Engineering Broader Specificity into an Antibiotic-Producing Polyketide Synthase

Andrew F. A. Marsden, Barrie Wilkinson, Jesús Cortés, Nicholas J. Dunster, James Staunton, Peter F. Leadlay*

The wide-specificity loading module for the avermectin-producing polyketide synthase was grafted onto the first multienzyme component (DEBS1) of the erythromycin-producing polyketide synthase in place of the normal loading module. Expression of this hybrid enzyme in the erythromycin producer *Saccharopolyspora erythraea* produced several novel antibiotic erythromycins derived from endogenous branched-chain acid starter units typical of natural avermectins. Because the avermectin polyketide synthase is known to accept more than 40 alternative carboxylic acids as starter units, this approach opens the way to facile production of novel analogs of antibiotic macrolides.

The emergence of pathogenic bacteria multiply resistant to antibiotics represents a growing threat to human health (*1*) and has given added impetus to the search for new drugs and for more effective versions of those in current use. The antibacterial erythromycin A, like other macrocyclic polyketides, is derived from simple carboxylic acid precursors by stepwise chain assembly on a modular polyketide synthase (PKS). Such PKSs are giant multienzyme complexes (*2–5*) containing a different set or "module" of enzyme domains to accomplish each successive cycle of polyketide chain extension. The erythromycin-producing PKS [6-deoxyerythronolide B synthase (DEBS)] of *Saccharopolyspora erythraea* contains six such extension modules, together with a "loading module" comprising an acyltransferase (AT) and an acyl carrier protein (ACP), and an "off-loading" domain, a thioesterase/cyclase (TE) (*2*). The modularity of such systems suggests that splicing together of the structural genes for different PKSs should produce hybrid multienzymes that might synthesize novel molecules incorporating elements of different polyketide natural products (*6*).

We report the production of novel macrolide antibiotics by this method. Our strategy was to replace the loading module of DEBS, which accepts propionyl coenzyme A (CoA) and acetyl-CoA in vivo (*7*), with the loading module from the avermectin-producing polyketide synthase of *Streptomyces avermitilis*, which has an unusually broad specificity for branched carboxylic acids as starter units in vivo (*8*). The viability of the approach was demonstrated initially in a truncated PKS consisting of the first two extension modules of DEBS and the chain-terminating thioesterase (*9*). The equivalent module swap was then carried out on the intact modular PKS in *S. erythraea*, whereupon novel, biologically active erythromycin derivatives containing branched-chain starter units were produced. These results suggest that the promiscuity of the avermectin PKS loading module can be transferred to other modular PKSs, opening the way to the wholesale and convenient biosynthesis of novel analogs of important polyketide antibiotics, antifungals, and immunosuppressants.

The donor module in these experiments was the NH_2-terminal AT-ACP di-domain of AVRS1, the first multienzyme component of the avermectin-producing polyketide synthase from *S. avermitilis*. The avermectins are a family of 16-membered macrocyclic lactones which are potent antiparasitic agents through their action as agonists of a γ-amino butyric acid–insensitive chloride channel (*10*). The pentacyclic ring structure of avermectin B1 is assembled from five propionate units and seven acetate units, with either an isobutyrate (B1b) or a 2-methylbutyrate (B1a) starter unit (*11*). The clustered genes encoding the biosynthetic enzymes have been cloned, and the locations of most of the constituent domains of the PKS have been reported (*5*). Although the natural avermectins normally incorporate just two alternative starter acids, the avermectin loading module has been shown previously to accommodate a wide range of nonnatural starter units, and this has allowed the facile production of avermectin analogs with novel starter units (*8*).

The loading module of the erythromycin PKS, DEBS, is highly selective for propionate and acetate units both in vivo and in vitro (*12*). Recently, domain swaps have been used successfully to alter the extender specificity of a modular PKS from propionate to acetate (*13*), and to alter the starter unit specificity from acetate to propionate (*14*). Independently, a precursor-directed approach has recently succeeded in the production of erythromycin D analogs altered in the region of the starter unit, by aberrant incorporation of synthetic partial chains into the polyketide synthase (*15*). It is not known why these experiments did not give rise to fully active erythromycin A analogs. In contrast, the genetic alteration we describe was aimed at the conversion of DEBS into a hybrid enzyme of broader specificity, potentially capable of accepting the wide range of alternative branched-chain starter units characteristic of the *avr* PKS within a host organism potentially capable of fully processing the new macrolides into erythromycin A analogs.

First, a hybrid PKS was constructed in which the broad specificity *avr* PKS loading module was fused, in place of the natural propionate-specific loading module, to the NH_2-terminus of the truncated PKS, DEBS1-TE (*9*), which contains only the first two extension modules of the *ery* PKS and the chain-terminating thioesterase. The predicted δ-lactone products for this hybrid PKS would have the normal erythromycin triketide lactone structure (*9*), but the alkyl substituent at carbon 5 (C-5) would be specified by the *avr* PKS loading module and therefore would be predicted to be derived from either isobutyrate or a 2-methylbutyrate (Fig. 1A) (*11*), because isobutyryl- and isovaleryl-CoA are the only C-2 branched acyl-CoA esters normally found in actinomycete cells (*16*). The region of the *avr* gene cluster of *S. avermilitis* encoding the loading module was identified from published data (*5*), cloned, and sequenced. The loading module was confirmed to consist of an AT-ACP di-domain showing significant sequence similarity to the loading module of DEBS1. The *avr* PKS loading module was inferred to start within the first multienzyme AVRS1 at the sequence ^{108}VVFVFPGQ (*17*) and to end some 150 amino acid residues later at the sequence HGGTAAAD^{556} (numbering refers to corresponding amino acid residues in DEBS1) (*18*). The DNA encoding the loading module was amplified by polymerase chain reaction (PCR) and cloned in the place of the equivalent fragment in the DEBS1-TE expression plasmid pRMTE (*13*) to create plasmid pIG1 (*19*), which was used to transform *Streptomyces coelicolor* CH999 (*20*).

In this system, the expression of the hybrid DEBS1-TE is under the control of the *actI* promoter and the specific activator

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protein ActII-ORF4 (*21*). When *S. coelicolor* CH999/pIG1 was grown for 5 days in liquid medium and the fermentation broth was extracted with ethyl acetate, several polyketides not produced by a control fermentation of *S. coelicolor* CH999 were identified and were subsequently purified from the extract. The products included the triketide lactones **3** and **4** arising from the use of the isobutyrate and 2-methylbutyrate starter units, respectively. The loading module swap does indeed generate a functional hybrid PKS and one that accepts starter units characteristic of the transplanted broad-specificity *avr* PKS loading module. The triketide lactones **1** through **4** (Fig. 1A) were obtained in the approximate ratio of 2:8:2:5 (total yield 10 mg/liter). The fermentation conditions were not optimized, and yields might also have been improved by addition of suitable carboxylic acids to the fermentation (*8*). All of the compounds were fully characterized by gas chromatography, high-pressure liquid chromatography (HPLC), two-dimensional (2D) nuclear magnetic resonance (NMR), electrospray mass spectrometry (ESMS), and high-resolution ESMS (*22*), and by comparison with synthetic standards (*23*). The production of **1** and **2** by the hybrid PKS was not unexpected: The *avr* PKS loading module accepts both acetate and propionate starter units (*24*).

The efficacy of the hybrid DEBS1-TE multienzyme encouraged us to examine whether the significant changes in starter unit would perturb either the later stages of polyketide chain assembly or the further processing steps required to produce active erythromycin antibiotics. In the first three post-PKS steps, the macrolide ring is hydroxylated at C-6, and then glycosylated successively with mycarose on the hydroxyl group at C-3 and with desosamine on the C-5 hydroxyl to give erythromycin D, the first compound on the pathway to show antibiotic activity (*6*). Subsequent hydroxylation at C-12 yields erythromycin C, which rapidly undergoes a final C-3″ O-methylation of the mycarose moiety to give the clinically effective erythromycin A. Alternatively, the last two steps may take place in the reverse order, in which case, erythromycin B is formed as a shunt intermediate in place of erythromycin C (*25*).

The cloned DNA for the *avr* PKS loading module, flanked by *ery* PKS sequence,

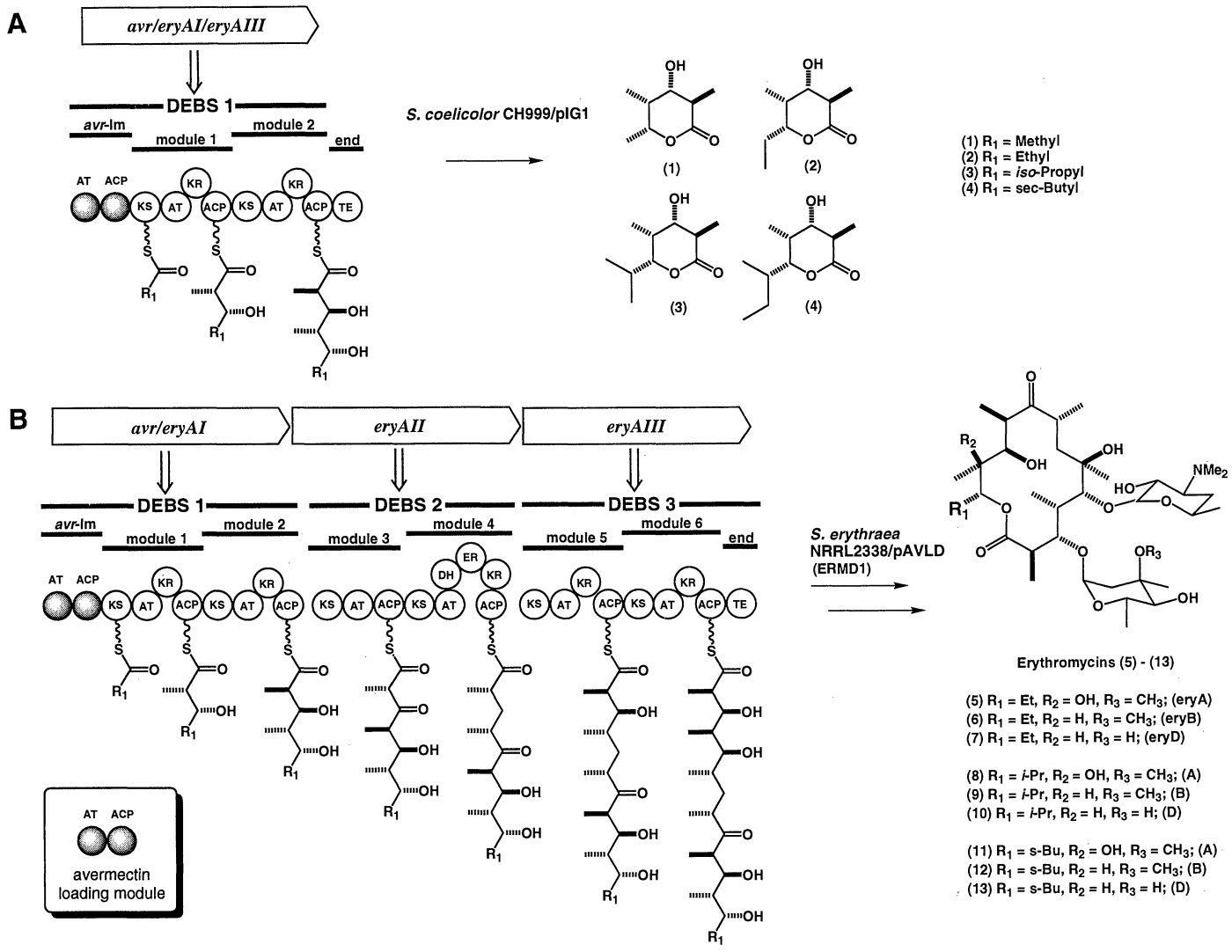

Fig. 1. The products of hybrid modular PKSs containing an altered loading domain. (**A**) Expression plasmid pIG1 in *S. coelicolor* CH999 (*20*) encodes the loading domain from the avermectin PKS (*avr*-lm) in place of the natural loading module, fused to a truncated PKS (DEBS1-TE) consisting of modules 1 and 2 and the chain-terminating thioesterase (end) of DEBS (*9*). (**B**) Mutant strain NRRL2338/pAVLD (ERMD1) of *S. erythraea* contains the hybrid *avr*-lm-DEBS1 multienzyme, DEBS2, DEBS3, and the auxiliary genes required to convert 6-deoxyerythronolide into erythromycin A (*6*). The active sites in each domain of DEBS are ketosynthase (KS), acyl carrier protein (ACP), acyltransferase (AT), dehydratase (DH), ketoreductase (KR), enoylreductase (ER), and thioesterase (TE). The erythromycins produced include analogs of erythromycins A, B, and D.

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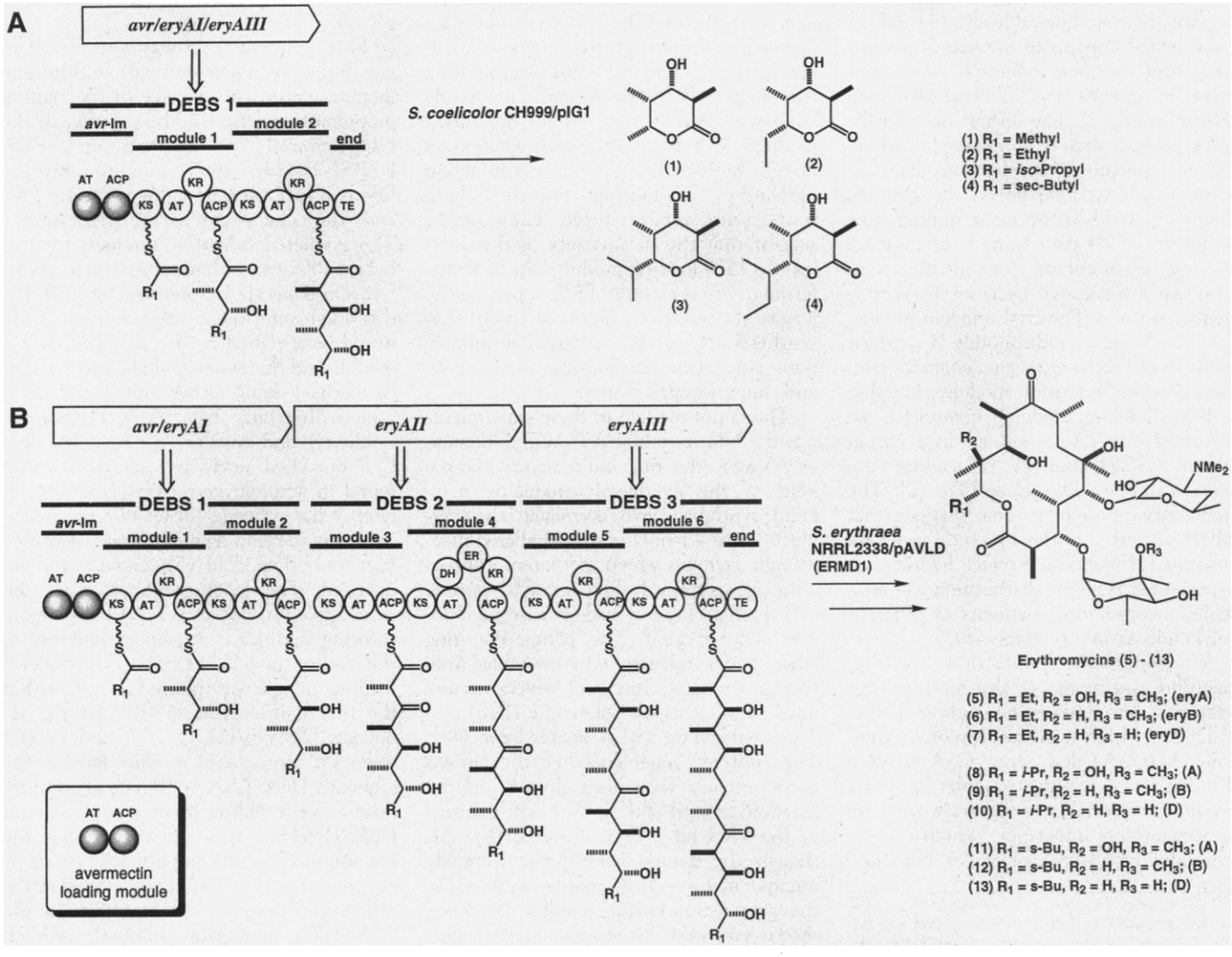

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Femtosecond Dynamics of Electron Localization at Interfaces

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The dynamics of two-dimensional small-polaron formation at ultrathin alkane layers on a silver(111) surface have been studied with femtosecond time- and angle-resolved two-photon photoemission spectroscopy. Optical excitation creates interfacial electrons in quasi-free states for motion parallel to the interface. These initially delocalized electrons self-trap as small polarons in a localized state within a few hundred femtoseconds. The localized electrons then decay back to the metal within picoseconds by tunneling through the adlayer potential barrier. The energy dependence of the self-trapping rate has been measured and modeled with a theory analogous to electron transfer theory. This analysis determines the inter- and intramolecular vibrational modes of the overlayer responsible for self-trapping as well as the relaxation energy of the overlayer molecular lattice. These results for a model interface contribute to the fundamental picture of electron behavior in weakly bonded solids and can lead to better understanding of carrier dynamics in many different systems, including organic light-emitting diodes.

Polarization interactions and the localization of charge carriers in condensed media continue to represent a challenge for theoretical descriptions of the interaction of a carrier with its environment. Understanding charge localization is important in determining the electronic and optical properties of applied materials and in the development of new materials. Despite extensive study of charge localization phenomena, electron localization at interfaces between dissimilar materials, such as metal-dielectric interfaces, remains largely unexplored. Electrons in metals are usually free-electron–like, whereas electrons in dielectric solids tend to be localized as small polarons (*1*). Small polarons form when charges become localized in self-induced potentials as a result of strong carrier-lattice interactions (*2*). These self-trapped carriers and the associated lattice relaxation affect a wide range of phenomena, such as photochemical defect formation (*3*), atomic desorption from solid surfaces (*4*), and various properties of high-temperature superconducting oxides (*5*). Here, we show how the transition from delocalized to localized electronic behavior near the metal-dielectric interface occurs dynamically.

The combination of recently developed angle-resolved two-photon photoemission (TPPE) (*6*) and femtosecond laser techniques provides a unique opportunity to study the dynamics of carrier localization at interfaces. On bare metal surfaces such as Ag(111), the image potential supports a Rydberg series of bound states characterized by the principal quantum number n with the electron density residing largely in the vacuum. Excess electrons in these image potential states are localized in hydrogenic wave functions normal to the surface, but are delocalized as plane waves parallel to the surface (*7*). If dielectric layers are grown on the metal substrate, one can explore the layer-by-layer evolution of the potential and the electronic structure of the interface by measuring the changes in the binding energy, effective mass, and lifetime of electrons excited into image potential states (*8*). With femtosecond time resolution, we can directly observe that these delocalized electrons become localized in the presence of dielectric layers. We address the underlying physical principles that lead to localization for excess electrons at multiple n-alkane layers on Ag(111).

In an angle-resolved TPPE experiment (Fig. 1A), a pump pulse excites electrons from occupied metal states into unoccupied interfacial states, and a probe pulse ejects the excited electrons into the vacuum where the electron kinetic energy, E_{kin}, is measured at various angles, θ. From the angular dependence of the kinetic energy spectrum, one can determine both the binding energy and the effective mass, m^*, of the interfacial electrons. The relation between m^*, E_{kin}, and the electron wave vector parallel to the surface, $k_{\|}$, is given by

$$E_{kin} = \hbar^2 k_{\|}^2/2m^* + E_0 \qquad (1)$$

where

$$k_{\|} = (2m_e E_{kin}/\hbar^2)^{1/2}\sin\theta \qquad (2)$$

$\hbar$ is Planck's constant ($\hbar = h/2\pi$), and E_0 is the kinetic energy for emission normal to the surface. An electron behaving like a free particle parallel to the interface will exhibit a dispersive band with an m^* close to the free electron mass, m_e. A spatially localized electron results in a nondispersive feature characterized by a flat energy band with a very large m^* (Fig. 1A).

The details of the TPPE experimental apparatus have been reported in (*9*). In n-heptane bilayer data taken at 120 K with two different pump-probe delays, a dispersive feature with $m^* = 1.2\ m_e$ appears at zero time delay (Fig. 1B), whereas a nondispersive feature appears in the spectra taken at a 1670-fs delay (Fig. 1C). These two features correspond, respectively, to the delocalized and localized states (*10*). The femtosecond TPPE data clearly reveal a delay in the formation of the localized state. Similar behavior is found for a monolayer and a trilayer. Lingle *et al.* studied various alkane-Ag(111) interfaces (*10*), investigating the effects of different preparation methods and annealing procedures on localization, and found no significant effects. In addition, the proportion of monolayer patches interspersed with bilayer (apparent in Fig. 1B) can be varied without variation in the bilayer dynamics. These experiments indicate that the observed localization phenomenon and dynamics are not controlled by defects in the layer, but rather are linked to the intrinsic two-dimensional (2D) properties of the layer.

We measured the dynamics of the localized and delocalized features at 120 K for various angles (Fig. 2). The delocalized fea-

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