A Novel Method for Improving *Streptomyces coelicolor* A3(2) for Production of Actinorhodin by Introduction of *rpsL* (Encoding Ribosomal Protein S12) Mutations Conferring Resistance to Streptomycin

Sir:

Streptomycetes are mycelial soil bacteria that produce a high proportion of agriculturally and medically important antibiotics. The development of rational approaches for the improvement of *Streptomyces* strains with respect to antibiotic production is therefore of considerable industrial and economic importance.

The initiation of antibiotic biosynthesis usually occurs at the transition between vegetative growth and morphological development of the organism, and considerable effort has been directed towards gaining a detailed understanding of the mechanisms involved. A potentially significant bacterial regulatory system is the stringent response which is initiated by nutrient limitation and causes an immediate cessation of RNA accumulation and of other cellular reactions (reviewed by CASHEL and RUDD[1]). The guanine nucleotides ppGpp (guanosine 5′-diphosphate 3′-diphosphate) and pppGpp (guanosine 5′-triphosphate 3′-diphosphate) are believed to be responsible for the stringent response. When uncharged tRNA, whose level would increase under amino acid limitation, enters the A site on a ribosome the *relA* gene product (stringent factor = ppGpp synthetase I) catalyses the synthesis of ppGpp and pppGpp. A functional 50S ribosomal protein L11 encoded by the *relC* gene (= *rplK* gene in *E. coli*) is required for activation of ppGpp synthetase I and thus for synthesis of (p)ppGpp. Mutants with mutations in the *relA* or *relC* genes fail to synthesize (p)ppGpp[1]. From analysis of *relC* mutants of several *Streptomyces* spp. it was proposed that ppGpp plays a central role in triggering the onset of antibiotic production in these organisms[2~5]. Results from subsequent studies in a *relC* mutant of *S. antibioticus*[6], *S. coelicolor*[7~9], *S. hygroscopicus*[10], *S. griseus*[11] and in *relA* mutants of *S. coelicolor*[12,13] have supported this hypothesis. More recently, an unambiguous correlation was established between ppGpp synthesis and antibiotic production from the isolation and analysis of a *relC* deletion mutant of *S. coelicolor*[14]. This mutant completely lacked the ability to produce the antibiotic actinorhodin, and was unable to accumulate ppGpp following a nutritional shiftdown. Introduction of the wild-type *relC* gene on a low copy number vector restored the ability of the mutant to accumulate ppGpp, and was accompanied by a complete restoration in actinorhodin productivity. Interestingly, it was also found that the impairment in actinorhodin production resulting from the *relC* mutation could also be completely restored by introduction of mutations conferring resistance to streptomycin (*str*) which result in the alteration of the Lys-88 amino acid in ribosomal protein S12 (*rpsL* gene product) to Glu or Arg. No accompanying restoration of ppGpp synthesis was detected in these *relC str* mutants. This confirmed previous observations which demonstrated that the presence of an altered ribosomal protein S12 resulting from certain mutations within the *rpsL* gene conferring resistance to streptomycin activates actinorhodin production by *S. lividans* TK24, and circumvents the detrimental effects on antibiotic production in *relA* and *brgA* mutants of *S. coelicolor* and *relC* mutants of *S. griseus*[15]. From these results it is therefore apparent that acquisition of certain *str* mutations by these organisms allows antibiotic production to be initiated

Table 1. Actinorhodin productivity of various drug-resistant mutants of *S. coelicolor*.

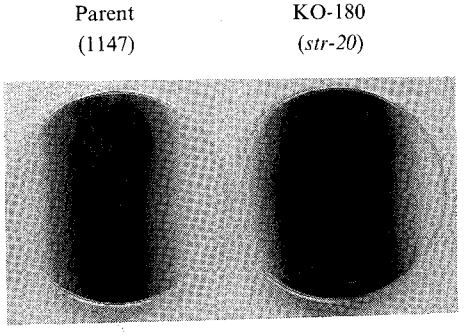
Drug	MIC[a] ($\mu g\,ml^{-1}$)	Concentration used for obtaining mutants ($\mu g\,ml^{-1}$)	Number of mutants		
			Total	Actinorhodin productivity relative to parent strain[b]	
				>5-fold	>10-fold
Chloramphenicol	30	60	120	0	0
Erythromycin	10	30	160	1	0
Lincomycin	20	60	160	0	0
Spectinomycin	30	60	150	2	0
Streptomycin	1	5	100	30	28
Tetracycline	15	50	80	0	0

[a] Determined 5 days after incubation on GYM agar.
[b] Determined 7 days after incubation on R3 agar.

without the requirement for ppGpp. This offers a possible strategy for improving wild-type *Streptomyces* strains for antibiotic productivity. This paper describes the effect of the introduction of *str* mutations into *S. coelicolor* A3(2) on actinorhodin production in this organism. The effect of resistance mutations to a range of other common antibiotics is also reported.

A range of spontaneous drug-resistant mutants of *S. coelicolor* A3(2) were isolated and their ability to produce the blue pigmented antibiotic actinorhodin was assessed (Table 1). Resistant mutants were obtained as colonies that grew within 7 days at 30°C after spores were spread on GYM agar containing 2~5 times the MIC of the drug (see Table 1). Strikingly, more than half (58%) of the mutants resistant to streptomycin (*str*) produced 5-fold or higher quantities of actinorhodin on R3 agar compared to that produced by the parental strain 1147. These actinorhodin-overproducing mutants grew as well as the parental strain, exhibiting only a slight reduction in the rate of growth, and produced aerial mycelia as well as, and in some cases more abundantly than, the parental strain. A representative *str* mutant KO-180 is shown as an example (Fig. 1). Of the other drugs tested, whose antibiotic activities are also attributed to the inhibition of protein synthesis by ribosome binding, only spectinomycin and erythromycin produced mutants which exhibited a significant increase in actinorhodin production but these occurred at a low frequency (Table 1). No mutants with a significantly increased actinorhodin productivity were detected among isolates resistant to chloramphenicol, lincomycin or tetracycline. Four representative streptomycin-resistant mutants (KO-178~KO-181) with an increased ability to produce actinorhodin were selected for analysis of the DNA sequence of the *rpsL* gene encoding ribosomal protein S12. As summarised in Table 2, three isolates possessed a point mutation within the *rpsL* gene resulting in an alteration in the amino acid at position 88 from Lys to Glu. When cultured in R3 liquid medium these mutants produced 8 to 14 times more actinorhodin than the parental strain 1147 following 8 days incubation at 30°C. Mutant KO-179, with low level resistance to streptomycin, also exhibited a similar increase in actinorhodin production (8 fold), but no mutation within the *rpsL* gene was detected. The *strA1* mutant KO-192 harbours a *rpsL* gene mutation at position 43 (Lys to Asn) but not at 88. This mutation resulted in little (if

Fig. 1. Ability of parental and *str* mutant strains of *S. coelicolor* to produce actinorhodin.



Spores were spread on R3 agar[15] and then incubated at 30°C for 5 days. The reverse of the plates are shown.

Table 2. Location of mutations in *rpsL* gene and the resulting amino acid exchange in ribosomal protein S12.

Strain	Position of mutation in *rpsL* gene[a]	Amino acid position	Amino acid exchange	Resistance to streptomycin[b] ($\mu g\,ml^{-1}$)	Actinorhodin productivity (OD_{600})[c]
1147 (wild-type)	—[d]			1	0.21 (0.11~0.27)[e]
KO-178 (*str-18*)	A-262→G	88	Lys→Glu	100	1.81
KO-179 (*str-19*)	None[f]			5	1.63
KO-180 (*str-20*)	A-262→G	88	Lys→Glu	100	1.69
KO-181 (*str-21*)	A-262→G	88	Lys→Glu	100	2.80
KO-192 (*strA1*)[g]	G-129→A[h]	43	Lys→Asn	200	0.38

[a] Cloning and sequencing procedures are described in detail in a previous paper[14]. Numbering is originated from start codon (GTG) of the open reading frame.
[b] Determined 3 days after incubation on GYM agar.
[c] Determined 8 days after incubation in R3 liquid medium.
[d] —, wild-type *rpsL* gene.
[e] Ten single colony isolates were tested. The mean value was 0.21.
[f] Mutations were not detected within the *rpsL* gene.
[g] This prototrophic *strA1* mutant was obtained by conjugation between strains 1147 and J1508 (*uraA1 hisA1 strA1* NF SCP2$^-$).
[h] Data from reference 15.

any) increase in actinorhodin productivity, consistent with a previous report that *strA1* mutation has no effect on antibiotic production[15].

The point mutation identified in the *rpsL* gene in KO-178, KO-180 and KO-181 corresponds to the *str-6* mutation previously reported as being responsible for inducing actinorhodin production by *S. lividans* TK24[15], and to a *str* mutation recently reported to be capable of blocking the inhibitory effect of *relA*, *relC* and *brgA* mutations on actinorhodin production by *S. coelicolor*[14,15]. The molecular basis for the observed role of the altered ribosomal protein S12 is unclear, but it is apparent that it exerts its effect at the translational level. It is possible that the streptomycin-resistant mutant identified in the present study which did not possess a lesion in the S12 protein (*i.e.* KO-179) harbours a mutation in rRNA or in a ribosomal protein other than S12. Three strains KO-178, KO-180 and KO-181 harbour identical mutations in *rpsL*: however, KO-181 produced considerably more actinorhodin than the other two strains. This raises the formal possibility that strain KO-181 might be a double mutant with a mutation similar to that in strain KO-179.

In conclusion, introduction of the specified *str* mutation into *S. coelicolor* A3(2) gave rise to an approximately 15 fold increase in actinorhodin production and breeding this trait into other *Streptomyces* species should therefore offer a convenient and effective method for improving antibiotic productivity in these organisms. Indeed, as will be reported in detail elsewhere, this method was effective for strain improvement as examined in several *Streptomyces* species producing actinomycin, formycin or fredericamycin.

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