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Acquisition of Certain Streptomycin-Resistant (*str*) Mutations Enhances Antibiotic Production in Bacteria†

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Physiological differentiation (including antibiotic production) in microorganisms usually starts when cells encounter adverse environmental conditions and is frequently accompanied by an increase in the accumulation of intracellular ppGpp. We have found that the acquisition of certain streptomycin-resistant (*str*) mutations enables cells to overproduce antibiotics, demonstrating an increase in productivity 5- to 50-fold greater than that of wild-type strains. The frequency of such antibiotic-overproducing strains among the *str* mutants was shown to range from 3 to 46%, as examined with several strains of the genera *Streptomyces*, *Bacillus*, and *Pseudomonas*. Analysis of *str* mutants from *Bacillus subtilis* Marburg 168 revealed that a point mutation occurred within the *rpsL* gene, which encodes the ribosomal protein S12, changing Lys-56 (corresponding to Lys-43 in *Escherichia coli*) to Asn, Arg, Thr, or Gln. Antibiotic productivity increased in a hierarchical manner depending upon which amino acid residue replaced Lys at this position. The *strA1* mutation, a genetic marker frequently used for mapping, had no effect on antibiotic productivity even though it was found to result in an amino acid alteration of Lys-56 to Ile. Gene replacement experiments with the *str* alleles demonstrated unambiguously that the *str* mutation is responsible for the antibiotic overproductivity observed. These results offer a rational approach for improving the production of antibiotic (secondary metabolism) from microorganisms.

One of the significant bacterial regulatory systems is the stringent response which is initiated by nutrient limitation and causes an immediate cessation of RNA accumulation and other cellular reactions. The guanine nucleotides ppGpp (guanosine 5′-diphosphate 3′-diphosphate) and pppGpp (guanosine 5′-triphosphate 3′-diphosphate) are believed to be responsible for this stringent response. Mutants with mutations in the *relA* or *relC* (=*rplK*) genes (which code for the ppGpp synthetase and the ribosomal L11 protein, respectively) fail to synthesize (p)ppGpp (for reviews, see references 3 and 14). Recently, through working with *Streptomyces coelicolor* A3(2), an unambiguous correlation was established between ppGpp synthesis and antibiotic production as demonstrated from the results of three independent laboratories using various approaches at the molecular level (4, 28, 35). We have found (35, 43) that the impairment in antibiotic production resulting from a *relA* or *relC* mutation in *S. coelicolor* A3(2) could be completely restored by introducing mutations conferring resistance to streptomycin (*str*). These mutations result in the alteration of the Lys-88 amino acid in ribosomal protein S12 (*rpsL* gene product) to Glu or Arg (35, 43). No accompanying restoration of ppGpp synthesis was detected in these *relA str* or *relC str* mutants. It is therefore apparent that acquisition of certain *str* mutations allows antibiotic production to be initiated without the requirement for ppGpp. This offers a possible strategy for improving the antibiotic productivity of wild-type prokaryotic microorganisms.

The initiation of antibiotic biosynthesis (so-called secondary metabolism) usually occurs at the transition between vegetative growth and morphological development, such as the sporulation stage of the organism, and considerable effort has been directed towards gaining a detailed understanding of the mechanism involved (reviewed in references 5–7, 22, 26, 38, and 58). Members of the genera *Streptomyces*, *Bacillus*, and *Pseudomonas* are soil bacteria that produce a high proportion of agriculturally and medically important antibiotics. The development of rational approaches to improve the production of antibiotics from these organisms is therefore of considerable industrial and economic importance. This paper describes the effect of the introduction of *str* mutations on antibiotic production in these organisms.

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† Dedicated to the late Edward Katz for his pioneering work regarding the implication of antibiotics in microbial secondary metabolism.

MATERIALS AND METHODS

Bacterial strains and preparations of mutants. The strains of *Streptomyces* spp. and other bacteria used in this study are listed in Table 1. *Bacillus subtilis* 168 is a standard strain frequently used for studying sporulation. Strains 60009, 61884, 61883, and 61953 are derivatives of strain 168. The spontaneous streptomycin-resistant mutants of each organism were obtained as colonies that grew within 7 to 14 days (for *Streptomyces* spp.) or within 3 to 7 days (for other bacteria) after spores or cells were spread on glucose-yeast extract-malt extract (GYM) agar containing streptomycin at a stated concentration (see below). The mutants obtained were used for subsequent study after single-colony isolation was done.

Media and growth conditions. GYM medium, nutrient sporulation medium, and synthetic medium (S7 medium) were described previously (32, 33). NG medium, a medium developed for antibiotic production by *Bacillus subtilis*, contained (per liter) 10 g of nutrient broth (Difco), 10 g of glucose, 2 g of NaCl, 5 mg of $CuSO_4 \cdot 5H_2O$, 7.5 mg of $FeSO_4 \cdot 7H_2O$, 3.6 mg of $MnSO_4 \cdot 5H_2O$, 15 mg of $CaCl_2 \cdot 2H_2O$, and 9 mg of $ZnSO_4 \cdot 7H_2O$ (adjusted to pH 7.2 with NaOH). All strains were stocked after growing on GYM agar at 30°C.

Production and determination of antibiotics. All strains, including *B. subtilis*, were incubated at 30°C with shaking. The culture conditions for the production of each antibiotic were optimized as described below.

(i) Fredericamycin. *Streptomyces chattanoogensis* ISP 5002 was precultured in galactose-glycerol-corn steep liquor medium for 2 days. Then, cells were inoculated into the production medium followed by 4 days of cultivation. The production medium consisted of (per liter) 5 g of L-phenylalanine, 1.5 g of ammonium sulfate, 0.5 g of K_2HPO_4, 0.5 g of KH_2PO_4, and 2 g of $CaCO_3$ (adjusted to pH 7.2 with NaOH). Concentration of fredericamycin produced was determined by high-performance liquid chromatography analysis.

(ii) Actinomycin. *Streptomyces antibioticus* 3720 (= ATCC 14888) was grown in peptone (NZ-amine; type A) medium for 30 h. Cells were inoculated into a production medium (galactose-glucose-glutamate medium) and cultivated for 4

TABLE 1. Antibiotic-producing microorganisms used in this study

Strain	Antibiotic produced	Description	Source or reference
S. antibioticus 3720	Actinomycin	Prototrophic wild type	31
S. chattanoogensis ISP5002	Fredericamycin	Prototrophic wild type	JCM 4299 (= ATCC 19739)
S. lavendulae MA406 A-1	Formycin	Prototrophic wild type	30
B. subtilis 168	NI[a]	*trpC2*	Standard strain for studying sporulation
B. subtilis 60009	NI	*strA1*	From E. Freese; originally isolated by Goldthwaite et al. as *str-1* (15) and now designated *rpsL* (39)
B. subtilis 61884	NI	*aspB66 trpC2*	33
B. subtilis 61883	Impaired antibiotic production	*aspB66 trpC2 relA1*	Isogenic *relA* mutant to 61884 (33)
B. subtilis 61953	Impaired antibiotic production	*thr-5 trpC2 relC*	34
B. cereus 2045	FR900493	Prototrophic wild type	29a
P. pyrrocinia 2327	Pyrrolnitrin	Prototrophic wild type	25

[a] NI, not identified, but inhibits growth of *S. aureus*.

days as described previously (31). The concentration of actinomycin produced was determined spectrometrically at 443 nm after extraction with ethyl acetate.

(iii) Formycin. *Streptomyces lavendulae* MA406 A-1 was cultivated in maltose-polypeptone-yeast extract (MPY) medium for 2 days as described previously (30). Concentrations of formycin were determined by bioassay using *Xanthomonas oryzae* as a test organism.

(iv) Pyrrolnitrin. *Pseudomonas pyrrocinia* 2327 (=ATCC 15958) was precultured in glucose-polypeptone-meat extract medium (2) for 2 days. Cells (0.1 ml) were then inoculated into the same medium (5 ml) and cultivated for 3 days. Concentrations of pyrrolnitrin were determined by a bioassay using *Candida albicans* as a test organism.

(v) FR900493. *Bacillus cereus* 2045 (=FERM BP-1791) was precultured in bouillon medium for 10 h. Cells (0.1 ml) were inoculated into 5 ml of production medium consisting of (per liter) 20 g of polypeptone, 20 g of corn steep liquor, and 5 g of NaCl (adjusted to pH 7.5 with NaOH). Concentrations of FR900493 {3-[(5-aminomethyl-3,4-dihydroxytetrahydrofuran-2-yl)oxy]-2-[(3-aminopropyl) methylamino]-3-[5-(3,4-dihydro-2,4-dioxo-2H-pyrimidin-1-yl)-3,4-dihydroxytetrahydrofuran-2-yl]propionic acid} were determined by a bioassay using *Staphylococcus aureus* 209P.

(vi) *B. subtilis* antibiotic. Strains of *B. subtilis* were grown in NG medium (see above) plus requirements (Trp, Thr, or Asp [each at 50 μg/ml]) by direct inoculation of spores into the medium. Cultivation was carried out at 30°C for 30 h. Antibiotic production was determined by a bioassay using *S. aureus* 209P as described previously (34). Antibiotic activity is expressed as units per milliliter. One unit per milliliter produced a 1.5-mm-wide halo (diameter of clear area, 11 mm). Test solutions were diluted appropriately to produce haloes of this diameter.

Sporulation conditions for *B. subtilis*. The cells were inoculated at an optical density at 600 nm (OD_{600}) of 0.01 to 0.02 into flasks containing sporulation medium (33) plus requirements (Trp, Thr, or Asp [each at 50 μg/ml]) and were incubated at 37°C for the indicated times with vigorous shaking. The spore titer was measured by first heating the culture for 20 min at 75°C and then plating after appropriate dilution.

Nutritional shift-down and assay of ppGpp. *B. subtilis* cells were grown in 20 ml of synthetic medium (33) plus 1% (wt/vol) vitamin-free Casamino Acids (Difco) and requirements (Trp, Thr, or Asp [each at 50 μg/ml]). When cells had grown to mid-exponential phase (OD_{600} = 2 to 2.5), they were collected on a membrane filter (0.45-μm pore size), immediately washed with 10 ml of synthetic medium, and the filters with cells were quickly transferred to 20 ml of the synthetic medium without Casamino Acids. After incubation for 15 min, cells were rapidly collected as just described, and the filters were then laid upside down on 10 ml of formic acid (1 M) in a plastic petri dish. After a 60-min incubation at 4°C, cells were removed by centrifugation. The supernatant was filtered through a syringe and vacuum evaporated. The residue was dissolved in 200 μl of water and used for the determination of ppGpp.

ppGpp was determined by high-performance liquid chromatography as described previously (32). Intracellular ppGpp concentrations were expressed in picomoles per AM unit (1 AM unit [or 1 AM_{600}] is the amount of cells which produced an OD_{600} of 1 when suspended in 1 ml). One AM unit was equivalent to 0.425 mg of cells (dry weight).

Detection of the *rpsL* gene mutation in *B. subtilis*. The *rpsL* gene of *B. subtilis* (as shown in the sequence obtained by H. Yoshikawa [DDBJ accession no. D64127]) was amplified from *B. subtilis* genomic DNA by PCR using synthetic oligonucleotide primers P1 (5′-CCACCTGGGTATGTGGGTT-3′) and P2 (5′-GCATCTGAATATCCTCCCT-3′). The PCR products were directly sequenced manually with α-^{35}S-dCTP or automatically by using the ABI PRISM 310 Genetic Analyzer. The chosen primers amplify the whole *rpsL* gene so that we can evaluate whether not finding a mutation in a particular gene indicates that the defect must be in another gene.

Replacement of the *rpsL* gene. Mutant *rpsL* genes were amplified by PCR with mutant genomic DNA (*str-5*, *str-9*, or *str-10*) as a template. The primers used were P3 (5′-CCTTGAATTCGCCTACAATTAATCAGCTAA-3′, corresponding to positions 3 to 22 of the *rpsL* open reading frame [underlining indicates the cleavage site for *Eco*RI]) and P4 (5′-CCTTAAGCTTGCATCTGAATATCCT CCCT-3′, corresponding to positions 27 to 47 of the gene outside the C-terminal end of the *rpsL* open reading frame [underlining indicates the cleavage site for *Hin*dIII). The resulting 479 bp of PCR product were digested with *Eco*RI and *Hin*dIII and then cloned into the corresponding sites of pAG58 (25). pAG58 is an expression vector which can integrate into the *B. subtilis* chromosome if the plasmid contains a DNA fragment homologous to part of the chromosome. This plasmid contains the chloramphenicol resistance gene (*cam*) as a selective marker in *B. subtilis*. The resulting plasmids, named pSR1, pSR150, and pSR87, were used to transform wild-type *B. subtilis* 168, and the transformants were first selected for streptomycin resistance. The concentration of streptomycin used for the selection of transformants was 100 μg/ml. The resulting *str* transformants were then screened for chloramphenicol sensitivity to obtain the clones with the mutant alleles, in which the gene replacement occurred by a double-crossover event (see Fig. 2).

RESULTS

Effect of *str* mutation on *Streptomyces* spp. We first examined the effect of a *str* mutation on antibiotic production in three *Streptomyces* spp., *S. chattanoogensis* (which produces fredericamycin), *S. antibioticus* (which produces actinomycin), and *S. lavendulae* (which produces formycin). When the spores of these *Streptomyces* spp. were spread and incubated on GYM agar containing 5 or 30 μg of streptomycin per ml, streptomycin-resistant (*str*) mutants developed after 7 to 14 days at a frequency of 10^{-6} to 10^{-8}. These spontaneous *str* mutants were characterized from a wide variety of colonies by size, morphology, and pigment formation. *S. lavendulae* and *S. chattanoogensis* are shown as examples (Fig. 1). We examined antibiotic production using each of 50 to 100 *str* mutants that were selected randomly. Strikingly, in *S. chattanoogensis* nearly half of the *str* mutants tested exhibited a significantly increased ability (greater than fivefold) to produce fredericamycin. The highest productivity detected was 26-fold higher than that of the wild-type strain (Table 2). The level of streptomycin resistance of the high-level-antibiotic-producing (high-producing) strains ranged from 50 to 200 μg/ml. Similarly, strains producing high levels of actinomycin and formycin could be detected at a relatively high frequency (3 to 4%) among *str* mutants of *S. antibioticus* and *S. lavendulae*, respectively (Table 2). Thus, like actinorhodin production by *S. coelicolor* A3(2) (19), introduction of mutations conferring resistance to streptomycin was shown to be effective for improving the antibiotic productivity of the *Streptomyces* spp. tested.

Effect of *str* mutations on *Bacillus* and *Pseudomonas*. Introduction of the *str* mutation also improved antibiotic productiv-

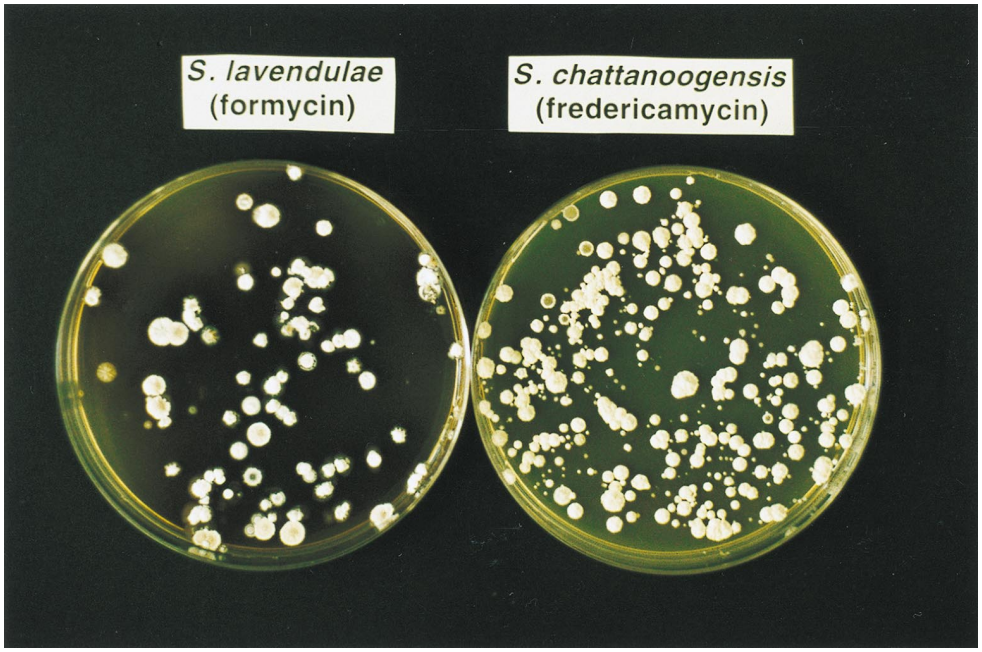

FIG. 1. Spontaneously generated streptomycin-resistant colonies of *S. lavendulae* and *S. chattanoogensis*. Spores (ca. 10^9) of wild-type *S. lavendulae* and *S. chattanoogensis* were spread on GYM agar plates containing 5 or 30 μg of streptomycin per ml, respectively, and incubated at 30°C for 10 days.

ity of bacteria such as *Bacillus* spp. and *Pseudomonas* spp. (Table 2). In *B. cereus* (which produces the nucleoside antibiotic FR900493) and *P. pyrrocinia* (which produces pyrrolnitrin), the frequency of antibiotic overproducing strains among *str* mutants ranged from 7 to 30%.

B. subtilis 168 and its derivatives produce an antibiotic (23, 34). *str* mutants of the organism developed on GYM agar containing either a low (5 μg/ml) or a high (400 μg/ml) concentration of streptomycin were examined. As shown in Table 2, antibiotic-overproducing strains were detected at a higher frequency among *str* mutants selected at a high concentration, rather than a low concentration, of streptomycin (19 versus 3%). Antibiotic production was found to be 50-fold greater than that of the wild-type strain in one case (380 U/ml for the *str* mutant compared with 8 U/ml for strain 168). These *str* mutants exhibit only a slight decrease in growth rate compared to the wild-type strain.

We examined whether acquisition of resistance to drugs other than streptomycin can also give rise to an increase in antibiotic-producing ability in *B. subtilis* 168. None of the mutants (we tested 60 mutants for each drug resistance) resistant to chloramphenicol, tetracycline, lincomycin, or spectinomycin exhibited increased antibiotic production.

Characterization of *str* mutants derived from *B. subtilis*. We focused on *B. subtilis* for further investigation, since this organism offers a feasible system for genetic analysis at the molecular level.

str mutants generated on GYM agar containing a high concentration (400 μg/ml) of streptomycin (we examined 11 representative strains) were all found to have a mutation within the *rpsL* gene, as was the case for strain 60009, which was previously known to contain the *str* mutation *strA1* (Table 3). It should be emphasized that the altered amino acid positions detected were all located at Lys-56 (corresponding to Lys-43 in *Escherichia coli*) of the ribosomal protein S12 encoded by the *rpsL* gene. These *str* mutants are classified in three groups according to the ability to produce antibiotic. The first group (KO-263 to KO-266), which alters Lys-56 to Asn, is characterized by a lack of increase in antibiotic-producing ability. The second group (KO-267 to KO-271), which alters Lys-56 to Arg

TABLE 2. Antibiotic productivity of bacterial streptomycin-resistant mutants

Microorganism	MIC (μg/ml)	Concn of streptomycin used for selecting *str* mutants (μg/ml)	Antibiotic (production [μg/ml]) in parental (wild-type) strain	Frequency (%) of *str* mutants producing increased antibiotic[c]	Highest productivity detected (μg/ml)
S. chattanoogensis	1	30	Fredericamycin (10)	46 (46/100)	260
S. antibioticus	1	5	Actinomycin (12)	4 (2/50)	63
S. lavendulae	1	5	Formycin (25)	3 (2/60)	130
P. pyrrocinia	100	300	Pyrrolnitrin (1.5)	30 (30/100)	15
B. cereus	1	3	FR900493 (76)	7 (9/70)	550
B. subtilis[a]	1	5	*B. subtilis* antibiotic (8)[b]	3 (7/240)	380[b]
	1	400	*B. subtilis* antibiotic (8)[b]	19 (37/194)	80[b]

[a] Strain 168 was used.
[b] Expressed in units per milliliter.
[c] Mutants producing more than fivefold more antibiotic than the wild type. Numbers in parentheses are number of mutants producing increased antibiotic/number of mutants tested.

TABLE 3. Summary of mutations on the *B. subtilis rpsL* gene resulting in amino acid exchange in the ribosomal protein S12

B. subtilis strain	Amt of antibiotic produced (U/ml)[a]	Spores/ml[b] in sporulation medium		Position of mutation in *rpsL* gene[c]	Amino acid position (exchange)	Resistance to streptomycin (μg/ml)[d]
		Alone	Plus 0.5% glucose			
168 (parent strain)	8	1.5×10^9	2.5×10^8			1
60009 (*strA1*)	6	3×10^8	5×10^7	A-167→T	56 (Lys→Ile)	1,000
KO-263 (*str-1*)	8	4×10^8	2×10^7	A-168→T	56 (Lys→Asn)	500
KO-264 (*str-2*)	6	6×10^8	3×10^8	A-168→T	56 (Lys→Asn)	500
KO-265 (*str-3*)	6	8×10^8	3×10^8	A-168→T	56 (Lys→Asn)	500
KO-266 (*str-4*)	6	9×10^8	2×10^8	A-168→T	56 (Lys→Asn)	500
KO-267 (*str-5*)	64	1.2×10^9	1×10^8	A-167→G	56 (Lys→Arg)	2,000
KO-268 (*str-6*)	64	1.5×10^9	3×10^7	A-167→G	56 (Lys→Arg)	1,000
KO-269 (*str-7*)	80	1×10^9	4×10^7	A-167→G	56 (Lys→Arg)	1,000
KO-270 (*str-8*)[e]	80	8×10^8	1×10^8	C-64→T A-167→G	22 (Pro→Ser) 56 (Lys→Arg)	1,000
KO-271 (*str-9*)	80	1.5×10^9	1×10^7	A-167→C	56 (Lys→Thr)	1,000
KO-272 (*str-10*)	42	1×10^8	1×10^7	A-166→C	56 (Lys→Gln)	400
KO-273 (*str-11*)	30	1×10^8	1×10^7	A-166→C	56 (Lys→Gln)	400
KO-274 (*str-12*)	380	3×10^9	3×10^9	None[f]		20
KO-275 (*str-13*)	240	2.5×10^9	1.5×10^9	None		5
KO-276 (*str-14*)	80	8×10^8	1×10^8	None		20
KO-277 (*str-15*)	64	2×10^9	2×10^8	None		5
KO-278 (*str-16*)	80	8×10^8	3×10^8	None		5

[a] Determined after 2 days of culture at 30°C in NG medium.
[b] Determined after 20 h of culture at 37°C in sporulation medium with or without glucose as indicated.
[c] Numbering originates from the start codon (ATG) of the open reading frame.
[d] Determined after 24 h of incubation at 37°C on NG agar.
[e] This mutant revealed double mutation (C-64 and A-167) within the *rpsL* gene.
[f] Mutations were not detected within the *rpsL* gene.

or Thr, is characterized by antibiotic production 10-fold greater than that of the parent strain. The third group (KO-272 and KO-273) alters Lys-56 to Gln and produced a four- to fivefold increase of antibiotic. Thus, the ability to produce antibiotic correlates tightly with which amino acid species is altered at position 56, although these mutations all confer a high level of resistance to streptomycin (400 to 2,000 μg/ml). The *strA1* mutation (39), a frequently used genetic marker for *B. subtilis* genetics, was found to alter Lys-56 to Ile. This mutation had no effect on antibiotic productivity (Table 3). The *str* mutants with a high level of resistance sporulated as well as, or somewhat less than, the parent strain (Table 3).

We next analyzed *str* mutants with the ability to resist a low level of streptomycin (5 to 20 μg/ml). Mutant colonies were initially generated on GYM agar containing 5 μg of streptomycin per ml. Five *str* mutants (KO-274 to KO-278) were capable of an antibiotic productivity boost of 10- to 50-fold. As summarized in Table 3, no mutation was detected within the *rpsL* gene from any of these *str* mutants, even though certain mutations within the *rpsL* gene are known to result in low-level resistance to streptomycin (18). Strikingly, strains KO-274 and KO-275 showed an increased ability to produce antibiotic accompanied with a greater sporulation ability than the parent strain, especially when cultured in sporulation medium plus 0.5% glucose (Table 3). We therefore conclude that the *str-12* and *str-13* mutations affect positively both the production of antibiotic and the formation of spores.

As determined by nutritional shift-down experiments, the *str* mutants accumulated 29 to 114% of the maximum amount of ppGpp accumulated by parent strain 168 (47, 125, and 32 pmol/AM_{600} for mutants KO-267, KO-271, and KO-274 respectively, compared with 110 pmol/AM_{600} for strain 168). The *str* mutants KO-274 and KO-275, which exhibit low-level resistance to streptomycin, revealed a fourfold-increased sensitivity to thiostrepton, tetracycline, and spectinomycin.

Gene replacement between *str* and *str*$^+$ alleles. In order to clearly demonstrate that the *str* mutations discussed above are responsible for the observed phenotype, i.e., the increased antibiotic-producing ability, gene replacement experiments were performed (see Materials and Methods). The plasmids pSR1, pSR150, and pSR87, which contain the *Eco*RI-*Hin*dIII fragment of the mutant *rpsL* gene from either strain KO-267 (*str-5*), KO-271 (*str-9*), or KO-272 (*str-10*), respectively, was used to transform the parent strain 168. Our strategy for gene replace-

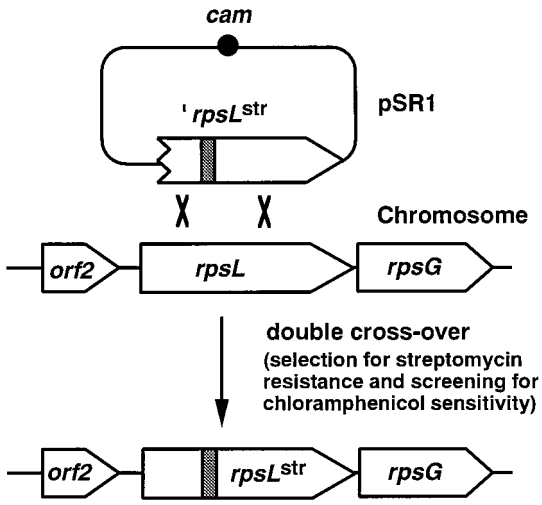

FIG. 2. Replacement of the *rpsL* locus by double-crossover recombination between chromosome and pSR1 containing a mutated *rpsL* allele. *rpsL*^{str} indicates the mutated *rpsL* gene (Lys-56 to Arg). The shaded area represents the mutation site. *cam* and *rpsG* indicate the chloramphenicol resistance gene and the ribosomal protein S7-encoding gene, respectively.

ment of *rpsL* with mutant alleles by a double-crossover event is illustrated in Fig. 2. Streptomycin-resistant but chloramphenicol-sensitive recombinants all overproduced antibiotic as much as the original mutant strains. Gene replacement of the mutant *rpsL* genes was confirmed by directly sequencing the PCR products. Thus, we concluded that *str-5*, *str-9*, and *str-10* mutations (altering Lys-56 to Arg, Thr, and Gln, respectively) are responsible for the observed increase in antibiotic productivity.

DISCUSSION

Our principal finding in this study was that the introduction of a specified *str* mutation into any bacterial genera gives rise to a marked increase in antibiotic productivity, thus expanding the previous preliminary work with *S. coelicolor* A3(2) (19). This novel breeding approach not only results in yielding high-producing strains but also makes it possible to generate these overproducing strains at a surprisingly high frequency (3 to 50% in general). Transferring this trait into other procaryotic microorganisms should therefore offer a convenient and effective method for improving antibiotic productivity. Indeed, as reported previously (43), introduction of a certain *str* mutation (altering Lys-88 to Glu in ribosomal protein S12) was effective even for activating the silent gene(s) in *Streptomyces lividans*, which is involved in production of the blue-colored antibiotic actinorhodin in this organism.

Elucidating the mechanism of initiation of so-called secondary metabolism has been the subject of a large number of publications, but very few have focused on ribosomal function or on the translational machinery. It is important to point out that among the ribosome-binding drugs tested so far, only streptomycin has been shown to be effective for improving antibiotic productivity (reference 19 and this study). The action of streptomycin on bacterial ribosomes has been studied in great detail (reviewed by Wallace et al. [54] and Cundliffe [8]), and among the numerous effects attributed to this drug, the misreading of mRNA codons is the best known. Positions in S12 affected by amino acid alterations which have previously been reported to confer streptomycin resistance in *E. coli* and *Mycobacterium tuberculosis* are Lys-43, Lys-88, and Pro-91 (11, 12, 21, 29, 53, 56). In *S. coelicolor* the alteration in Arg-86 also confers resistance (albeit low-level resistance) to streptomycin (43). The alteration in Lys-56 (corresponding to Lys-43 in *E. coli* and *S. coelicolor*) identified in *B. subtilis* in the present study therefore corresponds to one of these recognized positions (Table 3). Unlike those in *E. coli* and *M. tuberculosis*, the *str* mutations detected within the *rpsL* gene in *B. subtilis* were all represented by changes at the Lys-56 position (Table 3). As reported previously (43), mutation at Lys-43 in *S. coelicolor* does not result in an increase in antibiotic productivity, although it elicits a high level of resistance to streptomycin. Of particular interest is the fact that antibiotic productivity of *B. subtilis* increased in a hierarchical manner depending upon which species of amino acid replaced Lys at position 56 (Table 3). The set of mutants described in this paper therefore may offer a feasible system for studying the regulation of bacterial secondary metabolism at the translational level, together with the *bldA* (encoding leucine-tRNA) mutant of *S. coelicolor* as studied by Guthrie and Chater (17). We have thus demonstrated that an altered ribosomal protein S12, resulting from specific mutations in the *rpsL* gene which confer high level resistance to streptomycin, elicits the ability to overproduce antibiotics not only in streptomycetes but also in members of the family *Bacillaceae*. Although DNA sequencing was not attempted in this study for the gram-negative bacterium *P. pyrrocinia*, it is possible that the pyrrolnitrin overproducing *str* mutants (Table 2) also have a mutation in the *rpsL* gene.

In *B. subtilis*, in addition to the *relA* and *relC* mutations, a number of other mutations affecting the ability to produce antibiotic have been reported. These include *abrA* (which may be same as *rev-4*) and *abrB* (which is probably the same as *absA* and *absB*) (summarized by Piggot and Hoch [39]). *sco* mutations (*scoA*, *scoB*, *scoC*, and *scoD*) affecting sporulation control give rise to various degrees of overproduction of alkaline proteases (9). Mutations *abrA*, *abrB*, *absA*, and *absB* were all originally found by their ability to resist an antibiotic produced by the wild-type sporulating *B. subtilis* strain 168 (16, 24, 52). Some of these mutations compensate for the detrimental pleiotropic effects resulting from a *spo0A* mutation by restoring antibiotic and protease production, competence for transformation, and polymyxin resistance (51). The *abrB* locus has been cloned and sequenced (37). Mutations in this gene affect the transcription of a variety of genes, including *aprE* (which encodes subtilisin) (10), *tycA* (which encodes tyrocidine synthetase) (13, 27, 40), and *spo0E* and *spoVG* (57). Recent studies have demonstrated that the *abrB* gene encodes a 10.5-kDa protein which functions as either an activator or a repressor of the expression of a variety of genes by binding to the promoter regions of these genes (46, 55; reviewed briefly by O'Reilly and Devine [36]). Thus, the *abrB* locus appears to be the major locus responsible for regulating transition stage gene expression. Interestingly, all of the *abrB* mutants studied so far have been found to have alterations (missing or changed in polarity) in one or more of several different 50S ribosomal proteins (42, 50, 51). However, the causal relationship between the *abrB* mutation and the observed alteration in ribosomal proteins still remains obscure. The mutation *rev-4* (which may be the same as *abrA*) has been reported to restore the suppressed sporulation caused by other mutations in the RNA polymerase, ribosomal proteins, and the protein synthesis factor EF-G (41). The *rev-4* mutation has been located in *orfR* of the *spo0F* region (49). It is possible that the streptomycin-resistant mutants identified in the present study that do not have a mutation in the S12 protein (e.g., KO-274 − KO-278 [Table 3]) harbor a mutation in a ribosomal protein other than S12 (or in an rRNA component). This notion may be supported by the fact that those *str* mutants demonstrated an increased sensitivity to certain ribosome-binding antibiotics such as thiostrepton, and moreover *str* mutants (as examined with KO-274) revealed an impaired ability to accumulate ppGpp (see Results). In relation to this argument Staal and Hoch (44) reported a new class of *str* mutation, called *strB*, which confers conditional streptomycin resistance and is distinct from the classical *strA* (= *rpsL*) locus.

There are detailed studies from several laboratories that could shed light on the relationship of various phenotypes to ppGpp levels. On the basis of the isolation and analysis of relaxed (*relC*) mutants of several *Streptomyces* spp., ppGpp has been shown to play a central role in triggering the onset of antibiotic production (30–32). In agreement with our results, ppGpp accumulation was noted to coincide with transcription of the pathway-specific activation genes for undecylprodigiosin (*redD*) and actinorhodin (*actII*-ORF4) in *S. coelicolor* (45, 47, 48), and for bialaphos (*brpA*) in *Streptomyces hygroscopicus* (20). Antibiotic production by *B. subtilis* is also abolished by mutations such as *relA* and *relC*, which cause a deficiency in ppGpp synthetase and ribosomal L11 protein, respectively (34). As will be reported in detail elsewhere, acquisition of certain *str* mutations by *B. subtilis relA* and *relC* mutants (61883 and 61953, respectively) restores the antibiotic productivity lost in these mutants, without any accompanying ppGpp accu-

mulation. The dependence of *B. subtilis* on ppGpp for the initiation of antibiotic production is therefore apparently bypassed by certain *str* mutations, as was the case in *S. coelicolor* A3(2) (35, 43). The molecular basis for the observed role of the altered ribosomal protein S12 (and another putative ribosomal protein) is unclear. It is, however, conceivable that the specified ribosomal mutations led to a change in ribosomal structure which gave rise to the initiation of the secondary metabolism (by an entirely unknown mechanism), without the requirement of ppGpp. In fact, as reported by Allen and Noller (1), mutations in ribosomal proteins S12 and S4 may influence the higher-order structure of 16S rRNA. However, we cannot rule out the possibility that what we are observing is a novel regulation system, mediated by ribosomes, and not directly related with translation. We are now attempting to clarify, by analyzing protein and RNA formation and by studying mRNA and ppGpp levels, (i) whether certain *rpsL* alleles induce a stress response or not and (ii) how the combination of *rpsL* and *rel* affects these parameters.

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