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A Novel Erythromycin, 6-Desmethyl Erythromycin D, Made by Substituting an Acyltransferase Domain of the Erythromycin Polyketide Synthase

HRVOJE PETKOVIC[a], RACHEL E. LILL[a], ROSE M. SHERIDAN[a], BARRIE WILKINSON[a], ELLEN L. MCCORMICK[d], HAMISH A. I. MCARTHUR[d], JAMES STAUNTON[a,c], PETER F. LEADLAY[a,b] and STEVEN G. KENDREW[a,*]

[a] Biotica Technology Limited, 181A Huntingdon Road, Cambridge, CB3 0DJ, UK
[b] Department of Biochemistry, University of Cambridge, Cambridge, CB2 1GA, UK
[c] Department of Chemistry, University of Cambridge, Cambridge, CB2 1EW, UK
[d] Bioprocess Research and Development, Pfizer Inc., Groton, Connecticut, USA

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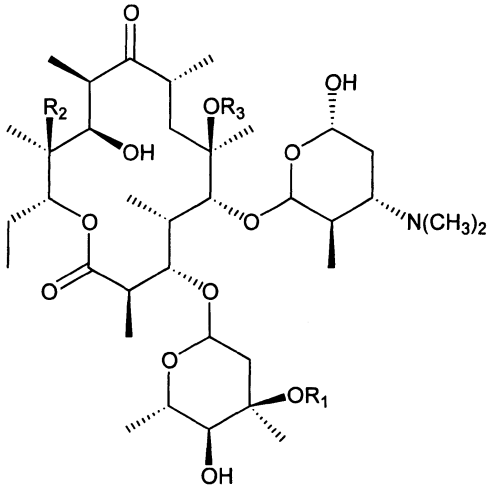
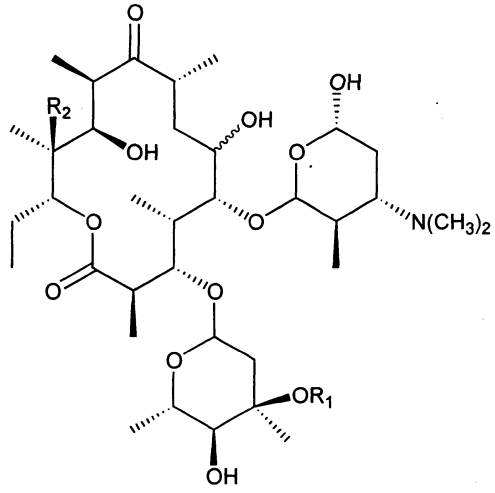
The acyltransferase (AT) domain in module 4 of the erythromycin polyketide synthase (PKS) was substituted with an AT domain from the rapamycin PKS module 2 in order to alter the substrate specificity from methylmalonyl-CoA to malonyl-CoA. The resulting strain produced 6-desmethyl erythromycin D as the predominant product. This AT domain swap completes the library of malonyl-CoA AT swaps on the erythromycin PKS and reinforces PKS engineering as a robust and generic tool.

Erythromycin A (**1**) and the derived semi-synthetic, second generation macrolides such as azithromycin (Zithromax) and clarithromycin (Biaxin) (**5**) (Figure 1) have been important antibiotics for the treatment of respiratory tract infections for many years. However, the rise in numbers of macrolide resistant pathogens has greatly increased the need to develop new derivatives of these highly effective molecules that combine both a better understanding of the different resistance mechanisms with novel approaches to changing the nature of the chemistry around the molecule, while maintaining the excellent safety and tolerability profile of the erythromycin series. In addition to utilising conventional chemical approaches, the well-established modularity of Type I polyketide synthase (PKS) multienzyme complexes readily lends itself to the use of genetic engineering to develop non-natural analogues of commercially valuable products[1]. Of the large numbers of modular PKS clusters sequenced during the past decade the erythromycin PKS (DEBS) is the most intensively studied and has remained the best model system for the demonstration of key technologies. A model system derived from the DEBS was used to demonstrate the first PKS heterologous AT domain swap, generating a desmethyl triketide lactone by replacing the methylmalonyl-CoA specific AT from module 1 with a malonyl-CoA specific AT from the rapamycin PKS[2]. Subsequent work in this PKS system has replaced the ATs from the loading module[3] and the extension modules 2, 3, 5, and 6 in a similar manner[4~6] to give the expected desmethyl products. However, it has been reported by a number of researchers that replacement of the DEBS AT4 by ATs specifying malonyl-CoA results in a non-productive PKS[4,7], although curiously, replacement of the AT4 with an AT specific for ethylmalonyl-CoA derived from the niddamycin cluster does result in a productive PKS[8], albeit with reduced synthetic activity. While ethylmalonyl-CoA specific ATs are often more similar to methylmalonyl-CoA specific ATs, this experiment demonstrates that the failure of the published malonyl-CoA AT swaps in module 4 is likely due to a combination of unsuitable protein/domain splice sites,

* Corresponding author: steve.kendrew@biotica.co.uk

Fig. 1. Structures of erythromycins (**1**~**4**), clarithromycin (**5**) and 6-desmethyl erythromycins (**6**~**9**).





Erythromycin A, **1**: $R_1 = CH_3$, $R_2 = OH$, $R_3 = H$
Erythromycin B, **2**: $R_1 = CH_3$, $R_2 = H$, $R_3 = H$
Erythromycin C, **3**: $R_1 = H$, $R_2 = OH$, $R_3 = H$
Erythromycin D, **4**: $R_1 = H$, $R_2 = H$, $R_3 = H$
Clarithromycin, **5**: $R_1 = CH_3$, $R_2 = OH$, $R_3 = CH_3$

6-desmethyl erythromycin A, **6**: $R_1 = CH_3$, $R_2 = OH$
6-desmethyl erythromycin B, **7**: $R_1 = CH_3$, $R_2 = H$
6-desmethyl erythromycin C, **8**: $R_1 = H$, $R_2 = OH$
6-desmethyl erythromycin D, **9**: $R_1 = H$, $R_2 = H$

incompatibility of the particular domains used, or limitations of product analysis and detection rather than an impossibility of making a productive swap at this point. Desire to produce this compound has remained strong; REEVES and co-workers having failed to develop an AT replacement strategy for production of the 6-desmethyl compound report the use of a site-directed mutagenesis strategy which produces the aglycone 6-deoxy-6-desmethyl erythronolide B in a mixture with 6-deoxy erythronolide B (6DEB)[7]. Here, we report the construction of a strain containing a hybrid DEBS PKS in which the AT4 has been replaced by the AT2 derived from the rapamycin PKS cluster and its use to produce a glycosylated and biologically active antibiotic 6-desmethyl erythromycin D (**9**).

Results

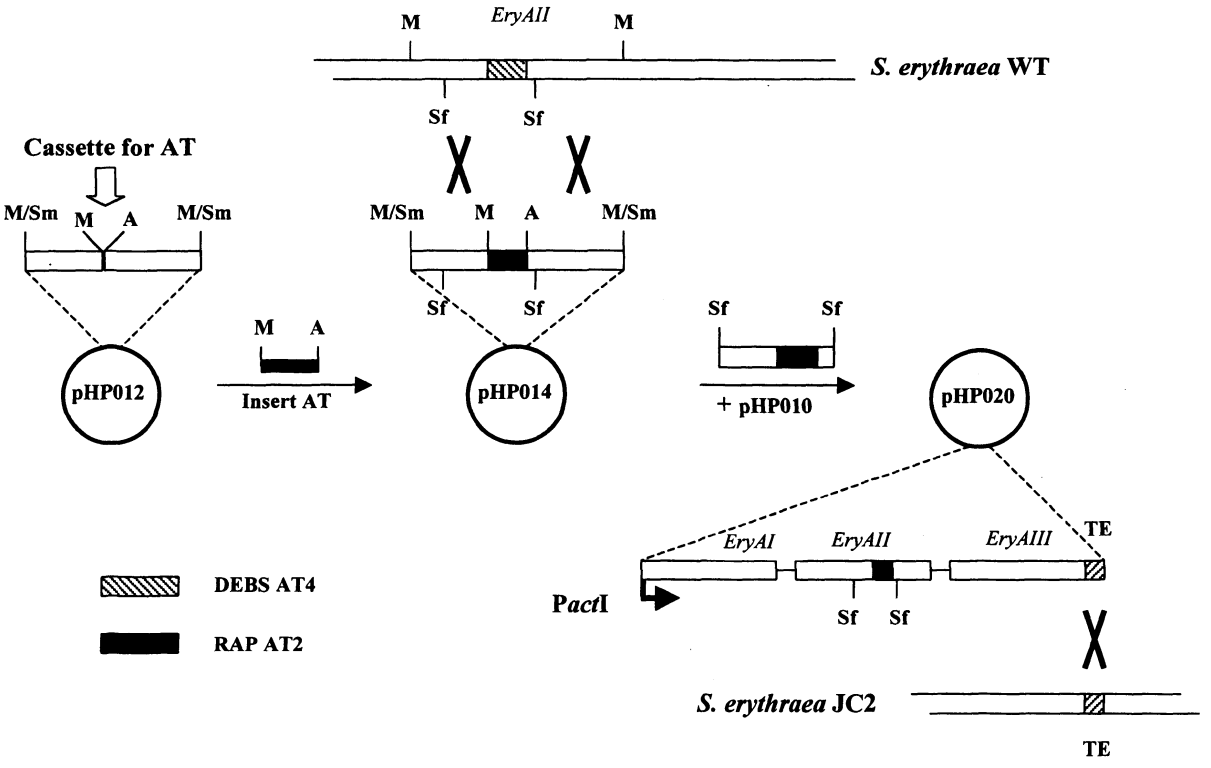
Construction of Recombinant Strains

Two strategies were employed to produce the desired strains. In the first, conventional gene replacement methodologies were used to substitute DEBS AT4 by RAP AT2 in the wild type *Saccharopolyspora erythraea* NRRL 2338 (Figures 2 and 3). The protein splice sites chosen to flank the donor AT were the same as those used by OLIYNYK *et al.*[2], although recombinant strains utilising a different set of splice sites and an alternative donor AT also produced the expected compound (Figure 3). The design of the constructs incorporated a cassette system that allowed us to introduce ATs from multiple donors in a relatively simple manner, although as our initial constructs were functional, it was not actually necessary to test a large number of further ATs. In the second strategy the replacement cassette containing the heterologous AT described above was excised and used to substitute the equivalent region in a plasmid containing *EryAI*, *EryAII* and *EryAIII* genes under the control of the *actI*/*act*II-Orf4 promoter-activator system of *Streptomyces coelicolor*. This plasmid pHP020 was then introduced into the strain JC2 which is deleted of the almost the entire PKS region[9], using the remaining TE portion of the PKS as a region of homology for integration (Figure 2).

Characterisation of Metabolites

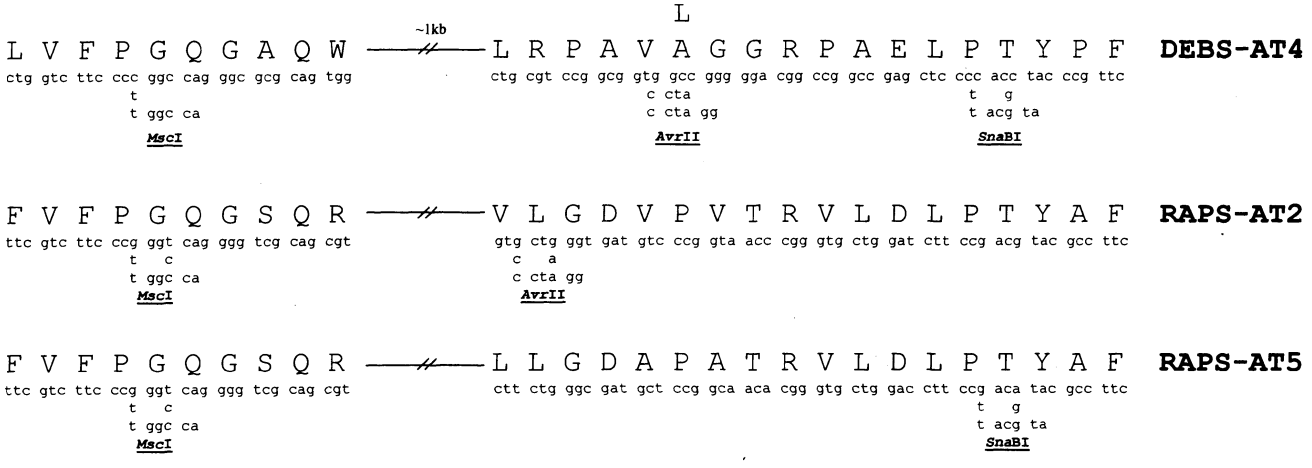
Recombinant strains made by replacement and single crossover strategies were grown under standard production

Fig. 2. Strategies used for construction of the 6-desmethyl erythromycin D producing strains.



Left hand side of diagram shows the cassette plasmid pHP012 used to introduce multiple ATs into AT4. Top of diagram shows the double crossover event required in the *S. erythraea* chromosome to replace the AT4 domain using replacement plasmid pHP014. Bottom of diagram shows the conversion of the replacement cassette into pHP020, containing the modified PKS under the *act*I/*act*II-Orf4 promoter-activator system and subsequent integration into *S. erythraea* JC2 chromosome. M=*Msc*I, Sm=*Sma*I, A=*Avr*II, Sf=*Sfi*I.

Fig. 3. Splice sites used for the construction of the hybrid PKS.



The protein sequences at the domain boundaries of the respective ATs are shown above the corresponding DNA sequences. Below are the nucleotide sequence changes required to introduce the *Msc*I, *Avr*II or *Sna*BI restriction sites used in the experiments. The mutation introduced by the *Avr*II site is marked.

conditions for *S. erythraea* and the culture extracts analysed by LC-MS. Two main components were observed that displayed mass spectra consistent with the presence of a desosamine derived fragment (m/z=158.5). The LC retention time of the first, in conjunction with the parent mass (m/z=690.5) and fragmentation pattern (m/z=546.5; loss of a mycarose moiety, m/z=144), is consistent with the structure for 6-desmethyl erythromycin D (**9**). The LC-MS data for the less polar, minor component (m/z=704.5), in particular the observation of a fragmentation with loss of a cladinose rather than mycarose moiety (m/z=546; loss of cladinose moiety m/z=158), indicated that the structure was most likely 6-desmethyl erythromycin B (**7**). Peaks consistent with the presence of 6-desmethyl erythromycin A and C were also identified.

Structure Determination

To verify the structure, compound **9** (4.2 mg) was isolated from a 14 litres fermentation of *S. erythraea* JC2/pHP020 (BIOT0861) in Ery-P medium[10]. The molecular formula [MH^+] of $C_{35}H_{64}NO_{12}$ was verified by FTICR-MS/MS. The ^1H and ^{13}C NMR data for **9** are shown in Table 1. The majority of the spectrum was readily assigned by comparison to that of erythromycin D with the regularly spaced methyl groups greatly aiding analysis of the 2D spectra. A broad singlet resonating at δ_H 3.09 was assigned as 6-OH. No signals were observed to indicate the presence of a C12 hydroxy group. A COSY correlation was observed between 6-OH and H6 (δ_H 4.26). This proton was also correlated to H5 (δ_H 3.59) and H7b (δ_H 1.92). Coupled to the H7b proton was H7a (δ_H 1.84) which, in turn, was

Table 1. ^1H and ^{13}C NMR data for 6-desmethyl erythromycin D (**9**).

Position	δ_H	Multiplicity	Coupling	δ_C
1				176.4
2	2.67	qd	7.0, 5.1	44.8
3	4.43	dd	5.1, 1.5	82.5
4	2.02	m		40.5
5	3.59	m		81.8
6	4.26	m		74.4
7	1.92	m		33.1
	1.84	m		
8	2.64	m		47.7
9				217.5
10	3.03	qd	7.0, 1.7	41.5
11	3.91	d	9.6	70.0
12	1.75	m		38.7
13	5.18	ddd	8.1, 6.6, 1.7	75.8
14	1.80	m		25.1
	1.50	dqd	14.1, 7.3, 4.3	
15	0.89	dd	7.5, 7.5	10.1
16	1.21	d	7.0	13.6
17	1.10	d	7.0	9.7
18	1.18	d	7.0	17.9
19	1.00	d	6.8	8.5
20	0.91	d	7.0	9.1
1′	4.87	d	3.2	100.1
2′	1.79	m		40.5
	2.26	dd	14.7, 1.1	
3′				69.8
4′	2.98	d	9.6	76.3
5′	3.86	dq	9.6, 6.4	66.4
6′	1.33	d	6.4	17.8
7′	1.25	s		25.4
1″	4.25	d	7.3	105.6
2″	3.27	dd	10.2, 7.0	70.2
3″	2.61	m		65.4
4″	1.75	m		29.1
	1.29	m		
5″	3.54	m		69.5
6″	1.23	d	6.2	21.2
7″	2.35	s		40.3
6-OH	3.09	br. s		

Table 2. Antibacterial activities of erythromycin D and 6-desmethyl erythromycin D.

Microorganism	MIC (μg/ml)	
	Erythromycin D	6-desmethyl erythromycin D
Staphylococcus aureus 01A1046	3.12	12.5
S. aureus 01A1095	> 100	> 100
Streptococcus pyogenes 02C0203	< 0.2	< 0.2
S. pyogenes 02C1079	> 100	> 100
S. pneumoniae 02J1016	0.39	0.78
S. pneumoniae 02J1046	> 100	> 100
Enterococcus faecalis 03A1085	6.25	6.25
E. faecalis 03A1069	> 100	> 100
Haemophilus influenzae 54A1100	100	100

correlated to H8 (δ_H 2.64). The observation of an HMBC correlation between H5 and the anomeric C1″ (δ_C 105.6) and between H8 and C9 (δ_C 217.5) confirmed these assignments.

Biological Activity

6-Desmethyl erythromycin D had comparable antibacterial activity to erythromycin D suggesting that the methyl group at the 6-position does not play a significant role in antibiotic activity. As anticipated, 6-desmethyl erythromycin D showed no activity against macrolide-resistant strains (Table 2).

Discussion

6-Desmethyl erythromycin D (**9**) was isolated from a strain in which the natural acyltransferase of DEBS module 4, which specifies methylmalonyl-CoA, was replaced with an acyltransferase specifying malonyl-CoA. While AT domain replacements have been shown to produce the desired products in DEBS modules 1, 2, 3, 5 and 6[4~6], successful substitution of this particular domain by malonyl-CoA specific ATs has evaded a number of researchers[4,7]. The reasons for this are unclear but it has been postulated that the inserted domain might cause the PKS complex to misfold, or alternatively that the modified polyketide chain cannot be recognised by downstream domains of the PKS[4,7]. On the other hand products have been produced when AT4 has been substituted by an AT specific for the ethylmalonyl-CoA extender unit[8] although these ATs tend to be more similar to methylmalonyl-CoA ATs and are therefore less likely to affect the intrinsic PKS structure. We also designed a plasmid system that would allow multiple AT domain donors to be tested in an efficient manner. In addition to a conventional, but slower, replacement (double crossover) strategy the design of the system also included the use of a plasmid containing the entire erythromycin PKS. Following manipulation of the PKS, this plasmid was introduced into *S. erythraea* JC2 (which has been deleted of almost the entire PKS[9]), using the TE as a homology region for integration. This approach gave us the ability to construct multiple recombinant strains by single integration, and enabled us to take advantage of the enhanced polyketide production levels we have observed from PKS expressed from the *actI*/*actII* promoter-activator system in this strain.

The demonstration that substituting the natural acyl transferase domain of module 4 with an AT that specifies

incorporation of malonyl-CoA can produce 6-desmethyl erythromycins completes the library of malonyl-CoA AT domain substitutions around the erythromycin PKS. Recent publications have shown that substitution of natural ATs with ATs specifying ethylmalonyl-CoA or methoxymalonyl-CoA results in production of the expected compound when the cells are engineered to synthesize the required substrate[8,11], further expanding the repertoire of possible manipulations. However, the production of 6-desmethyl erythromycins using a rationally designed domain replacement strategy, where others have failed to detect such compounds from strains constructed in a similar manner, does illustrate that testing a number of alternative splice sites and donor domains can be an important factor in determining the success of a particular experiment, whatever AT domain is required. It is possible that because module 4 is the only module of DEBS containing a complete reductive loop the exact choice of splice sites and inserted domain has a greater effect on the levels of expected product than in other modules and justifies the care taken during the design of the PKS engineering.

The incorporation of an acetate unit in the fourth condensation cycle does not appear to affect the ability of the PKS to process the modified polyketide. The reductive loop of module 4 catalyses complete reduction to a methylene moiety and the condensation by downstream KS5 occurs. The reduced yield of compound *versus* non-recombinant strains may indicate a lowered catalytic activity of the PKS but our experiments cannot determine precisely at what point, or for what reason, this reduction in yield occurs. REEVES and co-workers[7] propose that the failure of their heterologous AT swaps in module 4 was due to incompatibility between the inserted domain and module 4 rather than the inability of the PKS to process the desmethyl-pentaketide by using a heterologous AT capable of incorporating both malonyl- and methylmalonyl-CoA, although these authors did not do extensive trials of alternative domain splice sites. The production of the 6-desmethyl-6DEB as a mixture using a site-directed mutagenesis strategy to alter AT specificity is a further indication that the PKS can tolerate the desmethyl pentaketide.

One of the important features of our experiments was to produce fully glycosylated, and hence biologically active modified erythromycins rather than a simple aglycone template. It is the post-PKS processing steps such as glycosylation, hydroxylation or methylation that give these molecules their biological activity, and it can be difficult to judge which of these reactions will be affected by changes to the macrolide core. In this sense the hydroxylation of C6 by EryF cytochrome P450 hydroxylase was unexpected because the engineered change in the polyketide directly affects the carbon that is hydroxylated by this enzyme. However, EryF has been shown to possess an enlarged active site in comparison to other bacterial enzymes and to display wide substrate specificity[12~14]. Key points of enzyme-substrate recognition appear to be the hydroxyl groups of C5 and C11 and the keto of the C9 position in addition to the hydrophobic contacts to the methyl substituents on C2, C8, C10, C12 and C14[12,13]. These contacts are retained in the engineered polyketide molecule and are presumably sufficient for binding and orientation in the active site to ensure hydroxylation at the C6 position. Thus, EryF seems capable of oxidizing a secondary carbon as well as a tertiary carbon although, as suggested previously, this may be energetically less favourable[4]. That 6-desmethyl erythromycin D lacks the hydroxylation at C12 was not surprising as EryK, the cytochrome P450 that catalyses this reaction, has been shown to possess a much narrower substrate range[4,10,15]. EryG, the mycarose *O*-methyltransferase that catalyses the final step in erythromycin biosynthesis also fails to act efficiently on the 6-desmethyl template, although we could detect production of 6-desmethyl erythromycin A and B at lower levels by LC-MS. It has been noted that although both the 10- and 12-desmethyl derivatives were methylated efficiently, EryG is sensitive to changes in the lactone ring with, for example, this enzyme displaying reduced activity for 2-desmethyl erythromycin and $\Delta^{6,7}$-anhydroerythromycin[4,16,17]. In contrast other sugar methyl transferases show a broad tolerance to the nature of the aglycone[18].

Perversely, our experiments were probably aided by our strong belief that we should concentrate on the production of processed, and hence biologically active, erythromycins rather than the simple aglycone core. The presence of an amino sugar in glycosylated products enhances detection limits through protonation and improved ionisation in the electrospray MS source. Low levels of erythromycin A can be detected (2~5% of the desmethyl compounds), showing the tight specificity of the introduced domain. This is in contrast to the switch in AT4 substrate specificity by introducing site-directed mutations which at best produced approximately equal amounts of the 6DEB or 6-desmethyl-6DEB[7]. A low level of extender unit mis-incorporation does occur in a number of engineered and natural (*i.e.* not engineered) PKS systems and is probably due to a combination of factors such as 'relaxed specificity' of the heterologous AT domain in a non-native environment, or differences in timing or concentration of CoA substrate

supply versus that normally experienced by the inserted domain. Recent publications have begun to probe these facets of PKS manipulation[11,19] and it is clear that a better understanding of these factors will be important as engineered PKS are moved through the development process. Production of mixtures is only an advantage when generating diverse libraries of molecules or for producing molecules for preliminary testing purposes where the effort required to separate compounds differing by a single methyl group can be justified. In the later stages of product development an engineered PKS producing essentially a single component is much preferred.

Experimental

Bacterial Strains and Plasmids

Saccharopolyspora erythraea NRRL2338 and derivative recombinant strains were plated on R2T20 agar and propagated in liquid TSB. Plasmid vectors were propagated in *Escherichia coli* DH10B grown in 2TY. Unmethylated DNA was prepared from *E. coli* strain ET12567.

Construction of the DEBS AT4 Replacement Cassette pHP012

The domain splice sites for the heterologous AT were chosen to be *Msc*I and *Avr*II in the equivalent positions in DEBS AT4 as defined for AT1 by OLIYNYK *et al.*[2]. To construct a replacement cassette for AT4 of DEBS2 the 1.2 kb flanking region upstream of the engineered *Msc*I site and 0.5 kb-flanking region downstream of the engineered *Avr*II site, AT4 flanking regions, were obtained by PCR amplification from pHP001 (containing DEBS AT4 on a 4.7 kb *Msc*I fragment sub-cloned from pIB023 into pUC19 digested with *Sma*I). Two oligonucleotide primers were used to amplify the *Msc*I-upstream region 5′-TTTTTCTGCAGCGCCCTGGCCAGGGAAGACCAGGA-CCG-3′ and 5′-TTTTTAAGCTTCCTGCGAGGCACCG-ACACCGGCG-3′, the former introducing the *Msc*I site plus a *Pst*I site located just before the *Msc*I site, and the latter introducing a *Hin*dIII site and priming across a *Sfi*I site. The amplified product was digested with *Pst*I and *Hin*dIII and sub-cloned into pUC19 that had been digested with *Pst*I and *Hin*dIII. The resulting plasmid was designated pHP004. Two oligonucleotide primers were used to amplify the *Avr*II-downstream region, 5′-TTTTTGAATTCCGTCCTCCGGCGGCCACTGCTCGG-3′ and 5′-TTTTTCTGCAGCCTAGGGGGACGGCCGG-CCGAGCTGCCCACC-3′, the former introducing an *Avr*II site plus a *Pst*I site located just after the *Avr*II site and the latter an *Eco*RI site. The 545 bp PCR product was cut with *Pst*I and *Eco*RI and subcloned into *Pst*I and *Eco*RI cut pUC19 to generate pHP003. The 1.2 kb derived fragment containing the *Msc*I-upstream region was excised from pHP004 with *Pst*I and *Hin*dIII and sub-cloned into pHP003 that had been linearised with *Pst*I and *Hin*dIII, to generate pHP007. All PCR fragments were sequenced to ensure errors were not introduced during the amplification process.

pHP001 was digested with *Eco*RI and *Xba*I to isolate the PKS derived fragment which was cloned into pCJR24 that had been digested with *Eco*RI and *Spe*I to generate pHP005. The 2.2 kb *Sfi*I fragment from pHP005 was then replaced with the 1.1 kb *Sfi*I fragment from pHP007 and the resulting vector designated as pHP012 which is a replacement cassette without the presence of AT4. Using this cassette, multiple heterologous AT's can be introduced into AT4 at the unique *Msc*I and *Avr*II sites.

Isolation of a *S. erythraea* Strain Containing Rap AT2 by Replacement

RAP AT2 specifying a malonyl-CoA extender unit was used to replace DEBS AT4 in *S. erythraea*. The 0.8 kb *Msc*I/*Avr*II fragment of RapAT2[2] was isolated and cloned into pHP012 that had been digested with the same enzymes to generate the final replacement construct pHP014. Plasmid pHP014 was transformed into *S. erythraea* NRRL2338 protoplasts using standard methods. Thiostrepton resistant transformants were patched onto R2T20 plates containing 25 $\mu g\,ml^{-1}$ thiostrepton. To promote the second recombination event, transformants were passaged twice in liquid TSB without antibiotic and plated onto R2T20, individual colonies arising from spores were screened for thiostrepton sensitivity and Southern blotting was used to verify the presence of the RAP AT2 domain. A strain in which DEBS AT4 was correctly replaced by RapAT2 was designated as BIOT0875.

RapAT2 Replacement Using the *S. erythraea* JC2/pHP020 System

Plasmid pHP010 is a pCJR24-based plasmid[9] containing DEBS1, DEBS2 and DEBS3 under the control of the *act*I promoter. pHP010 was constructed by insertion of an additional 1 kb *Eco*RI/*Xba*I DNA fragment (kindly provided by Chris Wilkinson, Department of Biochemistry, University of Cambridge) into pIB023 downstream of the TE domain in order to provide extra homology for transformation into *S. erythraea* JC2. This strain (a derivative of NRRL2338) has been deleted of almost the entire DEBS PKS but leaves the TE as a homology region for integration[9]. DEBS AT4 of this plasmid was replaced

by RapAT2 by removing a 2.2 kb *Sfi*I fragment (*Sfi*I site not methylated) from pHP010 containing AT4 of erythromycin PKS and replacing it with 2.0 kb *Sfi*I fragment containing RapAT2 from pHP014 to generate pHP020. pHP020 was transformed into *S. erythraea* JC2 using standard methods. The resulting strain *S. erythraea* JC2/pHP020 was designated as BIOT0861.

Fermentation of *S. erythraea* JC2/pHP020

S. erythraea JC2/pHP020 was cultured from a frozen vegetative working stock (1 : 1, TSB culture : cryopreservative; cryopreservative was 20% glycerol : 10% lactose w/v in distilled water). A primary pre-culture was grown in TSB (50 ml in 250 ml flask) shaken at 250 rpm and 30°C. After two days this was used to inoculate (5% v/v) a secondary pre-culture of TSB (400 ml in a 2 litre flask), which was cultured under the same conditions for a further two days. Twelve litres of Ery-P production medium[10] was inoculated with the secondary pre-culture (5% v/v) and allowed to ferment in a 20 litre stirred bioreactor (Applikon) for five days at 30°C with an aeration rate of 6 litres/minute.

Isolation of 6-Desmethyl Erythromycin D (**9**)

After 5 days the fermentation broth of *S. erythraea* JC2/pHP020 was clarified by centrifugation, the supernatant adjusted to pH ~9.5 with sodium hydroxide and then stirred with Amberlite XAD-16 resin (100 g) at room temperature. After 30 minutes the resin was isolated by centrifugation, washed with water (200 ml) and then eluted with methanol (3×200 ml). This procedure was repeated twice more, the resulting methanol eluates combined and the solvent removed under reduced pressure to yield an aqueous solution (~50 ml). This was diluted with water (150 ml) and extracted with ethyl acetate (3×100 ml). The extracts were combined and the solvent removed to yield a brown oil (6.9 g). The oil was partitioned between acetic acid/sodium acetate (1 : 1, 100 ml, pH 5) and ethyl acetate (100 ml), and the solvent removed to leave a concentrated extract (0.5 g).

The extract was purified by chromatography over reversed-phase silica (Hypersil 5 μm C_{18}-BDS, 21×150 mm), eluting at a flow rate of 21 ml/minute with a linear gradient of acetonitrile: 20 mM ammonium acetate (25%~75% over 19 minutes). Fractions were collected every 30 seconds and assayed by LC-MS. Significant loss was tolerated at this stage to ensure purity of the final product. Those containing 6-desmethyl erythromycin D (**9**) were combined and the solvent removed. This was then concentrated using an IsoElute ENV+ cartridge (200 mg), washed with water (10 ml) and eluted with methanol (2×6 ml) to yield 6-desmethyl erythromycin D (**9**) as an amorphous white solid (4.2 mg). The presence of 6-desmethyl erythromycin A, B, C (**6**, **7**, **8**) in alternative fractions was confirmed by LC-MS analysis, although insufficient material was available for isolation.

Bacterial Test Strains and MIC Determinations

Staphyloccoccus aureus 01A1046 (pen^r) and *S. aureus* 01A1095 (amp^r, cef^r, gent^r, imipenem^s, MLS^r_B, tet^r, van^s) are clinical isolates. *Streptococcus pyogenes* 02C0203 (ATCC 12384, serotype B, MLS^S) was obtained from the American Type Culture Collection. *S. pyogenes* 02C1079 is MLS^r_B. *S. pneumoniae* 02J1016 (serotype 3) is a susceptible clinical isolate, *S. pneumoniae* (serotype 6) 02C1046 is MLS^r_B and tet^r. *Enterococcus faecalis* 03A1085 is pen^r and van^s while *E. faecalis* 03A1069 is a clinical, multidrug-resistant strain (cef^r, ery^r, gent^r, chl^r, kan^r, tet^s, van^s), confirmed to have the *erm*B gene. *Haemophilus influenzae* 54A1100 is a non-type B, ery^s, clinical isolate. *Escherichia coli* 51A1073 (MC4100) is a wild-type, van^s strain. MIC determinations were performed as described previously[20].

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