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Genetic Modulation of the Overexpression of Tailoring Genes *eryK* and *eryG* Leading to the Improvement of Erythromycin A Purity and Production in *Saccharopolyspora erythraea* Fermentation[▽]

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Erythromycin A (Er-A) is the most potent and clinically important member in the Er family produced by *Saccharopolyspora erythraea*. Er-B and Er-C, which are biologically much less active and cause greater side effects than Er-A, serve as the intermediates for Er-A biosynthesis and impurities in fermentation processes of many industrial strains. In this study, systematical modulation of the amounts of tailoring enzymes EryK (a P450 hydroxylase) and EryG (an *S*-adenosylmethionine-dependent *O*-methyltransferase) was carried out by genetic engineering in *S. erythraea*, including alterations of gene copy number ratio and organization and integrating the locus on the chromosome by homologous recombination. Introduction of additional *eryK* and *eryG* genes into *S. erythraea* showed significant impacts on their transcription levels and enhanced the biotransformation process from Er-D to Er-A with gene dose effects. At the *eryK*/*eryG* copy number ratio of 3:2 as well as their resultant transcript ratio of around 2.5:1 to 3.0:1, Er-B and Er-C were nearly completely eliminated and accordingly converted to Er-A, and the Er titer was improved by around 25% in the recombinant strain ZL1004 (genotype *PermK*-K-K-G + PermE*-K + PermA*-G*) and ZL1007 (genotype *PermK*-K-G-K + PermE*-K + PermA*-G*). This study may contribute to the continuous efforts toward further evaluation of the Er-producing system, with the aims of improving Er-A purity and production at the fermentation stage and lowering the production costs and environmental concerns in industry.

Erythromycins (Ers), produced by an actinomycete, *Saccharopolyspora erythraea*, are a group of important antibiotics with broad-spectrum activity against pathogenic gram-positive bacteria (13, 27). Er-A, the most potent and most widely clinically used member in this family, contains a characteristic 14-membered macrolide that is decorated by two unusual deoxysugars, mycarose and desosamine (Fig. 1). Its importance in infectious disease therapy is further highlighted by the fact that several Er-A-based semisynthetic derivatives known as expanded-spectrum and broad-spectrum products (e.g., azithromycin, flurithromycin, and telithromycin) are commercially successful and widely used in the clinic (8), continuously prompting efforts to improve Er-A production by *S. erythraea*. To obtain overproducing strains for industrial production of a certain antibiotic, wild-type actinomycete strains have been often subjected to multiple rounds of random mutagenesis followed by large-scale screening of the improved biological activities (1, 19). Although many cycles of such a classical program were carried out on *S. erythraea* and yielded strains with more than 100-fold improvement, the Er-A titer in fermentation culture remains limited and 5- to 10-fold lower than those of several other secondary metabolites, such as penicillin and cephalosporin C (4).

Due to their model molecular architecture and commercial importance, Ers have long been the focus of intensive research activity in the field of natural product biosynthesis (27). Briefly, as shown in Fig. 1, the biosynthesis of Ers can be divided into two phases. In the first, constructive phase, a set of multifunctional type I polyketide synthases (PKSs), namely, 6-deoxyerythronolide B (6-DEB) synthases 1 to 3, catalyze the assembly of the polyketide backbone from one propionyl coenzyme A (CoA) and six methylmalonyl-CoAs by sequentially decarboxylative condensations. The resulting polyketide chain then undergoes an intramolecular cyclization to give the first macrocyclic lactone intermediate, 6-DEB. In the second phase, a series of tailoring proteins sequentially carry out elaborate modifications, including regiospecific hydroxylations, glycosylations, and a methylation, on 6-DEB to finally produce Er-A. While the PKSs are responsible for the Er skeleton formation in phase I, the tailoring modifications in phase II are essential for producing the active antibiotics. Based on this characterized biosynthetic logic, genetic engineering of the Er-A pathway has produced substantial novel macrolide analogs that are not available by chemical synthesis (31). Furthermore, the genome sequence of *S. erythraea* NRRL2338 (which is the original soil isolate but phylogenetically related to the improved-production strains used by many companies) was released very recently (18) and is undoubtedly of considerable practical value for understanding this globally regulatory and biosynthetic machinery and boosting production of Ers and their analogs in *S. erythraea* (11).

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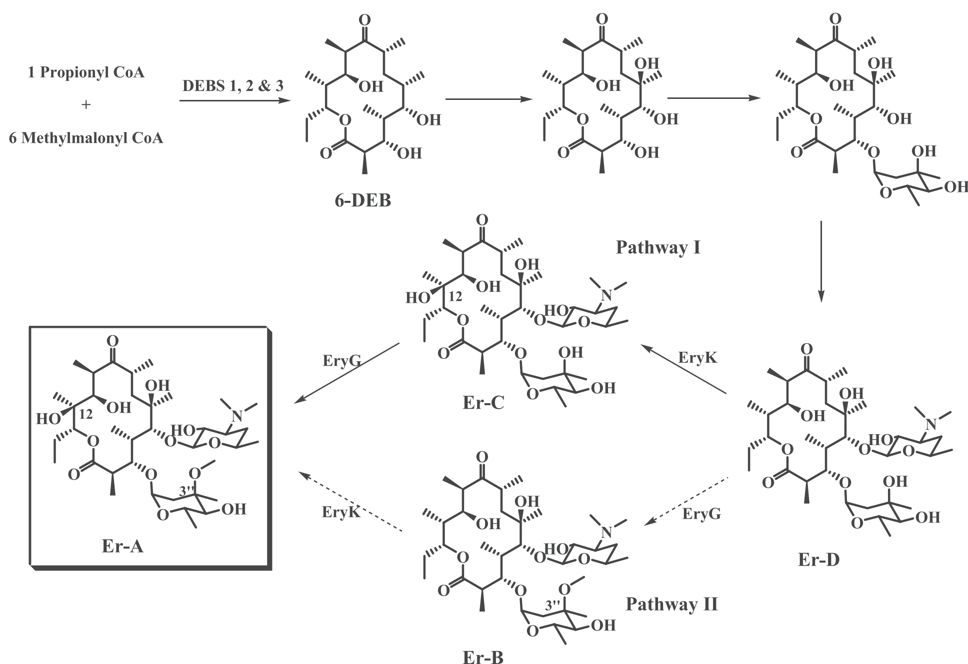

FIG. 1. Biosynthetic pathway and structures of Er-A, Er-B, Er-C, and Er-D. The dashed arrows indicate the shunt pathway (II).

A few members in the Er family, including Er-B, Er-C, and Er-D, occur as the intermediates in the biosynthetic pathway of Er-A (Fig. 1). The biotransformation of Er-D into the desired major component Er-A involves two final enzymatic reactions, EryK-catalyzed hydroxylation at the C-12 position of the aglycone (26) and EryG-catalyzed O methylation at the C-3′ position of macrose (10, 20). Previous biochemical and kinetic studies with EryK confirmed its 1,200- to 1,900-fold preference for Er-D over Er-B (14), supporting the idea that the C-12 hydroxylation catalyzed by EryK on Er-D proceeds to form Er-C, the "natural" substrate of EryG for the C-3′ O methylation to produce Er-A (pathway I), and the competitive action of EryG on Er-D results in the shunt metabolite Er-B, which might be an unsuitable substrate for EryK for further conversion to Er-A in vivo (pathway II). Since Er-B and Er-C are biologically much less active and cause >2-fold-greater side effects than Er-A, the proportion of Er-B plus Er-C in the commercial production of Ers is limited to within 5% according to the criteria of the European Pharmacopoeia (8a). However, many industrial strains produce a mixture of Ers in a ratio of Er-A to Er-B plus Er-C of around 3:1 in the fermentation culture, suggesting that the final two tailoring modifications involving C-12 hydroxylation and C-3′ O methylation are not as efficient as other enzymatic steps in the Er biosynthetic pathway. An amount of Er-B and Er-C needs to be removed during the postfermentation stage as they are impurities, potentially complicating the downstream process of isolation, increasing the production cost, and causing environmental concerns.

In recent years, the emerging biotechnique of rational genetic engineering (2, 9) has been applied to improving the production of Ers and fulfilled the potential that might be hard to attain solely by the still-used traditional methods based on random mutagenesis and selection over 50 years in *S. erythraea*. For example, heterologous expression of the *Vitreoscilla* hemoglobin gene (*vhb*) in an industrial Er-producing strain of *S. erythraea* produced a >60%-higher Er titer due to the increase of oxygen supply and consequent enhancement of the Er biosynthetic flux (4, 17), and engineering of the methylmalonyl-CoA metabolite node of the wild type of *S. erythraea* by inactivation or duplication of the methylmalonyl-CoA mutase gene also led to a significant increase in Er production in a high-performance carbohydrate-based or oil-based fermentation medium (23, 24). In contrast to extensive efforts toward improving total Er production, to our knowledge there have been few reports on the elimination of by-products such as Er-B and Er-C and improvement of Er-A purity at the fermentation stage by using the tools of genetic engineering.

In this study, we report systematical modulation of the amounts of tailoring enzymes EryK (the P450 hydroxylase) and EryG (the *S*-adenosylmethionine-dependent *O*-methyltransferase) by genetic engineering in *S. erythraea*. Increase of the copy numbers of *eryK* and *eryG* by homologous recombination significantly enhanced their transcript levels and the process of biotransformation from Er-D to Er-A due to gene dose effects. At the *eryK*/*eryG* copy number ratio of 3:2 as well as their resultant transcript ratio of around 2.5:1 to 3.0:1, Er-B and Er-C were nearly completely eliminated, and Er-A production was improved around 25% in the recombinant strain ZL1004 (genotype *PermK*-K-K-G* + *PermE*-K* + *PermA*-G*) and ZL1007 (genotype *PermK*-K-G-K* + *PermE*-K* + *PermA*-G*). The improvement of the Er-A purity and production at the fermentation stage resulted from the efficient biotransformation of the previous by-products Er-B and Er-C into Er-A and enhancement of the biosynthetic flux of Er-A by overcoming the limitations of tailoring modification.

MATERIALS AND METHODS

Bacterial strains, plasmids, and reagents. Bacterial strains and plasmids used in this study are summarized in Table 1. Biochemicals, chemicals, media, restric-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Reference or source
Strains		
E. coli		
DH5α	Host for general cloning	Invitrogen
ET12567(pUZ8002)	Donor strain for conjugation between *E. coli* and *Streptomyces*	12
S. erythraea		
HL3168 E3	Industrial Er-producing strain	30
ZL1001	*eryK*-duplicated derivative of HL3168 E3, with the genotype *PermK*-K + PermE*-K + PermA*-G*	This work
ZL1002	*eryK* and *eryG*-duplicated derivative of HL3168 E3, with the genotype *PermK*-K-G + PermE*-K + PermA*-G*	This work
ZL1003	*eryK* and *eryG*-duplicated derivative of HL3168 E3, with the genotype *PermK*-K + PermA*-G + PermE*-K-G*	This work
ZL1004	*eryK*-triplicated and *eryG*-duplicated derivative of HL3168 E3, with the genotype *PermK*-K-K-G + PermE*-K + PermA*-G*	This work
ZL1005	*eryK*-triplicated and *eryG*-duplicated derivative of HL3168 E3, with the genotype *PermK*-K-G + PermE*-K-K + PermA*-G*	This work
ZL1006	*eryK*-triplicated and *eryG*-duplicated derivative of HL3168 E3, with the genotype *PermK*-K + PermA*-G + PermE*-K-K-G*	This work
ZL1007	*eryK*-triplicated and *eryG*-duplicated derivative of HL3168 E3, with the genotype *PermK*-K-G-K + PermE*-K + PermA*-G*	This work
ZL1008	*eryK*-triplicated and *eryG*-duplicated derivative of HL3168 E3, with the genotype *PermK*-K + PermE*-K-G-K + PermA*-G*	This work
ZL1009	*eryK*-triplicated and *eryG*-duplicated derivative of HL3168 E3, with the genotype *PermK*-K + PermA*-G-K + PermE*-K-G*	This work
Plasmids		
pGEM-T Easy	*E. coli* subcloning vector	Promega
pGEM-7zf	*E. coli* subcloning vector	Promega
pANT841	*E. coli* subcloning vector	32
pWMH3	*E. coli-Streptomyces* shuttle vector; Thio^r	29
pKC1139	*E. coli-Streptomyces* shuttle vector; Apr^r; temperature-sensitive replication origin carrier	3
pZL1001	1.7-kb PCR product that contains *eryK* and its downstream native terminator in pGEM-T Easy	This work
pZL1002	2.2-kb fragment that contains the *PermE**-controlled *eryK* and its downstream native terminator in pWHM3	This work
pZL1003	2.2-kb fragment that contains the *PermE**-controlled *eryK* and its downstream native terminator in pKC1139	This work
pZL1004	1.3-kb PCR product that contains *eryK* without its downstream native terminator in pANT841	This work
pZL1005	1.8-kb fragment that contains the *PermE**-controlled *eryK* in pANT841	This work
pZL1006	1.5-kb PCR product that contains *eryG* in pANT841	This work
pZL1007	1.5-kb fragment that contains *eryG* in pET-28a	This work
pZL1008	3.3-kb fragment that contains the *PermE**-controlled *eryK-G* in pANT841	This work
pZL1009	3.3-kb fragment that contains the *PermE**-controlled *eryK-G* in pKC1139	This work
pZL1010	1.3-kb PCR product that contains *eryK* without its downstream native terminator in pGEM-7zf	This work
pZL1011	4.6-kb fragment that contains the *PermE**-controlled *eryK-K-G* in pANT841	This work
pZL1012	4.6-kb fragment that contains the *PermE**-controlled *eryK-G-K* in pANT841	This work
pZL1013	4.6-kb fragment that contains the *PermE**-controlled *eryK-K-G* in pKC1139	This work
pZL1014	4.6-kb fragment that contains the *PermE**-controlled *eryK-G-K* in pKC1139	This work

tion enzymes, and other molecular biological reagents were from standard commercial sources.

DNA isolation, manipulation, and sequencing. DNA isolation and manipulation in *Escherichia coli* and *S. erythraea* were carried out according to standard methods (12, 25). PCR amplifications were carried out on an Authorized Thermal Cycler (Eppendorf AG 22331; Hamburg, Germany) using either *Taq* DNA polymerase or *Pfu* Ultra High-Fidelity DNA polymerase (Promega). Primer synthesis and DNA sequencing were performed at Shanghai GeneCore Biotechnology Inc.

Plasmid constructions and recombinant strain generations. To duplicate *eryK* alone in *S. erythraea*, a 1.7-kb DNA fragment that contains the 1,194-bp complete sequence of *eryK* and the 358-bp downstream sequence involving a native terminator was amplified by PCR using the primer pair 5′-AAA CTG CAG CAC CGC GGA AGT CTC GAC ACC-3′ and 5′-TTT AAG CTT CGC CGG GGC AGT GCA AGT ACG-3′ and cloned into the pGEM-T Easy vector, yielding pZL1001. The identity of the PCR product with *eryK* (GenBank accession number AM420293) was confirmed by sequencing. The 1.7-kb PstI/HindIII fragment and a 0.5-kb EcoRI/XbaI fragment that contains a *PermE** promoter were sequentially inserted into the corresponding sites of pWHM3, yielding pZL1002. Consequently, the resultant 2.2-kb fragment was cloned into the EcoRI/HindIII site of pKC1139 (a temperature-sensitive vector), yielding pZL1003 for overexpression of *eryK* alone.

To duplicate both *eryK* and *eryG* in *S. erythraea*, a 1.3-kb fragment that contains the entire *eryK* sequence without its native terminator was reamplified by PCR using the primer pair 5′-AAA CTC GAG CAC CGC GGA AGT CTC GAC ACC-3′ and 5′-TTT ACT AGT GCT GCC CGA CTA CGC CGA CTG-3′ and

cloned into the pANT841 vector, yielding pZL1004. After sequencing of the PCR product confirmed its identity, a 0.5-kb *PermE**-containing fragment was cloned into the EcoRI/SacI site of pZL1004, yielding the recombinant plasmid pZL1005, in which *eryK* is under the control of *PermE**. On the other hand, a 1.5-kb fragment that contains the entire *eryG* sequence was amplified by PCR using primers 5′-TTA CCA TGG GGT GCT GTT GCC GTC CCT GCG-3′ and 5′-TCG ACT AGT CTA CCG CGT GCT GCG CTC CTA-3′ and cloned into the pANT841 vector, yielding pZL1006. After sequencing of the PCR product confirmed its identity with *eryG* (GenBank accession number AM420293), the 1.5-kb fragment was cloned into the NcoI/HindIII site of the pET-28a vector, yielding pZL1007. The 1.5-kb *eryG*-containing fragment was recovered and inserted into the SpeI/HindIII site of pZL1005, yielding pZL1008, which contains the *eryK-eryG* copy under the control of *PermE**. Finally, the resultant 3.3-kb EcoRI/HindIII fragment was cloned into pKC1139, yielding pZL1009 for overexpression of both *eryK* and *eryG*.

To triplicate *eryK* and duplicate *eryG* in *S. erythraea*, two recombinant plasmids that carry genes in the orders *eryK-K-G* and *eryK-G-K*, respectively, were constructed. The 1.3-kb XhoI/HindIII fragment that contains the entire *eryK* sequence was recovered from pZL1004 and cloned into the pGEM-7zf vector, yielding pZL1010. Subsequently, the 1.3-kb *eryK*-containing fragment from pZL1010 and the 1.5-kb *eryG*-containing fragment from pZL1006 were successively cloned into the SpeI/HindIII sites of pZL1005, yielding pZL1011 and pZL1012, respectively, dependent on the insertion order. Finally, the resultant 4.6-kb fragment was recovered and cloned into pKC1139, yielding pZL1013 or pZL1014, in which genes are organized in the order *eryK-K-G* or *eryK-G-K*, respectively, under the control of *PermE**.

In summary, for overexpressing *eryK* alone, the "long" version of *eryK* with the native terminator (containing a 358-bp downstream sequence) was chosen to shorten the length of mRNA. For overexpressing *eryK-G*, *eryK-G-K*, and *eryK-K-G*, the "short" version of *eryK* without the native terminator (containing only a 39-bp downstream sequence) for each was chosen to maintain the translational continuity of downstream genes. Both versions of *eryK* contain a 105-bp upstream flanking sequence of *eryK*. The version of *eryG* in this study contains a 308-bp upstream sequence and a 226-bp downstream sequence of *eryG*.

These constructs were introduced into *S. erythraea* by intergeneric conjugation from *E. coli* ET12567(pUZ8002), following the procedures described previously (15). Colonies that were apramycin resistant at 37°C were identified as the recombinant strains, genotypes of which were further confirmed by Southern hybridization.

Production and analysis of Ers in fermentation cultures. *S. erythraea* HL3168 E3 and recombinant strains were grown on agar plates (with appropriate antibiotics for recombinant strains) of the medium [consisting of 1% cornstarch, 1% corn steep liquor, 0.3% NaCl, 0.3% $(NH_4)_2SO_4$, 0.5% $CaCO_3$, and 2% agar, pH 7.0] at 34°C for sporulation. For fermentation, an agar piece around 1 cm^2 was inoculated into a 500-ml flask containing 50 ml of the seed medium [consisting of 5% cornstarch, 1.8% soybean flour, 1.3% corn steep liquor, 0.3% NaCl, 0.1% $(NH_4)_2SO_4$, 0.1% NH_4NO_3, 0.5% soybean oil, and 0.6% $CaCO_3$, pH 6.8 to 7.0] and incubated at 34°C and 250 rpm for 2 days. To a 500-ml flask containing 50 ml of the fresh fermentation medium [consisting of 4% cornstarch, 3% soybean flour, 3% dextrin, 0.2% $(NH_4)_2SO_4$, 1% soybean oil, and 0.6% $CaCO_3$] was then added 5 ml of the seed culture, and incubation was continued at 34°C and 250 rpm for 6 days. The mixture was supplemented with an additional 0.5 ml of *n*-propanol after 1 day of cultivation. Er isolation from the fermentation culture was carried out according to the methods described previously (28), which were slightly modified by using *n*-heptane instead of *n*-hexane.

High-pressure liquid chromatography (HPLC) analysis of Ers was carried out on a Nucleosil 100–5 CN column (250 × 4.6 mm; catalog no. 720090.46; Macherey-Nagel Inc., Germany), which was equilibrated with 69% solvent A (32 mM potassium phosphate buffer, pH 8.0) and 31% solvent B (acetonitrile/methanol ratio of 75/25). An isocratic program (7) was carried out at a flow rate of 1 ml/min, and UV detection was performed at 215 nm using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA). Liquid chromatography-mass spectrometry analysis of Ers was carried out on a Microsorb-MV C_{18} column (250 × 4.6 mm; catalog no. 281505; Varian Inc.), which was equilibrated with 62% solvent A (30 mM ammonium acetate, pH 4.8) and 38% solvent B (acetonitrile). An isocratic program (28) was carried out on an LCMS-2010 A liquid chromatograph-mass spectrometer (Shimadzu, Japan) at a flow rate of 1 ml/min, and UV detection was performed at 215 nm.

Bioassay-based titration of the Er production. From the liquid culture, fermentation supernatant (250 μl) was added to stainless steel cylinders on agar plates containing the test medium (consisting of 0.5% peptone, 0.3% beef extract, 0.3% K_2HPO_4, and 1.5% agar), which was preseeded with an overnight *Bacillus pumilus* culture at a concentration of 0.8% (vol/vol). The plates were incubated at 37°C for 16 h, and the Er production was estimated by measuring the sizes of the inhibition zones and calculated according to the standard curve made by using the commercially available Er as a control. Since Er-A is biologically much higher in activity than other Er components, the production of Er-A is nearly equal to the total Er production according to the titration done by assaying antibacterial activity against *Bacillus pumilus* CMCC(B)63 202.

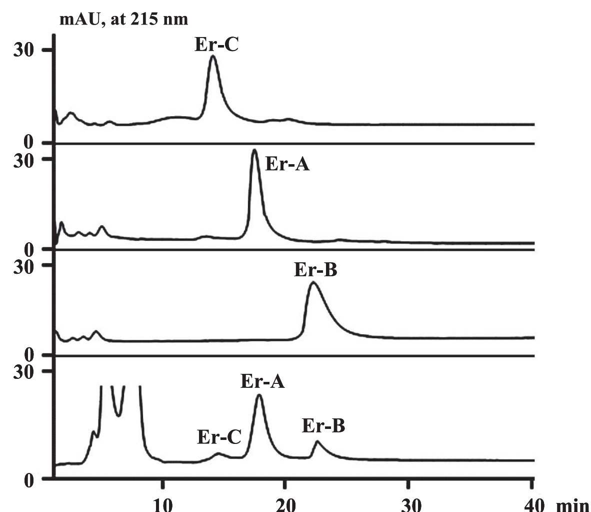

FIG. 2. HPLC analysis of Er production in fermentation of the controlled strain *S. erythraea* HL3168 E3, with standard Ers as controls.

Assay of transcript levels by reverse transcription-PCR (RT-PCR) amplification. Total RNAs of *S. erythraea* HL3168 E3 and its derivatives were isolated as described previously (6) from mycelia in the fermentation cultures, and an additional purification step was performed using the RNeasy Mini kit (Qiagen, Valencia, CA).

The transcription levels of *eryK* and *eryG* were assayed on an FTC-2000 (FengLing BioTech. Co., Shanghai, China) instrument, using real-time, quantitative PCR and the $2^{-\Delta\Delta C_T}$ method (16, 21). DNase treatment and cDNA synthesis were carried out according to the manufacturer's instructions. RT-PCR amplification was performed on each 25 μl of mixture (consisting of 1 μg/ml of template cDNA, 2× SYBR Premix EX *Taq* mix buffer [TaKaRa, Japan], and 0.3 μM of forward and reverse primers) with the following program: 5 min at 95°C and 40 cycles of 15 s at 95°C followed by 60 s at 60°C. For assessing the transcript level of *eryK*, the 115-bp internal fragment was amplified using the following primers: 5′-ACC TGA TCT CCC GCC TTG TC-3′ and 5′-AGG ACG GTC GTG GTG ATG TG-3′. For assessing the transcript level of *eryG*, the 107-bp internal fragment was amplified using the following primers: 5′-CAA GAA GAG CGT GAG CAG GC-3′ and 5′-CTG AGG CGA TCA CGA AGT CG-3′. For assessing the transcript level of the PKS gene *eryAIII* (as a control) involved in the Er aglycone biosynthesis, the 102-bp internal fragment encoding the thioesterase domain of EryAIII was amplified using the following primers: 5′-CCT GAT CGA CGT CTA CCC GC-3′ and 5′-TCG TCC ATC CGC ACC GTC TC-3′.

RESULTS

Qualitative and quantitative analysis of Ers in fermentation. Efficient culture cleanup for Er recovery (nearly 100% [28]) was performed on the fermentation broth of *S. erythraea* HL3168 E3, an industrial Er-producing strain. As shown in Fig. 2, HPLC analysis of this sample revealed three sequentially eluted compounds with the same retention times as those of the standard Er-C (at 13.8 min), Er-A (at 17.5 min), and Er-B (at 23.8 min). Their identities were further confirmed by liquid chromatography-mass spectrometry analysis, showing $(M - H)^-$ ions at m/z = 720.3, 734.2, and 718.3 that are consistent with the molecular formula $C_{36}H_{65}O_{13}$ for Er-C, $C_{37}H_{67}NO_{13}$ for Er-A, and $C_{37}H_{67}NO_{12}$ for Er-B, respectively. According to

the standard curve of each reference Er, the concentration of Er-A, Er-B, or Er-C was individually calculated at 3.36 mg/ml, 0.91 mg/ml, or 0.26 mg/ml, respectively, in the fermentation broth of *S. erythraea* HL3168 E3, giving a ratio of Er-A to Er-B plus Er-C of around 2.9:1. Alternatively, the total Er production is further titrated at 3,258 U/ml by assaying the antibacterial activity against *B. pumilus*. The methods described here for qualitative and quantitative analysis of Ers were applied to the resultant recombinant *S. erythraea* strains in this study.

Elimination of Er-B by overexpression of *eryK* in *S. erythraea*. Within the Er biosynthetic gene cluster, *eryG*, which resides in the largest operon, spanning over 35 kb, is cotranscribed with the PKS genes *eryAI*, *eryAII*, and *eryAIII* and the sugar genes *eryCII*, *eryCIII*, and *eryBII* under the control of the native promoter *PermA**, whereas *eryK* constitutes a single gene operon under the control of the native promoter *PermK** (22). Since Er-B is a shunt metabolite resulting from the EryG-catalyzed C-3′ O methylation occurring prior to the C-12 hydroxylation, increasing the amount of the P450 hydroxylase EryK may enhance its action on Er-D to generate the "natural" intermediate Er-C and competitively block bypass pathway II (shown in Fig. 1). To validate this hypothesis, within the Er biosynthetic gene cluster, *eryK* was insertionally duplicated by a single-crossover homologous recombination event to generate the recombinant *S. erythraea* strain ZL1001, into which a second *eryK* copy under the control of *PermE** (the constitutive promoter of Er resistance gene *eryE*) was introduced. Southern analysis of the genomic DNA from the mutant strain by using the 1.7-kb *eryK*-containing fragment as a probe (Fig. 3B) confirmed that ZL1001 has the designed genotype (*PermK*-K* + *PermE*-K* + *PermA*-G* [Fig. 3A]). While there was no apparent difference in growth characteristics, morphologies, and even total Er production (Fig. 4) (according to the bioassay-based titration) between HL3168 E3 and the recombinant strain ZL1001, HPLC analysis of the fermentation broth of ZL1001 revealed that Er-B was nearly completely eliminated and Er-C was accordingly increased from 0.26 mg/ml to 0.88 mg/ml as shown in Fig. 3C. This phenotype indeed confirmed that the enzymatic activity of EryK was enhanced by duplication of *eryK* in *S. erythraea* and, more importantly, demonstrated that the industrial strain HL3168 E3 is amenable to genetic engineering without a decrease of the Er titer.

Increase of Er-A production by biotransformation of Er-B and Er-C into Er-A. The accumulation of Er-C in fermentation broths of both control strain HL3168 E3 and EryK-enhanced mutant strain ZL1001 clearly indicated the inefficiency of EryG; increasing the amount of EryG may enhance its activity and improve the production of Er-A by conversion of Er-C. Consequently, additional genes with the *eryK-G* order was constructed under the control of *PermE** and introduced into *S. erythraea* HL3168 E3, yielding two mutant strains, ZL1002 and ZL1003, in which both *eryK* and *eryG* are insertionally duplicated but organized in different manners, depending on the locus where the single-crossover homologous recombination event took place (Fig. 3A). With the 1.7-kb *eryK*-containing fragment as a probe, Southern analysis of the genomic DNAs from the recombinant strains confirmed that they have the designed genotypes (type *PermK*-K-G* + *PermE*-K* + *PermA*-G* for ZL1002 and type *PermK*-K* + *PermA*-G* + *PermE*-K-G* for ZL1003 [Fig. 3B]). As shown in Fig. 3C, the concentration of Er-B + Er-C remarkably decreased in fermentations of both ZL1002 and ZL1003, enhancing the Er-A/Er-B plus Er-C ratio up to 17.2:1. Upon the bioassay-based titration, although ZL1003 (resulted from the recombination occurring at the *eryG* locus) had an Er titer similar to that of the control strain HL3168 E3, the Er titer of ZL1002 (resulting from the recombination event occurring at the *eryK* locus) was accordingly improved 21.5% (Table 2; Fig. 4), consistent with the 20.3% decrease of the concentration of Er-B plus Er-C.

Further ratio enhancement and production improvement of Er-A by genetic modulation of the overexpression of *eryK* and *eryG*. Nearly complete conversion of Er-C to Er-A in fermentation broths of the recombinant strains ZL1002 and ZL1003 showed that duplication of *eryG* resulted in an amount of EryG sufficient for C-3′ O methylation; however, the recurrence of the shunt metabolite Er-B indicated the importance of the ratio of enzymatic activities of EryK and EryG. Further increasing the *eryK* copy number and modulating the amounts of tailoring enzymes at a certain ratio might lead to the complete abolishment of the biosynthesis of by-products Er-B and Er-C and improvement of the production and purity of Er-A at the fermentation stage. To validate this prediction, a construct that carries genes in the order *eryK-K-G* was introduced into *S. erythraea* HL3168 E3, yielding three types of recombinant strains that contain three copies of *eryK* and two copies of *eryG* in each. Using the 1.7-kb *eryK*-containing fragment as a probe, the genotypes of the recombinant strains were confirmed by Southern hybridization (Fig. 3A and B), well in agreement with their designed genetic patterns regarding the organizations of *eryK* and *eryG* according to the locus where the single-crossover homologous recombination event took place: type *PermK*-K-K-G* + *PermE*-K* + *PermA*-G* for ZL1004, type *PermK*-K-G* + *PermE*-K-K* + *PermA*-G* for ZL1005, and type *PermK*-K* + *PermA*-G* + *PermE*-K-K-G* for ZL1006. As shown in Fig. 3C, the concentration of Er-B plus Er-C in fermentation broth of each recombinant strain dramatically decreased (25.8% in ZL1004, 20.2% in ZL1005, and 17.9% in ZL1006), accompanying the increase of the yield of Er-A and enhancement of the Er-A/Er-B plus Er-C ratio. Particularly in ZL1004, no visible Er-B or Er-C was detected upon HPLC analysis, suggesting that these by-products in fermentation were nearly completely biotransformed into Er-A. This finding is further supported by the bioassay-based titration, which revealed that the Er titer was improved 24.6% in ZL1004, 14.7% in ZL1005, and 7.4% in ZL1006 (Fig. 4; Table 2).

To investigate the effects of genetic locus (for homologous recombination) and gene organization of *eryK* and *eryG* on Er production, an additional construct that carries genes in the order *eryK-G-K* was introduced into *S. erythraea* HL3168 E3, as shown in Fig. 3A and B, resulting in the recombinant strains ZL1007 (*PermK*-K-G-K* + *PermE*-K* + *PermA*-G*), ZL1008 (*PermK*-K* + *PermE*-K-G-K* + *PermA*-G*), and ZL1009 (*PermK*-K* + *PermA*-G-K* + *PermE*-K-G*), respectively. Again, the increase of Er-A accompanied the decrease of Er-B plus Er-C upon HPLC analysis (Fig. 3C), and the bioassay-based titration showed that the Er titer was improved 30.2% in ZL1007, 27.7% in ZL1008, and 11.5% in ZL1009. Combined with the above data, these results indicated that integration of the locus on the chromosome by homologous recombination has a more significant impact on the improvement of Er-A production

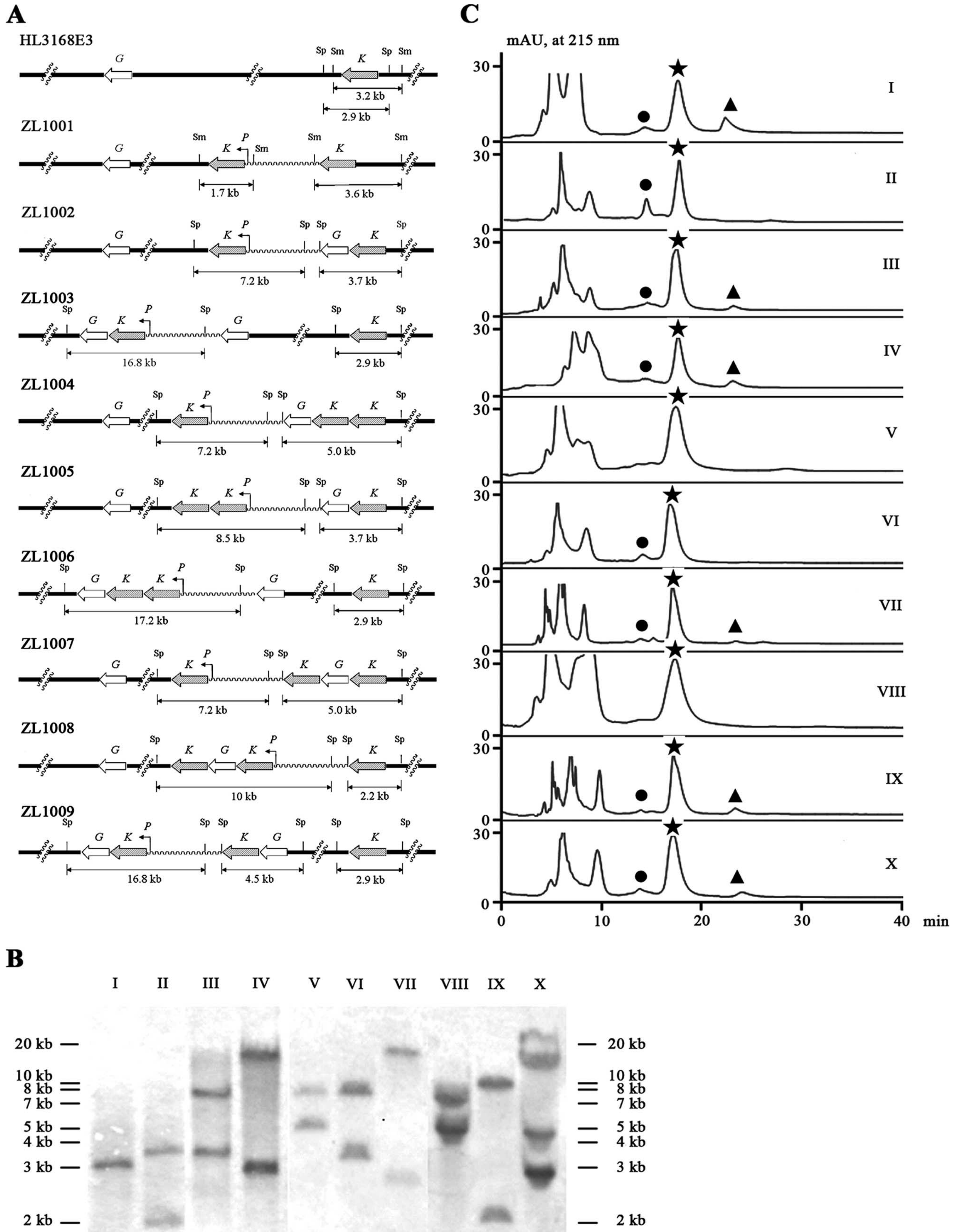

FIG. 3. Genotypes and phenotypes of *S. erythraea* HL3168 E3 and its recombinant strains. (A) Restriction maps showing predicted fragment sizes upon SmaI and/or SphI digestion. (B) Southern analyses of genomic DNAs digested with SmaI or SphI by using the 1.7-kb *eryK*-containing fragment as a probe. (C) HPLC analyses of the Er production in fermentations. I, HL3168 E3; II, ZL1001; III, ZL1002; IV, ZL1003; V, ZL1004; VI, ZL1005; VII, ZL1006; VIII, ZL1007; IX, ZL1008; X, ZL1009. Solid circles, stars, and triangles indicate Er-C, Er-A, and Er-B, respectively. Each wavy line indicates the DNA fragment of the vector integrated by single-crossover homologous recombination.

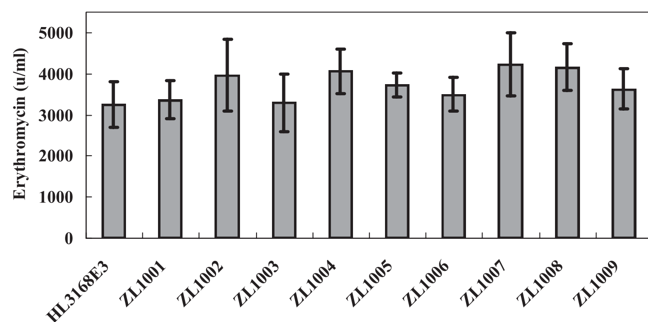

FIG. 4. Titration of Er-A production in fermentations of *S. erythraea* HL3168 E3 and its recombinant strains, by assaying the antibacterial activity against *B. pumilus*. Error bars represent standard deviations.

than the gene organization of *eryK* and *eryG* under the same promoter in each recombinant strain (Fig. 4; Table 2).

Assay of the transcript levels of *eryK* and *eryG* by RT-PCR amplification. To assess gene dose effects on the increase of enzyme amounts, RT-PCR amplification for analysis of the transcription levels of *eryK* and *eryG* was performed on the total RNAs that were extracted from 3-day fermentation cultures of *S. erythraea* HL3168 E3 and its recombinant derivatives. The transcript level of the gene fragment that encodes the thioesterase domain of the PKS EryAIII served as a control in this study. As shown in Fig. 5, introduction of an *eryK*, *eryK-G*, *eryK-K-G*, or *eryK-G-K* copy under the control of *PermE** into HL3168 E3 caused significant improvement of the transcript level of *eryK* or *eryG*. While duplication of *eryK* and *eryG* in each recombinant strain led to six- and two- to fivefold increases, respectively, triplication of *eryK* resulted in an increase of up to 8- to 13-fold. As shown in Table 2, in which the transcript level ratios of *eryK* to *eryG* for these recombinant strains are summarized, the recombinant strains ZL1004 and ZL1007, with the highest Er-A production and purity in fermentations, share a similar ratio of around 2.5:1 to 3.0:1.

DISCUSSION

In general, many microbial natural products, including antibiotics and other bioactive compounds, are secondary metabolites. Structurally related compounds are often produced as a complex and share a common biosynthetic pathway in a certain microorganism, exemplified by the biosynthesis of Ers in *S. erythraea*. Since a few components (i.e., Er-B, Er-C, and Er-D) in the Er complex serve as the intermediates, further tailoring modifications of them are required to obtain the clinically and commercially important major component Er-A. This phenotype represents two characteristic features of secondary metabolism: (i) enzymes involved in these tailoring steps, such as EryK and EryG, are not efficient enough for complete conversion of the substrates, and (ii) their relatively broad substrate specificities and competitive actions on the same intermediate lead to the formation of shunt products through a branched biosynthetic pathway.

The selective improvement of production of the desired components and elimination of the undesired components are always the goals in many projects of metabolic engineering (5). Based on the significant success in genetic and biochemical elucidation of the Er biosynthetic machinery, extensive efforts have been made in expanding Er structural diversity and improving total Er production. On the other hand, to reach the criteria of the European Pharmacopoeia regarding the quality of the commercial Er product, an amount of Er-B and Er-C needs to be removed during the postfermentation stage. In this study, we focused on systematically modulating the amounts of EryK and EryG by genetic engineering in an industrial Er-producing strain, *S. erythraea* HL3168 E3. The methods described here allowed us to biotransform the previous by-products Er-B and Er-C into Er-A and selectively improve the Er-A production and purity at the fermentation stage.

Introduction of a single *PermE**-controlled *eryK* copy into *S. erythraea* HL3168 E3 caused nearly complete elimination of Er-B, a corresponding increase of Er-C, and Er-A production comparable to that of the control strain. These results clearly showed that the improvement of the amount of EryK through

TABLE 2. Productions of Ers, ratios of Er-A to Er-B plus Er-C, and ratios of transcript levels of *eryK* to *eryG* in *S. erythraea* HL3168 E3 and its recombinant strains

S. erythraea strain	No. of independent cultures (no. of isolates tested)	Concn (mg/ml)			Avg Er-A/Er-B + Er-C ratio	Decrease of Er-B + Er-C[b] (%)	Total Er titer (U/ml)	Improvement of Er production[c] (%)	Avg transcript intensity ratio of *eryK* to *eryG*
		Er-A	Er-B	Er-C					
HL3168E3	196 (5)	3.36	0.91	0.26	2.9		3,258 ± 551		1.0
ZL1001	92 (8)	3.45	ND[a]	0.88	3.9	5.5	3,366 ± 460	3.3	5.1
ZL1002	92 (8)	3.96	0.15	0.08	17.2	20.3	3,960 ± 879	21.5	2.3
ZL1003	74 (7)	3.43	0.28	0.05	10.4	17.1	3,294 ± 693	1.1	1.4
ZL1004	96 (8)	4.15	ND	ND		25.8	4,061 ± 532	24.6	2.9
ZL1005	84 (8)	3.82	ND	0.23	16.6	20.2	3,738 ± 291	14.7	4.2
ZL1006	72 (6)	3.61	0.15	0.16	11.6	17.9	3,498 ± 407	7.4	3.4
ZL1007	58 (5)	4.29	ND	ND		25.8	4,243 ± 767	30.2	2.5
ZL1008	68 (6)	4.12	0.33	0.15	8.6	15.4	4,161 ± 574	27.7	2.1
ZL1009	62 (6)	3.67	0.31	0.18	7.5	14.1	3,632 ± 496	11.5	1.7

[a] ND, no product detected.
[b] Calculated as concentration of Er-B + Er-C/concentration of Er-A + Er-B + Er-C of HL3168 E3 − concentration of Er-B + Er-C/concentration of Er-A + Er-B + Er-C of each recombinant strain.
[c] Calculated as (Er titer of each recombinant strain − that of HL3168 E3)/Er titer of HL3168 E3.

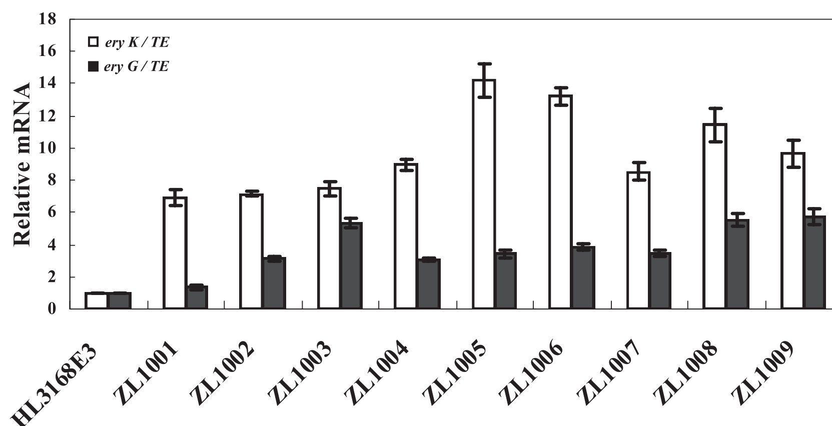

FIG. 5. Transcript levels of *eryK* and *eryG* in *S. erythraea* HL3168 E3 and its recombinant strains. Error bars represent standard deviations. For each strain, the experiment has been independently carried out three times.

a gene dose effect efficiently blocked the shunt Er-B biosynthetic pathway and enhanced the natural Er-C pathway by competitive action on the common substrate Er-D and, more importantly, demonstrated that the industrial strain HL3168 E3 is amenable to genetic engineering without decreasing the Er-A production.

To reach Er-A without accumulation of both Er-B and Er-C, we introduced an additional *eryG* copy into *S. erythraea* HL3168 E3 and modulated the increased amounts of EryK and EryG by altering the copy number ratio, gene organization, and integration locus for homologous recombination. In ZL1004 and ZL1007, the copy number ratio of *eryK* to *eryG* at 3:2 as well as their resultant transcript ratio at 2.5:1 to 3.0:1 led to the nearly complete biotransformation of Er-B and Er-C into Er-A and the highest improvement in titer of Er (around 25%), strongly supporting the idea that increase and modulation of the amounts of tailoring enzymes at a certain ratio would enhance the biotransformation process and biosynthetic flux and selectively improve the production and purity of the desired components at the fermentation stage. On the other hand, compared to the gene organization of *eryK* and *eryG* introduced under the same promoter in each recombinant strain, the integration of the locus on the chromosome by homologous recombination showed a more significant impact on the Er-A/Er-B plus Er-C ratio and an increase of Er-A production. To obtain the stable recombinant strains, we opted to integrate the additional gene copies into the chromosome of *S. erythraea* by homologous recombination between the introduced and genomic DNAs. This event happened at either the *eryK* or the *eryG* locus when both of them were introduced and caused distinct effects on Er production. In this study, the strains that harbor the integration at the *eryK* locus where the single gene constitutes an operon always produce more Er-A than do the strains with the integration at the *eryG* locus, probably due to the fact that *eryG* resides in the largest operon, and homologous combination occurring there may interfere with the transcription of other structural genes in it, including the PKS genes for Er skeleton formation.

Since Er-A is biologically much more highly active than other Er components, the production of Er-A is nearly equal to the total Er production according to titration by assay of antibacterial activity against *Bacillus pumilus*. As summarized in Table 2, the decreases of Er-B plus Er-C concentrations are close to the increases of Er titers in the recombinant strains ZL1002, ZL1004, and ZL1007, indicating the improvement of Er-A purity and production at the fermentation stage resulting from the efficient biotransformation of the previous by-products Er-B and Er-C into Er-A, and enhancement of the biosynthetic flux of Er-A could be achieved by rational engineering of the tailoring enzymes in the Er biosynthetic pathway.

In conclusion, systematically modulating the enzyme amounts of EryK and EryG by integrating additional *eryK* and *eryG* copies into the industrial strain *S. erythraea* HL3168 E3 significantly enhanced the process of biotransformation from Er-D to Er-A, competitively blocked the shunt biosynthetic pathway II, nearly completely eliminated the previous by-products Er-B and Er-C, and efficiently improved Er-A production and purity at the fermentation stage. In conjunction with other traditional and genetic ways to continuously evaluate the Er-A production system, this study may facilitate further attempts to improve Er-A production, simplify the downstream purification process, and lower the production costs and environmental concerns in industry.

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