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ORIGINAL PAPER

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Deletion of *scbA* enhances antibiotic production in *Streptomyces lividans*

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Abstract Antibiotic production in many streptomycetes is influenced by extracellular γ-butyrolactone signalling molecules. In this study, the gene *scbA*, which had been shown previously to be involved in the synthesis of the γ-butyrolactone SCB1 in *Streptomyces coelicolor* A3(2), was deleted from the chromosome of *Streptomyces lividans* 66. Deletion of *scbA* eliminated the production of the antibiotic stimulatory activity previously associated with SCB1 in *S. coelicolor*. When the *S. lividans scbA* mutant was transformed with a multi-copy plasmid carrying the gene encoding the pathway-specific activator for either actinorhodin or undecylprodigiosin biosynthesis, production of the corresponding antibiotic was elevated significantly compared to the corresponding *scbA*$^+$ strain carrying the same plasmid. Consequently, deletion of *scbA* may be useful in combination with other strategies to construct host strains capable of improved bioactive metabolite production.

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Introduction

Actinomycetes produce approximately two-thirds of all known antibiotics of microbial origin. Over 6,000 of these compounds are produced by *Streptomyces* species and many are commercially important medicinal products used therapeutically as anti-infective (antibiotics, antifungal and antiparasitic), anticancer or immunosuppressant agents (Champness 2000).

Many factors affect the level of production of a particular compound in a specific host. Classically, strain improvement has been achieved by empirical methods usually involving random mutation and subsequent screening for higher-producing progeny. With the information available from the *Streptomyces coelicolor* A3 (2) genome sequence (http://www.sanger.ac.uk/Projects/S_coelicolor/), it is now possible to attempt more rational approaches to increase yield (Chater et al. 1990). As a model system for strain improvement, we have used the closely related *Streptomyces lividans* 66, which has been engineered to overproduce either the polyketide actinorhodin (ACT) or the tri-pyrrole undecylprodigiosin (RED). This was achieved by introducing a multi-copy plasmid carrying the gene encoding the pathway-specific regulatory gene for either biosynthetic gene cluster. This leads to the activation of the normally silent *act* pathway in the *S. lividans* chromosome (Bruheim et al. 2002), and a substantial increase in the normally poorly expressed *red* pathway (Takano et al. 1992). Thus, these strains can be viewed as models for strains producing bioactive molecules that have already been subjected to some strain improvement to increase the expression of genes encoding biosynthetic enzymes, but in which some other aspect of host metabolism may still be limiting product formation.

Extracellular γ-butyrolactone signalling molecules are known to be required for streptomycin production and morphological differentiation in *Streptomyces griseus* (Horinouchi and Beppu 1994) and for virginiamycin production in *Streptomyces virginiae* (Kondo et al. 1989). Precocious production of ACT and RED was observed

when *scbA*, a homologue of a gene (*afsA*) involved in γ-butyrolactone biosynthesis in *S. griseus*, was deleted in *S. coelicolor* (Takano et al. 2001). Surprisingly, this phenotype is the opposite of that of the *S. griseus afsA* mutant (which is no longer able to produce streptomycin). To test whether the disruption of *scbA* could be a generally applicable method for increasing antibiotic production, we examined the effect of making the same mutation in *S. lividans* overproducing ACT or RED.

Materials and methods

Bacterial strains and plasmids

Escherichia coli strains DH5α (Sambrook et al. 1989) and ET12567 (MacNeil et al. 1992) were used for routine subcloning and conjugation into *S. lividans*, respectively. Growth was at 37 °C in Luria-Bertani medium and transformation was carried out by standard procedures (Sambrook et al. 1989). *E. coli* transformants were selected with carbenicillin (100 μg ml^{-1}), apramycin or hygromycin (both at 50 μg ml^{-1}). *S. lividans* 66 (referred to in this paper by its John Innes Centre strain number 1326) and *S. coelicolor* M145 were grown and manipulated as described previously (Kieser et al. 2000). Conjugation from *E. coli* used the helper plasmid pUZ8002 (Paget et al. 1999). *S. lividans* exconjugants were selected by adding 0.5 mg of nalidixic acid and 1 mg of apramycin in 1 ml of water to each agar plate. Strains were purified and maintained using 20 μg apramycin ml^{-1} or 50 μg of thiostrepton ml^{-1}.

The *scbA* deletion plasmid pIJ6140 (Takano et al. 2001) was based on pKC1132 (Bierman et al. 1992). The multi-copy plasmids pIJ68 (Passantino et al. 1991) and pIJ6014 (Takano et al. 1992), carrying the pathway-specific activator genes *act*II-orf4 and *redD*, respectively, were as described previously. The plasmid vector pIJ486 was described by Kieser et al. (2000).

Growth and fermentation conditions

Spores of the transformed strains were initially selected on R2 agar (containing 50 μg thiostrepton ml^{-1}). Spores were streaked on MS (mannitol soya flour medium) agar (containing 50 μg thiostrepton ml^{-1}) to generate dense spore suspensions for inoculation into 50 ml of a mixture of two parts YEME (yeast extract-malt extract medium) and one part TSB (tryptone soya broth) liquid medium (again containing 50 μg thiostrepton ml^{-1}) in a 250-ml conical spring-flask. After 2 days shaking at 30 °C, the mycelium was collected by centrifugation and resuspended in fresh spring-flasks containing phosphate-limited Evans medium (Evans et al. 1970) with 20 μg thiostrepton ml^{-1}. Incubation was continued at 30 °C for a further 7 days with 1 ml samples being removed for assessment of antibiotic production.

For stirred-tank fermentation experiments, strains were stored as frozen mycelium at −80 °C. The stock cultures were used to inoculate (1% v/v) 100 ml GG1 medium (per litre: 15 g glucose, 15 g glycerol, 15 g soy peptone, 3 g NaCl, 1 g $CaCO_3$, 0.005 g thiostrepton) in 500-ml baffled Erlenmeyer flasks. Cultures were grown for 48 h at 28 °C on a rotary shaker at 200 rpm. Eight ml of the culture were used to inoculate 100 ml GYB medium (per litre: 33 g glucose, 15 g yeast extract, 0.005 g thiostrepton) in 500-ml baffled flasks. Cultures were grown for 24 h at 28 °C on a rotary shaker at 200 rpm and 100 ml were used to inoculate 4-l fermenters containing 2.5 l of phosphate-limited Evans medium.

Cultivation conditions (using 3-l Applicon fermenters with a working volume of 1 l) for production of ACT by *S. lividans* 1326/pIJ68 in a phosphate-limited minimal medium (containing 10 g glucose per litre) were as described previously (Bruheim et al. 2002).

RED production was carried out in 4-l Chemap fermenters with 2.5 l of phosphate limited-Evans minimal medium containing 2 mM NaH_2PO_4 and 25 g glucose per litre(Butler et al. 2002). For determination of dry cell weight (DCW), 2 ml samples of culture were vacuum-filtered through a pre-weighed Whatman GF/C filter paper, washed with 10 ml distilled water, and dried at 90 °C to constant weight. All measurements were done in triplicate.

Antibiotic and metabolite estimations

ACT was measured as absorbance at 640 nm (ε640=25,320) as described by Bystrykh et al. (1996). RED production was analysed by HPLC following extraction with acidified methanol and using a sample of pure undecylprodigiosin as external standard (Butler et al. 2002).

Isolation of γ-butyrolactones and bioassay analysis

γ-Butyrolactones were isolated from liquid or solid media (SMMS, supplemented liquid minimal medium with 1.5% w/v agar) by extracting the culture supernatant or agar with ethyl acetate. The solvent was then evaporated and the samples were resuspended in methanol for use in bioassays as described previously (Takano et al. 2000).

Results

Construction of a *S. lividans scbA* deletion mutant

The deletion mutant allele of *scbA* from *S. coelicolor* (Takano et al. 2001) was introduced into *S. lividans* 1326 by conjugation of pIJ6140 from *E.coli* ET12567. Integration of the non-replicating plasmid was selected using apramycin. Purified apramycin-resistant exconjugants were grown without antibiotic selection for three rounds of sporulation (on MS agar). Colonies were screened for sensitivity to apramycin, indicating loss of the plasmid by a second homologous recombination event. Four apramycin-sensitive colonies were identified among 3,000 colonies screened. PCR analysis of chromosomal DNA produced amplified DNA fragments consistent with the wild-type chromosomal arrangement for three colonies, whereas the fourth colony yielded a smaller DNA fragment consistent with the in-frame deletion allele (Δ*scbA*). Southern hybridisation experiments on chromosomal DNA digested either with *Nco*I or a mixture of *Bgl*II and *Pst*I produced hybridizing bands consistent with the wild-type from the first three colonies and with the Δ*scbA* mutant from the fourth colony. The mutant was designated *S. lividans* M707.

No γ-butyrolactone with antibiotic stimulatory activity on *S. coelicolor* M145 was detected in ethyl acetate extracts of M707 grown on agar medium (Fig. 1), whereas stimulatory activity (visualised as a ring of pigmented mycelial growth in Fig. 1) was produced by *S. lividans* 1326 (*scbA*$^+$). Thus, the phenotype of the Δ*scbA* mutation in *S. lividans* with respect to the production of antibiotic stimulatory factors was indistinguishable from that observed previously in *S. coelicolor* (Takano et al. 2001).

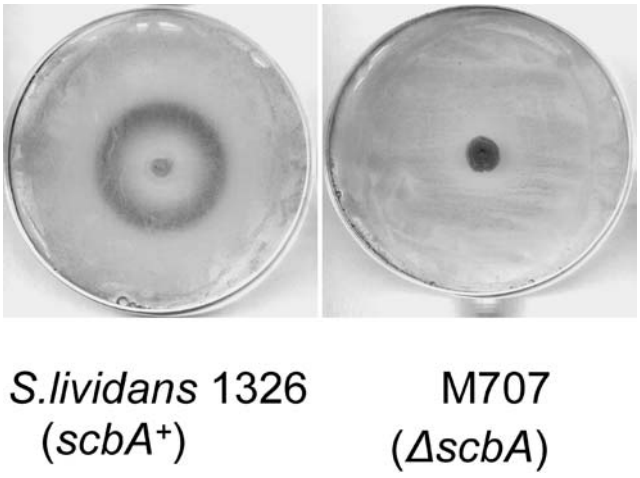

Fig. 1 Deletion of *scbA* eliminates production of the γ-butyrolactone antibiotic-stimulatory activity by *Streptomyces lividans*. Ethyl acetate extracts from SMMS agar cultures of *S. lividans* 1326 and M707 were spotted onto confluent lawns of *S. coelicolor* M145 spores on SMMS and incubated at 30 °C for 30 h

Production of antibiotics in shake flask fermentations using *S. lividans* M707

Analysis of growth and antibiotic production of an *scbA* mutant of *S. coelicolor* M145 had previously shown precocious production of both ACT and RED (Takano et al. 2001). We therefore examined whether increased levels of antibiotic production could be obtained in *scbA* mutants of *S. lividans* that had been engineered to overproduce either ACT or RED by cloning the relevant pathway-specific activator gene on a multi-copy plasmid.

Protoplasts of *S. lividans* strain M707 were transformed with pIJ68 (*act*II-orf4), pIJ6014 (*redD*) or pIJ486 (vector control), selecting in each case for the plasmid-borne thiostrepton-resistance gene. The effect of the *scbA* mutation on ACT production was assessed initially in shake-flask fermentation experiments using Evans liquid medium. No ACT was detected in either 1326 or M707 containing the control vector pIJ486 (data not shown). M707/pIJ68 produced ACT at an eight-fold higher concentration (12.1±1.0 mg ACT per g of mycelial DCW) than did 1326/pIJ68 (1.5±0.8 ACT per g of DCW), at a faster rate, and for a longer period of time (Fig. 2).

RED production in shake-flasks was also assessed (Fig. 3). RED production was approximately three times higher in M707/pIJ6014 than in 1326/pIJ6014 (biomass levels were not significantly different, data not shown). As for ACT production, RED synthesis occurred at a faster rate for a longer time period in the Δ*scbA* mutant. M707 and 1326 carrying the control vector (pIJ486) did not produce RED under the experimental conditions used (data not shown). Replicate experiments produced essentially the same levels of RED (±10%) as those shown in Fig. 3.

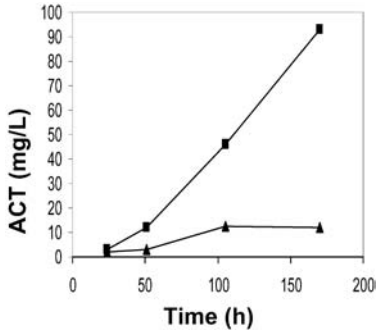

Fig. 2 ACT production in shake-flask fermentations. *Triangles* 1326/pIJ68, *squares* M707(Δ*scbA*)/pIJ68

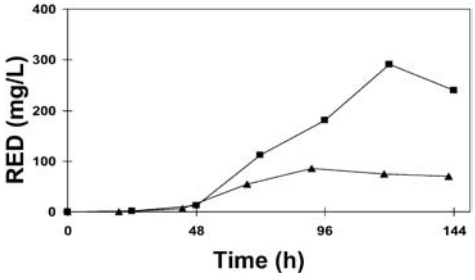

Fig. 3 RED production in shake-flask fermentations. *Triangles* Strain 1326/pIJ6014, *squares* strain M707(Δ*scbA*)/pIJ6014

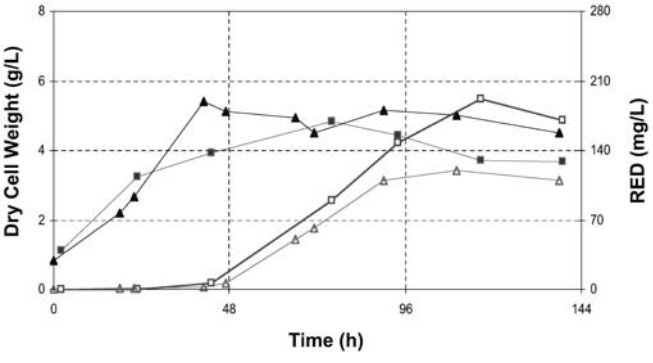

Fig. 4 RED production in 4-l fermenters. The values for RED production are shown as *open triangles* for strain 1326/pIJ68 and *open squares* for strain M707(Δ*scbA*)/pIJ6014. Dry cell weights are represented as *closed triangles* for 1326/pIJ6014 and *closed squares* for M707(Δ*scbA*)/pIJ6014

Production of antibiotics in stirred-tank fermenters using *S. lividans* M707

The strains were further tested for their ability to produce antibiotics in stirred-tank batch fermentations. M707/pIJ6014 and 1326/pIJ6014 were cultured in 4-l stirred-tank fermenters in phosphate-limited Evans medium (Fig. 4). The two strains showed similar growth kinetics and reached a maximum biomass concentration of approximately 5 g per litre. The onset of RED production coincided with the beginning of stationary phase. While the initial rates of RED synthesis were similar for both

strains, the production phase of M707/pIJ6014 continued for an additional 20 h, resulting in approximately 50% more RED production in the Δ*scbA* mutant.

Similarly, in three independent experiments (data not shown), an approximately two-fold improvement in ACT production was observed in 1 l fermentations with M707/pIJ68 (10±0.5 g ACT per litre) compared to 1326/pIJ68 (5±0.5 g per litre).

Discussion

Eliminating the ability of *S. lividans* to synthesise γ-butyrolactone signalling molecules capable of stimulating antibiotic production in *S. coelicolor* resulted in improved production of both ACT and RED in strains already engineered to produce substantial amounts of each antibiotic. The molecular events involved in the control of antibiotic biosynthesis by γ-butyrolactones (and their associated binding proteins) in *S. coelicolor*, *S. lividans*, and other streptomycetes are not known (e.g. Takano et al. 2001; Stratigopoulos and Cundliffe 2002). *S. coelicolor scbA* mutants show markedly reduced levels of transcription of the putative repressor protein ScbR (Takano et al. 2001), but there is currently no evidence to suggest that this mutation results in elevated levels of transcription of the *act* and *red* genes. Conceivably, the *scbA* mutation might reduce the level of expression of metabolic pathways that compete with the *act* and *red* biosynthetic enzymes for metabolic precursors. In this context, it is interesting to note that deletion of *scbA* in *S. coelicolor* results in reduced expression of a type I polyketide synthase gene cluster (Kotowska et al. 2002; E. Takano, unpublished results). The product of this gene cluster is not known, but the overproduction of ACT and RED observed in the *scbA* mutant may at least partially reflect its reduced expression and elevated levels of metabolic precursors. This interpretation would also be consistent with results obtained for *S. coelicolor*, in which deletion of *act*II-orf4 resulted in enhanced RED production, and deletion of *redD* resulted in increased ACT synthesis (B. Floriano and M. Bibb, unpublished results). This effect was subsequently confirmed for RED production in fermenters by comparing the levels produced by *S. coelicolor* M145 (28 mg per litre) with those of its Δ*act*II-orf4 derivative M511 (120 mg per litre; S. Jovetic and F. Marinelli, unpublished results). Alternatively, it is conceivable that *scbA* plays a role in regulating aspects of primary metabolism that influence levels of secondary metabolite production.

Irrespective of the physiological role of *scbA*, it may be useful to combine mutations in this gene with other approaches that have been used to enhance antibiotic production (Butler et al. 2002; Chen et al. 2000; Lombo et al. 2001; Minas et al. 1998; Pfeifer et al. 2001), thereby improving *S. lividans* as a host for the production of a wide range of secondary metabolites.

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