable to believe that by converting the hybrid motif to either of HAFH/YASH we could alter AT selectivity.

As described above our results demonstrate that mutating the HASH mixed motif to YASH does not appear to provide a statistically significant increase in the proportion of products derived from incorporation of methylmalonyl-CoA. The productivity of the PKS bearing this mutation was also severely compromised (although such a loss in product yields has been observed in previously reported experiments of this type). The parallel experiment in which the epoAT3 active site was mutated to HAFH in an attempt to alter the selectivity towards malonyl-CoA, and thus potentially directing the production towards exclusively epothilone A production, resulted in a non-productive PKS. Although a large number of clones were analysed none of **1–4** could be detected. To a certain degree, the failure of this mutation was surprising as this mutation makes this domain more similar to epoAT2, which is functional in the heterologous context.

Our data indicate that while a clear correlation between the active site HAFH/YASH motifs and malonyl-/methylmalonyl-CoA selectivity exists,[14–16] the assumption that a mixed form of this motif could directly explain the promiscuity observed for the EpoC AT domain was incorrect, and other factors are involved. Indeed, when the YASH motif of AT domains in the erythromycin PKS were mutated to HAFH in an attempt to switch their selection of substrate from methylmalonyl-CoA to malonyl-CoA, the resulting PKSs were shown to utilise both substrates.[14–16] Thus, as, in retrospect may be expected for some examples, mutations in the active site that change AT selectivity appear to need to be complemented with other mutations in or around the active site in order to alter substrate selectivity towards the desired extender unit, and to preserve the catalytic activity.[14] The high similarity between the two AT domains in EpoAT2 and AT3 means that further candidate amino acids may be readily

identified from protein sequence alignments (bold residues; Fig. 2). These differences were not investigated within this current study but provide direction for further investigations. When examined further two of these differences are located in regions which others have suggested may also play a role in substrate selectivity. The residues serine and lysine (EpoA2 and 3 respectively; shown as white on black background in Fig. 2) are located within what has been termed the 'hyper-variable' region of the AT domain.[26] The residues isoleucine and alanine are present in EpoAT2 and 3 respectively of the active site GHS**X** active site motif (shown as white on black background in Fig. 2); this **X** residue was suggested to potentially play a role in substrate selectivity.[15]

In summary, we have shown that functional hybrid PKSs can be generated by swapping myxobacterial AT domains from the second and third extension modules of the epothilone PKS with the AT1 domain of DEBS1-TE. Furthermore, site directed mutation of the specificity conferring motif at the active site of the promiscuous AT3 of the epothilone PKS had no statistical effect upon substrate selection and product distribution. These data indicate that in order to use active site motif alterations within epoAT3 of the epothilone PKS directly to improve the product ratio of *S. cellulosum* fermentations, additional mutation of alternative residues within this domain will be required.

Experimental

DNA manipulation

The nucleotide sequences of epoAT2 and epoAT3 from *epoC* show high (98%) identity to each other.[17] To ensure the amplification of both AT domains, cosmid22 from a *Sorangium cellulosum* So ce90 cosmid library[27] (RM, unpublished results) was used as a template for amplification *via* PCR. After *Bam*HI digestion of cosmid22, the epoAT2 domain was located on a 2 kb DNA fragment, whereas the epoAT3 domain could be isolated on a 4 kb fragment. PCR was carried out using *Pfu* DNA-polymerase (Stratagene) according to the manufacturer's protocol. Conditions for amplification with the Eppendorf Mastercycler gradient (Eppendorf) were as follows: denaturation for 30 s at 95 °C, annealing for 30 s at 55 °C and extension for 60 s at 68 °C. Primers used for the amplification were AS1 (5′-TCA CTG CGC AGG GCG CGC AGA CGC C-3′) and AS2 (5′-GTG CCT AGG ACG CCC GGC CAG CTG AC-3′). The PCR amplification products were cloned into the pCR®2.1 TOPO vector (Invitrogen). Resulting plasmids pAS1.5 (containing epoAT2 and pAS2.2 (containing epoAT3)), were re-sequenced employing primers AS3 (5′-GTG TTC TCG CTG GAA GAT GG-3′), AS4 (5′-GGT CGA GCA ACA ACG ACT CG-3′) and AS5 (5′-ACG GCG TTC ACC CAG CCC GCG-3′). After verification, fragments were cloned into pCJR26[19] replacing the rapAT2 domain in this vector. Plasmid pCJR26 was digested using *Avr*II–*Msc*I, phenol–chloroform purified and ligated with the *Avr*II–*Fsp*I digested fragments from pAS1.5 and pAS2.2, respectively. Resulting plasmids pABS61 (epoAT2) and pABS62 (epoAT3), were verified by restriction analysis.

Site directed mutagenesis

The alteration of the HASH motif of epoAT3 to HAFH and YASH was accomplished by oligonucleotide-mediated mutagenesis and the DNA fragments were amplified *via* overlap extension PCR,[28] using plasmid pAS2.2 as the template for the PCR reaction. PCR was carried out using the *Pfu* DNA polymerase under the following amplification conditions: denaturation for 30 s at 95 °C, annealing for 30 s at 55 °C and extension for 40 s at 68 °C. The resulting fragments were used as the template DNA in the second round of PCR reaction with the following differences: annealing for 30 s at 60 °C and extension for 1 min at 68 °C. Primer combinations used for the amplifications were as follows: For the first parallel PCR reactions the primer pairs (A) AS1/BIOT6HY (5′-TGG GAC GC***G TA***C GAG ACA TGC AGC CGC TTG G-3′) and (B) AS2/BIOT5HY (5′-CAT GTC TCG ***TAC*** GCG TCC CAC TCG CCG CTG-3′) or rather (C) AS1/BIOT8SF (5′-TG***G AA***C GCG TGC GAG ACA TGC AGC CGC TTG G-3′) and (D)AS2/BIOT7SF (5′-CAT GTC TCG CAC GCG ***TTC*** CAC TCG CCG CTG-3′) were used. The bold letters represent point mutations leading to the amino acid exchanges in the target protein. In the subsequent overlap extension PCR reactions the fragments of A + B and C + D were used with the primers AS1/AS2. The products were cloned into the pCR®2.1 TOPO vector. After sequencing the resulting plasmids pASHY.6 and pASSF4 with primers AS3, AS4 and AS5 (see above), the fragments were subcloned into pCJR26 to generate the final constructs pCJRHY.6 and pCJRSF.4, respectively. Verification of the sequences from pCJRHY.6 and pCJRSF.6 were performed again by sequencing using the primers AS3, AS4 and AS5.

Strain construction and growth

Saccharopolyspora erythraea JC2[19] was transformed by PEG mediated protoplast transformation following standard techniques adapted from Weber and Losick,[29] as described by Gaisser *et al.*[30] This strain lacks the erythromycin PKS (*eryA*) genes and individual colonies were patched further onto R2T20 plates containing thiostrepton. To remove colony-to-colony variation of yields as a problem (this is a characteristic problem of *S. erythraea* mutants produced by protoplast transformation), we conducted a pre-screen to identify and select the most productive and reproducible transformants from each experiment in order to achieve a more robust data set for analysis. Approximately 60 transformant colonies of each construct were examined in this pre-screen. On this basis, seven individual mutants derived from the introduction of each construct were selected for further quantitative study (Table 1). These were cultivated on TWM agar medium from which they were inoculated into TSB medium (7 mL) containing thiostrepton (5 μg mL^{-1}) and grown for 3 days at 28 °C. This culture (0.5 mL) was used to inoculate SM3 medium[31] (7 mL) and grown for a further 6 days at 28 °C. The resulting samples were extracted by the addition of acetonitrile (1 : 1, v/v) containing formic acid (0.1%) to ensure lactonisation. These were shaken on a Vibrax for 30 min and then clarified by centrifugation. Samples were examined for TKL content and diluted appropriately to ensure response with the dynamic linear range of the LC-MS instrument as determined by the production of calibration curves obtained by the analysis of authentic samples of TKLs **1–4**. Generally, samples were measured at several concentrations to ensure the reliability of data.

LC-MS was performed on an integrated Agilent HP1100 HPLC system in combination with a Bruker Daltonics Esquire 3000+ electrospray mass spectrometer operating in positive ion mode.

Chromatography was achieved over reversed-phase BDS-C_{18} silica in a Phenomonex (Macclesfield, UK) Luna column (250 × 4.6 mm, 5 micron particle size) eluting with the following gradient: t = 0 min, 25% B; t = 15 min, 100% B. Mobile phase A: 10% acetonitrile : 90% water, containing 10 mM ammonium acetate and 0.1% formic acid; Mobile phase B: 90% acetonitrile : 10% water, containing 10 mM ammonium acetate and 0.1% formic acid.

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