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Transposon Tn*916* Mutagenesis in *Bacillus anthracis*

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Mutagenesis of *Bacillus anthracis* by the streptococcal tetracycline resistance transposon Tn*916* is described. Tn*916* was transferred from *Streptococcus faecalis* DS16C1 to *B. anthracis* VNR-1 by conjugation in a standard filter mating procedure. Tetracycline-resistant (Tc^r) transconjugants were obtained at a frequency of 1.6×10^{-8} per donor CFU. When donor and recipient cells were treated with nafcillin before conjugation, the frequency was increased nearly 10-fold. Nafcillin pretreatment of donor and recipient strains was used in all subsequent conjugation experiments. *S. faecalis* CG110, containing multiple chromosomal insertions of Tn*916*, transferred the transposon to *B. anthracis* VNR-1 at a frequency of 9.3×10^{-5}. A Tc^r *B. anthracis* transconjugant, strain VNR-1-tet-1, transferred Tn*916* to *B. anthracis* UM23-1 and *Bacillus subtilis* BST1 at frequencies of 2.1×10^{-4} and 5.8×10^{-6}, respectively. The transfer of Tn*916* occurred only on membrane filters, since no Tc^r transconjugants were obtained when strains VNR-1-tet-1 and UM23-1 were mixed and incubated in broth culture. The presence of the Tn*916*-associated *tetM* gene in Tc^r *B. anthracis* and *B. subtilis* transconjugants was confirmed in hybridization experiments by using a 5-kilobase-pair DNA fragment containing the *tetM* gene as a probe. Of 3,000 *B. anthracis* UM23-1 Tc^r transconjugants tested, 21 were phenylalanine auxotrophs and 2 were auxotrophic for phenylalanine, tyrosine, and tryptophan.

An important focus of research on the pathogen *Bacillus anthracis* has been the generation of prototype, live-vaccine strains for immunization against anthrax. Transposon mutagenesis would appear to be a potentially useful tool in this research. Indeed, T. M. Koehler and C. B. Thorne have transferred Tn*917* into *B. anthracis* (C. B. Thorne, personal communication). We therefore undertook the development of a transposon mutagenesis system in *B. anthracis*. We chose Tn*916*, a 15-kilobase-pair tetracycline resistance (Tc^r) transposon originally detected in *Streptococcus faecalis* DS16 (9, 10), for this purpose. It seemed a particularly good candidate for two reasons: (i) it encodes conjugative functions which facilitate its transfer from one cell to another in the absence of plasmid DNA (9, 10), and (ii) it has been shown to insert into different sites on the recipient chromosome, with some transconjugants containing more than one copy of the transposon (11). Tn*916* has mediated its own transfer to other strains of *S. faecalis*, as well as to *Streptococcus mutans*, *Streptococcus agalactiae*, and *Streptococcus lactis*, during mixed incubation on membrane filters (5). It has also transferred itself from *S. faecalis* to *Staphylococcus aureus* (19). However, to our knowledge, the self-transmission of Tn*916* from streptococci into *Bacillus* species has not been reported, although Christie et al. (4) reported that Tn*925*, which is similar to Tn*916*, transferred itself from *S. faecalis* to *Bacillus subtilis*.

Our primary goals in this initial work were to develop methodology for Tn*916* mutagenesis in *B. anthracis* and to identify mutants, specifically mutants deficient in their ability to synthesize aromatic amino acids, which may serve as candidate live vaccines.

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MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used in this study are listed in Table 1. Brain heart infusion (BHI), brain heart infusion agar (BHIA), nutrient broth (NB), nutrient agar, and tryptic soy agar were obtained from Difco Laboratories, Detroit, Mich., and were prepared as recommended by the manufacturer. Sheep blood agar plates contained 5% sheep blood in blood agar base (Difco). Horse blood agar plates contained 5% horse blood in Columbia blood agar base (Difco). The preparation of R agar, which consisted of the defined, synthetic *B. anthracis* growth medium (R medium) with 1.5% Bacto-Agar (Difco) added, has been described previously (23). Plates of Brewer agar (BA) consisted of the synthetic *B. anthracis* growth medium described by Brewer et al. (3) with 1.5% agar. For isolation of specific *B. anthracis* auxotrophic mutants, phenylalanine, tyrosine, and tryptophan, together or independently, were omitted from BA. Antibiotics added to the media were used at the following concentrations: tetracycline, 10 μg/ml; streptomycin, 500 μg/ml; kanamycin, 10 μg/ml; nafcillin, 100 μg/ml.

Conjugation experiments. All incubations were at 37°C. Before being mixed, donor and recipient cultures were grown with gentle shaking (100 rpm) in 250-ml flasks containing 100 ml of either BHI or BHI with the appropriate antibiotic. Overnight cultures were diluted 1:10 in fresh medium and allowed to incubate for an additional 2 h. When nafcillin was used, it was added to donor and recipient cultures after the first hour of incubation. Donor and recipient cultures (1 ml of each) were mixed and collected on a cellulose acetate membrane (pore diameter, 0.45 μm; Gelman Sciences, Inc., Ann Arbor, Mich.). After being washed with 10 ml of medium, the filter was incubated, cell side up, on a sheep blood agar plate for 18 h. Cells were scraped from the filters with a bent glass rod and suspended in 0.5 ml of sterile phosphate-buffered saline. This procedure was repeated, and the cell suspensions were pooled in a sterile tube. Tenfold dilutions (0.1 ml of each) were spread on

TABLE 1. Bacterial strains used

Strain	Chromosome marker(s)[a]	Plasmid[b]	Source or derivation
S. faecalis			
DS16C1	*tet* (Tn*916*)	pAD2 (Km^r Sm^r Em^r [Tn*917*])	D. Clewell; 7, 27
JH2-2	*rif fus*	None	D. Clewell; 18
CG110	*rif fus tet* (Tn*916*)	None	D. Clewell; 10, 11; *S. faecalis* DS16C3 × *S. faecalis* JH2-2
E. coli			
DH1	F *recA1 endA1 gyr-96 thi-1 hsdR17 supE44*	pJI3 (Tc^r [*tetM*])	16; pACYC177 carrying *tetM* on a 5-kb *Hin*cII fragment
CG120	Same as *E. coli* DH1	pAM120 (Ap^r Tc^r [Tn*916*])	D. Clewell; 12
CG120LT	Same as *E. coli* DH1	pAM120LT (Ap^r)	D. Clewell; 12
B. anthracis			
VNR-1	None	pXO1 (Tox^+)	14; cured of pXO2 by novobiocin
UM23-1	*str*	pXO1 (Tox^+)	C. B. Thorne
VNR-1-tet-1	*tet* (Tn*916*)	pXO1 (Tox^+)	This study; *S. faecalis* DS16C1 × *B. anthracis* VNR-1
VNR-1-tet-2	*tet* (Tn*916*)	pXO1 (Tox^+)	This study; *S. faecalis* CG110 × *B. anthracis* VNR-1
VNR-1-tet-3	*tet* (Tn*916*)	pXO1 (Tox^+)	This study; *S. faecalis* CG110 × *B. anthracis* VNR-1
VNR-1-tet-4	*tet* (Tn*916*)	pXO1 (Tox^+)	This study; *S. faecalis* CG110 × *B. anthracis* VNR-1
VNR-1-tet-B	*str tet* (Tn*916*) *phe*	pXO1 (Tox^+)	This study; *B. anthracis* VNR-1-tet-1 × *B. anthracis* UM23-1
VNR-1-tet-C	*str tet* (Tn*916*) *phe tyr trp*	pXO1 (Tox^+)	This study; *B. anthracis* VNR-1-tet-1 × *B. anthracis* UM23-1
B. subtilis			
BST1	Δ*spo0A677*	pUB110 (Km^r)	17; *B. subtilis* BGSC 1S53 containing pUB110
KT1	Δ*spo0A677 tet* (Tn*916*)	pUB110 (Km^r)	This study; *B. anthracis* VNR-1-tet-1 × *B. subtilis* BST1

[a] Antibiotic resistance markers; *rif*, rifampin; *fus*, fusidic acid; *tet*, tetracycline; *str*, streptomycin.
[b] Antibiotic resistance phenotypes: Km^r, kanamycin; Sm^r, streptomycin; Em^r, erythromycin; Tc^r, tetracycline; Ap^r, ampicillin.

appropriate selective plates, which were incubated for 1 to 3 days in 5% CO_2. Conjugation frequencies were calculated as the number of Tc^r transconjugants divided by the number of donor CFU obtained at the end of the mating.

The procedure used for the broth mating of *B. anthracis* VNR-1-tet-1 with *B. anthracis* UM23-1 was a modification of the technique described by Gonzalez et al. (13). Overnight cultures of VNR-1-tet-1 in NB with tetracycline and UM23-1 in NB with streptomycin were each diluted 1:100 into 10 ml of fresh NB. After a 3-h incubation at 37°C with gentle shaking, tubes containing 2.0 ml of NB were inoculated with 20 μl of the donor and recipient cultures. These fresh cultures were incubated for 20 h with gentle shaking at 37°C and then plated onto nutrient agar containing tetracycline and streptomycin to screen for transconjugants. Donor and recipient viabilities were determined by plating onto nutrient agar containing tetracycline or streptomycin, respectively.

Isolation of *B. anthracis* auxotrophic mutants. *B. anthracis* Tc^r transconjugants (from the mating of strains VNR-1-tet-1 and UM23-1) were inoculated onto BA-plus-tetracycline plates, which also contained phenylalanine, tyrosine, and tryptophan or which lacked one or more of the three amino acids. After incubation at 37°C for 48 h, auxotrophic clones were selected which grew on BA but not on BA lacking the indicated amino acid(s).

Determination of frequency of spontaneous mutation to antibiotic resistance. Bacterial strains were inoculated onto BHIA containing either kanamycin (*B. subtilis* BST1), tetracycline (*B. anthracis* VNR-1-tet-1), streptomycin (*B. anthracis* UM23-1), or no antibiotic (*B. anthracis* VNR-1). Growth was scraped from each plate and suspended in phosphate-buffered saline. Samples (0.1 ml) were spread onto plates of BHIA containing the appropriate antibiotic as indicated above and tetracycline for *B. anthracis* UM23-1 and VNR-1 and *B. subtilis* BST1 or streptomycin for *B. anthracis* VNR-1-tet-1. Serial dilutions of other 0.1-ml samples were spread onto tryptic soy agar plates for determination of total viability. After incubation for 16 to 20 h at 37°C, colony counts were made, and the frequencies of spontaneous mutation to antibiotic resistance were determined.

Purification of DNA. Plasmid DNA was purified from *Escherichia coli* by a modification of the alkaline lysis methods of Birnboim and Doly (2), as described by Maniatis et al. (22), and then ultracentrifugation in CsCl-ethidium bromide gradients. *B. subtilis* and *B. anthracis* DNA was prepared by the sodium dodecyl sulfate-NaOH-heat method of Green et al. (14), except that the cells were incubated with lysozyme (15 mg/ml) for 30 min before the addition of lysis buffer. Similarly, before purification of streptococcal whole-cell DNA by the procedures of Anderson and McKay (1), the suspended cells were treated with lysozyme as described above. The DNA preparations were stored at 4 or −70°C, either in Tris-EDTA buffer (25) or as ethanol-precipitated pellets.

Detection of DNA by hybridization. For slot blotting procedures, total-cell DNA was prepared for hybridization by the procedure described by Kafatos et al. (20). The DNA was denatured by heating in 0.3 M NaOH at 65°C for 30 min. After cooling to room temperature, samples were neutralized by the addition of 2.0 M ammonium acetate. For each preparation, approximately 500 ng of DNA (determined by A_{260}) in 0.4 ml of 0.9 M sodium chloride–0.09 M sodium citrate solution (pH 7.0) (21) was applied to nitrocellulose membrane strips by using the Minifold Slot Blot System (Schleicher & Schuell, Inc., Keene, N.H.). The strips were dried and heated at 70°C for 30 min in a vacuum oven.

The *tetM* DNA probe (16) was prepared by digestion of plasmid pJ13 DNA with *Hin*cII restriction endonuclease

(International Biotechnologies, Inc., New Haven, Conn.). A 5-kilobase (kb) *Hin*cII fragment containing the *tetM* gene of Tn*916* was purified from the pACYC177 vector by electrophoretic elution from an agarose gel (22). One microgram of the *tetM* gene fragment was labeled with [α-^{32}P]dCTP (New England Nuclear Corp., Boston, Mass.) by nick translation. Reagents for the nick translation reaction were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md., and were used as recommended by the supplier. Unincorporated nucleotides were removed by filtration through Sephadex G-50 (Sigma Chemical Co., St. Louis, Mo.) minicolumns (26). The nitrocellulose membrane strips containing the samples of total-cell DNA to be probed were placed in a solution containing 50% formamide, 5× Denhardt solution, 5× SSPE (sodium chloride, sodium phosphate, EDTA), 0.1% sodium dodecyl sulfate, and 10 μg of denatured calf thymus DNA per ml, as described by Maniatis et al. (22). The membranes were prehybridized by incubation overnight at 42°C in a shaking water bath. The solution was removed, and the membranes were hybridized in 10 ml of the prehybridization solution containing heat-denatured, ^{32}P-labeled probe DNA (3×10^6 cpm) and calf thymus DNA (100 μg/ml). The membranes were then incubated at 42°C for 16 to 24 h. After hybridization, the strips were washed and subjected to autoradiography as described previously (17).

For probing with pAM120 and pAM120LT DNA, 1 μg each of total-cell DNA from the *B. anthracis* strains to be probed was digested with *Hin*dIII and analyzed by electrophoresis in 0.8% agarose gels as described previously (17, 22). The two plasmids (10 ng of each) and bacteriophage lambda DNA digested with *Hin*dIII and *Eco*RI were included in the gels. The gels were then dried in preparation for direct hybridization by the method of Tsao et al. (28).

One microgram each of lambda DNA and plasmid pAM120 DNA and 250 ng of plasmid pAM120LT DNA were labeled and prepared for probing as described above for the *tetM* DNA probe. The agarose gels were prepared, hybridized to labeled lambda DNA and either pAM120 or pAM120LT DNA, washed, and evaluated by autoradiography as described above for the nitrocellulose membranes and as described previously for agarose gels (17).

RESULTS

Effect of nafcillin on frequency of Tc^r transconjugants from mating of *S. faecalis* DS16C1 with *B. anthracis* VNR-1. The first two filter mating experiments tested the effect of nafcillin on the frequency of conjugation. The transconjugant frequencies when the donor (*S. faecalis* DS16C1) and recipient (*B. anthracis* VNR-1) were treated with nafcillin before mating were 4.8×10^{-8} (experiment 1) and 1.9×10^{-7} (experiment 2), with a mean of 1.2×10^{-7}. The frequencies of Tc^r transconjugants when untreated cells were mated were 1.7×10^{-8} (experiment 1) and 1.5×10^{-8} (experiment 2), with a mean of 1.6×10^{-8}. Since nafcillin pretreatment appeared to increase the frequency of conjugation, the antibiotic was used in all further filter matings.

Filter mating. The data shown in Table 2 demonstrate that mating Tn*916* donor *S. faecalis* DS16C1 with *B. anthracis* VNR-1 resulted in the appearance of Tc^r *B. anthracis* clones. Furthermore, the mating of one of the transconjugant clones, VNR-1-tet-1, with a streptomycin-resistant (Sm^r) strain of *B. anthracis* (UM23-1) and a kanamycin-resistant (Km^r) strain of *B. subtilis* (BST1) yielded Tc^r *B. anthracis* and *B. subtilis* transconjugants. In all mating experiments the frequencies with which Tc^r transconjugants appeared were similar to those reported for the transfer of Tn*916* among streptococci (5). The mean frequency when the two *B. anthracis* strains were mated was especially high, 2.1×10^{-4}. This value is similar to the highest reported Tn*916* transconjugant frequency of 2.4×10^{-4} during matings of donor *S. agalactiae* with recipient *S. faecalis* (5). When *S. faecalis* CG110 was mated with *B. anthracis* VNR-1, Tc^r transconjugants appeared at a frequency over 100 times higher than that observed when *S. faecalis* DS16C1 was mated with VNR-1. No Tc^r transconjugants were recovered after mating of *B. anthracis* VNR-1-tet-1 and UM23-1 in broth culture. The data from all of the mating experiments suggest that transfer of Tn*916* was responsible for the appearance of the Tc^r *B. anthracis* and *B. subtilis* clones.

Mutation to antibiotic resistance. The frequency of spontaneous mutation to tetracycline resistance for *B. anthracis* VNR-1 and UM23-1 and *B. subtilis* BST1 was less than 4.0×10^{-10}. For *B. anthracis* VNR-1-tet-1, the frequency of mutation to streptomycin resistance was 3.7×10^{-9}. It thus appeared unlikely that mutation to antibiotic resistance was responsible for the appearance of Tc^r transconjugant clones after mating.

Auxotrophic mutants. Three thousand transconjugant clones from the mating between *B. anthracis* VNR-1-tet-1 and UM23-1 were tested for auxotrophy. Twenty-one were Phe$^-$, and two were Phe$^-$ Tyr$^-$ Trp$^-$. No mutants isolated were only Tyr$^-$ or Trp$^-$. The finding that different *B. anthracis* mutants were generated by using Tn*916* mutagenesis suggests that the transposon can insert into multiple sites within the cell DNA.

Hybridization studies. The results of probing for the presence of *tetM* DNA in total-cell DNA from Tc^r and Tc^s strains are shown in Fig. 1. The Tc^r strains, *B. subtilis* KT1 (blot A), *S. faecalis* DS16C1 (blot B), and *B. anthracis* VNR-1-tet-1 (blot C), all hybridized strongly with the probe. In contrast, the Tc^s strains, *B. subtilis* BST1 (blot D), *S. faecalis* JH2-2 (blot E), and *B. anthracis* VNR-1 (blot F), exhibited no hybridization with the probe. These hybridization data clearly indicate the presence of *tetM* DNA in the transconjugants and further demonstrate that the presence of Tn*916* was responsible for the acquisition of tetracycline resistance.

Total *B. anthracis* cell DNA from the Tc^s recipient strain VNR-1 and from Tc^r transconjugants isolated in three independent mating experiments was digested with *Hin*dIII, which cuts Tn*916* at a single site in the tetracycline resis-

TABLE 2. Filter mating transfer of tetracycline resistance[a]

Donor × recipient	No. of replicate samples	Mean frequency ± SEM[b]
S. faecalis DS16C1 × *B. anthracis* VNR-1[c]	8	$1.6 \times 10^{-7} \pm 0.5 \times 10^{-7}$
B. anthracis VNR-1-tet-1 × *B. anthracis* UM23-1[d]	4	$2.1 \times 10^{-4} \pm 0.8 \times 10^{-4}$
B. anthracis VNR-1-tet-1 × *B. subtilis* BST1[e]	3	$5.8 \times 10^{-6} \pm 2.6 \times 10^{-6}$
S. faecalis CG110 × *B. anthracis* VNR-1[c]	1	9.3×10^{-5}

[a] For experimental details, see the text.
[b] The frequency is expressed as the number of Tc^r transconjugants divided by the number of Tc^r donor cells at the end of the mating.
[c] The selection medium was R agar (to select against the donor) with tetracycline (to select against the recipient).
[d] The selection medium was BHIA with streptomycin and tetracycline.
[e] The selection medium was BHIA with kanamycin and tetracycline.

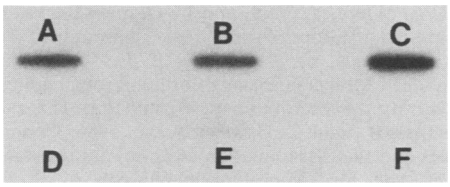

FIG. 1. Slot blot hybridization of total-cell DNA with a 5-kb probe for the *tetM* determinant of Tn*916*. Total-cell DNA (500 μg per sample) from Tc^r and Tc^s strains was transferred to a nitrocellulose membrane and hybridized to the ^{32}P-labeled probe. Blots: A, *B. subtilis* KT1; B, *S. faecalis* DS16C1; C, *B. anthracis* VNR-1-tet-1; D, *B. subtilis* BST1; E, *S. faecalis* JH2-2; F, *B. anthracis* VNR-1.

tance determinant (*tetM*) portion of the transposon, about 5 kb from one end (6). The fragments were separated by agarose gel electrophoresis, and after drying, the gel was incubated for hybridization with plasmid pAM120, which contains the entire Tn*916* transposon. Different-size fragments, all greater than 5 kb, from each Tc^r strain whose DNA was tested hybridized to the probes (Fig. 2), confirming several possible insertion sites from Tn*916* in the *B. anthracis* DNA. We saw no hybridization with the probe for the Tc^s parental strain VNR-1 (lane H). Prototrophic strains VNR-1-tet-1 (lane E), VNR-1-tet-2 (lane D), VNR-1-tet-3 (lane C), and VNR-1-tet-4 (lane B), Phe$^-$ strain tet-B (lane G), and Phe$^-$ Tyr$^-$ Trp$^-$ strain tet-C (lane F) all hybridized with the pAM120 probe. None of the strains which were tested hybridized with the pAM120LT probe from which Tn*916* had been excised (data not shown). For a single insertion of Tn*916* in the cell DNA, it would have been expected that Tc^r *B. anthracis* strains would exhibit two bands on the autoradiogram, one greater than 5 kb and the other greater than 10 kb, with the exact sizes depending upon the location of the nearest *Hin*dIII site on each end of the transposon. The presence of more than two bands for the Tc^r *B. anthracis* strains indicated that multiple insertions of Tn*916* were present in the DNAs.

DISCUSSION

The data presented here provide evidence for (i) the transfer of Tn*916* from *S. faecalis* to *B. anthracis* and the subsequent transfer of this transposon from *B. anthracis* to another *B. anthracis* strain and *B. subtilis*, (ii) the enhancement of Tn*916* transfer from *S. faecalis* to *B. anthracis* by the growth of donor and recipient strains in the presence of nafcillin, (iii) the insertion of Tn*916* into more than one site in the *B. anthracis* DNA, and (iv) the generation of specific mutants by transposon mutagenesis of *B. anthracis*. The range of hosts for Tn*916* is unknown, but the transposon may prove to be an effective mutagenic tool in numerous microbial genera. In this regard, Tn*916* was recently transferred from *S. faecalis* to *S. aureus* by mating on filter membranes (19). Furthermore, Tn*916* has been transferred to *Mycoplasma* species by transformation with pAM120 (8) and by mixed incubation of *S. faecalis* and *Mycoplasma hominis* on solid media (24). In these studies Tn*916* was shown to have inserted into the *Mycoplasma* chromosome at several locations. Gawron-Burke and Clewell (12) noted expression of tetracycline resistance in *E. coli* after transformation with a pBR322-derived plasmid, pGL101, into which Tn*916* had been ligated.

Nafcillin is a synthetic penicillin used clinically against microorganisms which are resistant to penicillin due to the production of beta lactamase. Pretreatment of donor and recipient strains with the antibiotic clearly increased the frequency of transfer of Tn*916* from *S. faecalis* to *B. anthracis*. We did not investigate the mechanism by which the antibiotic augmented transfer of the transposon; however, it was previously found (P. A. W. Martin, Abstr. Annu. Meet. 13th Int. Congr. Microbiol., p. 47, 1982) that cell wall-active antibiotics such as ampicillin and nafcillin increased the rate of transfer in matings on filter membranes of plasmid pAMβ1 from *S. faecalis* to *Bacillus thuringiensis*.

It was not surprising that *S. faecalis* CG110 transferred Tn*916* to *B. anthracis* at a much higher frequency, over 500-fold, than did *S. faecalis* DS16C1. Gawron-Burke and Clewell (11) reported a similar enhanced conjugation frequency when strain CG110 was used as a donor strain, compared with *S. faecalis* DS16C3 and CG130. The investigators suggested (11) that DNA sequences near Tn*916* may affect the rate at which the transposon is donated. It was also not unexpected that mating *B. anthracis* VNR-1-tet-1 and UM23-1 in broth yielded no transconjugants, since transfer of Tn*916* in broth between streptococci has never been reported.

Hoiseth and Stocker (15) used transposon Tn*10* mutagenesis to obtain *aro* mutants of *Salmonella typhimurium* whose growth required phenylalanine, tyrosine, and tryptophan (as well as 2,3-dihydroxybenzoate and *p*-aminobenzoate under very stringent nutritional conditions). These *aro* mutants were reduced in virulence and were effective as live vaccines in experimental animals. As a result of their inability to synthesize 2,3-dihydroxybenzoate and *p*-aminobenzoate or to obtain them in the host, infections by the *aro* mutants were self-limiting. It was the intent in our studies to similarly generate *B. anthracis* strains which would produce protective antigen and other anthrax toxin antigens but be safer in

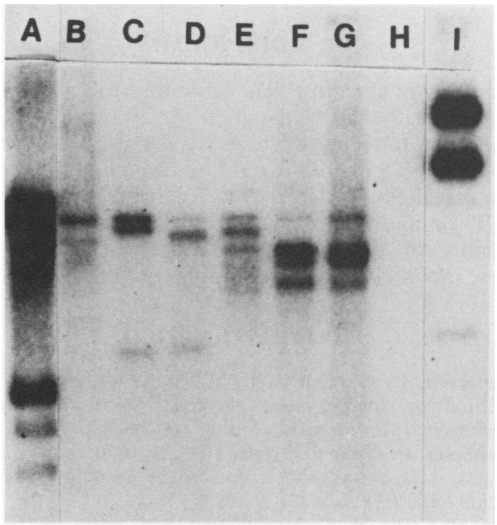

FIG. 2. Hybridization of digested, total *B. anthracis* cell DNA with ^{32}P-labeled lambda DNA and pAM120 DNA. Total-cell DNA was digested with *Hin*dIII enzyme, subjected to electrophoresis in 0.8% agarose, and probed (see the text). Lanes: A, lambda digested with *Hin*dIII and *Eco*RI; B, *B. anthracis* VNR-1-tet-4; C, *B. anthracis* VNR-1-tet-3; D, *B. anthracis* VNR-1-tet-2; E, *B. anthracis* VNR-1-tet-1; F, *B. anthracis* tet-C; G, *B. anthracis* tet-B; H, *B. anthracis* VNR-1; I, pAM120 (undigested). The total-cell DNA digests did not hybridize with a pAM120LT probe.

the immunized host than is the current Sterne veterinary vaccine strain. The frequency of excision of Tn*916* from the *B. anthracis* chromosome in the absence of tetracycline and the rates of reversion to prototrophy of the *B. anthracis* auxotrophic mutants are unknown. In streptococci, however, the frequency of excision and transposition within the cell appears to be related to the frequency of excision and transfer to another cell (11). Franke and Clewell (10) estimated the frequency of transposition of Tn*916* in *S. faecalis* to be approximately 10^{-5}. In *E. coli*, Tn*916* may excise precisely from DNA, leaving an intact functional gene (12). Thus, some in vivo revertants to prototrophy might be expected after immunization with the two *B. anthracis* Phe^- Tyr^- Trp^- strains isolated in the present study. Nevertheless, the strains should be substantially more attenuated than the nonencapsulated Sterne strain, and they will be tested in experimental animals as prototype, live-vaccine candidates against virulent anthrax spore challenge.

It is clear from these data that Tn*916* inserts at different loci in the *B. anthracis* DNA and that it can be used to obtain specific mutants. It is not clear, however, whether Tn*916* inserts preferentially into certain DNA sites. The relative frequency of appearance of Phe^- mutants compared with that of Phe^- Tyr^- Trp^- mutants suggests that such may be the case. Further hybridization studies with the various mutants will be required to resolve this question. Other investigations will be conducted to determine the utility of the transposon as a tool for investigating the molecular biology of the bacillus, as well as a means of generating specifically attenuated strains for vaccine studies.

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ADDENDUM

Since this manuscript was submitted for publication, we have learned that J. G. Naglich, R. E. Andrews, Jr., and P. A. Pattee, Iowa State University, Ames, have transferred Tn*916* from *S. faecalis* to *B. thuringiensis* by mating on membrane filters (R. E. Andrews, Jr., personal communication). Their investigations and those reported here confirm the ability of *S. faecalis* to transfer Tn*916* to *Bacillus* species, in which the Tc^r phenotype is then expressed.

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