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Streptococcus-Escherichia coli Shuttle Vector pSA3 and Its Use in the Cloning of Streptococcal Genes

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A shuttle vector that can replicate in both *Streptococcus* spp. and *Escherichia coli* has been constructed by joining the *E. coli* plasmid pACYC184 (chloramphenicol and tetracycline resistance) to the streptococcal plasmid pGB305 (erythromycin resistance). The resulting chimeric plasmid is designated pSA3 (chloramphenicol, erythromycin, and tetracycline resistance) and has seven unique restriction sites: *Eco*RI, *Eco*RV, *Bam*HI, *Sal*I, *Xba*I, *Nru*I, and *Sph*I. Molecular cloning into the *Eco*RI or *Eco*RV site results in inactivation of chloramphenicol resistance, and cloning into the *Bam*HI, *Sal*I, or *Sph*I site results in inactivation of tetracycline resistance in *E. coli*. pSA3 was transformed and was stable in *Streptococcus sanguis* and *Streptococcus mutans* in the presence of erythromycin. We have used pSA3 to construct a library of the *S. mutans* GS5 genome in *E. coli*, and expression of surface antigens in this heterologous host has been confirmed with *S. mutans* antiserum. A previously cloned determinant that specifies streptokinase was subcloned into pSA3, and this recombinant plasmid was stable in the presence of a selective pressure and expressed streptokinase activity in *E. coli*, *S. sanguis* (Challis), and *S. mutans*.

Streptococcal plasmid vectors have proven useful for the molecular cloning of DNA fragments into *Streptococcus sanguis* (3, 5, 18, 20), but the mechanism of transformation in this organism requires the interaction of two plasmid molecules and makes their use inefficient in the shotgun cloning of chromosomal DNA (1, 17, 23, 26). One approach to circumventing this problem is the use of a shuttle vector that can replicate in both *Escherichia coli* and *S. sanguis*, thus allowing chromosomal fragments to be cloned in *E. coli* and then transferred to *S. sanguis* or other streptococci for further study. Chimeric plasmids obtained from the fusion of streptococcal and *E. coli* plasmids have been found to be functional in *E. coli* with expression of streptococcal erythromycin and lincomycin resistance determinants (22; H. Malke and J. J. Ferretti, manuscript in preparation), as well as to increase the efficiency of cloning streptococcal DNA fragments into *S. sanguis* via *E. coli* (16, 19).

In the present communication, we describe the construction of a *Streptococcus-E. coli* shuttle vector, pSA3, and its use in the shotgun cloning of *Streptococcus mutans* chromosomal DNA into *E. coli*. Expression of *S. mutans* genes in *E. coli* was confirmed by immunoassays with rabbit antiserum to *S. mutans* surface antigens. Additionally, the cloned streptococcal determinant that specifies streptokinase (21) was used to test the effectiveness of cloning with pSA3 in *E. coli*, *S. sanguis* (Challis), and *S. mutans*.

MATERIALS AND METHODS

Bacterial strains and media. The *E. coli* K-12 strains used in this study were HB101 (7) and 294 (6). The streptococcal strains included *S. mutans* GS5, *S. sanguis* (Challis), and the Challis strain harboring pGB305 (5). The basic media used were Luria Bertani for *E. coli* (14) and brain heart infusion for streptococci. When required, antibiotics were added at the following concentrations: tetracycline at 12.5 μg/ml, chloramphenicol at 100 μg/ml, and erythromycin at 50 μg/ml for *E. coli* 294 and 250 μg/ml for HB101; and erythromycin at 10 μg/ml for *S. sanguis* and *S. mutans*.

Chemicals and reagents. Restriction endonucleases and T4 DNA ligase were obtained from Bethesda Research Laboratories. Protease, lysozyme, goat anti-rabbit immunoglobulin G (IgG)-peroxidase conjugate, goat anti-rabbit IgG-alkaline phosphatase conjugate, 4-chloro-1-naphthol, alkaline phosphatase substrate, and agarose were from Sigma Chemical Co.

Isolation of plasmid and chromosomal DNA. Plasmid and chromosomal DNAs were isolated from streptococci as described by Behnke and Gilmore (4). Briefly, the cells were grown overnight in the presence of DL-threonine (9) and treated with glycine, lysozyme, and protease before lysis by sodium dodecyl sulfate (SDS). The cleared lysate was phenol extracted (2), and the DNA was purified by dye-buoyant density gradient centrifugation and stored at 4°C in 10 mM Tris–1 mM EDTA (pH 7.5). Large-scale isolation and storage of plasmid DNA from *E. coli* was conducted by an adaptation of this technique and a technique previously described (25). Briefly, cells from 100 ml of overnight culture were washed in 15% sucrose–50 mM Tris–50 mM EDTA (pH 8.5) and resuspended in 10 ml of the same buffer. Lysozyme (3 ml of a 6-mg/ml solution) in the same buffer was added, and the tube was incubated at room temperature for 15 min. After the incubation time, 0.75 ml of 20% SDS (wt/vol in distilled water) was added and mixed gently by inverting the tube until obtention of a viscous lysate, to which 3.5 ml of 5 M NaCl was added. The tube was mixed gently and placed in a crushed ice slurry for at least 30 min. The tube was next centrifuged at 15,000 rpm for 30 min at 4°C in a Sorvall SS34 rotor. The clear lysate obtained was phenol extracted and purified as described above. Rapid plasmid screening from *E. coli* was performed by the alkaline lysis method of Ish-Horowicz and Burke (12).

Molecular cloning. Restriction enzyme analysis, buffers, and conditions for DNA ligation were as described by Maniatis et al. (25). Analysis of DNA cleavage and ligation

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was accomplished by electrophoresis on 1% agarose gels in 0.04 M Tris-acetate–0.002 M EDTA (pH 8.0).

Transformation. Transformation of *E. coli* HB101 and 294 was performed as described by Mandel and Higa (24). Transformation of *S. sanguis* (Challis) and *S. mutans* was performed with plasmid DNA isolated from *E. coli* by the rapid plasmid screening procedure and by the procedure of LeBlanc and Hassel (13) as modified by Behnke (1).

Shotgun cloning of chromosomal DNA. Chromosomal DNA of *S. mutans* was incompletely digested with *Eco*RI, *Eco*RV, *Bam*HI, or *Sph*I, and was ligated to pSA3 that had been cleaved with the corresponding enzyme at an equal concentration of vector to *S. mutans* chromosome. The resulting ligated DNA was used to transform *E. coli* HB101, and transformants were selected based on their anticipated antibiotic resistance phenotypes. The final DNA concentration was 20 ng/μl, and transformation was performed with 5, 10, and 15 μl of ligation mixture for each enzyme. The total volume of transformation mixture consisting of cells, DNA, and medium was 1.3 ml, and from each transformation mixture, 20- and 50-μl samples were plated per plate containing the appropriate antibiotic. Random transformant clones were screened for their plasmid content, and the isolated plasmids were cleaved with the equivalent enzyme used for cloning to analyze the sizes of the DNA inserts obtained.

Subcloning and expression of a streptokinase determinant. Plasmid pMF1, composed of pBR322 with a 7.4-kilobase DNA fragment from *Streptococcus equisimilis* containing a cloned streptokinase gene (21), was cleaved and ligated to pSA3 at the *Sal*I site. After transformation as described above, transformants were selected on chloramphenicol-containing plates and tested for streptokinase activity by overlaying the plate with soft agarose containing skim milk and human plasminogen as described previously (21) and incubating the plates overnight at 37°C. Colonies containing and expressing the cloned streptokinase determinant were surrounded by a cleared zone of the skim milk casein.

Expression of *S. mutans* genes in *E. coli*. Rabbit antisera to *S. mutans* GS5 cell wall and membrane were prepared previously in this laboratory (C. Shea, Ph.D. dissertation, University of Oklahoma, Oklahoma City, 1980). The antiserum was absorbed extensively with a homogenate of *E. coli* containing the plasmid pSA3. This absorption was carried out by incubation of the antiserum with an equal volume of *E. coli* (pSA3) homogenate for 1 h at 37°C and for 3 to 5 days at 4°C. The mixture was centrifuged, and the supernatant was incubated again with an *E. coli* (pSA3) homogenate as described above. This process was repeated until no reaction with *E. coli* was detected by a dot immunobinding assay on nitrocellulose, as described below. Complete absorption was obtained after five incubation cycles, and the antiserum was tested again for reactivity with *S. mutans* GS5 cell wall and membrane preparations before use.

E. coli transformants containing *S. mutans* DNA inserts were inoculated into wells of 96-well microtiter plates containing 300 μl of Luria Bertani medium with the appropriate antibiotic. After overnight incubation at 37°C, crude cell extracts were prepared by an adaptation of the method described by Clarke et al. (10). The plates were centrifuged at 2,000 rpm for 5 min, the supernatant fluid was decanted, and the cells were incubated at 30°C for 30 min with 300 μl of lysozyme at 0.25 mg/ml in 0.05 M Tris (pH 7.5). The cells were lysed by three cycles of freeze-thaw treatment and centrifuged to remove cell debris. Nitrocellulose disks (Bio-Rad Laboratories) of 0.6 cm diameter were cut off, placed into each well, and allowed to react for 1 h at room temperature. The cell extracts were transferred to another plate, and the original plate containing the nitrocellulose disks was used in an enzyme-linked immunosorbent assay (ELISA) as described by Voller et al. (27). The unbound surface of the nitrocellulose disks was blocked with 10% (vol/vol) heat-inactivated horse serum before incubation with rabbit antiserum to *S. mutans* surface antigens, followed by a goat anti-rabbit IgG-alkaline phosphatase conjugate. The nitrocellulose disks were washed extensively before and after the addition of the second antiserum. Transformant clones expressing the *S. mutans* genes were detected by a yellow reaction after incubation with alkaline phosphatase substrate prepared as described by the manufacturer (Sigma). Extracts from transformant clones giving positive reactions were tested further by the dot immunobinding assay as described by Hawkes et al. (11). In this assay, the antigens or extracts from the transformant clones were dotted on nitrocellulose to which the ELISA was performed. The second antibody in this assay was goat anti-rabbit IgG-peroxidase conjugate, and the biological staining solution for peroxidase was 0.5 mg of 4-chloro-1-naphthol per ml, 0.01% (vol/vol) H_2O_2 in Tris (pH 7.4), and 0.2 M NaCl. A positive reaction resulted in a purple-blue dot over a white background.

Stability of pSA3. The stability of pSA3 in *E. coli* and streptococci in the absence of an antibiotic selective pressure was determined by transferring an overnight culture of bacteria grown in the presence of the appropriate antibiotic to a liquid broth culture, without any antibiotic added, at a dilution of 10^5. The culture was incubated at 37°C for 24 h for *E. coli* and 48 h for streptococci. This culture was then plated on antibiotic-free solid medium and incubated at 37°C for 24 h. One hundred of the colonies obtained were replica plated onto plates containing the appropriate antibiotic (erythromycin for streptococci and chloramphenicol, tetracycline, or erythromycin for *E. coli*). The numbers of sensitive and resistant colonies were compared.

RESULTS

Construction of pSA3. Streptococcal plasmid pGB305 (4) (6.2 kilobases) and *E. coli* plasmid pACYC184 (8) (4 kilobases) were cleaved at their unique *Ava*I sites, ligated, and transformed into *E. coli* HB101. Transformant clones with the chloramphenicol, erythromycin, and tetracycline resistance phenotypes were selected, and a plasmid designated pSA3 was isolated from one of these clones (Fig. 1). Digestion of the chimeric plasmid pSA3 with *Ava*I gave two fragments corresponding to pACYC184 and pGB305 (Fig. 2). A physical map of pSA3 was derived from restriction analysis with *Hin*dIII (Fig. 1) and confirmed by double digestion with enzymes cleaving unique sites on the plasmid (data not shown). Plasmid pSA3 has seven single sites for cloning: *Eco*RI, *Eco*RV, *Xba*I, *Bam*HI, *Sph*I, *Sal*I, and *Nru*I. Insertion of DNA segments into the *Eco*RI and *Eco*RV sites would inactivate chloramphenicol resistance of *E. coli*, and cloning into the *Bam*HI, *Sal*I, and *Sph*I sites would inactivate tetracycline resistance of *E. coli*.

Shotgun cloning of *S. mutans* chromosomal DNA into *E. coli*. Plasmid pSA3 and *S. mutans* chromosomal DNAs that had been cleaved with *Eco*RI, *Eco*RV, *Bam*HI, or *Sph*I were separately mixed at the same DNA concentration of vector to *S. mutans* chromosome and were ligated in separate tubes for each enzyme. With each of the enzymes used for cloning, transformants with the expected antibiotic phenotypes were

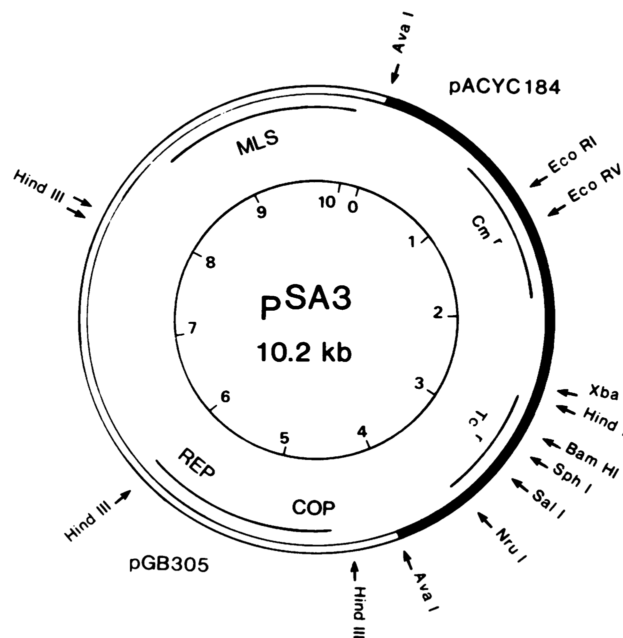

FIG. 1. Physical map of plasmid pSA3. Symbols and abbreviations: ■, pACYC184; □, pGB305; MLS, macrolide linocosamide streptogramin B resistance; Cm^r, chloramphenicol resistance; Tc^r, tetracycline resistance; REP, replication region; COP, copy control region.

obtained. There was no significant difference in the number of clones obtained with the doses of DNA used (100, 200, and 300 ng), and ca. 25% of the clones contained inserts in the *Bam*HI or *Sph*I site, whereas 55% of the clones contained inserts in either the *Eco*RI or *Eco*RV site. Random transformant clones were screened for their plasmid content, and various size inserts ranging from 2 to more than 15 kilobases were obtained (data not shown). The genome of streptococci contains ca. 2,000 genes (15); thus, 1,000 colonies with a 2-kilobase minimum size of insert would be sufficient to contain the entire genome. Since we consistently obtained larger inserts, such a gene library was easily available as described in these experiments. One transformant each of plasmid pSA3 with *S. mutans* DNA inserts at the *Eco*RI, *Eco*RV, *Bam*HI, or *Sph*I site was isolated and used to transform *S. sanguis*. Identical plasmids were recovered from *S. sanguis* transformant clones (data not shown).

Stability of pSA3 clones. *E. coli*, *S. sanguis*, and *S. mutans* harboring pSA3 were obtained in 1982 and were stored frozen or routinely transferred on antibiotic-containing plates without loss of the plasmids. *E. coli* containing pSA3 with *S. mutans* chromosomal DNA inserts, *S. sanguis* and *S. mutans* transformants containing pSA3 with an insert at the *Eco*RI, *Eco*RV, *Bam*HI or *Sph*I site, and pSA3 with a streptokinase determinant that had been shuttled from *E. coli* as described below, were also stable in the presence of antibiotics. In the absence of antibiotic, *E. coli* (pSA3) was relatively stable, with a curing frequency of 1% after 48 h in antibiotic-free medium as shown independently on chloramphenicol, tetracycline, or erythromycin plates. The same clone was sensitive on all three plates. We also observed a lag growth of the colonies on erythromycin plates as previously described (22). *S. sanguis* and *S. mutans* (pSA31) clones were cured of their plasmid after 72 h in antibiotic-free medium at a frequency of 25 to 30%.

Expression of *S. mutans* genes in *E. coli*. Transformant clones containing pSA3 with *S. mutans* DNA inserts were screened for reactivity with rabbit antiserum to *S. mutans* surface antigens. Crude cell extracts from 472 clones were analyzed by ELISA on nitrocellulose disks placed in the wells of 96-well microtiter plates. Positive reactions were observed with 43 clones, and these reactions were confirmed later with the dot immunobinding assay.

Subcloning and expression of the streptokinase gene in *E. coli* and streptococci. The availability of a cloned streptokinase determinant (21) was useful in evaluating the shuttle capabilities of plasmid pSA3. Plasmid pMF1 was cleaved into three fragments by *Sal*I and ligated to pSA3 that had been linearized by the same enzyme. The ligated mixture was used to transform *E. coli* 294, and transformant clones producing and secreting streptokinase were obtained.

The streptokinase-producing clones were screened for their plasmid content, and one of these clones consisting of pSA3 with the *Sal*I fragment of pMF1 containing the streptokinase determinant was designated *E. coli* pSA31. Plasmid pSA31 was isolated and used to transform *S. sanguis* and *S. mutans* with selection on erythromycin-containing plates. Among the *S. sanguis* transformant clones obtained, 84% were found to produce streptokinase. Under the same conditions of transformation, *S. mutans* did not transform well, and only eight clones were obtained, five of which produced streptokinase. Streptokinase produced by each of the pSA31-containing hosts (*E. coli, S. sanguis, S. mutans*) was easily detected by the streptokinase plate assay. Plasmid DNA isolated from the three hosts and subjected to restriction endonuclease analysis confirmed the presence of the vector and DNA insert (data not shown).

DISCUSSION

A new *E. coli-Streptococcus* shuttle vector has been constructed and has proven useful for the molecular cloning of streptococcal genes. This vector, pSA3, consists of com-

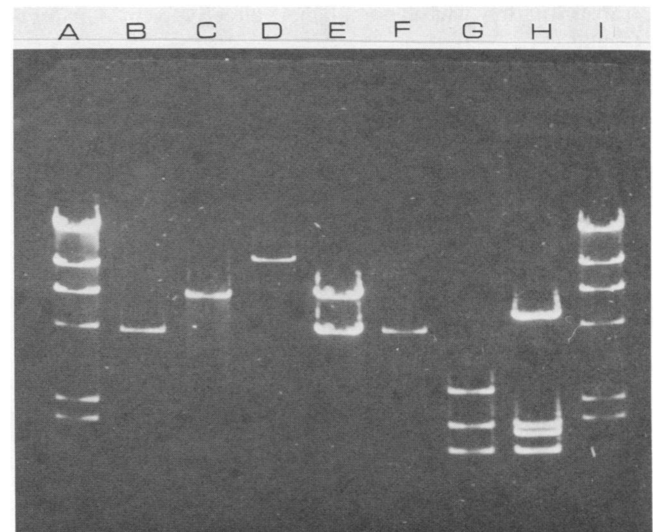

FIG. 2. Restriction enzyme analysis of shuttle vector pSA3. Bacteriophage lambda cleaved with *Hin*dIII (A and I), pACYC184 cleaved with *Ava*I (B), pGB305 cleaved with *Ava*I (C), pSA3 cleaved with *Eco*RI (D), pSA3 cleaved with *Ava*I (E), pACYC184 cleaved with *Hin*dIII (F), pGB305 cleaved with *Hin*dIII (G), and pSA3 cleaved with *Hin*dIII (H).

ponent plasmid pACYC184, which replicates in *E. coli*, and component plasmid pGB305, which replicates in streptococci. pSA3 is particularly useful because it contains two antibiotic resistance genes that can be insertionally inactivated in cloning experiments with *E. coli*. Thus, DNA can be inserted at single restriction sites in either the chloramphenicol (*Eco*RI, *Eco*RV) or tetracycline (*Bam*HI, *Sal*I, *Sph*I) resistance genes of the component plasmid pACYC184, and after transformation into *E. coli*, selection can be made for the phenotypic marker that remains functional. In this study, DNA fragments were inserted at each of these sites, and the inserts were shown to be expressed and stable in *E. coli*. The other two single sites, *Xba*I and *Nru*I, in component plasmid pACYC184 have not been used for cloning in the present study but are potentially useful sites for forced cloning since they allow segments of DNA to be cloned with two noncompatible ends. If one of the two different sites is in a resistance gene, then only recombinant clones containing DNA inserts will be formed and detected by insertional inactivation since the vector cannot self-ligate. The erythromycin resistance gene of component plasmid pGB305 does express in *E. coli*, although the basal level of erythromycin resistance in this organism is so high that selection for this phenotype is restricted to only certain *E. coli* strains. The *E. coli* strains used in this study were inhibited at the respective selected erythromycin concentrations, but the selection of transformants on erythromycin-containing plates was not practical due to the slower appearance of transformants as compared with either chloramphenicol- or tetracycline-containing medium.

The shuttle vector pSA3 has been used successfully in the shotgun cloning and establishment of a genomic library of *S. mutans* GS5. The initial cloning was performed in *E. coli* to circumvent the need for high concentrations of identical DNAs required to attain two-hit kinetics characteristic of transformation in streptococci. The recombinant clones were stable in *E. coli* in the presence of a selective antibiotic. Expression of *S. mutans* genes in this host was confirmed by the use of specific antibody to *S. mutans* surface antigens. After isolation of the recombinant plasmids from *E. coli*, transformation was accomplished effectively in *S. sanguis* Challis.

The shuttle capabilities of this vector were further demonstrated by inserting into pSA3 a cloned streptococcal determinant that specified the protein streptokinase (21). Transformation was first performed in *E. coli*, and then the recombinant plasmid was shuttled via transformation into *S. sanguis* (Challis) and *S. mutans*. Expression of streptokinase activity was detected in all three hosts, and restriction analysis of plasmid DNA showed that the shuttle vector and the cloned streptokinase gene remained intact.

In some instances, the streptokinase activity was lost in transformant clones; this was most likely due to damage occurring to the streptokinase gene during transformation by two-hit kinetics in streptococci. Malke and Ferretti (manuscript in preparation) have recently shown that in *E. coli*, inserts into the gene of pACYC184 are placed under the control of the *cat* chloramphenicol transacetylase gene promoter and in the sense orientation, expression of these mutants is greater than when the insert is under the control of its own transcription signals. The enhanced expression directed by the *cat* gene promoter is only operative in *E. coli* since this promoter is apparently not recognized in gram-positive organisms. Therefore, enhanced expression that is possible when genes are inserted in the correct orientation in the chloramphenicol resistance gene of the component plasmid pACYC184 may constitute an additional attribute of the pSA3 shuttle vector.

Since the streptococcal component plasmid pGB305 can replicate and is stable in *Bacillus subtilis* (M. S. Gilmore, personal communication), the new shuttle vector pSA3 may also have a place in the cloning of genes from other gram-positive species, first in *E. coli* and then shuttling the recombinant plasmids to gram-positive hosts. In addition, pSA3 can also be used as a subcloning vector in streptococci due to the availability of additional unique sites provided by component plasmid pACYC184. The determinant for type A streptococcal exotoxin isolated from streptococcal bacteriophage T12 was successfully subcloned by forced cloning into the *Eco*RI and *Sal*I sites of pSA3. Expression of this exotoxin in *S. sanguis* was also observed (28).

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