NOTE

Development of the ability to produce secondary metabolites in *Streptomyces* through the acquisition of erythromycin resistance

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The Journal of Antibiotics (2012) 65, 323–326; doi:10.1038/ja.2012.16; published online 11 April 2012

Keywords: antibiotic production; erythromycin resistance; secondary metabolites; *Streptomyces*

Members of the genus *Streptomyces* are gram-positive filamentous bacteria that produce various secondary metabolites, which are exemplified by antibiotics. Because more than two-thirds of clinically useful antibiotics are produced from *Streptomyces*,¹ members of this genus are of high pharmacological and industrial interest. Additionally, recent genome-sequencing projects have revealed that many biosynthetic gene clusters, producing unknown secondary metabolites, exist in *Streptomyces* genomes.^{2–4} The exploitation of the genomic potential of *Streptomyces* may lead to the isolation of new biologically active compounds.^{5,6} Therefore, it is extremely important for antibiotic discovery research to investigate their unexploited abilities to produce secondary metabolites and to clarify their activation mechanism. We previously demonstrated a method for activating unexpressed or poorly expressed *Streptomyces* genes to synthesize secondary metabolites through a mutation that confers resistance to drugs targeting the RNA polymerase and/or ribosomes.^{7–9} This led to the discovery of piperidamycin, a novel antibiotic produced by *Streptomyces* sp. 631689, which rarely produces antibiotics in any type of culture media.¹⁰ Here, we describe a new technique for advanced utilization of the above-mentioned method to take complete advantage of the ability of *Streptomyces* to produce secondary metabolites.

Erythromycin is a macrolide antibiotic that inhibits protein synthesis by binding to the bacterial 50S ribosomal subunit. Previous studies have demonstrated that generating a mutation that confers resistance to a drug, such as rifampicin, streptomycin, gentamicin, paromomycin or thiostrepton, is an effective approach to increasing the production of the blue-pigmented antibiotic actinorhodin (ACT) in *Streptomyces coelicolor* A3(2) and *Streptomyces lividans*.^{9,11–13} *S. coelicolor* A3(2) and *S. lividans* are well-characterized strains of *Streptomyces* from a physiological and genetic viewpoint; however, the effects of an erythromycin resistance mutation on antibiotic

production in *S. coelicolor* A3(2) and *S. lividans* have not been assessed. Therefore, we examined whether the acquisition of erythromycin resistance enables *S. coelicolor* A3(2) and *S. lividans* to overproduce antibiotics.

We isolated 259 and 300 spontaneous erythromycin-resistant (Ery^r) mutants of *S. coelicolor* A3(2) strain 1147 (SCP1⁺, SCP2⁺, prototroph) and *S. lividans* strain 1326 (*S. lividans* 66, SLP2⁺, SLP3⁺, prototroph), respectively, from colonies that grew within 4 weeks after the spores (approximately 10¹¹ spores) were spread on GYM agar plates containing 170 or 420 µg ml^{−1} of erythromycin, which corresponds to approximately 3- to 14-fold amount of minimum inhibitory concentration. These spontaneous Ery^r mutants were first characterized for the production of ACT and levels of resistance to erythromycin. The mutants were cultured using GYM, R4, R4C or modified R5 (MR5) agar medium, which revealed that 22 and 20% of spontaneous Ery^r mutants isolated from strains 1147 and 1326, respectively, exhibited improved ACT production (Supplementary Table S1). Table 1 and Figures 1a and b show the characteristics of the Ery^r mutants of *S. coelicolor* A3(2) 1147 and *S. lividans* 1326 that have a representative phenotype for ACT and undecylprodigiosin (RED) production or erythromycin resistance. In *S. coelicolor* A3(2), as in the Ery^r SCE71 and SCE234 mutants, the level of resistance to erythromycin in the high-level ACT-producing strains ranged from 200 to 400 µg ml^{−1}, whereas ACT productivity in strain SCE166, which exhibited a high-level of resistance to erythromycin (> 500 µg ml^{−1}), was on similar level as in the parent strain 1147. Strain SCE234 was the highest antibiotic-producing Ery^r mutant tested in liquid culture, and the strain demonstrated increased productivity of 22- and 4.5-fold (ACT and RED, respectively). As expected, transcription analysis by RT-PCR showed that expressions of the *actII*-ORF4, *redD* and *redZ* genes, encoding the pathway-specific positive regulatory protein for the biosynthesis of ACT and RED, respectively, were clearly enhanced

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Received 26 August 2011; revised 24 January 2012; accepted 21 February 2012; published online 11 April 2012

Table 1 Characteristics of spontaneous Ery^r mutants isolated from *S. coelicolor* A3(2) and *S. lividans*

Strain (relevant genotype)	ACT production [A ₆₄₀ (mg DCW) ⁻¹ ± s.d.] ^a	RED production [A ₅₃₀ (mg DCW) ⁻¹ ± s.d.] ^a	Resistance level (μg ml ⁻¹) to: ^b			
			EM	OLE	TYL	LIN
<i>S. coelicolor</i> A3(2)						
1147 (prototroph)	0.31 ± 0.04	0.06 ± 0.03	30	150	40	200
SCE71 (<i>ery</i> -1)	1.86 ± 0.35	0.12 ± 0.04	400	> 1000	80	> 1000
SCE166 (<i>ery</i> -2)	0.98 ± 0.25	0.11 ± 0.06	> 500	> 1000	400	> 1000
SCE234 (<i>ery</i> -3)	6.87 ± 0.92	0.27 ± 0.03	200	> 1000	150	> 1000
<i>S. lividans</i>						
1326 (prototroph)	0.30 ± 0.10	2.71 ± 0.33	60	200	40	300
SLE160 (<i>ery</i> -4)	1.97 ± 0.30	4.12 ± 0.47	200	> 1000	40	> 1000
SLE259 (<i>ery</i> -5)	2.56 ± 0.28	4.59 ± 0.65	> 500	> 1000	80	> 1000
SLE299 (<i>ery</i> -6)	0.12 ± 0.02	1.38 ± 0.18	> 500	> 1000	60	> 1000

Abbreviations: ACT, actinorhodin; EM, erythromycin; LIN, lincomycin; OLE, oleandomycin; RED, undecylprodigiosin; TYL, tylosin.

^aStrains were inoculated into GYM [for *S. coelicolor* A3(2)] or R4 [for *S. lividans*] liquid medium and incubated on a rotary shaker (200 r.p.m.) at 30°C for 8 days. ACT and RED production was determined spectrophotometrically as described by Kieser T. *et al.*¹ The values are expressed as the mean ± s.d. of triplicate or more samples.^bDetermined after incubation on GYM agar medium at 30°C for 3 days. **Figure 1** Activation of antibiotic production in *Streptomyces* spp. through the acquisition of erythromycin resistance. ACT production by parent and Ery^r mutant strains of *S. coelicolor* A3(2) 1147 (a) and *S. lividans* 1326 (b). Strains were inoculated onto GYM, R4C, MR5 or R4 agar medium and incubated at 30°C for 7 days. Production of antibacterial compounds by parent and Ery^r mutant strains of *S. griseus* IFO13189 (c) and *S. parvulus* ATCC12434 (d). Strains of *S. griseus* and *S. parvulus* were inoculated onto TSB agar medium and GYM agar medium, which were incubated at 30°C for 24 and 60 h, respectively. Agar plugs were cut from the plate and put on the assay plate that had been seeded with *Bacillus subtilis* ATCC6633, followed by 12 h incubation at 37°C.

in the Ery^r mutant SCE234 during the late growth phase (Supplementary Figure S1). In *S. lividans*, the Ery^r SLE160 and SLE259 mutants, which exhibited a moderate level (200 μg ml⁻¹) and a high level (> 500 μg ml⁻¹) of resistance to erythromycin, respectively, produced abundant ACT and RED. The Ery^r mutant SLE299, with a level of resistance to erythromycin as high as the Ery^r SLE259 mutant, exhibited lower antibiotic productivity compared with the parent strain 1326. These results suggest that many different types of erythromycin resistance mutations affect the ability of *S. coelicolor* A3(2) and *S. lividans* to produce antibiotics either positively or negatively.

Additionally, to assess the effects of a spontaneous mutation conferring erythromycin resistance on antibiotic production in other *Streptomyces* strains, a number of Ery^r mutants (> 100 of each) were isolated from *Streptomyces griseus* IFO13189 (streptomycin producer), *Streptomyces parvulus* ATCC12434 (actinomycin producer), and *Streptomyces antibioticus* 3720 (actinomycin producer), and their ability to produce antibiotics were examined. In *S. griseus* and

S. parvulus, 14 and 24% of the Ery^r mutants tested exhibited a significantly increased ability to produce antibiotics, although such a phenomenon was not found among the Ery^r mutants of *S. antibioticus* 3720 (Supplementary Table S1). We do not know the reason why erythromycin resistance mutations were ineffective for antibiotic overproduction by *S. antibioticus*. Figures 1c and d show the antibiotic productivity by the representative overproducing Ery^r mutants isolated from *S. griseus* IFO13189 and *S. parvulus* ATCC12434 as examples. Thus, spontaneous mutations conferring erythromycin resistance in *Streptomyces* spp. effectively increase the productivity of antibiotics.

Surprisingly, we found that an ACT-overproducing Ery^r mutant of *S. lividans* 1326, strain SLE259, produced abundant antibacterial compounds when it was grown on R4 agar medium. As shown in Figure 2a, the antibacterial activity of strain SLE259 was clearly detected in both the presence and absence of calcium nitrate, suggesting that this activity could be due to multiple antibacterial compounds as well as calcium-dependent antibiotics. We then

Figure 2 Detection and analysis of antibacterial compounds produced by Ery^r mutant of *S. lividans* 1326. **(a)** Production of antibacterial compounds by parent strain 1326 and Ery^r mutant SLE259. Strains were grown for 3–7 days on R4 agar medium, subsequently overlaid by Mueller Hinton agar medium (diluted two-fold) inoculated with *Staphylococcus aureus* 209P in the presence or absence of 12 mm calcium nitrate, and incubated at 37°C for 12 h. **(b)** Comparative metabolic profiling of the culture extracts. Samples were prepared and analyzed by HPLC as described in experimental procedures (in supplementary information). The absorbance of eluate was monitored at 190–800 nm (maximum absorbance at any wavelength). The arrows indicate the peaks that were scarcely detected in the sample prepared from the parent strain.

compared the metabolic profile of the culture from the parent strain 1326 and Ery^r mutant SLE259 by using HPLC with multi-wavelength monitoring (diode array detector). As a result, several peaks, which are indicated by arrows in Figure 2b, were identified only in the methanol-extractable fraction of the Ery^r SLE259 mutant. The peaks were lacking or scarcely detectable in the fractions of the parent strain. These interesting phenomena were also observed when the Ery^r SLE259 mutant was grown in R4 liquid medium (Supplementary Figures S2a and b). Although each peak detected in the samples of the Ery^r SLE259 mutant was not identified in this study, our results evidently show that the potential of *S. lividans* 1326 to produce secondary metabolites was dramatically activated by the acquisition of erythromycin resistance.

A mutation in the *rplD* and *rplV* genes, which encode ribosomal protein L4 and L22, respectively, confers resistance to erythromycin and other macrolide antibiotics in various bacteria.^{14–16} We, therefore, sequenced and compared these genes from the antibiotic-overproducing Ery^r mutants SCE234 and SLE259 to the parent strain of *S. coelicolor* A3(2) 1147 or *S. lividans* 1326. The mutation was, however, not detected in either *rplD* or *rplV* genes. As summarized in Table 1, the antibiotic-overproducing Ery^r mutants were found to exhibit comparatively high resistance to other macrolide antibiotics and lincomycin, which may provide a clue to identifying the mutated gene of the Ery^r mutants found in the present study.

In conclusion, we showed that antibiotic production in *Streptomyces* spp. is markedly increased through a spontaneous mutation conferring erythromycin resistance, although the mutated genes remain unknown. Most interestingly, an ACT-overproducing Ery^r mutant of *S. lividans* 1326, strain SLE259, produced abundant antibacterial compounds that could include various substances as well as calcium-dependent antibiotics. Indeed, comparative metabolic profiling of the culture extracts using HPLC identified several substances that were present in the culture medium of strain SLE259 but not in the culture medium of the parent strain. We propose that the acquisition of erythromycin resistance in *Streptomyces* by certain spontaneous mutations could be an effective strategy for the development of their ability to produce secondary metabolites. Our next research goal is to identify the mutation responsible for erythromycin resistance and antibiotic overproduction, and to clarify the mechanisms by which this mutation elicits the ability of *Streptomyces* strains to

produce secondary metabolites. We previously demonstrated, with antibiotic-overproducing ribosomal mutants of *S. coelicolor* A3(2) 1147, the importance of maintaining a high level of protein synthesis at the late growth phase for activating antibiotic production.^{8–10} Although the mutated gene of antibiotic-overproducing Ery^r mutants isolated from *S. coelicolor* A3(2) 1147 and *S. lividans* 1326 was not identified, it is highly probable that ribosomal properties and concomitant protein synthesis activity was modified extensively by an erythromycin resistance mutation, because erythromycin targets ribosomal component.

Experimental procedures are detailed in the supplementary information online.

ACKNOWLEDGEMENTS

This study was performed through the Program for Dissemination of Tenure-Track System funded by the Ministry of Education and Science, Japan, and partially supported by the Hokuto Bio-science Promotion Foundation. We are grateful to Drs Haifeng Hu and Yukinori Tanaka for discussions at the early stage of this study.

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