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New Chloramphenicol Resistance Locus in *Bacillus subtilis*

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A spontaneously occurring, noninducible, chloramphenicol-resistant mutant of *Bacillus subtilis* 168 has a mutation (*cam-2*) which maps in the ribosomal protein region of the chromosome near *dal*. Its presence does not confer dependence on chloramphenicol. Ribosomes of the *cam-2* strain remained sensitive to chloramphenicol in in vitro protein synthesis. No chloramphenicol acetyltransferase activity could be detected.

The mutant described here was obtained as part of a control experiment for a study using the chloramphenicol resistance (Cm^r) plasmid pC194 as part of a *Bacillus* insertional plasmid marker (2, 6, 10). The mutation locus itself is significant since it confers resistance to a higher level of chloramphenicol than any previously reported chromosomal mutation (but does not confer resistance to other antibiotics) and because the type of mutation is not a ribosome-associated change and may confer alteration in uptake or permeability.

Isolation of *cam-2*. After repeated attempts at plating high-density cultures of *Bacillus subtilis* 168 onto chloramphenicol plates (20 μg/ml), no Cm^r colonies could be isolated. One published report of Cm^r *B. subtilis* states that mutants of strain ATCC 6633 resistant to 5 μg of chloramphenicol per ml were obtained after treatment with the mutagen nitrosoguanidine (8). Plating several of our strains on 5 μg of chloramphenicol per ml gave a background haze of growth from which no distinct colonies could be isolated. We decided to replica plate this haze of growth from 5 to 20 μg of chloramphenicol per ml. After several days, some colonies were visible. These were streaked onto nutrient media with and without chloramphenicol (20 μg/ml). Strains that grew again at 20 μg/ml were checked for stability by streaking from the nonselective media to fresh chloramphenicol plates (20 μg/ml). An isolate was selected and designated 168 *cam-2* (saving *cam-1* as the designation for the isolate in reference 8).

Stability of the *cam-2* mutation. Dilutions of cultures which were grown to a high density in nonselective liquid media were plated on media containing several concentrations of chloramphenicol. The mutation was quite stable; 168 *cam-2* was able to grow vigorously at 10 μg of chloramphenicol per ml. Strain 168 *cam-2* grew equally well without chloramphenicol, demonstrating that the mutation does not render the cell chloramphenicol dependent. Similarly, the resistance level was not affected by prolonged incubation in the absence of selective pressure.

MIC. The *cam-2* mutation was introduced into strain BR151 by transformation (10) using a nonsaturating concentration of DNA. The parent and transformed strains were subcultured overnight in complete medium and inoculated into nutrient broth containing various concentrations of chloramphenicol. The MIC for parental strain BR151 was 2 to 3 μg/ml. When this same strain contained the mutation *cam-2*, the MIC was approximately 20 μg/ml (Fig. 1A).

Genetic mapping. The *cam-2* mutation could be efficiently transferred by either transformation or transduction (10) using selection levels of 10 μg of chloramphenicol per ml in tryptose blood agar base plates (Difco Laboratories). The mutation was localized to the ribosomal protein region of the chromosome near *dal* through the use of the kit strains of Dedonder et al. (5).

More detailed mapping positioned *cam-2* close to *aroI906* (5% cotransduction), *dal-1* (21% cotransduction), and *ddl-1475* (15% cotransduction) (strains from A. Wolfe and the Bacillus Genetic Stock Center [selection as described in the catalog]) (Tables 1 and 2). There were some discrepancies in the mapping data, most notably in the two-factor crosses, which preclude a clear determination of the map order. Unfortunately, no other selectable markers in this region of the chromosome are available (*furB* was not selectable in our hands).

These data, in conjunction with literature data for the position of *ddl* relative to *dal-1* and *aroI* (3) agree best with the order *aroI906 ddl-1475 dal-1 cam-2*. However, the disproportionately small number of transductants recovered when selecting directly for Cm^r and the anomolous appearance of 4% Dal$^-$ Aro$^+$ Cm^r isolates when selecting for Aro$^+$ made precise determination of the order difficult.

Ribosomal sensitivity to chloramphenicol. Ribosomes purified from BR151 and BR151 *cam-2* were tested for the ability to translate phage SPO1 mRNA in vitro (4) in the presence of chloramphenicol (Fig. 1B). Stimulation of incorporation by template addition was 7- to 15-fold. No difference in sensitivity to chloramphenicol was observed between ribosomes of BR151 and BR151 *cam-2*.

Polyacrylamide gel electrophoresis of ribosomal proteins. No difference was observed in two-dimensional polyacrylamide gel electrophoresis patterns (7) of basic 70S ribosomal proteins from BR151 and BR151 *cam-2*. Migration of 70S ribosomal proteins in sodium dodecyl sulfate-polyacrylamide gradient gels (1) was also identical for the two strains (data not shown).

Lack of chloramphenicol acetyltransferase activity of BR151 and BR151 *cam-2*. The possibility of the presence of a

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TABLE 1. Three-factor PBS1 transduction

Selected marker	Recombinant class[a]			No. of recombinants
	arol-906	*dal-1*	*cam-2*	
Aro$^+$	1	0	0	380
	1	1	0	95
	1	0	1	21
	1	1	1	4
Dal$^+$	0	1	0	272
	1	1	0	205
	0	1	1	19
	1	1	1	1
Cm^r	0	0	1	86
	0	1	1	19
	1	0	1	0
	1	1	1	5

[a] The donor was 168 *cam-2* (designated 1); the recipient was QB 928 (*aroI906 trpC2 metB5 dal-1*) (designated 0).

chloramphenicol transacetylase in the BR151 *cam-2* mutant extracts was also examined by using a spectrophotometric assay (9). Cell extracts containing plasmid-generated chloramphenicol acetyltransferase showed good activity, whereas highly concentrated BR151 *cam-2* extracts, regardless of induction with chloramphenicol during growth, were consistently negative.

We have isolated a stable mutant of *B. subtilis* 168 that is capable of growing in the presence of 10 μg of chloramphenicol per ml. The mutation maps near *dal*-1, and for the strains containing it, the MIC is approximately 20 μg/ml; the MIC for the parental strain is approximately 2 μg/ml. The consistent lack of demonstrable in vitro resistance to chloramphenicol in extracts and ribosomes of this mutant leads us to suggest that the mechanism of resistance is not the result of a ribosomal alteration.

Several types of chloramphenicol resistance loci can be envisioned. The fact that there is no alteration of the electrophoretic pattern in one- and two-dimensional gels of ribosomal proteins and no change in in vitro sensitivity to chloramphenicol suggests that the *cam-2* locus is distinct from Cm^r mutants described by Osawa and Takata (8). The absence of chloramphenicol acetyltransferase activity, the most prevalent chloramphenicol-inactivating enzyme in gram-positive organisms, leaves altered uptake of chloramphenicol as a possible explanation for the increased resistance.

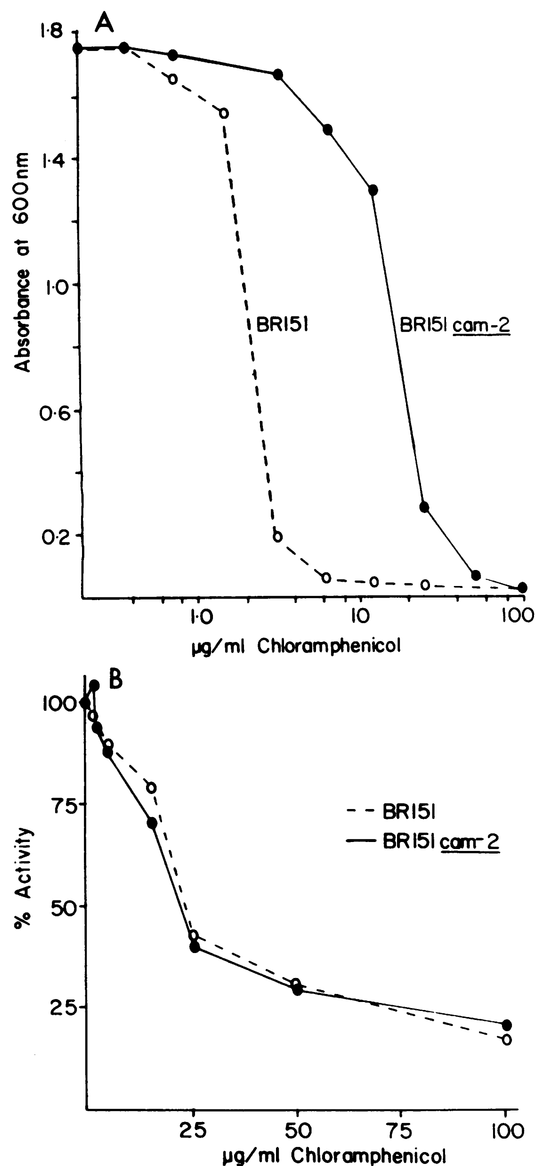

FIG. 1. Effect of chloramphenicol on (A) the growth of BR151 and BR151 *cam-2* expressed as the optical density at 600 nm after 8 h at 37°C in nutrient broth and on (B) in vitro ribosome activity as measured by incorporation of [^{14}C]phenylalanine (14 cpm/pmol). Activity at different concentrations of drug is expressed relative to activity in the absence of chloramphenicol, which for BR151 was 375 pmol incorporated; for BR151 *cam-2* it was 360 pmol incorporated.

TABLE 2. Two-factor PBS1 transduction[a]

Selected marker	No. of recombinants	% Cotransduction
Ddl$^+$	479 Cm^s	4.2
Cm^r	21 Cm^r	
Cm^r	262 *ddl*	15
Ddl$^+$	44 *ddl*$^+$	

[a] The donor was 168 *cam-2*; the recipient was RB1949 (*purA16 metB5 ilvA1 ddl-1475*).

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