

## Cloning of the *Bacillus subtilis* Sulfanilamide Resistance Gene in *Bacillus subtilis*

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A recombinant plasmid was constructed by ligation of chromosomal DNA from a sulfanilamide-resistant strain of *Bacillus subtilis* to the plasmid vector pUB110 which specifies neomycin resistance. Recombinant molecules generated in vitro were introduced into a *B. subtilis* recipient strain which carried the *recE4* mutation, and selection was for neomycin-sulfanilamide-resistant transformants. A single colony was isolated containing the recombinant plasmid pKO101. This 6.3-megadalton plasmid simultaneously conferred resistance to neomycin and sulfanilamide when transferred into sensitive Rec<sup>+</sup> or Rec<sup>-</sup> cells by either transduction or transformation.

*Bacillus subtilis* has emerged as a major organism for exploration of genetics, physiology, and industrial applications of bacilli. Development of a molecular cloning system in *B. subtilis* promises to further expand the usefulness of this organism.

For examination of the utility of this system for cloning specific regions of the *B. subtilis* chromosome, a well-mapped and easily selected chromosomal gene, *sul*, was inserted into a *Staphylococcus aureus* plasmid vector. The *sul* gene specifies chromosomally determined sulfanilamide resistance in *B. subtilis*. In *Escherichia coli*, this antibiotic resistance is thought to be the result of a chromosomal mutation which gives rise to an altered form of the sulfanilamide target enzyme, dihydropteroic acid synthetase. This form is much less sensitive to sulfanilamide but retains a normal affinity for the natural substrate, *p*-aminobenzoic acid (8). The exact mechanism of sulfanilamide resistance in *B. subtilis* has not been determined.

This report describes the construction, selection, and characterization of a recombinant plasmid carrying the *B. subtilis sul* gene.

All strains used in these experiments were *B. subtilis* 168 derivatives. *B. subtilis* ASB327 was used as the transformable recipient. Chromosomal DNA was isolated from the sulfanilamide-resistant strain VB157. These and other strains used are listed in Table 1.

Chromosomal DNA was isolated from strain VB157, using the technique previously described by Burke and Spizizen (3).

The cloning vector pUB110 is a 3.0-megadalton (Mdal) plasmid coding for resistance to neomycin and was isolated from *B. subtilis* as described by Gryczan et al. (6) with the following exception. After the sodium dodecyl sulfate-

salt precipitation step, the supernatant was carefully decanted, warmed at 37°C, and incubated with 20 µg of proteinase K per ml in a water bath shaker at 37°C and 150 rpm. The DNA was then ethanol precipitated and purified as previously described (6).

Restriction endonucleases *Bam*HI, *Bg*III, *Hind*III, and *Hpa*II were obtained from New England Biolabs, as was T4 DNA ligase. *Eco*RI was purchased from Sigma Chemical Co.

With the exception of *Eco*RI, restriction endonuclease cleavage was performed with a modification of digestion buffers described previously (6). The modification involved the omission of bovine serum albumin from the buffers. *Eco*RI cleavage was performed using a buffer consisting of 100 mM Tris-hydrochloride (pH 7.5), 5 mM MgCl<sub>2</sub>, and 50 mM NaCl. In all cases, restriction endonuclease cleavage was accomplished using 1 to 2 U of restriction endonuclease per µg of DNA and incubating the mixture at 37°C for 2 to 3 h. The reaction was terminated by heating at 65°C for 10 min. The ratio of *Eco*RI-digested pUB110 plasmid DNA to *Eco*RI-digested VB157 chromosomal DNA in the ligation mixture was 1:10. Ligation of DNA was performed with T4 ligase; the ligation mixture consisted of 50 µM ATP, 7.5 mM MgCl<sub>2</sub>, 10 mM Tris-hydrochloride (pH 7.5), and 30 µg of *Eco*RI-digested DNA in a total volume of 200 µl. The mixture was incubated for 15 h at 4°C before the addition of 200 U of ligase and further incubation at 4°C for 24 h.

Transformations were performed as described previously (1), using a final DNA concentration of 5 µg/ml. Transformants were selected on minimal plates containing 1,500 µg of sulfanilamide per ml.

The procedure for the transduction experi-

TABLE 1. Strains of *B. subtilis*

Strain	Genotype	Reference
168	<i>trpC2</i>	1
VB157	<i>trpC2 sul</i>	9
BD224	<i>trpC2 thr-5 recE4</i>	6
BD408	<i>dnaA13 metB5 ile</i> (pUB110)	10
ASB327	<i>pheA1 recE4 spoOH81</i>	This laboratory
ASB338	<i>pheA1 recE4 spoOH81</i> (pKO101)	This laboratory
ASB340	<i>trpC2 thr-5 recE4</i> (pUB110)	This laboratory
ASB341	<i>trpC2 thr-5 recE4</i> (pKO101)	This laboratory
ASB342	<i>trpC2 sul</i> (pKO101)	This laboratory

ments was similar to that described by Hoch et al. (7) as modified by Young et al. (12). Motile variants were grown in motility medium (2).

Agarose gels, varying in concentration from 0.8 to 1.5%, were used to resolve plasmid and chromosomal DNAs. The Tris-borate buffer of Greene et al. (5) was used throughout. Vertical gels were performed at a constant voltage of 80 V for 4 h. Horizontal gels were prepared and run as described by Davis et al. (4). Gels were stained in ethidium bromide (1 µg/ml) for 30 min at room temperature and photographed using shortwave UV light with Polaroid type 57 film and a Kodak 23A red filter. The molecular weight of the recombinant plasmid pKO101 was determined using *Hind*III and *Hind*III-*Eco*RI fragments as standards (11).

*Eco*RI-digested pKO101 plasmid DNA was separated electrophoretically on an 0.8% agarose gel, and fragments were recovered from gel slices by electrophoretic elution as described by Southern (11). The ethidium bromide in the DNA samples was removed by dialysis, and these fragments of plasmid DNA were used in subsequent transformations.

Minimal inhibitory concentrations (MICs) of sulfanilamide, sulfathiazole, and sulfamerazine were determined by replica plating to minimal plates containing antibiotic. Strains to be tested were transferred to a tryptose blood agar base plate with 0.5% glucose and 50 µg of thymine per ml. After 3 h of incubation at 37°C, this plate was used as a master plate for replication to minimal plates containing various concentrations of the appropriate antibiotic in addition to the auxotrophic requirements of the strains. These plates were incubated at 37°C and scored for growth at 24, 48, and 72 h.

A single neomycin-sulfanilamide-resistant clone was isolated when 5 µg of a ligated mixture of *Eco*RI-digested pUB110 and VB157 chromosomal DNAs was used to transform competent cells of the Rec<sup>-</sup> *B. subtilis* ASB327.

Examination of a cleared lysate of the transformant by agarose gel electrophoresis revealed that the host had acquired a plasmid with a larger molecular weight than that of the vector plasmid pUB110. The recombinant plasmid was designated pKO101.

PBS1 was propagated on ASB338 carrying the recombinant plasmid pKO101 and used to transduce the *recE4* strain BD224. Selection for transductants that were resistant to both neomycin and sulfanilamide at concentrations of 5 and 1,500 µg/ml, respectively, resulted in  $1.1 \times 10^3$  transductants per ml.

Competent BD224 cells were transformed with pKO101, and transformants were selected with either neomycin at a final concentration of 5 µg/ml or sulfanilamide at a final concentration of 1,500 µg/ml. Selection with neomycin resulted in a transformation frequency of 0.43%, and selection with sulfanilamide resulted in a transformation frequency of 0.62%. Transformants selected with neomycin were replica plated to sulfanilamide selective medium, and colonies selected with sulfanilamide were replica plated to neomycin selective medium. All of the transformants selected for sulfanilamide resistance were also neomycin resistant, whereas approximately 80% of the transformants selected for neomycin resistance were also sulfanilamide resistant.

For an estimation of the stability of pKO101 in *recE4 B. subtilis*, the plasmid-bearing strain was grown at 37°C for 3 h in MM2 medium (2× Penassay broth plus 0.5% yeast extract). The culture was diluted and plated on tryptose blood agar base medium and incubated at 30°C overnight. Colonies were then transferred to tryptose blood agar base, incubated at 37°C for 3 h, and replica plated to minimal plates containing either 1,500 µg of sulfanilamide per ml, 5 µg of neomycin per ml, or both. Of 860 colonies examined, approximately 40% (338) were found to be sensitive to both antibiotics. This is especially noteworthy when compared with the extreme stability of the vector plasmid pUB110 (6). No loss of either of the resistance characters of pKO101 was detected when sulfanilamide selection alone was maintained.

Purified hybrid plasmid was digested with a number of restriction endonucleases and found to have single cleavage sites for endonucleases *Bam*HI (Fig. 1, lane D) and *Xba*I and *Bgl*III (data not shown). All of these sites are located within the cloning vehicle pUB110. *Hind*III was unable to convert the plasmid DNA from a covalently closed circular form to a linear form, indicating the absence of the corresponding recognition site in the cloned chromosomal DNA.

Digestion with *Eco*RI resulted in a release of linearized pUB110 (slowest-migrating band) and

two additional fragments (Fig. 1, lane B). The fragments, designated A and B, were determined to be 2.4 and 0.9 Mdal, respectively. Therefore, the recombinant plasmid pKO101 is 6.3 Mdal and consists of the 3.0-Mdal vector plasmid pUB110 and a 3.3-Mdal cloned fragment of *B. subtilis* chromosomal DNA.

The recombination-proficient strain 168 was transformed to sulfanilamide resistance with *EcoRI*-digested fragments of pKO101. The transformation frequency obtained using the A fragment was  $2.4 \times 10^{-5}$ , whereas a transformation frequency of less than  $1.2 \times 10^{-8}$  was obtained using the B fragment. These results indicated that the sulfanilamide resistance was most likely associated with the A fragment. However, *EcoRI*-digested pUB110 ligated to fragment A was unable to transform recombination-deficient BD224 cells to the sulfanilamide-resistant phenotype.

Sensitivity to sulfanilamide was determined for various strains in the presence and absence of the recombinant plasmid (Table 2). The MIC of sulfanilamide was increased by over threefold in cells carrying pKO101. The presence of the cloning vector pUB110 in cells did not affect their sensitivity to sulfanilamide. Further, the cells harboring the recombinant plasmid were found to exhibit levels of resistance to sulfamerazine (MIC = 400  $\mu\text{g/ml}$ ) and sulfathiazole (MIC = 100  $\mu\text{g/ml}$ ) equal to those of the parent sulfanilamide-resistant strain.

The fact that the chromosomally determined

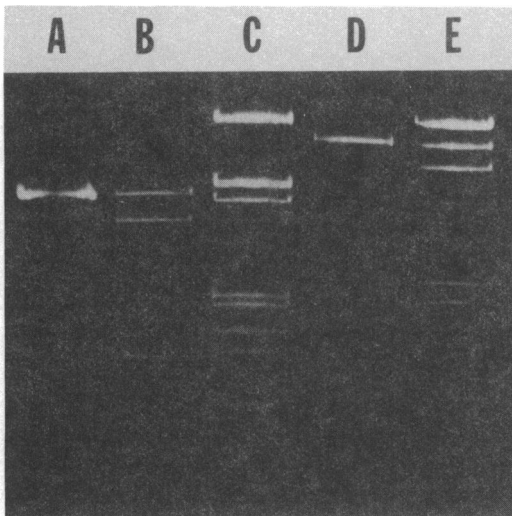


FIG. 1. Restriction endonuclease analyses of various plasmid DNAs by agarose gel electrophoresis. Restriction digests were separated on a 1.5% gel at 80 V for 4 h. (A) *EcoRI*-digested pUB110; (B) *EcoRI*-digested pKO101; (C) *HindIII*-plus-*EcoRI*-digested  $\lambda$  c1857 S7 DNA; (D) *Bam*HI-digested pKO101; (E) *HindIII*-digested  $\lambda$  c1857 S7 DNA.

TABLE 2. MIC of sulfanilamide

Strain	Plasmid	MIC ( $\mu\text{g/ml}$ )
168	None	500
BD224	None	500
ASB327	None	500
ASB340	pUB110	500
VB157	None	1,100
ASB338	pKO101	>1,700
ASB341	pKO101	>1,700
ASB342	pKO101	>1,700

resistance to sulfanilamide was expressed in the *recE4* recipient in both transduction and transformation experiments strongly suggests that the *sul* gene is located on the recombinant extrachromosomal element. Spontaneous loss of the sulfanilamide phenotype occurred in approximately 40% of the recombinant cells when selection for the *sul* gene was not maintained, also indicating that this characteristic is located extrachromosomally.

The cloned 3.3-Mdal chromosomal DNA probably was generated by incomplete *EcoRI* digestion of VB157 DNA. The region of the cloned DNA essential for the sulfanilamide-resistant phenotype appears to be associated with the A fragment, since this fragment was capable of transforming *Rec*<sup>+</sup> competent cells to sulfanilamide resistance at high frequency. However, when purified A fragment was ligated to pUB110 vector DNA and used to transform *Rec*<sup>-</sup> host strains, no sulfanilamide-resistant colonies resulted, despite the fact that the vector-coded neomycin resistance was expressed. This suggests that the B fragment codes for some essential function. The B fragment may contain the chromosomal promoter for the *sul* gene or may contain an integral portion of the *sul* gene not included on the A fragment. It appears that the sulfanilamide resistance phenotype can only be obtained in *Rec*<sup>-</sup> cells if the internal *EcoRI* site of the *sul* gene is not cut with this restriction enzyme. This may explain why only one *Sul*<sup>r</sup> clone was obtained from 5  $\mu\text{g}$  of ligated DNA in the initial cloning experiments.

The level of resistance to sulfanilamide was greater for the strains harboring the recombinant plasmid pKO101 (ASB338, ASB341, and ASB342) than for the sulfanilamide-resistant VB157 which was the source of the cloned *sul* gene (Table 2). This may be due to the presence of multiple copies of the recombinant plasmid, although this gene dosage effect has not been experimentally confirmed. The inclusion of neomycin in the media did not alter the MIC for the strains harboring plasmid pKO101.

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#### LITERATURE CITED

1. Anagnostopoulos, C., and J. Spizzen. 1961. Requirements for transformation in *Bacillus subtilis*. *J. Bacteriol.* **81**:741-746.
2. Burke, W. F., Jr., and M. McCammon. 1978. Ultraviolet sensitivity of *Bacillus subtilis* *citD* mutants. *Curr. Microbiol.* **1**:167-170.
3. Burke, W. F., Jr., and J. Spizzen. 1977. Isolation, characterization, and activation of the magnesium-dependent endodeoxyribonuclease from *Bacillus subtilis*. *Biochemistry* **16**:403-410.
4. Davis, R. W., D. Botstein, and J. R. Roth. 1980. Advanced bacterial genetics, p. 148-152. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. Greene, P. J., M. C. Betlach, H. M. Goodman, and H. W. Boyer. 1974. The *EcoRI* restriction endonuclease, p. 87-111. In R. B. Wickner (ed.), *DNA replication and biosynthesis*, vol. 7. Marcel Dekker, Inc., New York.
6. Gryczan, T. J., S. Contente, and D. Dubnau. 1978. Characterization of *Staphylococcus aureus* plasmids introduced by transformation into *Bacillus subtilis*. *J. Bacteriol.* **134**:318-329.
7. Hoch, J. A., M. Barat, and C. Anagnostopoulos. 1967. Transformation and transduction in recombination-defective mutants of *Bacillus subtilis*. *J. Bacteriol.* **93**:1925-1937.
8. Hugo, W. B., and A. D. Russell. 1980. *Pharmaceutical microbiology*, p. 151. Blackwell Scientific Publications, London.
9. Racine, F. M., and W. Steinberg. 1974. Genetic location of two mutations affecting the lysyl-transfer ribonucleic acid synthetase of *Bacillus subtilis*. *J. Bacteriol.* **120**:384-389.
10. Shivakumar, A. G., and D. Dubnau. 1978. Plasmid replication in *dna* Ts mutants of *Bacillus subtilis*. *Plasmid* **1**:405-416.
11. Southern, E. 1979. Gel electrophoresis of restriction fragments. *Methods Enzymol.* **68**:152-176.
12. Young, F. E., C. Smith, and B. E. Reilly. 1969. Chromosomal location of genes regulating resistance to bacteriophage in *Bacillus subtilis*. *J. Bacteriol.* **98**:1087-1097.