Mutation Affecting Expression of Spectinomycin Resistance in *Bacillus subtilis*

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A mutation that affects the expression of spectinomycin resistance in a spectinomycin-resistant (*spcA*), conditionally asporogenic strain of *Bacillus subtilis* has been designated *srm* (spectinomycin resistance modifier). This mutation resulted in altered colony morphology and increased growth rate and sporulation efficiency in the presence of spectinomycin.

Studies of bacterial mutants resistant to ribosome-directed antibiotics have provided information on the mechanism of action of antibiotics and on the structure and function of the bacterial ribosome (3, 10). Antibiotic-resistant mutants of *Bacillus subtilis* have been used in investigations of possible translational regulation of sporulation (11). We have isolated spectinomycin-resistant mutants of *B. subtilis* that have alterations in their sporulation ability (2, 4). High-level spectinomycin resistance of *B. subtilis* occurs by a *spcA* mutation, which causes an alteration of ribosomal protein S5 (2, 7, 9). This report describes a mutation that affects the expression of a *spcA* mutation in *B. subtilis*.

Strain SPC1-11 contains a spontaneous *spcA* mutation, resulting in a conditional sporulation phenotype. Cultures of this strain grow vegetatively in the presence of antibiotic and can sporulate in the absence of antibiotic, but do not form spores in the presence of spectinomycin (2, 4). When grown on agar medium containing 100 µg of spectinomycin per ml, strain SPC1-11 forms small colonies, with smooth surfaces, and even, circular edges. When a culture of SPC1-11 was grown for five generations in the presence of spectinomycin and plated on medium containing antibiotic, 3% of the colonies were of a different colony morphology, being larger in size, with rough surfaces and irregular edges. The two colony types of SPC1-11 maintained their distinctive colony morphologies only when grown on agar medium containing spectinomycin; when grown on medium without antibiotic, both types formed large colonies. Similar observations have been made by I. Smith with a different *spcA*-containing strain (personal communication).

Figure 1 shows the effect of spectinomycin on the exponential growth rate of SPC1-11 and one large-colony variant of it, which was designated strain SPC1-12. In the absence of antibiotic, both strains grew at the same rate (generation time = 45 min). In the presence of 100 µg of spectinomycin per ml, the growth rate of SPC1-11 was significantly decreased (generation time = 110 min), whereas the effect on SPC1-12 was much less severe (generation time = 55 min). Both SPC1-11 and SPC1-12 grew and maintained the appropriate colony morphology on medium containing up to 3 mg of spectinomycin per ml, which was the highest concentration tested. Neither SPC1-11 nor SPC1-12 showed any cross-resistance to streptomycin.

SPC1-12 did not show the spectinomycin sensitivity of sporulation of the parent strain. The level of sporulation of SPC1-12 in the presence of 100 µg of spectinomycin per ml was 80% (determined by the method of Sterlini and Mandelstam [12]). Sporulation of SPC1-11 under the same conditions was 0.1%.

DNA from SPC1-11 and SPC1-12 was used to transform strain 168 *trp* (wild type) to spectinomycin resistance (2). Transformants were scored for colony morphology and for ability to sporulate in the presence of spectinomycin with agar plates of glutamic acid medium (1). On this medium, sporulating (Sp⁺) colonies of *B. subtilis* turn brown, but asporogenic (Sp⁻) colonies remain white (Table 1). When SPC1-11 was the donor, all of the transformants were of the small colony type, with spectinomycin-sensitive sporulation. When SPC1-12 was the donor, transformants were of both the large- and small-colony types. All large-colony transformants were able to sporulate in the presence of spectinomycin; all small-colony transformants had spectinomycin-sensitive sporulation. DNA from several of the large-colony-type transformants was used to transform strain 168 *trp* to spectinomycin resistance (Table 2). In each of the seven transformation experiments, two classes of transformants resulted. The percentage of transformants with the large-colony phenotype was
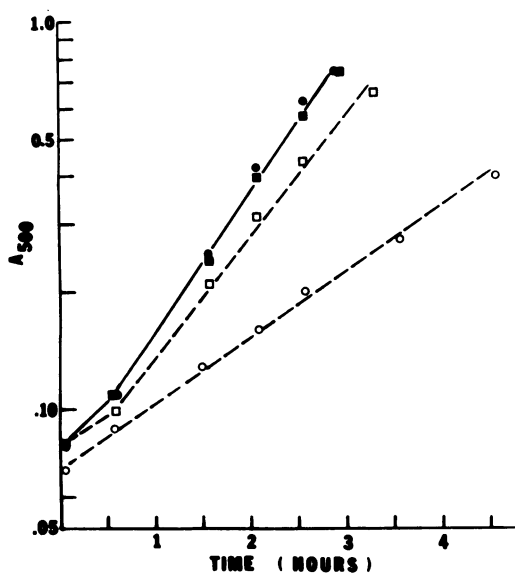
FIG. 1. *Exponential growth rate of SPC1-11 and SPC1-12 in the presence and absence of spectinomycin. Cultures were grown in the medium of Sterlini and Mandelstam (12). After exponential growth had begun, spectinomycin was added to one culture of each strain to a concentration of 100 µg/ml at zero time. A parallel culture of each strain received no antibiotic. Symbols:* ●, *SPC1-11, no spectinomycin;* ○, *SPC1-11 + 100 µg of spectinomycin per ml;* ■, *SPC1-12, no spectinomycin;* □, *SPC1-12 + 100 µg of spectinomycin per ml.* A_{500}, *absorbance at 500 nm.*

TABLE 1. *Transformation of 168 trp to spectinomycin resistance*

Donor DNA	Spectinomycin-resistant transformants[a]	
	Small colony	Large colony
SPC1-11 (small-colony type; Sp⁻ on spectinomycin)	138	0
SPC1-12 (large-colony type; Sp⁺ on spectinomycin)	270	182

[a] Small colony and large colony refer to colony morphology on agar plates containing spectinomycin.

approximately equal to that found in the original cross with DNA from SPC1-12. These observations suggest that strain SPC1-12 contains a second mutation, responsible for both the increased growth rate and ability to sporulate in the presence of spectinomycin. This mutation probably does not in itself confer spectinomycin resistance, and it can be genetically linked to the *spcA* genetic marker. This mutation has been designated *srm*, for spectinomycin resistance modifier. If *srm* could confer Spc^r by itself, then

TABLE 2. *Transformation of 168 trp to spectinomycin resistance*

Donor DNA	Total no. of transformants	No. of spectinomycin-resistant transformants of type:		
		Small colony (Sp⁻)	Large colony (Sp⁺)	% Large colony (Sp⁺)
1	193	90	103	53.4
2	202	110	92	45.5
3	180	89	91	50.5
4	210	109	101	48.1
5	210	117	93	44.3
6	249	146	103	41.1
7	187	109	78	41.7
Total for all crosses	1431	770	661	46.2 (avg)

[a] Transforming DNA was prepared from each of seven independent large-colony-type transformants resulting from SPC1-12 × 168 *trp.*

some of the transformants from a SPC1-12 × 168 transformation would contain *srm* alone, without the *spcA* marker. When those transformants were used as donors of spectinomycin resistance, only a single class of spectinomycin-resistant transformants would result: large colony, Sp⁺ or Spc. Clearly, Table 2 shows this is not the case with each of the seven transformants tested.

Polyacrylamide gel electrophoresis (2) of the ribosomal proteins of strain SPC1-12 showed that this strain contains the altered 30S protein resulting from the *spcA* mutation of the parent strain. This protein alteration was also seen in ribosomes from a large-colony-type transformant from a SPC1-12 × 168 *trp* transformation (Fig. 2). This result is consistent with the interpretation that large-colony-type transformants contain both the *spcA* spectinomycin resistance mutation and the *srm* mutation. No difference between the ribosomal proteins of SPC1-11 and SPC1-12 could be detected in either the 30S or 50S ribosomal subunit (data not shown).

The *spcA* genetic marker of strain SPC1-11 is located in a cluster of genes for ribosomal components near *cysA* on the *B. subtilis* chromosome (2, 9, 10). The finding that the *srm* mutation could be genetically linked to *spcA* with a cotransformation frequency of 0.46 (Table 2) suggested that this mutation might also be located in that cluster of ribosomal genes. A transformation cross was performed between SPC1-12 (*spcA srm*) and OSB 192 (*cysA*). Selection was for Cys⁺. Of 240 Cys⁺ transformants, 42 (17%) were spectinomycin resistant; 34 of these were able to sporulate in the presence of spectinomycin. These results suggest that the *srm* mutation is located between *cysA* and *spcA*,
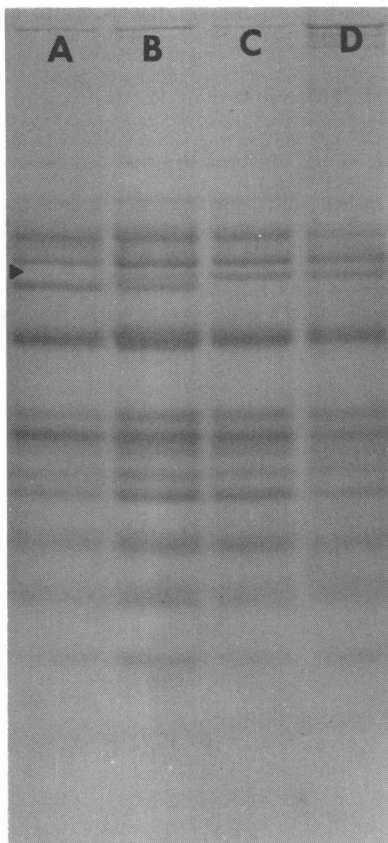
FIG. 2. *Electrophoresis of the proteins of the 30S ribosomal subunit from 168 trp, SPC1-11, and a large-colony-type transformant resulting from a SPC1-12 × 168 trp transformation. Salt-washed ribosomal subunits were prepared, digested with RNase, and electrophoresed at pH 4.3 in the presence of 8 M urea (2). Each sample contains 0.85 absorbance units of 30S subunits at 260 nm. (A) 168 trp (wild type). (B) Mixture of 168 trp + SPC1-11. (C) SPC1-11. (D) Large-colony-type transformant from SPC1-12 × 168 trp.*

in additional mapping experiments. However, due to the lack of reciprocity and additivity in genetic maps constructed for this region of the chromosome (2, 5, 10), and due to the tightness of the linkage between *srm* and *str-1*, it was not possible to order *srm* relative to *str-1* (data not shown). We have previously shown (2) that when *str-1* is the selected marker, the cotransformation frequency between *str-1* and *spcA* is 0.15. Therefore, *str-1* is closer to *srm* than it is to *spcA*. The order of these genetic markers is *cysA-(str-1-srm)-spcA*, and *srm* is thus located in the area of the chromosome that has been shown to contain the genes coding for many ribosomal components.

The *srm* mutation provides a new example of the complex nature of antibiotic resistance mechanisms. It has been shown that spectinomycin dependence in *B. subtilis* is the result of two mutations, one conferring resistance (*spcA*) and one making the resistant mutant drug dependent (*spcD*) (6). Thus, *srm* represents a third locus which can affect the response of *B. subtilis* to spectinomycin. The interaction between *srm* and *spcA* is similar to the functional interactions that have been described for other pairs of markers located in this region of the *B. subtilis* chromosome (5, 10). It cannot be concluded that the *srm* mutation is expressed only in the presence of a *spcA* mutation. There may be other phenotypic differences caused by *srm*, not necessarily related to spectinomycin resistance, but these have not yet been identified. Whatever the alteration caused by the *srm* mutation, it is able to overcome some of the functional constraints, including the inability to sporulate, imposed on the spectinomycin-resistant, conditional strain by the addition of antibiotic. Identification of the component altered in a *srm* mutant could prove to be a useful means of distinguishing between some of the possible models for the inability of the conditional strain to sporulate in the presence of spectinomycin.

since the cotransformation frequencies between *cysA* and *spcA* (0.17) and between *spcA* and *srm* (0.46) and the ability to genetically link *cysA* and *srm* are inconsistent with a *cysA-spcA-srm* gene order. *srm* was also mapped relative to the *str-1* mutation, which is located between *cysA* and *spcA* on the chromosome (2, 10). A strain with the *str-1*, *spcA*, and *srm* mutations was constructed by transformation and used as the source of DNA in a transformation with strain SPC1-11 as the recipient. Of 150 streptomycin-resistant transformants, 137 were able to sporulate in the presence of spectinomycin, giving a cotransformation frequency between *str-1* and *srm* of 0.91. An attempt was made to determine the order of the *str-1* and *srm* genetic markers

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