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ORIGINAL ARTICLE

Improved precursor-directed biosynthesis in *E. coli* via directed evolution

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Erythromycin and related macrolide antibiotics are widely used polyketide natural products. We have evolved an engineered biosynthetic pathway in *Escherichia coli* that yields erythromycin analogs from simple synthetic precursors. Multiple rounds of mutagenesis and screening led to the identification of new mutant strains with improved efficiency for precursor-directed biosynthesis. Genetic and biochemical analysis suggested that the phenotypically relevant alterations in these mutant strains were localized exclusively to the host-vector system, and not to the polyketide synthase. We also demonstrate the utility of this improved system through engineered biosynthesis of a novel alkynyl erythromycin derivative with comparable antibacterial activity to its natural counterpart. In addition to reinforcing the power of directed evolution for engineering macrolide biosynthesis, our studies have identified a new lead substance for investigating structure–function relationships in the bacterial ribosome.
The Journal of Antibiotics (2011) **64**, 59–64; doi:10.1038/ja.2010.129; published online 17 November 2010

INTRODUCTION

Polyketides form an important class of natural products having a wide range of uses, including as anti-infective agents, antitumor drugs, immunosuppressants and veterinary products.[1,2] The emergence of antibiotic resistance is a serious problem[3] and is fueling the demand for new antibiotics. The multi-modular structure of certain bacterial polyketide synthases (PKS) has made them attractive targets for protein engineering to produce improved drug candidates.[4]

Precursor-directed biosynthesis[5–7] is a powerful approach to expand the range of engineered macrolide antibiotics, especially in conjunction with semisynthesis[8] and hybrid PKS construction.[9,10] Precursor-directed biosynthesis combines the complementary power of synthetic chemistry, which allows a virtually unlimited range of simple compounds, and biosynthesis, which routinely creates complex molecules[11] from simple precursors. Although the range of natural precursors for polyketide biosynthesis is relatively limited,[12] precursor-directed biosynthesis enables the incorporation of unnatural precursors with diverse functional groups, expanding the pool of 'unnatural' natural products. Additionally, regio- and chemo-selectively modified and modifiable functional groups can be introduced easily using this approach.

Notwithstanding its promise, there are significant limitations to precursor-directed biosynthesis. The feeding of labile precursors to *Streptomyces* or *Saccharopolyspora* bacteria that naturally produce bioactive polyketides is fraught with challenges.[11,13–15] By contrast, incorporation of precursors into heterologous polyketide biosynthetic

pathways in *E. coli* is relatively efficient,[16] but such systems typically yield inactive polyketide intermediates, which have not undergone post-PKS modifications. Furthermore, due to the low affinity of the acceptor PKS module for unnatural thioester substrates,[16] precursors are often required to be fed in high concentration to the engineered strains to effectively transform precursors into polyketide products. The substrate specificity of one or more downstream PKS modules may also cause kinetic bottlenecks in the production of polyketides by precursor-directed biosynthesis.[11,14]

Recently, the entire erythromycin biosynthetic pathway has been reconstituted in *E. coli*,[17,18] enabling the production of active antibacterial agents in this heterologous host. A rapid and sensitive screen for detecting erythromycin from precursor-directed biosynthesis was also established.[17] Here, we have modified the erythromycin biosynthetic pathway in a directed manner increasing titers in *E. coli*.

The feasibility of producing inactive macrolides aglycones in *E. coli* by precursor-directed biosynthesis was originally demonstrated using an engineered host BAP1 containing plasmids pBP175, which encodes for 6-deoxyerythronolide B synthase (DEBS) module 2 and propionyl-CoA carboxylase, and pBP130, which encodes for DEBS2 and DEBS3 (Figure 1; Table 1).[16] Subsequently, we engineered two additional compatible plasmids: pHL80, which encodes mycarose biosynthetic and glycosyl transfer functions and pHL74, which encodes desosamine biosynthetic and glycosyl transfer functions (Table 1) to produce bioactive glycosylated macrolides.[17] *E. coli* BAP1/pBP175/pBP130/pHL80/pHL74 produces the antibiotic 6-deoxyerythromycin D

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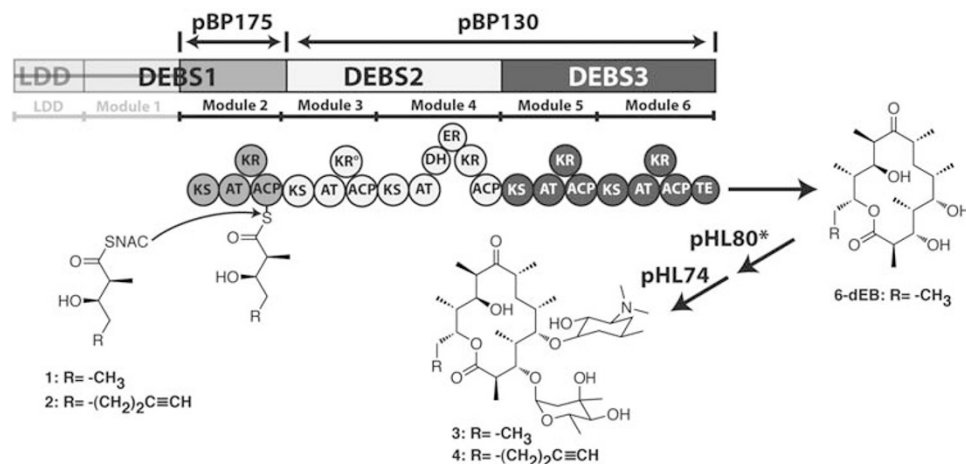E-mail: khosla@stanford.edu

Figure 1 Precursor-directed biosynthesis of 6-deoxyerythromycin D and analogs. A truncated derivative of the 6-deoxyerythronolide B synthase was constructed lacking the loading module and module 1 of DEBS1.[14] *E. coli* cells harboring this truncated DEBS, along with plasmids responsible for mycarosyl and desosaminyl sugar biosynthesis and transfer, are able to synthesize 6-deoxyerythromycin D (**3**) or its analog (**4**) when fed with an appropriate diketide analog precursor (**1** or **2**).

Table 1 Plasmids used

Plasmid	Encoded proteins	Function	Vector
pBP130	DEBS2–DEBS3	Biosynthesis of 6dEB	pET21
pBP144	PccAB-DEBS1		pET28
pBP175	PccAB-DEBS mod2		pET28
pBP190	thioesterase II (TEII)		pGZ119
pHL74	EryCIII-EryCII-TylAI-DesIV-DesI-DesII-DesV-DesVI	TDP-desosamine biosynthesis and transfer	pGZ119
pHL80	GroES-GroEL-TylCVII-EryBIV-EryBVI-EryBV-TylCIII-EryBII	TDP-mycarose biosynthesis and transfer+chaperone	pCDF-Duet

Abbreviation: 6dEB, 6-deoxyerythronolide B.

(6d-EryD) when fed with compound **1** (Figure 1), a synthetic cell permeable mimic of the natural diketide intermediate in erythromycin biosynthesis.[17] Through directed evolution, we also isolated a mutant plasmid pHL80*, which substantially improves mycarosylation of the aglycone compared with wild-type pHL80, facilitating improved precursor-directed biosynthesis.[17] A simple, visual single colony screening assay was used to isolate a mutant of *E. coli* BAP1/ pBP175/pBP130/pHL80*/pHL74 (designated mutant D), which showed a >3-fold improvement in the amount of 6-deoxyerythromycin D production with high (>0.3 mM) concentrations of diketide **1**.[17] Here we have analyzed mutant D with the goal of understanding the molecular basis of this phenotype, and have extended this finding through additional rounds of directed evolution toward improved mutants. We identified and analyzed several new mutants to explain the improved erythromycin biosynthesis from simple precursors, and demonstrated their utilities for the engineered biosynthesis of a novel derivative of erythromycin with promising antibacterial properties.

MATERIALS AND METHODS
Analysis of mutant D
Mutants showing improved conversion of diketide **1** into erythromycin were grown in liquid Luria-Bertani (LB) medium containing kanamycin, carbenicillin, streptomycin and chloramphenicol, and the plasmids were isolated from each culture. Each plasmid was separated by re-transforming a dilute sample of the plasmid mixture into *E. coli* XL1-Blue and isolating transformants that

expressed only the desired antibiotic resistance gene. Each purified plasmid was then co-transformed along with other wild-type plasmids to obtain *E. coli* HYL3/pBP175(*)/pBP130(*)/pHL80*(*)/pHL74(*) (* refers to plasmid derived from the mutant strain). These transformants were analyzed by plate-based assays to identify the plasmid responsible for the desired phenotype.

Quantifying relative protein expression in BAP1 vs HYL3
To compare the relative expression levels of DEBS module 2 in the wild-type (BAP1) and evolved (HYL3) hosts, pBP175 was introduced into each via transformation. As described earlier,[19] cells were grown at 37 °C in LB medium with 50 μg ml^{-1} kanamycin with an $OD_{600}=0.6$, then chilled on ice for 10 min, and induced at 20 °C with 0.2 mM isopropyl β-D-1-thiogalactopyranoside for 15 h. Thereafter, the cells were harvested by centrifugation (4500 *g*, 15 min) and disrupted by sonication (10×30 s, buffer: 50 mM sodium phosphate, pH 8.0, 10 mM imidazole, 0.3 M NaCl, 1 mM dithiothreitol, 20% glycerol). Cellular debris was removed by centrifugation (17 000 *g*, 45 min), and DEBS module 2, as well as PccA and PccB (both have similar molecular masses and therefore migrate as a single band) were purified using nickel affinity chromatography, with protein elution at 200 mM imidazole (buffer: 50 mM sodium phosphate, pH 8.0, 200 mM imidazole, 0.3 M NaCl, 1 mM dithiothreitol, 20% glycerol).

Generation of pBP175 library
Plasmid pBP175 was mutagenized using a mutator strain XL1-Red. The wild-type plasmid was transformed to XL1-Red and selected with kanamycin (50 mg l^{-1}) on LB plate. Approximately 200–2000 colonies were collected and grown to stationary phase in LB media with kanamycin. The culture

was used to re-inoculate LB medium with kanamycin (1% inoculum volume) and also to prepare pBP175 plasmid DNA. These steps were repeated seven times. At the end of the entire procedure, individual plasmid preparations from each step were combined in equal quantities to yield a mutagenized pBP175 sample, which was transformed into HYL3/pHL80*/pHL74 along with pBP130. Approximately 10^3 transformants were screened as described below.

Screening of pBP175 library
The library of *E. coli* HYL3/pBP175/pBP130/pHL80*/pHL74 was plated on a cellophane disk placed on LB agar containing $1\,mg\,ml^{-1}$ sodium propionate and $10\,\mu M$ diketide **1**. The plates were incubated at $30\,^{\circ}C$ for 60–68 h. The cellophane was then transferred onto a second plate while soft agar containing *Bacillus subtilis* was overlaid onto the original plate. After overnight incubation at $30\,^{\circ}C$, halos of growth inhibition were observed. Colonies producing the biggest halos were isolated from the secondary plate and reanalyzed to verify the phenotype.

Biosynthesis of macrolides in shake-flask cultures
E. coli BAP1/pBP175/pBP130/pHL80*/pHL74 (or any derivative mutant) was grown in LB medium containing $50\,\mu g\,ml^{-1}$ kanamycin, $100\,\mu g\,ml^{-1}$ carbenicillin, $50\,\mu g\,ml^{-1}$ streptomycin and $34\,\mu g\,ml^{-1}$ chloramphenicol with $OD_{600}=0.6$. The cells were chilled on ice for 10 min and centrifuged briefly. After washing with LB, the cell pellet was resuspended in 5% of the original volume of LB (that is; 20-fold concentration) without antibiotics. Following addition of 0.1–0.3 mM isopropyl β-D-1-thiogalactopyranoside and 0.1–1 mM diketide precursor, cell growth was allowed to proceed at $30\,^{\circ}C$ for additional 72 h. To quantify 6-deoxyerythromycin D (or analog) in the culture medium, serial (two-fold) dilutions of the $0.2\,\mu m$ filter-sterilized culture medium were added to freshly inoculated cultures of *B. subtilis*. No exogenous antibiotics were used during the growth of the *E. coli* culture to be tested. The growth inhibition of *B. subtilis*, monitored by measuring OD_{490} after $\sim 20\,h$ at $30\,^{\circ}C$ in a 96-well plate reader, was used to determine the titer of antibiotic. Any apparent discrepancy between the titers shown here in Figure 3 and those in previous reports[17] is a result of the lack, until recently, of an authentic standard of 6d-Ery D. All previously reported titers were calculated based on a similar, more potent, antibiotic.

Plasmid stability analysis
To quantify plasmid stability in a recombinant *E. coli* strain, the cell line was grown overnight in LB medium containing $50\,\mu g\,ml^{-1}$ kanamycin, $100\,\mu g\,ml^{-1}$ carbenicillin, $50\,\mu g\,ml^{-1}$ streptomycin and $34\,\mu g\,ml^{-1}$ chloramphenicol. The culture was diluted 10^6-fold, and $100\,\mu l$ was spread on an LB agar petridish containing each antibiotic individually as well as on control plates containing all antibiotics or lacking any antibiotic. Plasmid stability was estimated by comparing the number of colonies on plates with individual antibiotics with the control plates.

Construction of hybrid plasmids
To localize mutations on mutant plasmids pBP175#1 and pBP175#8, three different hybrid plasmids were constructed in each case using the unique *Pac*I, *Pst*I and/or *Xba*I sites on this plasmid.[17] The first hybrid contained the vector from wild-type pBP175 and the DEBS module 2 and *PccAB* genes from the mutant plasmid, whereas the second hybrid had the vector from the mutant plasmid and the wild-type genes. In the third hybrid, the *PccAB* genes alone from the mutant plasmid were swapped with the corresponding genes from the wild-type plasmid. The phenotype of each hybrid plasmid was analyzed in *E. coli* HYL3 along with wild-type plasmids pBP130, pHL80* and pHL74.

Preparative production and isolation of 6-deoxyerythromycin D
E. coli HYL3/pBP130/ pBP144/pHL74/pHL80* cells were grown in LB media containing $50\,\mu g\,ml^{-1}$ kanamycin, $100\,\mu g\,ml^{-1}$ carbenicillin, $50\,\mu g\,ml^{-1}$ streptomycin and $34\,\mu g\,ml^{-1}$ chloramphenicol at $37\,^{\circ}C$ until $OD_{600}=0.4–0.5$. The cell pellets were collected by centrifugation and resuspended in 5% of the original volume of $4\,^{\circ}C$ LB media supplemented with antibiotics at concentrations mentioned above. The cells were induced with $500\,\mu M$ isopropyl

β-D-1-thiogalactopyranoside along with the addition of $2.5\,g\,l^{-1}$ sodium propionate and left to shake at $30\,^{\circ}C$ for 72 h.

After the 72 h production phase, the culture broth was clarified by centrifugation for 1 h at $4420\,g$. The supernatant was basified to pH=12 with 2 M NaOH and extracted with EtOAc. The organic phase was dried over Na_2SO_4, and the solvent was removed *in vacuo*. Preliminary purification through an acid–base back extraction was performed based on the presence of a tertiary amine on the target molecule. The crude extract resuspended in 10 ml water, acidified to pH=2 with 2 M HCl and washed with 10 ml Et_2O five times. The aqueous phase was then basified to pH=11 with 2 M NaOH and extracted with 10 ml EtOAc five times. The EtOAc extracts were combined, dried over Na_2SO_4 and the solvent was removed by evaporation. For the final purification, HPLC was used with a Zorbax RX-C8 column ($9.4\times250\,mm$, Agilent Technologies, Santa Clara, CA, USA) with a solvent system of CH_3CN/H_2O each with 0.1% formic acid. The desired product eluted in 45–50 min (CH_3CN gradient: 0–25% in 10 min, 25–40% in 80 min, 40–95% in 10 min, 95% for 10 min; detection at 210 nm). The identity of 6-deoxyerythromcin D was confirmed by high-resolution mass spectrometric (Supplementary Information) and NMR analysis (Supplementary Information). The MIC of this compound was determined to be 0.04 μg/ml against *B. subtilis*.

RESULTS AND DISCUSSION
Analysis of the mutant D
Individual plasmids were isolated from mutant D and re-transformed into *E. coli* BAP1 along with the remaining complement of wild-type plasmids. In addition, mutant D was cured of all plasmids by propagation in the absence of selection markers and was subsequently transformed with a full complement of wild-type plasmids. At least four clones of each lineage were independently isolated and compared with both mutant D and wild-type *E. coli* BAP1/pBP175/pBP130/pHL80*/pHL74 using single colony assays on plates that included $10\,\mu M$ diketide **1**. Unexpectedly, the overproduction phenotype could be exclusively assigned to the re-transformed derivative of the cured host cell derived from mutant D (data not shown). This new cured host, designated *E. coli* HYL3, was therefore assumed to carry the phenotypically relevant genetic change identified in mutant D, and was investigated further as described below.

To determine if the mutant strain only improved diketide-directed biosynthesis of 6-deoxyerythromyin D, or whether it could also enhance macrolide production upon expression of the complete biosynthetic pathway, *E. coli* HYL3/pBP144/pBP130/pHL80*/pHL74 was compared with its BAP1 counterpart using single colony assays. No statistical difference was observed between the two host cells. We also compared two related aglycone-producing systems, HYL3/pBP175/pBP130/pBP190 vs BAP1/pBP175/pBP130/pBP190. Plasmid pBP190 encoding the thioesterase II from *Saccharopolyspora erythraea* was included in these cell lines[19] (Table 1). The former strain produced more 6-deoxyerythronolide B than the latter strain in the presence of diketide **1** (Figure 2a). Taken together, these results highlight the ability of HYL3 to increase polyketide production while suggesting that the phenotypically relevant mutation functions either through increasing the efficiency of acylation of module 2 by compound **1** or by increasing the intracellular concentrations of stand alone module 2.

To evaluate these options, the expression levels of the biosynthetically relevant proteins were compared between the two hosts. *E. coli* HYL3/pBP175 showed a $\sim$threefold higher expression of soluble module 2 than BAP1/pBP175 (Figure 2b). Owing to their large size and codon usage differences between the native and heterologous hosts, type I polyketide synthase proteins are generally not expressed well in *E. coli*.[20] Previous work has shown the loading of diketide thioester substrates onto DEBS module 2 to be slow compared with
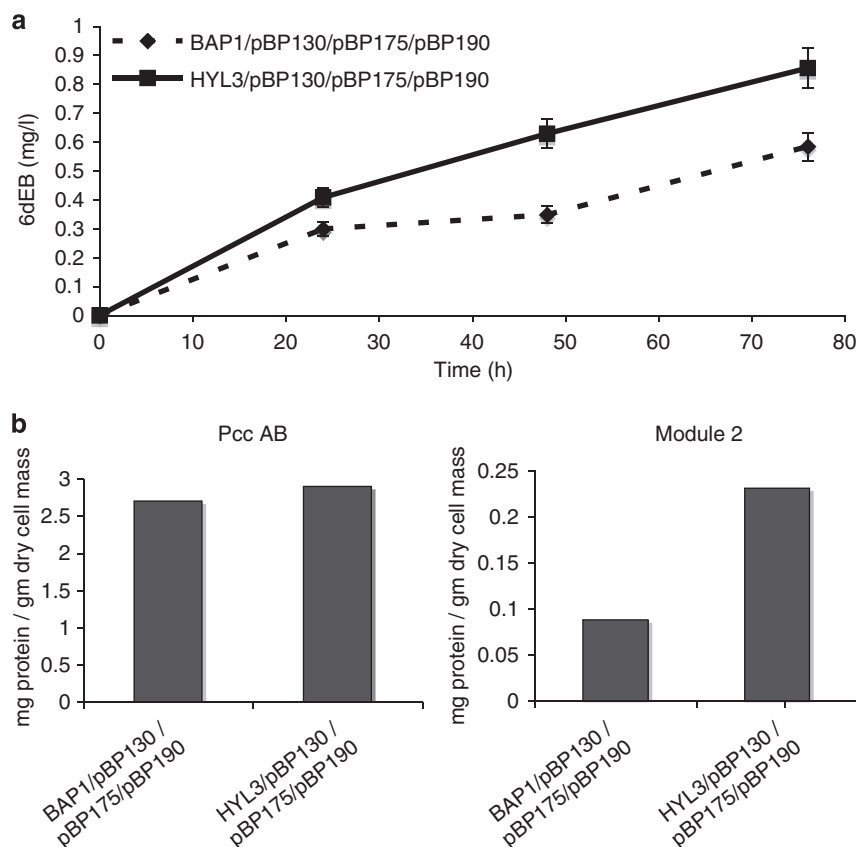
Figure 2 Analysis of *E. coli* HYL3 mutant: (**a**) biosynthesis of 6-deoxyerythronolide B by *E. coli* strains BAP1/pBP175/pBP130/pBP190 and HYL3/pBP175/pBP130/pBP190. (**b**) Densitometric quantitation of DEBS module 2 and PccAB expressed in BAP1/pBP175 and HYL3/pBP175.

subsequent steps, suggesting that the increased concentration of module 2 likely relieves a kinetic bottleneck in precursor-directed biosynthesis of erythromycin antibiotics.

As shown below, the ability of *E. coli* HYL3 to effect increased erythromycin production by precursor-directed biosynthesis is not exclusive to the wild-type system as it also led to increased titers of **4**, an unnatural alkynyl derivative. (Figures 3a and b).

Directed evolution of plasmid pBP175
The above result suggested that DEBS module 2 could be an attractive target for evolution aimed at further enhancing precursor-directed biosynthesis of macrolides. In particular, due to the high cost of synthetic precursors and the strong dependence of polyketide production on exogenous precursor concentrations[21] we sought to identify mutants capable of more efficient transformation of low concentrations of synthetic precursors into macrolides. We therefore subjected plasmid pBP175 to random mutagenesis by passing it through the mutator strain of *E. coli*, XL1-Red,[22] for multiple generations. Plasmid DNA was isolated and co-transformed into HYL3 along with unmutated plasmids pBP130, pHL80* and pHL74. Approximately 500–1000 colonies from the resulting library of transformants were screened in a single colony assay for mutants that showed larger zones of growth inhibition of *B. subtilis* than HYL3/pBP175/pBP130/pHL80*/pHL74 in the presence of limiting (10 μM) concentrations of diketide **1**. Two mutants, designated mutant #1 and mutant #8, were isolated and purified by re-streaking and re-testing. As discussed below, their enhanced ability to convert synthetic precursors into corresponding macrolides is not limited to diketide **1** alone (Figure 3a). By isolating

and characterizing the host cell and each plasmid from mutant #1 and mutant #8, it was deduced that both mutants contained phenotypically relevant changes in plasmid pBP175, which were designated pBP175 #1 and pBP175 #8, respectively.

Analysis of mutant plasmids pBP175 #1 and pBP175 #8
Because of the large size of plasmid pBP175, sequencing was not considered a practical strategy for decoding the mechanistic basis for enhanced precursor-directed biosynthesis of pBP175 #1 or pBP175 #8. Alternatively, we investigated several properties of each mutant plasmid, including copy number, stability and level of module 2 expression. Although these represented the pertinent altered phenotypes within previous studies of pHL80* and the HYL3 host,[17] no statistical differences in these properties were observed here between wild-type pBP175 and either mutant plasmid. We therefore constructed three hybrid plasmids each from pBP175 #1 and pBP175 #8, as described in the materials and methods section. Each hybrid plasmid contained a portion of the mutant plasmid with the remainder being derived from wild-type pBP175. In the case of both mutant #1 and mutant #8, the phenotypically relevant mutation was exclusively localized to the vector portion, as opposed to either the DEBS module 2 or *PccAB* genes.

Given the apparently contradictory observations that the vector portion of plasmid pBP175 had undergone mutation albeit without affecting its own copy number, stability or protein expression level, we speculated that these genetic changes may induce a phenotypic benefit for one or more of the other plasmids in the recombinant cell lines. Among plasmids pBP130, pBP175, pHL80* and pHL74,
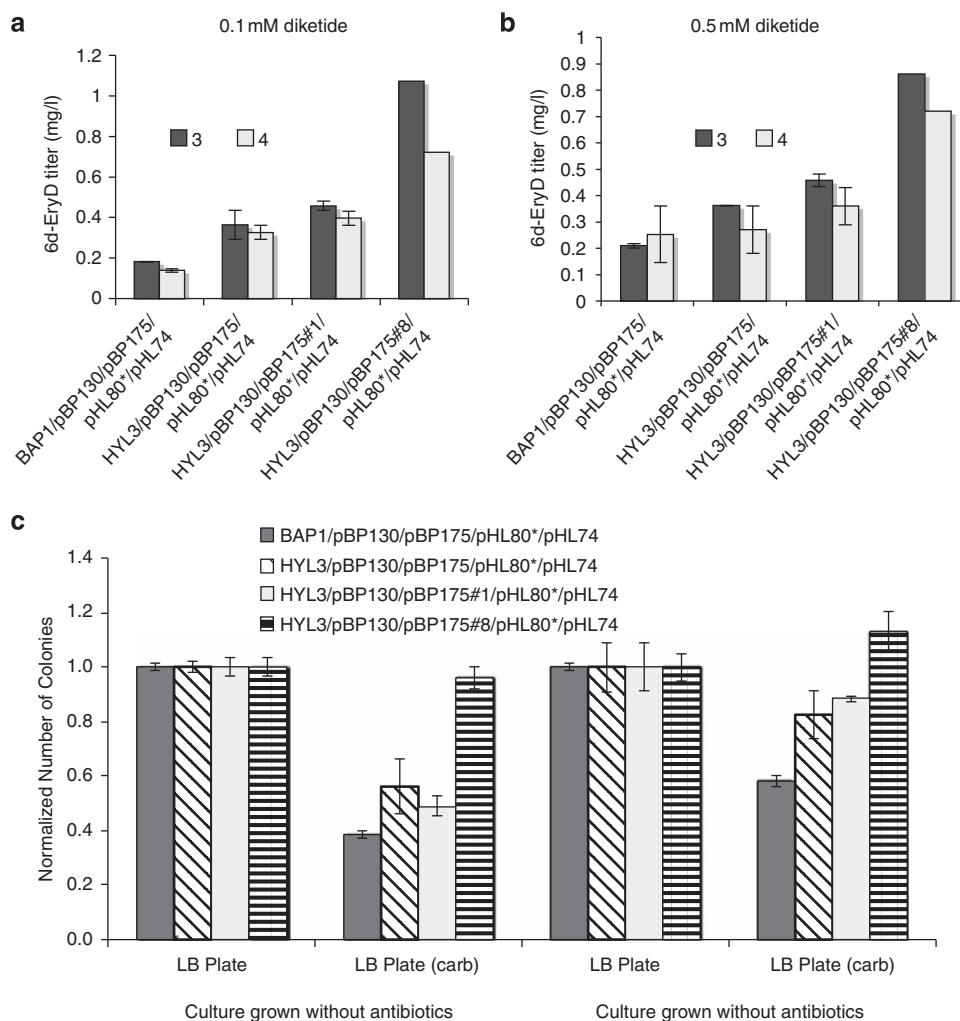
Figure 3 Characterization of mutant *E.coli* strains. (**a**, **b**) Comparison of macrolide production by wild-type and mutant *E. coli* cell lines: biosynthesis of 6-deoxyerythromycin D (**3**) and alkynyl-6-deoxyerythromycin D (**4**) by *E. coli* BAP1/pBP175/pBP130/pHL80*/pHL74, HYL3/pBP175/pBP130/pHL80*/pHL74, HYL3/pBP175 #1/pBP130/pHL80*/pHL74 and HYL3/pBP175 #8/pBP130/pHL80*/pHL74 when fed **1** and **2**, respectively at $100\,\mu M$ in (**a**) and $500\,\mu M$ in (**b**). For experimental details, see text. (**c**) Plasmid stability of pBP130 in *E. coli* BAP1/pBP175/pBP130/pHL80*/pHL74, HYL3/pBP175/pBP130/pHL80*/pHL74, HYL3/pBP175 #1/pBP130/pHL80*/pHL74 and HYL3/pBP175 #8/pBP130/pHL80*/pHL74 was compared by counting the number of cells having pBP130 (Carb[R], MP Biomedicals, Solon, OH, USA) after grown for 12 h at $37\,°C$.

plasmid pBP130 has the lowest stability with approximately 40% retention when a culture of *E. coli* BAP1/pBP175/pBP130/pHYL80*/pHYL74 is grown for 12 h in the absence of antibiotic (Figure 3c). Plasmids pBP130 and pBP175 have the same origin of replication (pBR322), a feature that is known to induce plasmid incompatibility.[23–25] The larger size of pBP130 presumably makes it less stable than pBP175 due to a slower rate of replication.[26] As shown in Figure 3c, the stability of pBP130 is enhanced significantly in the presence of pBP175 #1 and to an even greater extent when coexpressed with pBP175 #8. Taken together, these results suggested that the phenotypically relevant mutations were related to the origin of replication of mutant plasmids such that the competition between pBP175 and pBP130 is relieved.[27]

Sequencing of the origin of replication of pBP175 #8 revealed a single T to C mutation as compared with the wild-type pBR322 origin, supporting that the phenotypically relevant mutation is in this region of the plasmid (Supplementary Information).

Improved precursor-directed biosynthesis of novel macrolide antibiotics

To test the relevance of the above results for precursor-directed biosynthesis of *unnatural* erythromycin analogs, we targeted preparation of an unnatural macrolide bearing an alkynyl functional group. Alkynes can be used as particularly convenient handles for further semisynthetic modification using cycloaddition chemistry.[28–30] An alkynyl diketide substrate (**2**, Figure 1, Supplementary Information) was prepared and fed to the various cell lines as detailed in the materials and methods section. The presence of macrolide **4** and absence of compound **3** in liquid cultures that were fed diketide **2** was confirmed by high-resolution MS. This result suggests that the bioactivity observed in these experiments does arise from the production of a novel alkynyl erythromycin analog (Supplementary Information). The bioconversion efficiency of diketide **2** was comparable to that of diketide **1**, and the relative productivity trends observed between BAP1/pBP175/pBP130/pHL80*/pHL74 and various mutant

strains characterized in this study were also similar for the two precursors (Figures 3a and b). These results reinforce the utility of *E. coli* HYL3/pBP175#8/pBP130/pHL80*/pHL74 as a superior cellular biocatalyst for precursor-directed biosynthesis of erythromycin analogs.

In summary, we have evolved a system capable of improved production of erythromycin-type macrolide antibiotics by precursor-directed biosynthesis in *E. coli*. The parent producer of 6-deoxyerythromycin D was subjected to two rounds of random mutagenesis and with the improved mutants identified via activity-based single colony screening procedures. Phenotypic characterization revealed no changes in the biosynthetic proteins themselves, but rather a genetically more stable system in which altered protein expression relieved a kinetic bottleneck present in the wild-type system. The best of these mutants is not only able to produce four-fold more antibiotics, but also does so at a relatively low precursor concentration. Finally, we have demonstrated the utility of this system by, for the first time, reporting the production of a novel erythromycin analog derived from a simple synthetic precursor in a single *E. coli* cell line. This new antibiotic not only displays promising antibacterial activity, but also possesses an attractive handle for semisynthetic modification.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)