JOURNAL OF BACTERIOLOGY, Mar. 1979, p. 1452-1455
0021-9193/79/03-1452/04$02.00/0

Vol. 137, No. 3

Spectinomycin Dependence in *Bacillus subtilis*

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Received for publication 21 December 1978

Spectinomycin dependence in *Bacillus subtilis* involves two mutations, one conferring drug resistance and the other producing a requirement for spectinomycin for growth.

Mutants resistant to or dependent on antibiotics that specifically inhibit protein synthesis have been usefully employed in the characterization of the structure and function of the bacterial ribosome. Antibiotic-resistant mutants are easily obtained, and have been studied extensively in both *Escherichia coli* and *Bacillus subtilis*; antibiotic dependence is apparently a rarer phenomenon, and has been reported for only a few of the antibiotics to which resistant mutants have been found. Mutants of *E. coli* dependent for growth on streptomycin (2, 14, 16, 17), spectinomycin (6), and erythromycin (21) have been reported. Streptomycin dependence results from a specific mutation in the *strA* gene, which codes for ribosomal protein S12 of the 30S subunit (18). Spectinomycin and erythromycin dependence in *E. coli* each require two mutations, one causing resistance to the antibiotic and the other conferring dependence. The only antibiotic for which dependence has been reported in *B. subtilis* is streptomycin (10); as in *E. coli*, streptomycin dependence is caused by a single mutation which maps in the *strA* region. In this report we describe the isolation of a spectinomycin-dependent, asporogenous mutant of *B. subtilis*.

The spectinomycin-dependent mutant, named M5, arose spontaneously in the course of selection for spectinomycin-resistant variants of our streptomycin-resistant, asporogenous strain SRB15 (*trpC2 lys-3 metB10 strA*) (5). Although mutant M5 retained the three auxotrophic markers of SRB15, it had acquired sensitivity to streptomycin. To determine if the streptomycin resistance mutation was still present in its genome, DNA was extracted from M5 (3) and used at saturating concentration to transform (4) strain BR151 (*trpC2 lys-3 metB10*). No Str^r transformants were obtained, indicating that the streptomycin resistance of SRB15 had been lost from M5. Spectinomycin-resistant transformants were readily obtained (strain BR151spcR).

DNA from M5 was then used to transform strain Kit 1 (*cysA14 purA16 trpC2*) (7), selecting for spectinomycin resistance. Of 210 transformants capable of growth on spectinomycin-containing medium, only two were dependent on the drug, while the remainder were spectinomycin resistant. This suggested that the spectinomycin dependence (Spc^d) phenotype requires two mutations, one conferring resistance and the other making the resistant mutant drug dependent.

PBS-1-mediated transduction was used to assign a map location to the *spcD* locus (11). Lysates prepared on M5 were used to transduce strain Kit 1 (*cysA14 purA16 trpC2*) and a Spc^r transformant of Kit 1 obtained in the transformation cross described above. The results of this analysis are presented in Table 1. The spectinomycin dependence locus was located in the region between *cysA* and *purA* (Fig. 1).

The majority of ribosomal genes in *B. subtilis* are located between *cysA* and *spcA* (20, 24), which is where the *spcR* mutation of M5 was mapped. However, Trowsdale et al. (22, 23) reported an additional region of ribosomal protein genes between *cysA* and *purA*, and it appears that the spectinomycin dependence mutation of M5 maps close to or within this region. Resistance to pactamycin, an antibiotic which inhibits protein synthesis, is also located in this region (8).

Mutant M5 required spectinomycin for growth at 30 and 37°C on solid medium. When cultures of M5 were spread on plates lacking spectinomycin, approximately 10^{-4} fewer colonies were formed than when spread on plates containing spectinomycin. At 45°C equivalent numbers of colonies were formed on solid medium with or without spectinomycin. The colonies that were formed at 30 and 37°C on medium lacking drug were apparently drug-independent revertants since subsequent characterization revealed no spectinomycin requirement for

† Paper no. 2290 from the Laboratory of Genetics.

growth. The cells growing at 45°C on medium without spectinomycin retained their drug-dependent phenotype. Spectinomycin dependence in M5 is therefore a temperature-dependent phenomenon. M5 is apparently cold sensitive on solid medium in the absence of spectinomycin.

In liquid medium, however, M5 does not require spectinomycin for growth at 30, 37, or 45°C; this was the case in both nutrient sporulation medium (19) and antibiotic assay medium no. 3 (Difco). The cells grown in liquid cultures in the absence of spectinomycin were shown to retain the dependence phenotype by plating on solid nutrient sporulation medium with and without spectinomycin and incubating at 37°C. Mutant M5 was asporogenous, sporulating at a frequency of less than 0.1%; the wild-type strain (BR151) sporulated at a frequence of about 40%.

At 30°C, M5 reverted to spectinomycin independence at a relatively high frequency, approximately 4×10^{-5} as determined by the Luria-Delbrück fluctuation test (15). Independent revertants were heterogeneous for a variety of characteristics, including colony morphology, sporulation phenotype and response to temperature. One characteristic common to all independent revertants examined was their retention of resistance to spectinomycin. The diversity of revertant types suggests that some may result from additional mutations suppressing the dependence phenotype.

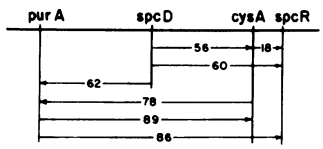

FIG. 1. *Genetic map of the localization of the spectinomycin dependence marker by PBS-1-mediated transduction. Numbers are 100 minus percent of cotransduction. Arrows indicate selected markers.*

To determine how the spectinomycin resistance and dependence mutations affected the activity of *B. subtilis* ribosomes, the ability of ribosomes from BR151, BR151spcR, and M5 to translate natural mRNA in vitro was assayed (Table 2). Ribosomes, initiation factors, and high-speed supernatant (S-150) were prepared according to Legault-Demare and Chambliss (13), as modified by Campbell and Chambliss (5). With no added spectinomycin, the three ribosome types showed similar activity in the translation of BR151 mRNA (total RNA extracted from exponentially growing cells [13]). The addition of 10 μg of spectinomycin per ml inhibited the activity of wild-type ribosomes by greater than 50%; this concentration also inhibits the growth of wild-type cells. Ribosomes from BR151spcR were quite resistant to added spectinomycin, and ribosomes from M5 were resistant to spectinomycin but were not dependent on the drug for activity. In *E. coli*, streptomycin dependence was more easily demonstrated in

TABLE 1. *Mapping of spectinomycin dependence mutation in mutant M5 by transduction*

Donor	Recipient	Selected marker	Recombinant classes[a]				No. of recombinants
			spcR	*cysA*	*purA*	*spcD*	
spcD	*cysA purA*	Spc^r	1	1	1	1	40
			1	1	1	0	4
			1	1	0	1	83
			1	1	0	0	125
			1	0	1	1	0
			1	0	0	1	0
			1	0	1	0	0
			1	0	0	0	56
							308
spcD	*cysA purA spcR*	Cys$^+$		1	1	1	18
				1	1	0	2
				1	0	1	61
				1	0	0	99
							180
spcD	*cysA purA spcR*	Pur$^+$		1	1	1	34
				1	1	0	0
				0	1	1	24
				0	1	0	94
							152

[a] "1" and "0" refer to donor and recipient phenotype, respectively.

TABLE 2. *Effect of spectinomycin on in vitro activity of M5 ribosomes*

Spectinomycin concn (μg/ml)	BR151		BR151spcR		M5	
	pmol Incorporated[a]	% Activity	pmol Incorporated	% Activity	pmol Incorporated	% Activity
0	150.5	100	109.6	100	166.8	100
1	81.2	54	105.7	96	155.9	93
10	55.1	37	103.1	94	147.6	89
100	41.1	27	100.7	92	129.4	76
1,000	33.9	23	78.8	72	125.4	75

[a] Amount of [^{14}C]phenylalanine incorporated per absorbancy unit at 260 nm of ribosomes with BR151 mRNA (300 μg per assay tube) as the template. Incubation was at 37°C for 30 min. S150 and initiation factors were from 168T$^+$ wild-type strain.

tro when Ca^{2+} replaced Mg^{2+} in the assay system (14), but in our system Ca^{2+} is a poor replacement for Mg^{2+}, giving very low activity, and no spectinomycin dependence was observed for M5 ribosomes. Two-dimensional polyacrylamide gel electrophoresis (9, 12) of the basic 70S ribosomal proteins (1) of mutant M5 revealed no differences from BR151 and BR151spcR. A possible explanation for the failure to demonstrate ribosomal protein changes and dependence in vitro is that spectinomycin may be required for some ribosomal function other than protein synthesis, e.g., effector molecule synthesis, or for ribosomal assembly.

This research was supported by grant PCM77-09326 from the National Science Foundation and by the College of Agriculture and Life Sciences of the University of Wisconsin-Madison. T.M.H. is a predoctoral trainee of National Institutes of Health training grant GM-07133. K.M.C. was a predoctoral fellow of the National Science Foundation.

We thank M. Sadowsky for preliminary work on this project. We also thank D. Dean and J. Cannon for their supply of much-needed transducing phage, and the Upjohn Co. and Abbott Laboratories for generously providing us with spectinomycin sulfate.

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