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Characterization of Chromosomal DNA Amplifications with Associated Tetracycline Resistance in *Bacillus subtilis*

CATHERINE L. IVES† AND KENNETH F. BOTT*

Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, CB no. 7290, Chapel Hill, North Carolina 27599

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Endogenous chromosomal DNA amplifications with associated tetracycline resistance (Tc^r) in *Bacillus subtilis* were first described by C. R. Wilson and A. E. Morgan (J. Bacteriol. 163:445–453, 1985). We have confirmed and extended their results, and we show that fusion of protoplasts from Tc^s *B. subtilis* 168 *trpC2* with polyethylene glycol and regeneration on medium containing 20 μg of tetracycline per ml induces Tc^r regenerants that contain amplified DNA. This phenomenon appeared to be *recE* dependent and requires the addition of polyethylene glycol. Along with three regenerants kindly provided by Wilson and Morgan (RAD1, RAD6, and RAD7), we characterized three strains (CLI20, CLI22, CLI30) isolated in this laboratory. All six contain an amplified region of DNA which was independently cloned on plasmid pCIS7. Integration of pCIS7 into the wild-type (Tc^s) *B. subtilis* chromosome and amplification of the plasmid sequences generated a Tc^r phenotype, even though the DNA on pCIS7 was cloned from Tc^s *B. subtilis* KS162 (Ives and Bott, J. Bacteriol. 171:1801–1810, 1989). The amplified DNA also showed homology (through hybridization analysis) with pAMα1Δ1, a gram-positive Tc^r plasmid, indicating that *B. subtilis* normally contains a silent integrated copy of the gene whose amplification confers Tc^r. The amplifications were determined to lie between *purA* and *gyrB* on the *B. subtilis* chromosome, and the endpoints were mapped. RAD6 and CLI30 may share the same left-hand endpoint, but the other endpoints are different in each isolate. The amplified DNAs of RAD1, RAD6, CLI20, and CLI30 end near known DNA membrane binding sites. The number of amplified units of DNA was determined through dot blot analysis to be approximately 80 to 100 copies per cell, with corresponding increases in transcription of RAD1, RAD6, CLI20, CLI22, and CLI30.

Gene amplification is recognized as a regulatory mechanism in both eucaryotes and procaryotes. In eucaryotes, amplifications can be developmentally regulated or a response to metabolic stress (28, 32). Examples include the amplification of rRNA genes of *Xenopus* oocytes and the amplification of dihydrofolate reductase genes in cultured cells that are resistant to methotrexate (16, 28). In procaryotes, gene amplification is viewed as an adaptive response (i.e., a response to stress) and as an important factor in the evolution of new genes (2); it has been studied extensively in gram-negative species. Gene amplification in procaryotes occurs through recombination between rRNA operons, IS1 sequences, or short DNA homologies (3, 9, 20). The rate-limiting event in gene amplification is the formation of an initial duplication. Gene duplications occur spontaneously from illegitimate unequal recombination between nonhomologous sequences on homologous chromosomes or through legitimate unequal recombination between homologous sequences located at different points on homologous chromosomes (2). All duplications examined in procaryotes have been tandem arrays of a repeated unit, although in *Streptomyces glaucescens* deletions sometimes accompany amplification (11).

In *Bacillus subtilis*, few endogenous gene amplifications have been documented. Instead, DNA amplification has been studied primarily as a laboratory-induced phenomenon through the integration of heterologous plasmid DNA (containing a selectable drug resistance marker) into the chromosome and selection for increased copy number of the plasmid sequences by increasing the level of antibiotic in the growth medium. Using this method, Young demonstrated the 15-fold amplification of the chloramphenicol acetyltransferase marker when this gene was integrated into the *B. subtilis* chromosome and the concentration of chloramphenicol in the growth medium was increased from 5 to 20 μg/ml (37). Similarly, Albertini and Galizzi amplified a chromosomal region in *B. subtilis* containing the α-amylase gene by integrating a plasmid containing this gene and a drug resistance marker. They reported that the increased expression of α-amylase correlated with the gene dosage (1).

Hashiguchi et al. (12) described an endogenous DNA amplification of the *tmrA* locus, which is associated with tunicamycin resistance (Tm^r) and hyperproduction of α-amylase. They later determined that the *tmrA* locus is actually the junction fragment between the amplified units of DNA (18). The Tm^r phenotype apparently results from the increased copy number of a wild-type gene whose amplification was estimated to be 10 to 15 copies (12).

The only other endogenous gene amplification identified in *B. subtilis* was reported by Wilson and Morgan (34). They observed that regenerated protoplasts gave rise to tetracycline-resistant (Tc^r) strains, a subset of which contained amplified DNA. Using phage PBS1 transduction, they genetically mapped the *tet* locus near *guaA* (the origin of replication). The amplified DNA was 100% cotransferred with the *tet* locus, suggesting that the DNA amplifications and Tc^r phenotype were linked.

Williams and Smith have also described chromosomal mutations conferring Tc^r in *B. subtilis* (33). The mutations, isolated after ethyl methanesulfonate mutagenesis, fell into two classes, *tetA* and *tetB*. *tetA* mutations showed altered mobility of the S10 ribosomal protein in two-dimensional

* Corresponding author.
† Present address: New England Biolabs, Beverly, MA 01915.

polyacrylamide gels and were genetically mapped near the *tuf* gene in the *B. subtilis* ribosomal protein gene cluster. Isolates with the *tetB* mutation did not show altered ribosomal proteins, and their ribosomes were sensitive in vitro to tetracycline. The *tetB* mutation was genetically mapped proximal to *cysA14* and postulated to be an alteration of a membrane protein affecting transport of the drug.

Recently, we reported that a cloned chromosomal fragment of *B. subtilis* DNA conferred Tc^r when present in multiple copies (13). Plasmid pCIS7, containing 11.5 kilobases (kb) of *B. subtilis* DNA, was isolated from a Tn*917* transposon insertion in tetracycline-susceptible (Tc^s) *B. subtilis* KS162 (24). When integrated into the chromosome of *B. subtilis* 168, this plasmid confers tetracycline resistance upon reiteration of the plasmid DNA sequences in the chromosome. We were able to physically map the *tet* locus through contour-clamped homogenous electric field gels between *purA* and *guaA* near the origin of replication (13).

In this report, we demonstrate that the endogenous DNA amplifications described by Wilson and Morgan contain DNA cloned on pCIS7 and that this DNA is related to other gram-positive tetracycline resistance determinants. We also discuss the copy number, the extent of the DNA amplifications, and the increased transcription of the amplified DNA.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains, plasmids, and recombinant phage are described in Tables 1 and 2.

Media, chemicals, and enzymes. Tryptose blood agar base was obtained from Difco Laboratories (Detroit, Mich.). Luria broth is 10 g of tryptone, 5 g of yeast extract, and 10 g of sodium chloride per liter. DM3 protoplast regeneration medium (5) consists of the following sterile solutions (per liter): 200 ml of 4% agar, 500 ml of 1 M sodium succinate (pH 7.3), 100 ml of 5% Difco Casamino Acids, 50 ml of 10% Difco yeast extract, 100 ml of 3.5% K_2HPO_4, 1.5% KH_2PO_4, and 25 ml of bovine serum albumin. SMMP contains equal volumes of 2× SMM (1 M sucrose, 0.04 M maleic acid, 0.04 M magnesium chloride) and 4× antibiotic medium 3 (A3; Difco). Ampicillin, chloramphenicol, and tetracycline were purchased from Sigma Chemical Co. (St. Louis, Mo.) and used at concentrations of 50, 5, and 20 μg/ml, respectively, unless otherwise stated. All other chemicals were purchased from Sigma, except ultrapure sodium dodecyl sulfate (BDH Chemicals Ltd., Poole, England). Restriction endonucleases were obtained from International Biotechnology Inc. (New Haven, Conn.), Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), or Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and used as recommended by the manufacturers. [α-^{32}P]dCTP was purchased from Dupont, NEN Research Products (Boston, Mass.).

Endonuclease restriction digests were analyzed by electrophoresis in horizontal 0.7 or 1.0% agarose gels in Tris-acetate buffer (40 mM Tris [pH 8.0], 20 mM ammonium acetate, 2.5 mM disodium EDTA) or Tris-borate buffer (89 mM Tris hydrochloride, 89 mM boric acid, mM EDTA). *InCert* agarose was purchased from FMC Corp. (Rockland, Maine). Nitrocellulose and Optibind membranes were obtained from Schleicher & Schuell Co. (Keene, N.H.).

Isolation of CLI strains. *B. subtilis* CLI20, CLI22, and CLI30 were isolated by the protoplast formation and regeneration procedure of Chang and Cohen (5). An overnight culture of *B. subtilis* 168 *trpC2* was used to inoculate 50 ml of A3 medium at a 1:100 dilution. Cells were grown at 37°C with shaking until the optical density at 500 nm was 0.35 and then centrifuged at 5,000 rpm for 5 min and suspended in 5 ml of SMMP. Lysozyme was added to a concentration of 2 mg/ml, and cells were incubated at 37°C with gentle shaking. The extent of protoplasting was monitored by phase-contrast microscopy. After 70 to 80% protoplasting, cells were pelleted in a table-top centrifuge at 2,600 × *g* for 15 min. Cells were washed in 1× SMMP, pelleted again, and then suspended in 0.5 ml of SMMP.

TABLE 1. Bacterial strains

B. subtilis strain	Genotype	Source
168	*trpC2*	This laboratory
KS162	Tn*917* (*erm*)	K. Sandman (24)
168(pCIS7)	Cat *tet*	Ives and Bott (13)
BC92	*purA argA ile*	Wilson and Morgan (34)
RAD2	*purA argA ile tet*	Wilson and Morgan (34)
RAD1	*purA argA ile tet* AUD	Wilson and Morgan (34)
RAD6	*purA argA ile tet* AUD	Wilson and Morgan (34)
RAD7	*purA argA ile tet* AUD	Wilson and Morgan (34)
CLI7	*trpC2 tet*	This study
CLI20	*trpC2 tet* AUD	This study
CLI22	*trpC2 tet* AUD	This study
CLI30	*trpC2 tet* AUD	This study
IS176	*trpC2 thr-5 tetB*	Williams and Smith (33)

To 500 μl of protoplasts, 50 μl of distilled H_2O and 50 μl of 2× SMM were added. Then 1.5 ml of polyethylene glycol (PEG, 8,000; molecular weight, 40% in 1× SMM) was added and mixed, and the cells were incubated at room temperature for 2 min. SMMP (5 ml) was added and mixed, and then cultures were centrifuged at 150 × *g* for 20 min. The supernatant was carefully removed, the pellet was suspended in 1 ml of SMMP, and the protoplasts were incubated at 37°C for 90 min with gentle shaking. Cells were plated on DM3 regeneration medium with and without tetracycline (20 μg/ml).

DNA isolation. Chromosomal DNA was isolated from *Bacillus* cells growing exponentially in A3 or SMMP by the method of Saito and Miura (22) and purified on an equilibrium CsCl gradient or by the detergent extraction chromosomal isolation procedure of Wilson and Morgan (34). DNA used for restriction mapping experiments was prepared from protoplasted cells immobilized in agarose blocks by a modification of the method of Schwartz and Cantor for DNA isolation for pulse-field gel electrophoresis (29). An overnight culture was used to inoculate 25 ml of SMMP to an optical density at 600 nm of approximately 0.1. This culture was grown at 37°C to an optical density at 600 nm of 0.8; 5 ml

TABLE 2. Plasmids and phage used in this study

Plasmid or phage	Selectable markers	Vector	Source
Plasmids			
pCIS7	Tet[a] Cat Amp	pTV20	Ives and Bott (13)
pCIS2	Cat Amp	pTV21Δ2	Ives and Bott (13)
pMS31	Tet Cat	pJAB1	Sargent and Bennett (25)
pAMα1Δ1	Tet	pAMα1	Perkins and Youngman (19)
Phage			
529		Lambda	Sargent et al. (26)
F3		Lambda	Ives and Bott (13)

[a] pCIS7 is Tc^r when integrated into the *B. subtilis* chromosome and amplified.

was centrifuged at 3,000 × *g* for 10 min, and the pellet was suspended in 1 ml of SMMP buffer. Lysozyme was added to a final concentration of 2 mg/ml, and the mixture was incubated at 37°C for 30 to 60 min. The extent of protoplasting was examined under a phase-contrast microscope. *In-Cert* agarose (2%, 1 ml) was added; 100-μl portions were quickly dispensed into a Plexiglas mold (1 by 6 by 8 mm) and chilled on ice. The blocks of immobilized protoplasts were added to lysis buffer (25 mM Tris chloride [pH 8], 25 mM EDTA, 0.3 M sucrose, 20 μg of DNase-free RNase per ml, 1 mg of lysozyme per ml) and incubated at 37°C for 4 to 8 h with gentle shaking. The blocks (100-μl volume) were then transferred to proteinase K buffer (0.05 M EDTA [pH 9.0], 1% Sarkosyl, 2 mg of proteinase K per ml) and incubated at 50°C for 14 to 18 h with gentle shaking. The blocks were then washed in 10 mM Tris hydrochloride (pH 7.5)–0.1 mM EDTA containing 1 mM phenylmethylsulfonyl fluoride at 4°C overnight. Blocks were stored in 10 mM Tris chloride (pH 7.5)–0.1 mM EDTA (TE) at 4°C.

For restriction digests, one-third of a block was washed once in 1 ml of TE at 37°C for 1 h and then equilibrated in 100 μl of 1× restriction buffer at 37°C for 30 min. The 1× restriction buffer was supplemented with bovine serum albumin at 100 μg/ml. The buffer was removed, and the blocks were placed at 65°C for 20 min to melt the agarose. Thirty units of the appropriate enzyme was added to the liquified blocks, and the digests were incubated at 37°C for 2 to 4 h.

Determination of copy number of AUD. The number of amplified units of DNA (AUD) was determined by the dot blot technique of Detetic et al. (7). The dilutions of DNA tested were 1 μg (undiluted), 0.1 μg (1:10), 0.05 μg (1:20), 0.025 μg (1:40), 0.17 μg (1:60), 0.0125 μg (1:80), and 0.001 μg (1:100). Purified DNA was denatured in 300 μl of 0.2 M sodium hydroxide–30 mM Tris hydrochloride (pH 7.5) and heated for 10 min at 80°C. Then samples were cooled on ice and neutralized with 40 μl of 2 M Tris hydrochloride (pH 7.0). Samples were immediately spotted onto an Optibind filter in a Schleicher & Schuell Minifold II Slot Blotter. The DNA was UV cross-linked onto the filter with a UV Stratalinker (Stratagene, La Jolla, Calif.) and probed with a radiolabeled restriction fragment.

Hybridization conditions. The modified Southern procedure described by Smith and Summers was used to transfer DNA restriction fragments from agarose gels to nitrocellulose or Optibind membranes (30). DNA was radiolabeled with [α-^{32}P]dCTP by nick translation by the method of Maniatis et al. (17) or by the random priming method of Feinberg and Vogelstein (10) with the Boehringer random-primed DNA labeling kit.

Low-stringency hybridization (70% homology) was conducted in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with 0.5% sodium dodecyl sulfate at 52°C for 16 to 20 h. The filters were washed extensively with 0.1× SSC–0.05% sodium dodecyl sulfate at room temperature, dried, and autoradiographed. The same procedure was followed for high-stringency hybridization (80% homology), except that the filters were hybridized at 65°C and washed at 52°C after a rinse at room temperature.

RNA isolation. All glassware was baked overnight in a 250°C oven. All solutions were treated for 12 h with 0.1% diethylpyrocarbonate and then autoclaved. The appropriate *B. subtilis* strains were inoculated into 100 ml of 4× A3 or SMMP medium from overnight cultures. Cells were grown to the late-log phase (optical density of 600 nm, 0.8) at 37°C and then pelleted at 5,000 rpm for 10 min. The pellet was suspended in 5 ml of Tris-sucrose buffer (50 mM Tris hydrochloride [pH 8.0], 50 mM EDTA, 15% sucrose). Five milligrams of lysozyme was added, and the cells were incubated at 37°C for 1 h. Proteinase K was added to a final concentration of 2 μg/ml, and diethylpyrocarbonate was added to a final concentration of 0.08%. Twenty-five milligrams of ultrapure sodium dodecyl sulfate was added, and the cells were incubated for 30 to 60 min at 37°C. Potassium acetate (pH 5.5) was added to a final concentration of 0.5 M and incubated on ice for 1 h. The mixture was centrifuged for 45 min at 7,500 rpm, and the pellet was discarded. The RNA was phenol extracted twice, phenol-chloroform extracted once, and chloroform extracted once. Ethanol (2.5 volumes) was added, and RNA was precipitated at −20°C overnight. The RNA was pelleted at 6,000 rpm for 1 h. The pellet was dried under vacuum and suspended in 1 ml of distilled H_2O. The RNA was treated with ultrapure DNase (Boehringer), phenol-chloroform extracted, precipitated, and quantitated by UV A_{260}.

For Northern RNA blot analysis, RNA samples (15 μg) were added to 15 μl of freshly made formamide-formaldehyde buffer [0.5 ml of formamide, 184 μl of 37% formaldehyde, 0.1 ml of 10× running buffer; 10× running buffer is 100 mM 3-(*N*-morpholine)propanesulfonic acid, 50 mM sodium acetate, and 10 mM EDTA (pH 7.0)]. Samples were denatured at 65°C for 5 min, placed on ice, mixed with 4 μl of loading dye (50% sucrose, 0.4% bromphenol blue, 0.1% iodoacetate), and loaded onto a formaldehyde-agarose gel.

RNA was electrophoresed in gels containing 0.8% agarose, 2.2 M formaldehyde, and 10% 10× running buffer. Gels were run in 1× running buffer at 50 V for 10 h. After electrophoresis, the gel was stained with ethidium bromide (EtBr) for 30 min, destained for 30 min, and photographed. The gel was soaked for 30 min in 20× SSC, and RNA was transferred to an Optibind filter in 10× SSC.

Transcriptional analysis was also studied by using RNA slot blots. With a Schleicher & Schuell slot blot apparatus, 50 μg of RNA was spotted onto a nitrocellulose filter after samples were treated with formamide-formaldehyde buffer and denatured for 5 min at 65°C. The filter was UV cross-linked and then hybridized under the conditions described above.

RESULTS

Generation of Tc^r amplified strains by protoplasting. Wilson and Morgan first reported a Tc^r phenotype with associated DNA amplifications after protoplasting fusing with PEG, and regenerating normally Tc^s *B. subtilis* in the presence of 20 μg of tetracycline per ml (34). DNA prepared from many, but not all, of these Tc^r regenerants contained amplified restriction fragments in EtBr-stained gels. Some of the amplified restriction fragments were common to all strains, but others were unique. Through PBS1 transductional mapping, the Tc^r determinant was localized near the origin of replication and showed that the amplified DNA was genetically transferred along with the Tc^r marker.

These experiments were successfully repeated in this laboratory. *B. subtilis* 168 *trpC2* was protoplasted, fused with PEG, and regenerated on DM3 medium containing 20 μg of tetracycline per ml. Tc^r revertants were recovered at a low frequency (approximately 10^{-8}). Chromosomal DNA from these Tc^r regenerants was prepared by the modified method of Schwartz and Cantor (29). DNA prepared in agarose plugs was completely digested with restriction endonucleases; for unknown reasons, DNA prepared by con-

ventional means was often refractory to digestion (Ives and Bott, unpublished observations). Totally digestable DNA was necessary to map the endpoints of the amplifications (see below). Chromosomal DNA was digested, fractionated through 0.8 to 1.0% agarose gels, and stained with EtBr. A number of the Tc^r regenerants contained amplified DNA. Like Wilson and Morgan, we were able to recover Tc^r regenerants that did not contain amplified DNA after the protoplast formation-regeneration regimen on tetracycline-containing medium (Ives and Bott, unpublished results). The resistance of these strains was as great as that of strains with amplified DNA, and this Tc^r determinant was localized through transductional mapping to the same general region of the chromosome (13; Ives and Bott, unpublished results). Apparently two similar types of Tc^r determinants are induced through protoplast formation, PEG fusion, and regeneration; one is associated with DNA amplifications, whereas the other is not. Here, we examined only strains that contained amplified DNA.

Amplified *Eco*RI restriction fragments could be seen in an EtBr-stained agarose gel (Fig. 1, lanes 6, 7, and 8) when compared with *B. subtilis* 168 *trpC2* chromosomal DNA digested with the same enzyme (Fig. 1, lane 2). These chromosomal digests were compared with *Eco*RI-digested chromosomal DNA from strains isolated by Wilson and Morgan (Fig. 1, lanes 3, 4, and 5) (34). Similar restriction fragments were amplified; for example, RAD6, CLI22, and CLI30 (Fig. 1, lanes 4, 7, and 8) contained an amplified 11.5-kb *Eco*RI restriction fragment, and all the strains amplified a 1.1-kb *Eco*RI restriction fragment. However, some restriction fragments were unique; RAD7 (Fig. 1, lane 5) contained an amplified 9.6-kb *Eco*RI restriction fragment.

Similar results were obtained when the chromosomal DNA was digested with restriction endonuclease *Pst*I. For example, a 1.8-kb *Pst*I fragment was amplified in all strains when compared with strain 168 chromosomal DNA (Fig. 1, lanes 10 through 16), and an 8.6-kb *Pst*I fragment was amplified in RAD6, CLI22, and CLI30 (Fig. 1, lanes 12, 15, and 16), whereas a 9.2-kb *Pst*I fragment was amplified in RAD6, RAD7, CLI20, CLI22, and CLI30 (Fig. 1, lanes 12, 13, 14, 15, and 16). However, RAD1 and CLI20 contained unique *Pst*I amplified restriction fragments of 11 and 4.5 kb, respectively.

We have therefore confirmed the initial findings of Wilson and Morgan; that is, that protoplasting, fusing, and regenerating Tc^s *B. subtilis* 168 *trpC2* gives rise to Tc^r regenerants containing amplified DNA. Disparity in the size and number of amplified fragments suggests that the extent of the duplicated DNA is different in all six isolates chosen for further study. Using PBS1 transduction, we also genetically mapped the Tc^r determinant of CLI20 near the origin region (data not shown), confirming that both of these phenomena occur in the same area of the chromosome.

The protoplast formation, fusion, and regeneration were attempted with an *recE4* strain of *B. subtilis* (BD224). No Tc^r revertants were recovered (Ives and Bott, unpublished observations), suggesting that the phenomenon is dependent on recombination functions. (However, the possibility that the *recE4* mutation in the presence of amplification acts synergistically to result in nonviable cells cannot be excluded, since we did not isolate nonamplified Tc^r strains in the *recE4* background either.) We were able to demonstrate that the addition of PEG is apparently an essential step in generating these amplifications. CLI7, a Tc^r revertant, was isolated by protoplasting and regenerating on DM3 semisolid agar containing 20 μg of tetracycline per ml (no PEG treatment). This isolate, although Tc^r, contained no amplified DNA, suggesting that fusion of protoplasts is necessary for isolation of amplified Tc^r regenerants.

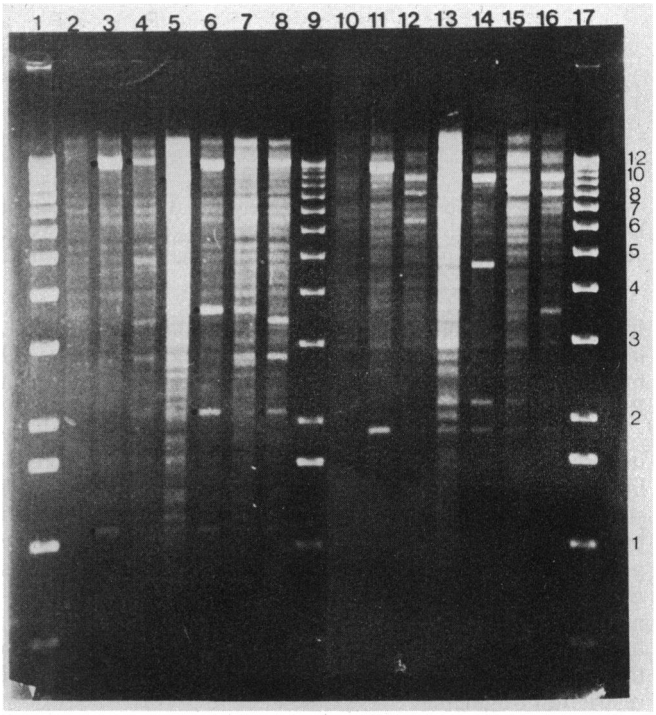

FIG. 1. DNA restriction endonuclease digestion profiles of Tc^r regenerants. The purified DNAs were prepared as described in Materials and Methods by the modified protocol of Schwartz and Cantor (29). The DNA was incubated with *Eco*RI or *Pst*I for 2 to 4 h, electrophoresed on a 1% agarose gel, and stained with EtBr. RAD1, RAD6, and RAD7 were acquired from R. Wilson. CLI20, CLI22, and CLI30 were obtained by the protoplasting procedure detailed in Materials and Methods. EcoRI digests (lanes): 2, 168; 3, RAD1; 4, RAD6; 5, RAD7; 6, CLI20; 7, CLI22; 8, CLI30. *Pst*I digests (lanes): 10, 168; 11, RAD1; 12, RAD6; 13, RAD7; 14, CLI20; 15, CLI22; 16, CLI30. Lanes 1, 9, and 17 contained a 1-kb ladder (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), whose sizes in kilobase pairs are indicated on the right. Subsets of amplified restriction fragments are indicated with black dots.

Hybridization of core amplification unit to pCIS7. A subset of the amplified DNA observed by EtBr staining of agarose gels was contained within plasmid pCIS7. Plasmid pCIS7 was isolated from Tc^s *B. subtilis* KS162 (one of a collection of strains containing Tn*917* insertions in the *oriC* region [24]) by the procedure of Youngman et al. (38) with the pTV20 integrative vector. It contained 11.5 kb of *B. subtilis* DNA (Fig. 2) and, when integrated and amplified, confers Tc^r (13) even though the parental strain was Tc^s.

DNA fractionated in the agarose gel of Fig. 1 was transferred to an Optibind membrane and probed with pCIS7 radiolabeled by the random priming method (10). Against an *Eco*RI chromosomal digest of 168, RAD1, RAD6, RAD7, CLI20, CLI22, and CLI30 (Fig. 3, lanes 1 through 7), pCIS7 hybridized to an 11.5-kb *Eco*RI restriction fragment in all strains but also hybridized to a subset of the amplified DNA fragments that could be visualized on the EtBr-stained gel. Intensely hybridizing restriction fragments (indicative of amplification) included an 11-kb *Eco*RI fragment in RAD1 and CLI20 (Fig. 3, lanes 2 and 5) and a 9.6-kb *Eco*RI fragment in RAD7 (lane 4). The 11.5-kb *Eco*RI restriction

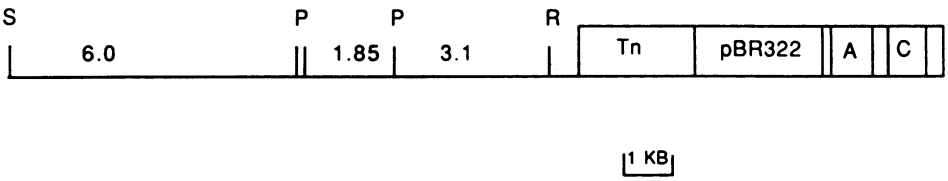

FIG. 2. Restriction map of pCIS7. The plasmid was constructed as described previously (13). Vector DNA is indicated by a box. *B. subtilis* DNA is indicated by a single line. Distances between cleavage sites are indicated in kilobases. Abbreviations: C, chloramphenicol acetyltransferase gene from pC194; A, β-lactamase gene of pBR322; Tn, Tn*917* DNA; S, *Sph*I; P, *Pst*I; R, *Eco*RI.

fragment was amplified in RAD6, CLI22, and CLI30 (Fig. 3, lanes 3, 6, and 7). Upon longer exposure, a 0.8-kb *Eco*RI fragment hybridized in all strains and was amplified in RAD6, CLI22, and CLI30 (data not shown).

These data suggest that the Tc^r phenotype conferred by integrating plasmid pCIS7 into the chromosome and increasing its copy number is closely related to the Tc^r phenotype of strains with endogenous amplifications obtained through protoplasting, fusing with PEG, and regenerating in the presence of tetracycline. These were also the same sizes as *Eco*RI restriction fragments that hybridized to an endogenous gram-positive Tc^r plasmid, pAMα1Δ1 (see below), further indicating that the DNA sequences that encode Tc^r in these amplifications are located on the amplified *Eco*RI restriction fragments.

Hybridization to pAMα1Δ1. pAMα1 is a 9.6-kb nonconjugative plasmid isolated from *Streptococcus faecalis* that confers amplifiable tetracycline resistance to several gram-positive species (20). The tetracycline resistance determinant is located on a 4.2-kb segment of pAMα1, which is flanked by homologous sequences of approximately 380 base pairs (6, 36). Intramolecular recombination at these repeats dissociates pAMα1 into two circular molecules, each containing a single repeat sequence. One of the resolved molecules, pAMα1Δ1, contains the Tc^r determinant and replicates in *B. subtilis* (19). pAMα1Δ1 is virtually identical in its profile of restriction endonuclease recognition sites to a Tc^r plasmid of the same size, pBC16, originally isolated from *Bacillus cereus* (8).

Chromosomal DNA of 168, BC92, RAD1, RAD2, RAD6, RAD7, and CLI20 was digested with *Eco*RI restriction endonuclease and fractionated through a 0.8% agarose gel. The DNA was denatured, transferred to nitrocellulose, and hybridized to nick-translated pAMα1Δ1. With high-stringency conditions (see Materials and Methods), an 11.5-kb *Eco*RI restriction fragment common to all strains, an 11.0-kb *Eco*RI restriction fragment in RAD1 and CLI20, and a 9.6-kb *Eco*RI restriction fragment in RAD7 hybridized poorly. Upon lowering of stringency conditions, these same restriction fragments hybridized more clearly (Fig. 4). The 11.5-kb *Eco*RI restriction fragment of RAD6 (Fig. 4, lane 5), the 11-kb *Eco*RI restriction fragment of RAD1 and CLI20 (Fig. 5, lanes 3 and 7), and the 9.6-kb *Eco*RI restriction fragment of RAD7 (Fig. 4, lane 6) hybridized more intensely than the 11.5-kb *Eco*RI restriction fragments in 168, BC92, and RAD2 (Fig. 4, lanes 1, 2, and 4), indicating amplification of these DNA sequences in the chromosome. The chromosomal DNA from the amplified strains contained sequences that showed homology with an endogenous gram-positive plasmid. However, the normal function of these sequences in the chromosome is unknown, since the parental strains, 168 and BC92, are normally Tc^s. Hybridization of pAMα1Δ1 to chromosomal *Pst*I digests of RAD6 and CLI20 has shown that 1.8- and 9.2-kb *Pst*I restriction fragments hybridize intensely to this probe (data not shown), suggesting that the DNA sequences that confer Tc^r are localized in the middle of pCIS7 (which contains a 1.8-kb *Pst*I restriction fragment [Fig. 2]).

Copy number determination of the DNA amplifications. Two methods were undertaken to determine the copy number of the AUDs. Cultures of *B. subtilis* 168, RAD1, RAD6, RAD7, CLI20, CLI22, and CLI30 were grown in 40 ml of SMMP containing 20 μg of tetracycline per ml where appropriate. The cultures were harvested after 16 to 18 h, and chromosomal DNA was prepared by the detergent extraction procedure of Wilson and Morgan (34), and was quantitated by optical density at 260 nm in a Gilford 250 spectrophotometer. First, dilutions of restriction digests were electrophoresed through agarose, transferred to nitrocellulose, and hybridized with nick-translated pCIS7 (data not shown). Second, 1 μg of chromosomal DNA from each of the strains was denatured in 0.2 M sodium hydroxide–30 mM Tris and heated, and 10-fold dilutions were spotted onto an Optibind filter in a Schleicher and Schuell Minifold II slot

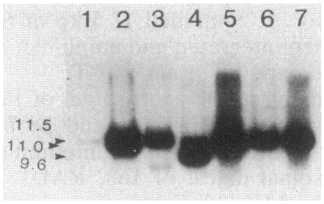

FIG. 3. Hybridization of pCIS7 to the chromosomal DNA of amplified Tc^r strains. The agarose gel from Fig. 1 was transferred to a nitrocellulose filter. Plasmid pCIS7 was radiolabeled by the random priming method and hybridized to the filter as described in Materials and Methods. Lanes: 1, 168; 2, RAD1; 3, RAD6; 4, RAD7; 5, CLI20; 6, CLI22; 7, CLI30. The 11.5-, 11.0-, and 9.6-kb *Eco*RI restriction fragments are indicated; no other *Eco*RI bands hybridized.

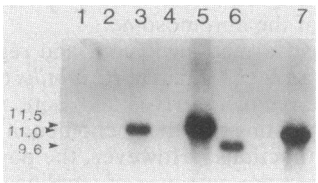

FIG. 4. Hybridization of a gram-positive tetracycline resistance determinant to the chromosomal DNA of amplified Tc^r strains. Chromosomal DNA (5 μg) was digested with *Eco*RI and electrophoresed through a 0.8% agarose gel. ^{32}P-labeled pAMα1Δ1 was used as a probe after transfer of the DNA to a nitrocellulose filter. Lanes: 1, 168; 2, BC92; 3, RAD1; 4, RAD2; 5, RAD6; 6, RAD7; 7, CLI20. The sizes of the *Eco*RI restriction fragments are indicated; no other *Eco*RI bands hybridized.

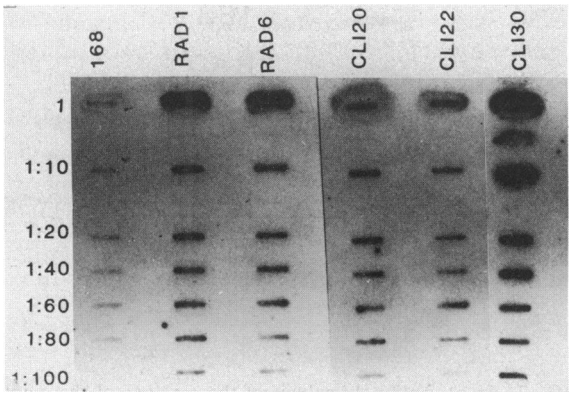

FIG. 5. Quantitative determination of the copy number of AUDs in the Tc^r strains. Slot blot analysis was done by immobilizing serial dilutions of chromosomal DNA starting from 1.0 μg (undiluted) onto Optibind filters. Filters were probed with the radiolabeled 3.1-kb *Pst*I-*Eco*RI restriction fragment of pCIS7. Samples: 1, undiluted (1 μg); 1:10, 0.1 μg; 1:20, 0.05 μg; 1:40, 0.025 μg; 1:60, 0.017 μg; 1:80, 0.0125 μg; 1:100, 0.001 μg.

blotter. The DNA was UV cross-linked onto the filter and probed with a 3.1-kb *Pst*I-*Eco*RI restriction fragment of pCIS7. Comparison of the band intensities of strain 168 and the dilutions of the amplified DNA indicates that CLI30 contains at least 100 copies of the amplified units and that RAD1, RAD6, CLI20, and CLI22 contain approximately 80 to 100 copies of the amplified units (Fig. 5). Hybridization of similar filters with a rRNA probe or a DNA gyrase probe did not disclose any increase in DNA copy number of these genes (data not shown). The high level of AUDs in CLI20 and RAD1 was confirmed by using the pulsed-field gel procedure of Piggot and Curtis (21) for the determination of the number of reiterated sequences in the *B. subtilis* chromosome (data not shown). Quantitation of copy number is difficult, since the photographic emulsion has such a narrow exposure range; however, the two methods taken together clearly indicate that more than 80 copies are present in most amplified strains. When chromosomal DNA was prepared from CLI20 grown in liquid cultures containing 100 μg of tetracycline per ml, no striking difference in the intensity of restriction fragments could be seen in EtBr-stained agarose gels compared with that in chromosomal DNA prepared from the same strain grown in cultures containing 20 μg of tetracycline per ml (data not shown).

Defining the endpoints of the AUDs. The protoplast-induced Tc^r amplifications of RAD1, RAD6, RAD7, CLI20, CLI22, and CLI30, although sharing a common core, possessed different endpoints, as could be seen in EtBr-stained gels. As a prelude to locating the endpoints of the AUDs, 27.2 kb of DNA surrounding pCIS7 was isolated, and a restriction map of the *B. subtilis* 168 chromosome was constructed (Fig. 6). These clones were used as hybridization probes to identify the endpoints of the DNA amplifications. pCIS2, a plasmid isolated by Tn*917* insertional cloning, is contiguous with pCIS7 (13). Phage F3 was isolated by screening a recombinant lambda Charon 30 library with pCIS7. Phage F3 partially overlaps pMS31, a subclone of phage 529 characterized by Sargent and Bennett (25). Phage 529 maps near *purA* and binds the membrane (26). Membrane binding studies with independently isolated DNA from this region have recently confirmed the linear restriction map

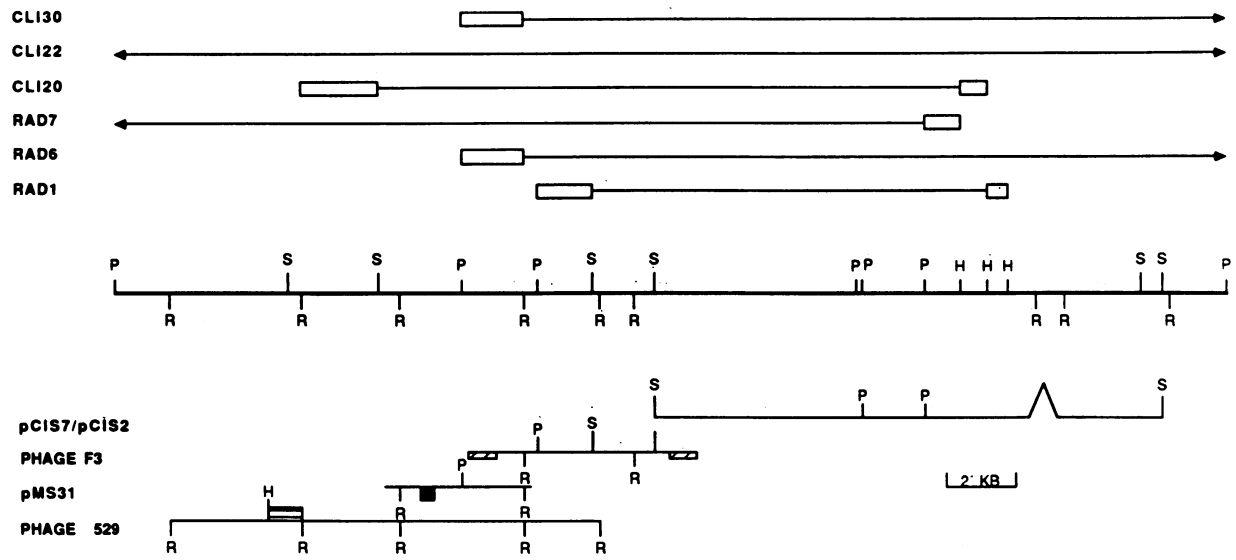

FIG. 6. Mapping of the endpoints of the AUDs of Tc^r regenerated protoplasts of *B. subtilis*. DNA from independent Tc^r regenerants was purified from cultures prepared as described in Materials and Methods. The DNA was digested with *Eco*RI, *Pst*I, *Sph*I, and *Hin*dIII restriction endonucleases and electrophoresed. The gel was transferred to an Optibind filter and probed with α-^{32}P-labeled fragments of the clones. When the next distal fragment was not observed to be amplified, it was assumed that the endpoint of the amplification unit was located within this fragment. The thick line in the middle of the figure indicates the restriction map of *B. subtilis* 168 chromosomal DNA. Clones used as probes are located beneath this thick line, and the strains whose endpoints were mapped are located above the thick line. The slashed boxes of phage F3 indicate uncertainty of the endpoints of cloned DNA. The black box on pMS31 indicates the membrane attachment site characterized by Sargent and Bennett (25). The horizontally lined box on phage 529 indicates a 1-kb *Hin*dIII-*Eco*RI restriction fragment of pCIS7 used as a hybridization probe. The 3.1-kb *Pst*I-*Eco*RI fragment of pCIS7 was used as a probe to map the endpoints of the amplifications of RAD1, RAD7, and CLI20. The open boxes on the amplified strains indicate the locations of the endpoints of the amplifications. Only pertinent restriction sites are shown: P, *Pst*I; H, *Hin*dIII; R, *Eco*RI; S, *Sph*I.

of phage 529 (T. McKenzie and N. Sueoka, personal communication).

Plasmid pMS31 hybridized to 3.6-, 2.8-, and 2.2-kb *Eco*RI restriction fragments (25), 10- and 2.2-kb *Pst*I restriction fragments, and a 6.2-kb *Sph*I restriction fragment in *B. subtilis* 168 chromosomal digests, whereas phage F3 hybridized to 2.2-, 1.1-, and 11.5-kb *Eco*RI restriction fragments, 2.2- and 9.2-kb *Pst*I restriction fragments, and 6.2-, 1.8-, and 14-kb *Sph*I restriction fragments (Fig. 6). Using these clones as hybridization probes and EcoRI, *Pst*I, and *Sph*I chromosomal digests of the amplified strains, we were able to delineate the left-hand endpoint (toward *purA*) of the amplifications in strains RAD1, RAD6, CLI20, and CLI30 (Fig. 6). RAD7 and CLI22 contain amplified DNA that extended beyond our clone collection. Using a 1-kb *Hin*dII-*Eco*RI fragment from within the 3.9-kb *Eco*RI fragment of phage 529 (kindly provided by T. McKenzie), we found that both RAD7 and CLI22 contained amplifications that extended beyond a 10-kb *Pst*I fragment in the chromosome toward the *purA* locus.

Plasmid pCIS7 hybridized to 11.5- and 0.8-kb *Eco*RI restriction fragments, 9.2-, 8.6-, and 1.8-kb *Pst*I restriction fragments, and a 14-kb *Sph*I restriction fragment in *B. subtilis* 168 chromosomal digests, whereas pCIS2 hybridized to 0.8- and 2.8-kb *Eco*RI restriction fragments, a 8.6-kb *Pst*I restriction fragment, and 14- and 0.6-kb *Sph*I restriction fragments in a *B. subtilis* 168 chromosomal digest (Fig. 6). Using these clones as probes and *Eco*RI, *Pst*I, and *Sph*I chromosomal digests of the amplified strains, we localized the right-hand endpoint (toward *gyrB*) of the amplified strains of RAD1, RAD7, and CLI20 to a 3.1-kb *Pst*I-*Eco*RI restriction fragment in pCIS7. Using this purified fragment as a hybridization probe and *Hin*dIII chromosomal restriction digests of the amplified strains, we further localized the endpoints of the DNA amplifications to a 1-kb *Pst*I-*Hin*dIII restriction fragment (for RAD7), a 0.8-kb *Hin*dIII restriction fragment (for CLI20), and a 0.5-kb *Hin*dIII restriction fragment (for RAD1) (Fig. 6). RAD6, CLI20, and CLI22 contained amplified DNA that extended beyond our clone collection. CLI22 is the only strain in which we could not position at least one amplification endpoint; this amplified unit must contain more than 30 kb of DNA.

Transcription of the Tc^r gene in the amplified strains. An important question to address is whether the increase in DNA is accompanied by an increase in the level of transcription in these Tc^r stains. Total RNA was prepared from exponentially growing *B. subtilis* 168, RAD1, RAD6, RAD7, CLI20, CLI22, and CLI30. RNA (15 μg) was electrophoresed on formaldehyde-agarose gels and transferred to Optibind membranes. The blots were probed with various purified restriction fragments of pCIS7. When these blots were probed with a ribosomal DNA gene or the *gyrA* gene, no increase in transcription was seen (data not shown). However, the purified restriction fragments of pCIS7 did indicate increased transcription.

Through sequence analysis of the *tet* gene on pCIS7 (Ives, Ph.D. dissertation, University of North Carolina, Chapel Hill, 1990), a 243-base-pair *Pst*I restriction fragment was shown to be internal to the coding region of the *tet* gene. Against Northern blots, this probe hybridized to an RNA species of approximately 1.4 kb and was amplified in the protoplasted strains (data not shown). This probe was then used against RNA immobilized on nitrocellulose with the Schleicher & Schuell slot blot apparatus (Fig. 7). RAD6, CLI20, CLI22, and CLI30 showed a large increase in transcription, whereas RAD1 showed a smaller increase in transcription when compared with that of 168. RAD7, which contains amplified DNA, did not show an increase in transcription with RNA prepared from late-log-phase cultures.

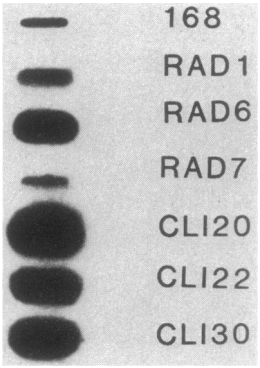

FIG. 7. Transcriptional analysis of the *tet* gene of the amplified strains. RNA was prepared as described in Materials and Methods. RNA (50 μg) from each strain was denatured and spotted onto an Optibind filter. The filter was probed with a radiolabeled 243-base-pair fragment of pCIS7, which is internal to the coding region of the *tet* gene. The filter was hybridized as described in Materials and Methods.

DISCUSSION

The phenomenon of protoplasting *B. subtilis* cells, fusing with PEG, and regenerating in the presence of tetracycline was first described by Wilson and Morgan (34). How the amplifications arise is still not understood, although the frequency is similar to that of random mutation. The addition of PEG appears to be a necessary step, since simple protoplast formation and regeneration alone does not generate amplified DNA. PEG addition causes fusion of bacterial protoplasts (27), perhaps allowing the formation of diploids. These diploids or partial diploids may be necessary for the recombination events that give rise to an initial duplication. In analysis of protoplast fusion products, Sanchez-Rivas et al. (23) showed that the most frequent recombinant protoplasts resulted from crossovers occurring in the vicinity of the origin of replication and terminus. The attachment of DNA to wall and membrane occurs in these two regions of the chromosome and seems essential for correct replication and cell division (4, 35). Perhaps because the DNA-wall association is lost in protoplasts, recombination is favored in these regions.

Although the Tc^r phenotype associated with AUDs is characterized in this paper, it appears that other Tc^r determinants can arise by stressing normally Tc^s *B. subtilis* cells. RAD2 is a Tc^r strain isolated by Wilson and Morgan with the same protoplasting procedure; this isolate does not contain amplified DNA. CLI7 is another Tc^r strain arising from nonfused protoplasts; this isolate is also nonamplified. It is not known whether these two Tc^r isolates are related to IS176, a Tc^r mutant characterized by Williams and Smith (33). This strain was isolated after ethyl methanesulfonate mutagenesis, does not contain amplified DNA (Ives and Bott, unpublished observations), and was postulated to be a membrane alteration, since no alterations of ribosomal proteins were seen on two-dimensional gels. The Tc^r determinant was inducible and located through transductional mapping near the origin of replication. Through transformational mapping experiments, we believe that this locus is not linked to the DNA encoded on pCIS7 and therefore does not

involve the same gene as the amplified Tc^r strains (Ives and Bott, unpublished data). Furthermore, RAD2, CLI7, and IS176 do not show an increase in transcription of the *tet* gene of pCIS7, suggesting that these isolates do not encode up-promoter mutations of the same gene (Ives and Bott, unpublished results). These Tc^r phenotypes must arise from another gene(s) located in this area of the chromosome. Along with the ribosomal protein alteration of the *tetA* mutation characterized by Williams and Smith (33), there now appear to be at least two other Tc^r determinants located on the *B. subtilis* chromosome. The amplified strains confer Tc^r only when a presumably wild-type gene is amplified, whereas IS176 (*tet*B), RAD2, and CLI7 are other mutations that confer a high level of inducible Tc^r and appear to be membrane alterations. It is unknown whether these later three Tc^r determinants are related.

Wilson and Morgan recovered Tc^r strains whose chromosomal DNA contained amplified restriction fragments when viewed on EtBr-stained gels (34). Through analysis of the restriction fragments seen on EtBr-stained gels, they suggested that the AUD differed in each isolate but contained a common core of amplified DNA. We have repeated and extended these experiments and show that the AUDs contain sequences cloned on pCIS7. Plasmid pCIS7 contains 11.5 kb of *B. subtilis* DNA isolated from Tc^s *B. subtilis* 168 which, upon integration and amplification in the chromosome, confers Tc^r (13). Therefore, it appears that these two Tc^r phenomena are related; namely, that amplification of a wild-type gene confers Tc^r in *B. subtilis*. Definitive evidence for this still remains to be collected. Cloning the *tet* gene from one of the amplified strains, sequencing it, and comparing it with the *tet* gene on pCIS7 would show that the two determinants are identical and that there is not a secondary mutation elsewhere on the amplified strains conferring Tc^r.

Previous gene amplification studies in *B. subtilis* have primarily involved the integration of plasmid sequences into the chromosome and the subsequent amplification of these sequences by increasing the concentration of antibiotic in the growth medium. Young (37) demonstrated a 15-fold increase in copy number of integrated plasmid sequences, Albertini and Galizzi (1) demonstrated a 10-fold increase in copy number, and Janniere et al. (14), using various plasmid constructs, demonstrated between 5 and 50 copies of integrated plasmid sequences. In the only other endogenous gene amplification characterized in *B. subtilis*, the *amyE-tmrB* region was replicated 5 to 10 times in strains transformed with the AUD (12).

The copy number of the AUD associated with Tc^r is approximately 80 to 100 in the protoplasted regenerants when compared with nonamplified Tc^s *B. subtilis* 168 *trpC2*. This number of AUDs is quite high in comparison with those in previous studies. However, we confirmed these results for RAD1 and CLI20 through contour-clamped homogenous electric field analysis (Ives and Bott, unpublished observations). Possibly due to the high copy number of AUD, the Tc^r strains have an altered metabolism. The strains grow poorly in liquid culture; an improvement in growth is seen when the strains are grown in SMMP, the protoplasting medium. This hypotonic medium (with 0.5 M sucrose) may serve as a stabilizing agent. The organization of the membrane-DNA complex may be altered, since so many copies of DNA are located in this region, resulting in the inability to correctly replicate and segregate the chromosomal DNA.

The increase in transcription seen in the amplified Tc^r strains corresponds to the increased gene dosage. It is unclear why RAD7, which contains amplified DNA, does not show an increase in transcription. It may be that in this strain the Tc^r determinant was not expressed at the time the RNA was harvested. However, we cannot rule out the possibility that a second Tc^r determinant exists that confers Tc^r upon RAD7. We have not conducted a temporal study of gene expression to determine when the gene is maximally expressed.

In gram-positive bacteria, five classes of Tc^r determinants have been distinguished on the basis of DNA and sequence homology: *tetK*, *tetL*, *tetM*, *tetN*, and *tetO* (15). There are three known mechanisms of resistance to tetracyclines. For the two major mechanisms of resistance, the antibiotic is not altered. The most common is an energy-dependent efflux of tetracycline from the cell and is mediated by the related classes L and K in gram-positive bacteria. Classes M, N, and O specify a cytoplasmic protein that mediates a different mechanism of resistance. The protein, which has a partial sequence similarity to ribosome elongation factors, protects ribosomes from inhibition by tetracycline. The Tc^r determinant of CLI20 was tested for its ability to inactivate tetracycline by the procedure of Speer and Salyers (31); tetracycline was not inactivated. Sequence analysis of the *tet* gene on pCIS7 indicates that it is of the class L type (Ives, Ph.D. dissertation). It is not clear why or how a silent *tet* gene became integrated into the *B. subtilis* chromosome or why it mediates Tc^r only when present in multiple copies.

The work described here raises a myriad of questions. What is the involvement of the *recE* system in the formation of the duplications and subsequent amplifications? Although we did not detect amplification events in a *recE* strain, we cannot say whether the *recE* dependence is due only to subsequent amplification after duplication or whether it results from reduced frequency of an initial duplication. The separation of each stage of the amplification process is required to evaluate the role of the *recE* system.

It is unknown whether short homologous sequences are required for generating the large tandem amplifications. We have recovered AUDs that range from 11 to greater than 30 kb; each AUD has unique endpoints. It seems unlikely that most of the original duplications occurred at a repeated sequence as large as 12 base pairs (as in *ampC* [9]) or 14 base pairs (as in the *amyE-tmrB* duplication of *B. subtilis* [12]). It is more likely that the duplications do not involve repeated sequences at all. Subcloning and sequencing the junction fragments of the amplifications would answer this question.

Is there a special mechanism for generating large duplications in bacteria? Anderson and Roth have suggested that many duplications may confer a selective advantage (2). Perhaps these amplifications are inducible under growth-limiting conditions such as those resulting from selective pressures exerted in the protoplast formation-fusion regimen described above. It is unknown whether the application of other metabolic stresses, such as heat shock or chemical mutagenesis, will induce these amplifications.

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