JOURNAL OF BACTERIOLOGY, Jan. 1979, p. 635-643
0021-9193/79/01-0635/09$02.00/0

Vol. 137, No. 1

Plasmid Copy Number Control: Isolation and Characterization of High-Copy-Number Mutants of Plasmid pE194

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Received for publication 14 October 1978

A plasmid, pE194, obtained from *Staphylococcus aureus* confers resistance to macrolide, lincosamide, and streptogramin type B ("MLS") antibiotics. For full expression, the resistance phenotype requires a period of induction by subinhibitory concentrations of erythromycin. A copy number in the range of 10 to 25 copies per cell is maintained during cultivation at 32°C. It is possible to transfer pE194 to *Bacillus subtilis* by transformation. In *B. subtilis*, the plasmid is maintained at a copy number of approximately 10 per cell at 37°C, and resistance is inducible. Tylosin, a macrolide antibiotic which resembles erythromycin structurally and to which erythromycin induces resistance, lacks inducing activity. Two types of plasmid mutants were obtained and characterized after selection on medium containing 10 μg of tylosin per ml. One mutant class appeared to express resistance constitutively and maintained a copy number indistinguishable from that of the parent plasmid. The other mutant type had a 5- to 10-fold-elevated plasmid copy number (i.e., 50 to 100 copies per cell) and expressed resistance inducibly. Both classes of tylosin-resistant mutants were shown to be due to alterations in the plasmid and not to modifications of the host genome.

A plasmid, pE194 (molecular mass, 2.3 ± 0.1 megadaltons), was isolated from cells of an erythromycin-resistant strain of *Staphylococcus aureus* described originally by Iordanescu (7, 8). The erythromycin resistance phenotype is indistinguishable from that originally discovered by Chabbert (1), and the biochemical mechanism of this type of resistance has been determined to be specific N^6-dimethylation of adenine in 23S rRNA, as a consequence of which the affinity between erythromycin and the ribosome is markedly reduced (9, 10, 19). Plasmid pE194 specifies co-resistance to macrolide, lincosamide, and streptogramin type B ("MLS") antibiotics and, upon transfer to *Bacillus subtilis* by DNA-mediated transformation, the inducible resistant phenotype found is indistinguishable from that of the original *S. aureus* strain from which pE194 was isolated. In the present studies, the consequences of establishing this *S. aureus* plasmid in *B. subtilis* are described with special emphasis on the regulation of plasmid DNA synthesis in the new host background.

MATERIALS AND METHODS

Strains. *B. subtilis* BD170 (*trpC2 thr-5*) was used as the transformable recipient. *S. aureus* RN2442 carrying pE194 was obtained from R. P. Novick.

Gel electrophoresis. Agarose (0.8%; LE grade; Seakem Marine Colloids Inc., Portland, Maine) was used with tris(hydroxymethyl)aminomethane (Tris)-borate buffer (5). DNA samples were fractionated by electrophoresis in agarose slabs (16 by 16 cm, 80 V, 50 mA, 18 h). Gels were stained for 30 min with ethidium bromide (EtBr; 1 μg/ml), destained in water for 30 to 60 min, and photographed through combined 23A and 8 Kodak filters on Polaroid 667 film, using long-wavelength UV light.

To prepare crude lystates for electrophoretic analysis, overnight cultures (1 ml) of *B. subtilis* were grown at 32°C in VY medium (4), washed in TES (50 mM NaCl, 5 mM disodium ethylenediaminetetraacetate, 30 mM Tris; pH 7.5), suspended in 1 ml of TES containing lysozyme (0.5 mg/ml), ribonuclease A (50 μg/ml), and ribonuclease T1 (0.5 μg/ml), and incubated at 37°C for 30 min. An equal volume of predigested Pronase (1 mg/ml) in 1.6% Sarkosyl was added, and incubation continued for 2 h. Samples were sheared by mechanical agitation on a Vortex mixer for 30 s before electrophoresis. For determination of radioactivity in agarose gel slices, fluorescent bands were excised, dissolved in 0.2 ml of 30% H_2O_2–NH_4OH (19:1, vol/vol), and allowed to stand for 8 h at 50°C. Four milliliters of Liquiscint counting fluid (National Diagnostics Inc.) was added, and the samples were vigorously mixed before counting by liquid scintillation. Acrylamide gel electrophoresis was performed similarly, using 5% acrylamide–0.25% bisacrylamide gels.

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Transformation. Preparation of competent cells followed by transformation, using covalently closed circular plasmid DNA, was carried out as described previously (3, 6).

CsCl-EtBr centrifugation. To 6 ml of TES buffer containing the DNA, 7.5 g of CsCl and 2 ml of EtBr solution (1 mg/ml) were added, followed by centrifugation in a Ti 50 rotor at 42,000 rpm for 40 h. The covalently closed circular DNA was isolated and purified by dye-buoyant CsCl density gradient centrifugation (12). Analytical density gradient centrifugation in neutral CsCl solution, followed by an optical scan of the photographic record, was performed as described previously (17).

Restriction endonucleases. Enzymes were purchased from New England Biolabs or Bethesda Research Laboratories. The reaction conditions used have been described previously (6). The enzymes *Hae*III, *Hha*I, and *Alu*I used in some of the studies were prepared by published procedures (22, 15, 14 and 23, respectively).

Analysis of methylated adenine. Analysis of methylated adenine in rRNA was performed by two methods. For labeling with ^{14}C, [*methyl*-^{14}C]methionine was included in the growth medium. *methyl*-^{14}C-labeled 23S RNA was isolated and then digested with T1 ribonuclease, and the resultant digest was fractionated by two-dimensional electrophoresis (16). This method permits examination of the total 23S rRNA to ascertain the degree of altered methylation.

For labeling with [^{3}H]adenine, cells were incubated in a medium containing [8-^{3}H]adenine, followed by isolation of 23S rRNA, depurination with acid and heat, fractionation of adenine plus methylated adenine as a group free from pyrimidines and guanine(s) by column chromatography on Dowex 50, and fractionation of the resultant adenine group into adenine, N^6-monomethyl-adenine (m^{6}A), and N^6, N^6-dimethyl adenine (m_2^6A), as described previously (10). This method provides a measurement of the relative number of methylated adenine residues in 23S rRNA.

Hybridization studies. The concentrations of pE194 sequences in *B. subtilis* and *S. aureus* DNA preparations were determined with a ^{32}P-labeled complementary RNA probe prepared by transcription, using *E. coli* RNA polymerase, [α-^{32}P]ATP, and the other three ribonucleotides in unlabeled form. The incubation medium for preparation of the complementary RNA probe contained (total volume, 0.1 ml): 40 mM Tris-hydrochloride (pH 7.9); 4 mM $MgCl_2$; 150 mM KCl; 12 mM 2-mercaptoethanol; 1 mM $MnCl_2$; μM [^{32}P]ATP (specific activity, 250 Ci/mmol); 0.5 mM each unlabeled GTP, CTP, and UTP; 1 μg of pE194 form I DNA, and 5 U of *E. coli* RNA polymerase.

After incubation for 60 min at 37°C, 10 μl of 10% sodium dodecyl sulfate solution was added to the reaction mixture, and unincorporated substrates were removed by gel filtration over Sephadex G-50 equilibrated with water. The calculated specific activity of the ^{32}P-labeled complementary RNA probe was 10^8 cpm/μg. The ^{32}P-labeled complementary RNA was hybridized to *B. subtilis* or *S. aureus* DNA preparations affixed to nitrocellulose (Millipore Corp.) filters. The conditions used for preparation of the nitrocellulose filters and the hybridization reactions were as described by Denhardt (2). For preparation of the nitrocellulose filters, total cellular DNA (50 μg) was digested with endonuclease *Hae*III to convert pE194 form I DNA to open linear form. The volume of the incubation mixture was adjusted to 10 ml with water, boiled for 5 min, and chilled rapidly on ice, and the salt concentration was adjusted to 0.9 M NaCl and 0.9 M sodium citrate (6 × SSC). The mixture was adsorbed to a nitrocellulose filter (47 mm in diameter), dried, and baked as described previously (2). From each filter, approximately 20 disks (5 mm in diameter), each containing approximately 2 μg of DNA, were obtained with a paper punch.

RESULTS

Phenotypic expression of MLS antibiotic resistance in *B. subtilis*. Covalently closed circular pE194 DNA prepared from *S. aureus* RN2442 was used to transform sensitive *B. subtilis* BD170, followed by selection for resistance on solid medium containing 5 μg of erythromycin per ml. One *B. subtilis* erythromycin-resistant transformant selected for further study was tested with disks containing MLS antibiotics to determine the extent of similarity between the erythromycin-resistant strains of *S. aureus* and *B. subtilis*. The results in Fig. 1 show the pattern of resistance characteristic of induction on solid medium described and analyzed in detail in earlier studies (19–21). The asymmetric inhibition zone can be attributed to in situ induction by (initially) subinhibitory concentrations around the disk which contains erythromycin.

The biochemical alteration in resistant *S. aureus* cells responsible for the resistance phenotype has been identified as a specific N^6-dimethylation of adenine in 23S rRNA (9, 10). Similar results were found when 23S RNA from *B. subtilis* transformants was tested. First, an examination of the fractionated T1 ribonuclease digest of in vivo *methyl*-^{14}C-labeled 23S rRNA revealed the presence of a single component which was absent from the sensitive strain (Fig. 2). A similar finding for resistant cells of *S. aureus* was reported previously (9). To determine whether adenine was dimethylated in the resistant transformant, *B. subtilis* cells were grown with [8-^{3}H]adenine, and the relative amounts of m_2^6A and adenine were determined after purification of 23S rRNA. Results of the analysis presented in Fig. 3 show the absence of m_2^6A and m^{6}A in the sensitive and uninduced *B. subtilis* strains, whereas in the induced strain approximately 0.2% of the radioactivity present in the total sample comigrated as a distinct peak together with m_2^6A. On the basis of the foregoing determinations, we conclude that the genetic information encoded by pE194 is expressed similarly in both the *S. aureus* strain from which

the plasmid was isolated and the new *B. subtilis* transformant.

Physical state of pE194 in *B. subtilis* and *S. aureus*. Both *S. aureus*(pE194) and *B. subtilis*(pE194) were found to contain covalently closed circular DNA as judged by EtBr-CsCl centrifugation, and digestion of this purified plasmid fraction with several restriction endonucleases failed to show any differences. At the level of resolution afforded by these techniques, pE194 can be demonstrated in both the parent *S. aureus* and recipient *B. subtilis* strains.

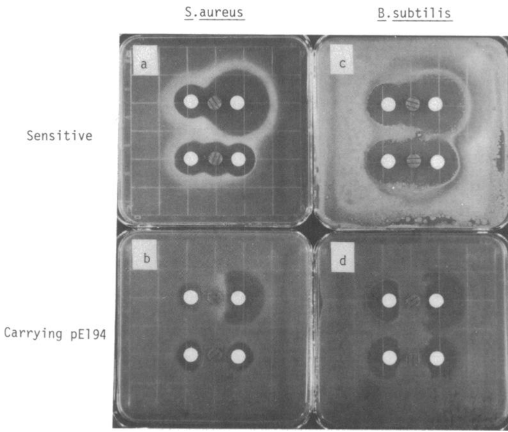


FIG. 1. *Erythromycin-inducible resistance in S. aureus and B. subtilis into which plasmid pE194 had been introduced. Induction on solid medium in situ was demonstrated with the use of erythromycin disks apposed to disks containing other MLS antibiotics. The test panel contained (in addition to erythromycin [15 μg per disk]) clindamycin, carbomycin, tylosin, and vernamycin B alpha (20 μg per disk each) as test MLS antibiotics. The relative locations of the disks on the plate are: (top row in each plate, from left to right) carbomycin, erythromycin, and clindamycin; (bottom row in each plate, from left to right) tylosin, erythromycin, and vernamycin B alpha. The truncated zones of inhibition seen in b and d are due to induction of resistance by erythromycin in situ to the MLS antibiotic contained in the respective test disks.*

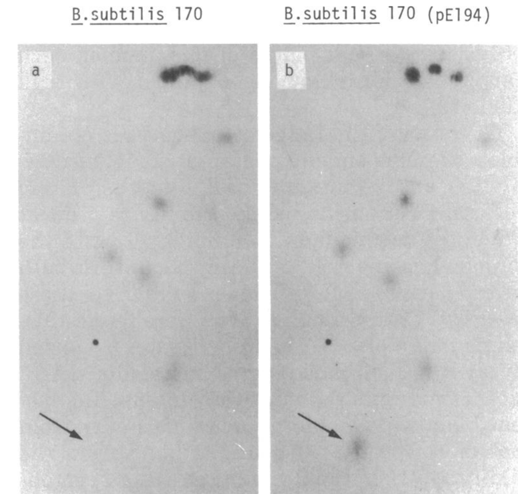


FIG. 2. *Altered methylation of 23S rRNA from erythromycin-resistant B. subtilis transformed with pE194. Growing B. subtilis cells were incubated with [methyl-^{14}C]methionine. 23S rRNA was prepared and digested with T1 ribonuclease, and the resultant digest was fractionated by two-dimensional separation on cellulose acetate and diethylaminoethyl-cellulose by the method of Sanger et al. (16); autoradiography was then performed.*

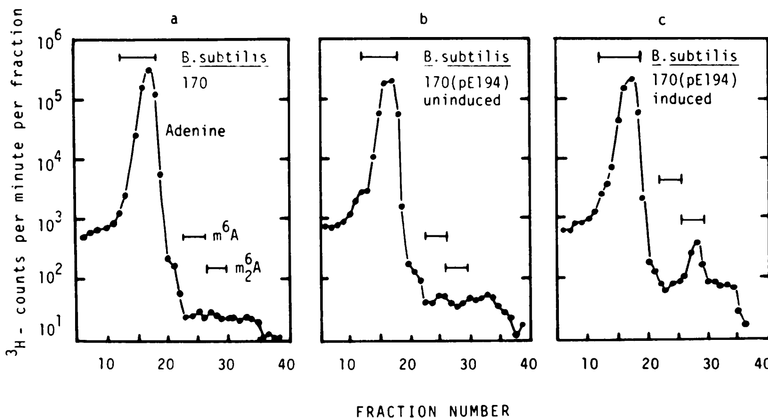


FIG. 3. *m^6_2A in 23S rRNA from erythromycin-resistant B. subtilis pE194 transformants. 23S rRNA in intact cells was labeled by growth in [^{3}H]adenine. m^6_2A was demonstrated and estimated quantitatively after purification of adenine plus methylated adenine and separation of adenine from m^6_2A by paper chromatography with isopropanol-concentrated ammonia-water (85:1.3:15, vol/vol/vol) as the solvent. The number of counts in m^6_2A relative to those in adenine provides a measure of the number of m^6_2A residues per 23S rRNA. This amounts to approximately 0.2%, or two residues per 23S rRNA.*

Constitutively resistant mutants have been found useful in previous studies of inducible erythromycin resistance (21). The rationale for the isolation procedure for such mutants is as follows. Whereas erythromycin can act as an inducer at subinhibitory concentrations, other MLS antibiotics normally cannot (Fig. 1). To survive, one class of such mutants must have m^6_2A-containing 23S rRNA at the time of plating on selective medium. Among survivors of inducible *S. aureus* cells plated in this manner, such constitutive forms can be obtained with high efficiency.

To compare the range of phenotypes obtainable, 20 independent mutants of *S. aureus*(pE194) and 20 mutants of *B. subtilis*(pE194) were selected on the basis of ability to grow at 37°C on solid medium containing 10 μg of the noninducing macrolide tylosin per ml. In both series, patterns of resistance similar to those described earlier for *S. aureus* were found (21). Examination of DNA in crude lysates extracted revealed two mutant types. Nineteen of the *B. subtilis* mutants contained an intense fluorescent band with mobility indistinguishable from that of the less-intense pE194 band from the wild-type strain. Similar mutants have been obtained by using clindamycin or carbomycin for selection (B. Weisblum, unpublished data). The plasmid profile of the remaining *B. subtilis* mutant was indistinguishable from that of the inducible parent. Figure 4 shows typical agarose gel electrophoresis patterns of *B. subtilis* strains carrying pE194 and one each of the two varieties of tylosin resistance plasmids. In similar gels prepared *S. aureus*(pE194) and its tylosin-resistant derivatives, only a faint plasmid band was visible, similar to that seen in band c of Fig. 4. A further test was performed by digesting with restriction endonucleases, covalently closed circular pE194 DNA prepared from *S. aureus* and total DNA from *B. subtilis tyl-6* (Fig. 5). Patterns obtained from digestion of the plasmid material present stood out strongly against a background of chromosomal DNA fluorescence, indicative of a high copy number in the *B. subtilis* tylosin-resistant strain used. From these observations, we infer that the intense band seen in the agarose gels corresponds to a high copy number of pE194 in 19 of 20 of the tylosin-resistant mutants of *B. subtilis*. For further study, one typical *S. aureus* mutant (*tyl-1*) and one typical *B. subtilis* mutant showing an apparently increased copy number (*tyl-6*) were chosen as representative strains for detailed comparative investigation.

To determine whether the tylosin resistance phenotype and the altered copy number were dependent on changes in the host cell or in the plasmid, covalently closed circular DNA purified by CsCl-EtBr centrifugation from *S. aureus*(pE194 *tyl-1*) and from *B. subtilis*(pE194 *tyl-6*) was used to transform *B. subtilis* BD170 to erythromycin resistance. Of 200 erythromycin-resistant colonies derived from each transformation, all were found to be tylosin resistant

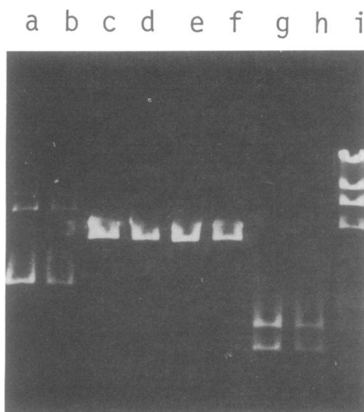


FIG. 4. *Restriction endonuclease digestion patterns of pE194 DNA isolated from S. aureus (a, c, e, g) and B. subtilis (b, d, f, h). (a and b) Undigested DNA. Results of digestion with XbaI (c and d), HaeIII (e and f), and HpaII (g and h). Lane i contains a HindIII digest of bacteriophage lambda.*

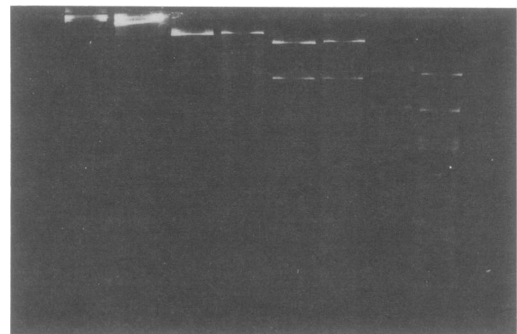


FIG. 5. *Comparative analysis of pE194 from S. aureus and B. subtilis. Total cellular DNA from B. subtilis(pE194 tyl-6) (lanes b, d, f, and h) and, for comparison, pE194 prepared as covalently closed circular DNA from S. aureus RN2442 (lanes a, c, e, and g) were digested with restriction endonucleases HaeIII, HhaI, and AluI, respectively, followed by electrophoretic fractionation of the resultant digest in 5% polyacrylamide gel. The high pE194 copy number in B. subtilis(pE194 tyl-6) can be inferred from the fluorescence intensity of plasmid bands relative to chromosomal DNA.*

when the *tyl-1* and *tyl-6* donors were used. None of those derived from pE194 was tylosin resistant. Lysates obtained from one transformant derived from each donor were examined by agarose gel electrophoresis. Only the transformant derived from pE194 *tyl-6* showed an intense fluorescent band corresponding to the mobility of pE194. Plasmid bands from all three transformants had indistinguishable mobilities. Thus, expression of tylosin resistance in both the *tyl-1* and *tyl-6* mutants is attributable to an alteration of the plasmid rather than of the host DNA. The plasmid-mediated tylosin resistance of the strains carrying pE194 *tyl-6* is correlated with increased copy number. The high copy number of pE194 in the *B. subtilis tyl-6* mutant could also be visualized directly if total DNA extracted from the *tyl-6* strain was digested with restriction endonucleases *Hae*III, *Hha*I, and *Alu*I. Our results are shown in Fig. 6. The fragments obtained by digestion of total *tyl-6* DNA contained a high multiplicity of fragments with mobilities

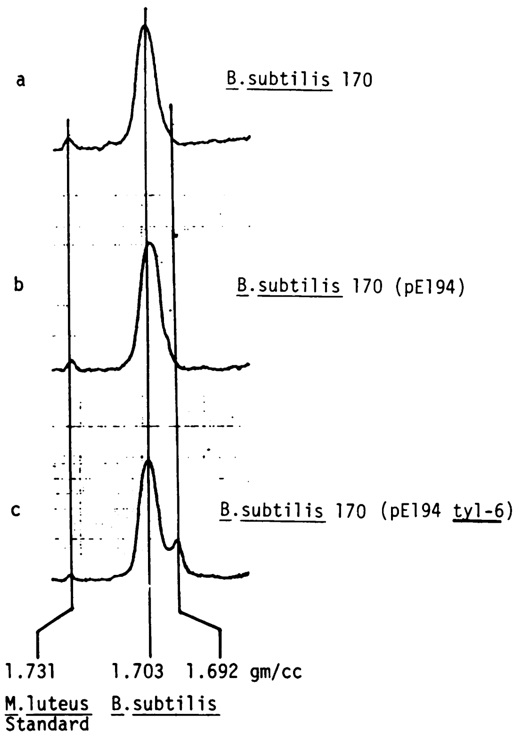


FIG. 6. *Analysis of total DNA extracted from B. subtilis BD 170(pE194) and (pE194 tyl-6) by density gradient centrifugation in CsCl solution. Total DNA extracted from the three named strains was spun to equilibrium in CsCl solution. Buoyant densities of the constituents and of the Micrococcus luteus internal reference standard are annotated directly on the figure.*

indistinguishable from those obtained by digestion of pE194 (form I DNA) purified from *S. aureus*, included for comparison. From visual inspection, we infer that pE194 is present at a high copy number and that no discernible rearrangements occurred which would produce an altered restriction pattern.

Estimation of pE194 copy number. pE194, with a buoyant density of 1.692g/cm^3, is easily resolvable from *B. subtilis* chromosomal DNA, which has a buoyant density of 1.703 g/cm^3 in CsCl. DNA extracted, respectively, from *B. subtilis*(pE194) and *B. subtilis*(pE194 *tyl-6*) grown at 37°C was banded to equilibrium by density gradient centrifugation in CsCl solution (Fig. 6). The DNA sample from the *tyl-6* mutant showed a main band at 1.703 g/cm^3, which was close to 1.702 reported for *B. subtilis* DNA (17), and a distinct satellite banding at 1.692 g/cm^3, which was undetectable in the photometric tracings of the DNA sample from the plasmid-free strain and was possibly seen as a minor shoulder in *B. subtilis*(pE194). We infer that *tyl-6* contains an elevated pE194 copy number, amounting to approximately 10% of the total cellular DNA, or 2.5×10^8 daltons. For a plasmid with a molecular weight of approximately 2.4×10^6, this implies the presence of about 100 copies of pE194 *tyl-6* per chromosome.

The satellite buoyant density, 1.692 g/cm^3, corresponds to that of pE194 extracted directly from erythromycin-resistant *S. aureus*, as well as to the density reported for total bulk chromosomal DNA from *S. aureus* (17). Since pE194 does not band as a distinct satellite in DNA samples extracted from *S. aureus*, further quantitative studies were performed by hybridizing ^{32}P-labeled complementary RNA probes prepared from form I pE194 (obtained by dye-buoyant CsCl density gradient centrifugation) to total DNA from various sources. To estimate the copy number of pE194 in the parental and the *tyl-6* strains, as well as in *S. aureus* strains carrying pE194, equal amounts of total cellular DNA from each source were denatured and fixed to nitrocellulose (Millipore Corp.) membrane filters. The extent of hybridization was then determined as a function of increasing concentrations of pE194 complementary RNA probe in the hybridization mixture (Fig. 7). For the *B. subtilis* strains, the *tyl-6* mutant contains 10 to 100 times as much pE194 complementary sequences as does *B. subtilis*(pE194). Hybridization to DNA from *S. aureus*(pE194) and DNA from *S. aureus*(pE194 *tyl-1*) shows that the *tyl-1* copy number is the same in both strains. This method appears to underestimate consistently the level of pE194 in *B. subtilis*, for reasons which are not yet clear.

Lysates of *B. subtilis*(pE194), *B. subtilis*-(pE194 *tyl-1*), and *B. subtilis*(pE194 *tyl-6*) which had been uniformly labeled with [*methyl*-^{3}H]-thymidine at 32°C were subjected to CsCl-EtBr centrifugation. Comparison of radioactivity present as covalently closed circular DNA and in the main chromosomal DNA band yielded the following estimates of copy number: 10, pE194; 10, pE194 *tyl-1*; and 65, pE194 *tyl-6*. Similarly, [*methyl*-^{3}H]thymidine-labeled lysates from cells grown at 32°C were sheared, and total DNA was examined by agarose gel electrophoresis. Plasmid (covalently closed circular and open circular) bands and chromosome bands were excised and radioactivity was determined. Copy numbers determined in this way were: 17, pE194; 18, pE194 *tyl-1*; and 84, pE194 *tyl-6*. It is apparent from these studies that the *tyl-6* mutant is altered in copy control and that the *tyl-1* mutant appears indistinguishable in this regard from the parent plasmid.

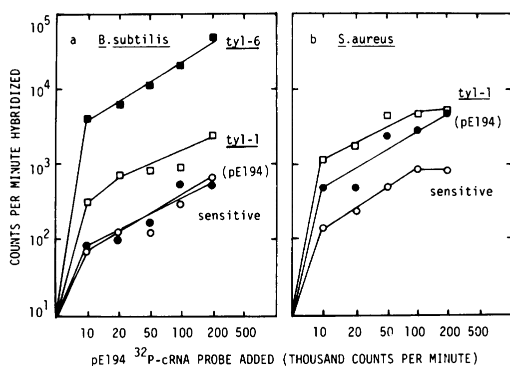


FIG. 7. *Comparison of pE194 complementary sequences in B. subtilis and S. aureus resistant to erythromycin and tylosin. Hybridization of pE194 complementary RNA (cRNA) to membrane filters carrying 2 μg of total cellular DNA purified from the strains indicated was measured as a function of increasing concentration of probe added. The extent of hybridization to DNA from both the pE194-carrying strains and the tyl-1 mutants of B. subtilis and S. aureus fall within one order of magnitude of each other, whereas the extent of hybridization to DNA from tyl-6 (measured only in B. subtilis) was at least one order of magnitude higher.*

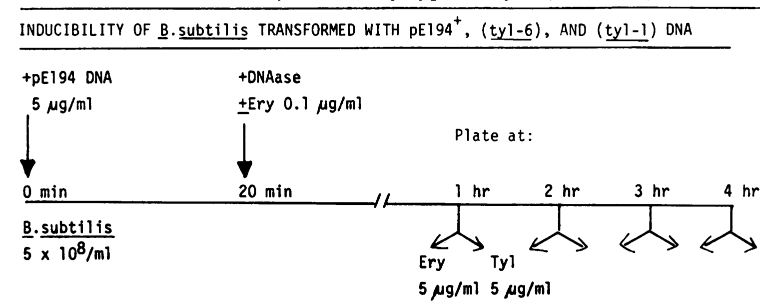
Inducibility of erythromycin resistance in *B. subtilis*(pE194 *tyl-6*). The causal relationship between high plasmid copy number seen in *B. subtilis tyl-6* and the resultant tylosin resistance phenotype is not apparent directly from the work described above. Especially pertinent is the question whether the information for rRNA methylation and the resistance phenotype is still inducible in *B. subtilis*(pE194 *tyl-6*) despite the high copy number. An experiment designed to provide a definitive answer (outlined in Table 2) was performed as follows. Plasmid DNA isolated from the two tylosin resistance plasmids pE194 *tyl-1* and pE194 *tyl-6*, as well as from the wild-type plasmid pE194, was used to transform the sensitive recipient strain of *B. subtilis*. All three covalently closed circular plasmid DNA preparations had been isolated from *B. subtilis* by CsCl-EtBr centrifugation. Erythromycin-resistant and tylosin-resistant transformants were selected after plating on agar with and without inducing levels (0.1 μg/ml) of eryth-

TABLE 1. *Estimation of pE194 copy number in B. subtilis*

Plasmid DNA[a] from:	Total circular DNA (cpm)	Chromosomal DNA (cpm)	Ratio	pE194 copy number
pE194	361	22,700	0.016	17
pE194 *tyl-1*	443	26,050	0.017	18
pE194 *tyl-6*	1,752	21,700	0.081	84

[a] *B. subtilis* strains carrying each of the three plasmids were used, and lysates were prepared and analyzed as described in the text. DNA bands were stained with EtBr and excised from the gel. Radioactivity associated with the various bands was counted. To calculate copy number, the molecular weights of pE194 and the *B. subtilis* chromosome were taken as 2.4×10^6 and 2.5×10^9, respectively.

TABLE 2. *Test of inducibility of pE194 tyl-6 (schematic)*[a]



[a] DNAase, Deoxyribonuclease; Ery, erythromycin; Tyl, tylosin.

romycin. Plates were incubated for various times at 37°C to allow expression with or without induction, followed by a challenge with the selecting antibiotic by overlay. Figure 8 shows the results. Few tylosin-resistant transformants were detected using pE194 DNA (Fig. 8) without induction, whereas the number of erythromycin-resistant transformants gradually increased to about 4×10^4/ml. The high initial level (2×10^4 transformants per ml) presumably represents induction on the plates even in the presence of a "selecting" concentration of erythromycin. With induction before challenge, equivalent numbers of both tylosin- and erythromycin-resistant transformants were obtained, and the numbers rose rapidly to about 2×10^5/ml. When pE194 *tyl-6* DNA was used (Fig. 8b), both erythromycin-resistant and tylosin-resistant transformants were obtained without induction. The latter increased 100-fold during the first 90 min of delay. If challenge is preceded by a period of induction, the number of both erythromycin-resistant and tylosin-resistant transformants increased markedly, and again a pronounced initial rise in the yield of tylosin-resistant colonies is evident. We conclude that in the pE194 *tyl-6* mutant resistance to both erythromycin and tylosin is inducible. It is likely that the high copy number of this plasmid allows sufficient "escape" synthesis of rRNA methylating activity to confer tylosin resistance without induction. The appearance of tylosin-resistant transformants probably reflects this escape synthesis together with the rapid replication of the plasmid to a high copy number.

pE194 *tyl-1* DNA was used in a similar experiment (Fig. 8c). Prior induction produced only a small increase in the number of tylosin- or erythromycin-resistant colonies. The kinetics of appearance and the number of these transformants are very similar to those seen for tylosin- and erythromycin-resistant transformants with induction using pE194 DNA. This pattern would be expected for a low-copy-number plasmid which specifies constitutive resistance to MLS antibiotics. We cannot rule out the possibility that pE194 *tyl-1* may be partially constitutive. Whatever the detailed explanation for the kinetic behavior shown in Fig. 8, the pE194 parent plasmid and the pE194 *tyl-6* mutant derivative confer inducible MLS antibiotic resistance, whereas the *tyl-1* derivative confers constitutive

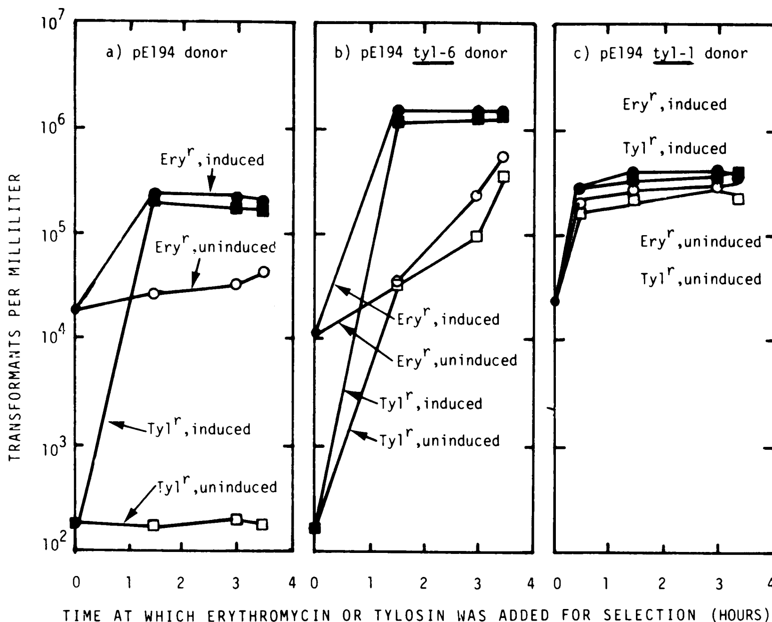


FIG. 8. *Expression of erythromycin and tylosin resistance after transformation of B. subtilis with (a) pE194, (b) pE194 tyl-6, and (c) pE194 tyl-1 DNA. B. subtilis BD170 competent cells were incubated for 20 min at 37°C with 5 µg of covalently closed circular DNA per ml. Deoxyribonuclease (50 µg/ml) was added to terminate uptake, and the transformed cells were plated at 37°C on medium either with or without an inducing concentration (0.1 µg) of erythromycin per milliliter. At the indicated times, the plates were overlaid with agar containing selecting concentrations of erythromycin or tylosin. The number of transformants per milliliter of transformed culture is shown for erythromycin selection with and without induction and for tylosin selection with and without induction as indicated directly in the labels.*

resistance. Thus, our data clearly indicate that at least two distinct genetic mechanisms of tylosin resistance can be established by mutations of pE194. The first, exemplified by pE194 *tyl-6*, results in a high copy number and may result from an alteration in initiation of replication. We propose to refer to these mutations as "*cop*" (e.g., pE194 *cop-6*) in accordance with a published recommendation (11). The second mechanism, exemplified by pE194 *tyl-1*, results in constitutive expression of the resistance phenotype and may result from altered control of transcription.

DISCUSSION

By use of the selection methods described above an unlimited number of mutants with altered copy number control (*cop*) can be obtained. We expect that these mutants will be useful in studies of the control of DNA replication. Progress in understanding the complexity of transcriptional control will likewise benefit from examination of constitutive mutants.

The relative numbers of *cop* and constitutive mutants selected with tylosin in *B. subtilis*(pE194) are a function of the selection temperature (A. G. Shivakumar, J. Hahn, and D. Dubnau, unpublished data). The steady-state copy number of pE194 can be lowered by growth at temperatures greater than 32°C. The probability of plasmid loss by segregation is a function of both growth temperature and the copy number; high-copy mutants have a lower probability of plasmid loss at elevated temperatures, and their isolation is thus favored by selection on tylosin plates at 37°C compared with that at 32°C. pE194 may be more stably maintained at 37°C under these conditions in *S. aureus* than in *B. subtilis*. This model is supported by the finding of tylosin-resistant *B. subtilis* mutants with constitutive R-determinant gene expression when selection is performed at 30°C, a temperature which favors stable maintenance of pE194 in *B. subtilis*. The complementary experiment, selection of *cop* mutants of *S. aureus* by cultivation at higher temperatures (e.g., 40 to 42°C), would provide additional support for this interpretation.

To date, pE194 is the smallest plasmid described which specifies inducible resistance. Shivakumar and Dubnau (unpublished data) have studied expression of pE194 in the *B. subtilis* minicell system (13). pE194 segregates readily into *B. subtilis* minicells, where it specifies between six and eight major peptides. One of these is clearly induced by exposure of the minicells to low concentrations (0.05 μg/ml) of erythromycin. This peptide is also expressed inducibly in minicells containing pE194 *cop-6*, but constitutively in the *tyl-1* mutant.

Part of the impetus for constructing *B. subtilis*(pE194) was to determine the genetic complexity of the erythromycin resistance mechanism in terms of the relative contributions of plasmid and chromosome to the resistance phenotype. From the present studies we infer that the determinants are specified by the plasmid exclusively in the sense that chromosomal contributions, if any, are present in all gram-positive organisms including naive strains such as *B. subtilis*. Moreover, these studies provide a means for development of general methods for amplification of plasmids which specify inducible functions, the expression of which can be selected and possibly utilized in recombinant systems.

ACKNOWLEDGMENTS

We thank R. P. Novick for the gift of strains and for valuable discussions, and Annabel Howard and Susan Crosbie for expert secretarial assistance. S. B. Holder provided expert technical assistance. D. Womble and R. H. Rownd assisted in the analytical ultracentrifugation studies, E. Lund and J. E. Dahlberg assisted in the oligonucleotide mapping studies, and R. Burgess provided RNA polymerase for probe preparation. S. Silver made many helpful suggestions for improvement of the manuscript.

This work was supported by Public Health Service grant AI-10311 to D.D., from the National Institute of Allergy and Infectious Diseases, and NSF grant PCM77-19390, as well as by research grants from The Upjohn Co. and Eli Lilly and Co. to B.W.

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