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Characterization and Construction of Molecular Cloning Vehicles Within *Staphylococcus aureus*

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Four chloramphenicol resistance (Cm) and four tetracycline resistance (Tc) plasmids from *Staphylococcus aureus* were characterized by restriction endonuclease mapping. All four Tc plasmids had molecular masses of 2.9 megadaltons (Mdaltons) and indistinguishable responses to seven different restriction endonucleases. The four Cm plasmids (pCW6, pCW7, pCW8, and pC221) had molecular masses of 2.6, 2.8, 1.9, and 2.9 Mdaltons, respectively. The four Cm plasmids also differed both in the level of resistance to Cm and in susceptibility to restriction endonucleases. Single restriction endonuclease sites contained within each plasmid included the following: in pCW6 for *Hin*dIII, *Xba*I, *Hpa*II, and *Bst*EII; in pCW7 for *Hin*dIII, *Bst*EII, *Bgl*II, *Hae*III, and *Hpa*II; in pCW8 for *Hin*dIII, *Hae*III, and *Hpa*II; in pC221 for *Hin*dIII, *Bst*EII, and *Eco*RI. The molecular cloning capabilities of pCW8 and pC221 were determined. Cm and erythromycin resistance (Em) recombinant plasmids pCW12, pCW13, and pCW14 were constructed and used to transform *S. aureus* 8325-4. A 2.8-Mdalton *Hin*dIII fragment from plasmid pI258 was found to encode Em resistance and contain single sites for the restriction endonucleases *Bgl*II, *Pst*I, *Hae*III, and *Hpa*II. The largest *Eco*RI fragment (8 Mdaltons) from pI258 contained the *Hin*dIII fragment encoding Em resistance intact. Cloning of DNA into the *Bgl*II site of pCW14 did not alter Em resistance. Cloning of DNA into the *Hin*dIII site of pCW8 and the *Hin*dIII and *Eco*RI sites of pC221 did not disrupt either plasmid replication or Cm resistance.

Recent developments in recombinant DNA research have led to a capability for the in vitro manipulation of genes. Restriction endonucleases and molecular cloning techniques have been employed with great success for the analysis of the structure and function of the chromosome and plasmids of *Escherichia coli* (6, 7, 32). The isolation via molecular cloning of biologically functional fragments of DNA has provided another means for studying gene organization and expression (2, 7, 14, 23, 33). Molecular cloning has also been used as a tool in the study of pathogenic *E. coli* (28).

Genetic analysis of *Staphylococcus aureus* has been hampered for several reasons, including the lack of a conjugation system and a poorly defined and low-level transformation system. One approach to an improved genetic analysis of this organism is to establish a molecular cloning system which could be used to analyze both plasmid and chromosomal genes.

This paper describes the characterization by restriction endonuclease mapping of several chloramphenicol resistance (Cm) and tetracycline resistance (Tc) plasmids of *S. aureus*. Two of the Cm plasmids were found to be suitable as cloning vehicles. In addition, several plasmids with improved cloning capabilities were constructed.

MATERIALS AND METHODS

Bacterial strains. Strains of *Staphylococcus aureus* used in this research are contained in the culture collection of J. N. Baldwin. Cultures were stored at −20°C in normal beef serum or a 50% vol/vol glycerol-PO_4 buffer (pH 7.0). The origins of strains used as donors of Cm and Tc plasmids are described in Table 1.

Media and chemicals. Trypticase soy agar contained Trypticase soy broth (BBL) plus 1.5% agar (Difco). All antibiotics were purchased from Sigma Chemical Co.

Phage propagation and typing. Propagating strains of *S. aureus* and typing phages utilized in this study were from the International Typing Series. The propagation of phages and typing of strains of *S. aureus* were performed by the method of Blair and Williams (3).

Antibiotic and heavy metal susceptibility testing. Antibiotic susceptibility was determined by a modification of the standardized disk agar diffusion test as published in the *Federal Register* (11). Tryp-

TABLE 1. *Description of plasmids*[a]

Plasmid	Molecular mass (Mdaltons)	Genotype[b]	Origin[c]	Description of cloned fragments
pCW2	2.9	*tet*	Transformed from JB48126	
pCW3	2.9	*tet*	Transformed from JB48827	
pCW4	2.9	*tet*	Transformed from JB48125	
pCW5	2.9	*tet*	Transformed from JB38	
pCW6	2.6	*cat*	Transformed from JB48126	
pCW7	2.8	*cat*	Transformed from JB48827	
pCW8	1.9	*cat*	Transformed from JB96	
pC221	2.9	*cat*	See reference 5	
pCW12	8.7	*cat erm*	In vitro recombination	*Hin*dIII, fragments B, D, and K from pI258 in pC221
pCW13	5.7	*cat erm*	In vitro recombination	*Hin*dIII, fragment B from pI258 in pC221
pCW14	4.7	*cat erm*	In vitro recombination	*Hin*dIII, fragment B from pI258 in pCW8
pCW17	12.6	*cat erm*	In vitro recombination	*Eco*RI, fragments C, I, J, and L from phage φ11 in pCW13
pCW18	9.6	*cat erm*	In vitro recombination	*Bgl*II, fragment C from phage φ11 in pCW14
pCW19	6.1	*cat erm*	In vitro recombination	*Bgl*II, fragment D from phage φ11 in pCW14
pCW20	5.6	*cat erm*	In vitro recombination	*Bgl*III, fragment E from phage φ11 in pCW14

[a] All plasmids were transformed into *S. aureus* 8325-4.

[b] Abbreviations indicate resistance to: *tet*, tetracycline; *cat*, chloramphenicol; *erm*, erythromycin.

[c] Date and site or source of isolation of each donor strain were: JB48126 (Maryland, 1972); JB48827 (New York, 1972); JB48125 (North Carolina, 1972); JB38 (S. Cohen, 1969); JB96 (Connecticut, 1975); pC221 (France, 1964).

ticase soy agar was used in place of Mueller-Hinton agar as the assay medium for *S. aureus*. Minimum inhibitory concentrations were determined by Trypticase soy broth. *S. aureus* 8325-4 was used as a reference control. Resistance to mercury was determined as described by Weiss et al. (34). Resistance to cadmium and arsenate was determined as described by Novick and Roth (21).

Isolation of plasmid DNA. Plasmid DNA was isolated as described by Stiffler et al. (29). Ethidium bromide was removed from plasmid DNA by at least two extractions with isopropanol saturated with CsCl. CsCl and residual isopropanol were removed by dialysis against 10 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid, and if necessary the plasmid DNA was concentrated by evaporation. DNA concentrations were estimated from absorbance readings at 260 nm.

Transformation. The phage superinfection transformation procedure of Thompson et al. (31) was modified by substituting 0.1 M $CaCl_2$ (pH 5.2) for the 0.1 M Tris-maleate–0.1 M $CaCl_2$ (pH 7.0) buffer used to resuspend cells before addition of transforming DNA and by using a heat-pulse treatment similar to that employed in the transformation of *E. coli* (17). After the addition of DNA, the cell suspension was placed in an ice bath for 45 min, then heat pulsed at 42°C for 2 min before returning to the ice bath for an additional 20 min. Then the cells were pelleted by centrifugation at 1,200 × *g* for 1 min. The $CaCl_2$ supernatant was not discarded. After the addition of 3.0 ml of Trypticase soy broth, the cells were resuspended and incubated without aeration at 37°C for 1.5 or 6 h depending on the resistance marker being selected. The times of incubation were 1.5 h for selection of both Cm- and Tc-resistant transformants and 6 h for selection of erythromycin (Em)-resistant transformants. After incubation the cells were pelleted by centrifugation and resuspended in 0.5 to 1.0 ml of Trypticase soy broth and plated on Trypticase soy agar containing either 10 μg of Cm, 5 μg of Em, or 6 μg of Tc per ml. Phage typing and antibiotic resistance patterns of transformants were determined and compared with that of the recipient strain to detect contaminants. In addition, the plasmid DNA content of the donor strain and transformant were compared by the mini-volume Brij-cleared lysate procedure (36).

Purification of *S. aureus* bacteriophage φ11 DNA. Phage φ11 was concentrated by the procedure of Sjöström et al. (27) and purified by the procedure of Thomas and Davis (30). φ11 DNA was isolated by a modification of the procedure of Sjöström et al. (27). The phage suspension was not treated with Pronase prior to the addition of sodium dodecyl sulfate. Phage DNA with a ratio of absorbancy at 260 to 280 nm greater than or equal to 1.9 was used in molecular cloning experiments.

Molecular weight standards. Molecular weight standards in agarose gel electrophoresis were generated by the digestion of lambda bacteriophage DNA with *Eco*RI and *Hin*dIII and *Pseudomonas* bacteriophage PM2 DNA with *Hin*dIII. Lambda DNA is cleaved by *Eco*RI into six fragments having molecular masses of 13.7, 4.74, 3.73, 3.48, 3.02, and 2.13 megadaltons (Mdaltons) (30) and by *Hin*dIII into seven frag-

ments having molecular masses of 14.4, 6.19, 4.16, 2.80, 1.57, 1.39, and 0.31 Mdaltons (18). *Pseudomonas* phage PM2 DNA is cleaved by *Hin*dIII into seven fragments having molecular masses of 3.46, 1.47, 0.62, 0.27, 0.24, 0.18, and 0.07 Mdaltons (22).

Restriction endonuclease digestion of plasmid and phage DNAs. Restriction endonucleases *Eco*RI and *Pst*I were a gift from D. Vapnek. Restriction endonucleases *Hin*dIII, *Hpa*II, *Bgl*II, *Hae*III, and *Xba*I were purchased from New England Biolabs, Beverly, Mass. Restriction endonucleases *Bst*EII and *Alu*I were a gift from R. Meagher. Restriction endonuclease *Bam*HI, a gift from B. Carlton, was purchased from BRL Laboratories, Rockville, Md. T4 polynucleotide ligase, a gift from S. Kushner, was purified according to the method of Weiss et al. (35).

The procedure for the single enzyme digestion of plasmid and phage DNAs was to expose 0.5 to 2.0 μg of DNA contained in 10 to 40 μl of the appropriate buffer to 1 to 5 U of a restriction endonuclease. Buffers were made up at 2×, 5×, and 10× concentrations, and a sufficient volume was added to DNA to produce a final buffer concentration of 1×. Buffers (1×) utilized for restriction endonuclease digestions were as follows: *Eco*RI, 100 mM Tris-hydrochloride (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 0.02% NP-40; *Hin*dIII, 20 mM Tris-hydrochloride (pH 7.4), 60 mM NaCl, 7 mM $MgCl_2$; *Hpa*I, *Hpa*II, and *Bgl*II, 10 mM Tris-hydrochloride (pH 7.4), 10 mM $MgCl_2$, 6 mM KCl, 200 μg of autoclaved gelatin per ml, 1 mM dithiothreitol; *Pst*I, 90 mM Tris-hydrochloride (pH 7.5), 10 mM $MgSO_4$; *Xba*I, 6 mM Tris-hydrochloride (pH 7.9), 150 mM NaCl, 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol; *Bam*HI, 20 mM Tris-hydrochloride (pH 7.5), 7 mM $MgCl_2$, 2 mM 2-mercaptoethanol; *Alu*I, 6 mM Tris-hydrochloride (pH 7.5), 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 200 μg of autoclaved gelatin per ml; *Hae*III, 6 mM Tris-hydrochloride (pH 7.4), 6 mM NaCl, 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol; *Bst*EII, 100 mM Tris-hydrochloride (pH 7.5), 5 mM $MgCl_2$, 7 mM 2-mercaptoethanol; and *Sal*I, 100 mM Tris-hydrochloride (pH 7.5), 5 mM $MgCl_2$, 7 mM 2-mercaptoethanol, and 150 mM NaCl. Reaction mixtures were incubated at 37°C for time intervals which varied from 1 to 3 h depending on the restriction endonuclease employed. Restriction endonuclease activity was stopped by heating at 63°C for 5 min. For digestions not involving exposure to a second enzyme, the heat treatment was followed by the addition of 10 μl of a stop dilution containing 50% (wt/vol) glycerol, 0.025% (wt/vol) bromothymol blue, and 5% (wt/vol) sodium dodecyl sulfate. In experiments involving digestion by two restriction endonucleases the DNA was exposed last to the enzyme requiring the higher salt concentration.

Molecular cloning. In cloning experiments the ratio of complementary ends of DNA to be cloned to complementary ends of the plasmid DNA cloning vehicle was kept at approximately 4 to 1. This was done to enhance the probability of selecting a transformant containing a hybrid cloning vehicle (8). DNAs (2 to 10 μg total) contained in a volume of 150 to 250 μl were digested with the appropriate restriction endonuclease, and the reaction was stopped by heating at 65°C for 5 min. Adenosine-5′-triphosphate, dithiothreitol, and $MgCl_2$ were added to the DNA digest to final concentrations of 0.05 mM, 10 mM, and 5 mM, respectively. The solution was equilibrated at 12.5°C, and then bacteriophage T4 DNA ligase was added, and the incubation of the ligation mixture was continued at 12.5°C for 24 to 36 h. At the end of the incubation period the degree of DNA ligation was determined by agarose gel electrophoresis of a small portion of the ligation mixture. The work described in this paper was performed under P-2 containment conditions for recombinant DNA research.

Agarose gel electrophoresis. Restriction endonuclease-digested DNA samples (10 to 50 μl) were applied to wells of a vertical agarose (Sigma Type II, medium EEO) slab gel (15 by 15 cm by 3 mm) and electrophoresed in Tris-borate buffer (0.089 M Tris, 0.089 M boric acid, 2 mM ethylenediaminetetraacetic acid, pH 8.5) overnight at 25 to 30 V (constant voltage). The agarose concentration used in different experiments ranged between 0.9 and 1.5% wt/vol.

Mini-volume Brij-cleared lysate procedure. The mini-volume Brij-cleared lysate procedure was as described by Wilson et al. (36).

Gel staining and photography. After electrophoresis, gels were stained for 45 min in electrophoresis buffer containing 1 μg of ethidium bromide per ml and then rinsed for 5 min in distilled water. DNA bands were visualized on a short-wave UV light box (Ultraviolet Products, Inc.) and photographed with a Polaroid MP-4 camera using Polaroid type 55 P/N film and a Wratten no. 23 filter.

RESULTS

Detection of plasmid DNA by the mini-volume Brij-cleared lysate-agarose gel electrophoresis procedure. Slot 1 of Fig. 1 contained a lysate of plasmid-free *S. aureus* 8325-4. The only visible band in slot 1 represents chromosomal DNA which serves to position the chromosomal bands of the remaining slots. Slots 2, 3, 4, and 5 contained lysates of Tc-resistant transformants of *S. aureus* 8325-4. Electron microscopy and limited digestion with pancreatic deoxyribonuclease I indicated that the major DNA band below the chromosomal band in slots 2, 3, 4, and 5 contains the supercoiled configuration of the Tc resistance plasmid (data not shown). Note that the lysates of all the Tc-resistant strains have identical DNA migration patterns. The molecular weight of the Tc resistance plasmid pCW5 was determined by electron microscopy and agarose gel electrophoresis of linear DNA. Values of 3.0 and 2.9 Mdaltons were determined, respectively. Slots 6, 7, 8, and 9 contained lysates of Cm-resistant transformants of *S. aureus* 8325-4. The predominant DNA band below the chromosomal band in slots 6, 8, and 9 represented the supercoiled configuration of the Cm resistance plasmids. An exception was the pC221 Cm plasmid seen in slot 7 where the

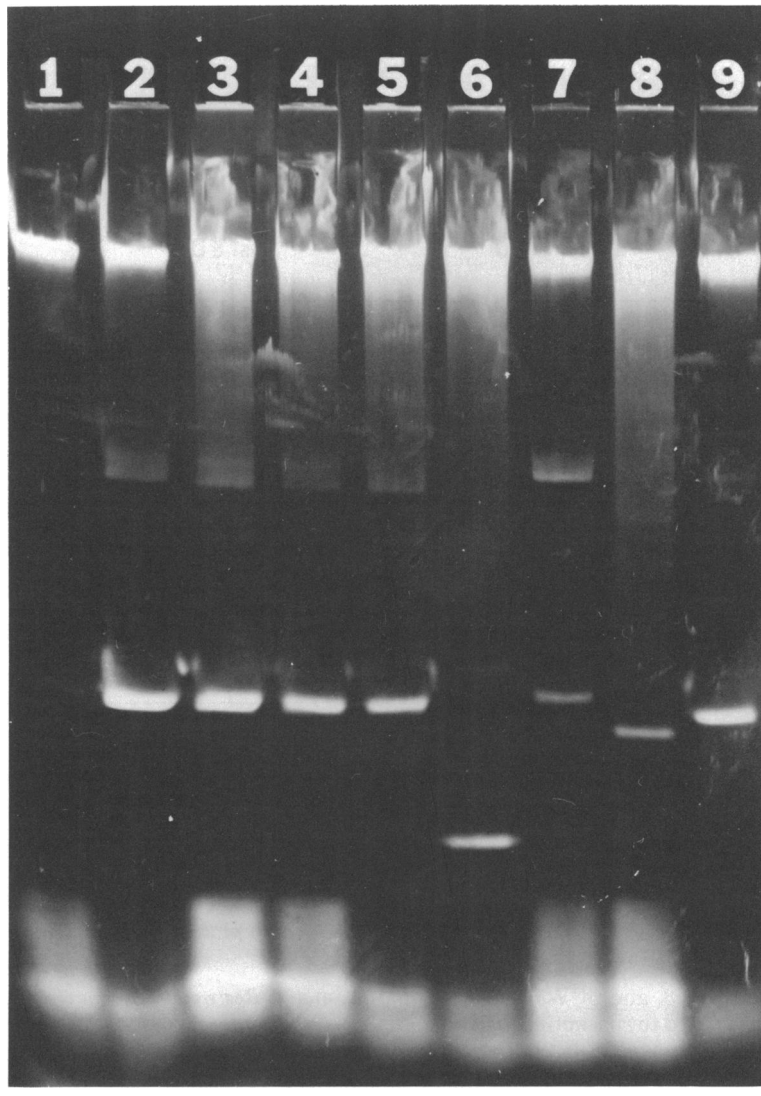

FIG. 1. *Mini-volume Brij lysis-agarose gel electrophoresis detection of plasmid DNAs. DNA samples were subjected to electrophoresis on a 1% agarose gel at 30 V for 15 h. Movement of DNA was toward the anode (bottom of gel). Mini-Brij lysates in slots 1-9 were: 1, S. aureus 8325-4 plasmid-free control; 2, 3, 4, and 5, S. aureus 8325-4 containing Tc resistance plasmids pCW5, pCW2, pCW4, and pCW3, respectively; 6, 8, and 9, S. aureus 8325-4 containing Cm resistance plasmids pCW8, pCW6, and pCW7, respectively. The lysate in slot 7 was from S. aureus 8325-4 containing the chloramphenicol-resistance plasmid pC221 (see text for discussion).*

open circular and supercoiled configurations each represented approximately 50% of the plasmid DNA. The RNA at the bottom of the gel includes 23S and 16S rRNA as observed by Barnes (1). Molecular masses of the Cm plasmids pCW8, pC221, pCW6, and pCW7 were subsequently determined to be 1.9, 2.9, 2.6, and 2.8 Mdaltons, respectively.

Mapping of restriction endonuclease sites. Restriction endonuclease maps were constructed for Cm plasmids pCW6, pCW7, pCW8, and pC221 (Fig. 2) and Cm and Em resistance recombinant plasmids pCW13 and pCW14 (see Fig. 4). The relative positions of substrate sites within a plasmid were determined from the molecular weights of DNA fragments produced by digestion with appropriate combinations of restriction endonucleases.

Restriction endonuclease digestions of Tc resistance plasmid DNAs. Tc resistance

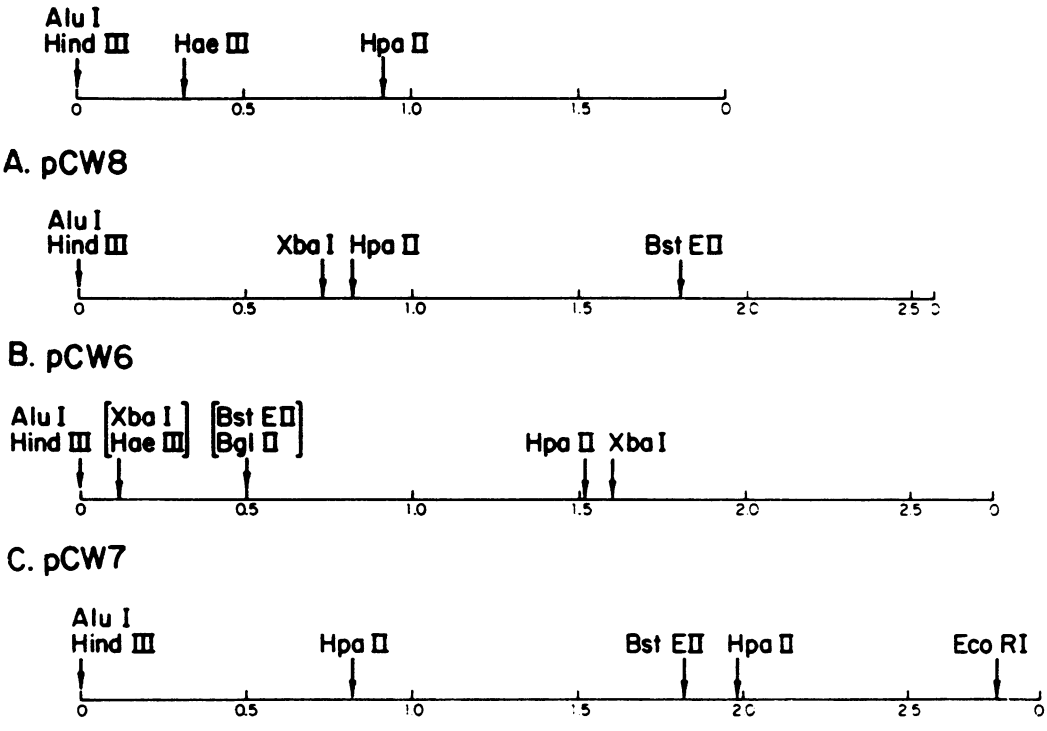

FIG. 2. *Linear restriction maps of four Cm resistance plasmids. Map units are in megadaltons (Mdaltons). These are linear representations of circular molecules. The four Cm resistance plasmids and their average molecular masses are: (A) pCW8, 1.9 Mdaltons; (B) pCW6, 2.6 Mdaltons; (C) pCW7, 2.8 Mdaltons; and (D) pC221, 2.9 Mdaltons. Restriction endonucleases which failed to cleave included: EcoRI, PstI, SalI, and BglII for pCW6; EcoRI, PstI, and SalI for pCW7; EcoRI, PstI, SalI, BstEII, XbaI, and BglII for pCW8; and PstI, SalI, BglII, and HaeIII for pC221. The presence of XbaI sites within pC221 was not determined.*

plasmids pCW2, pCW3, and pCW5 appeared to be identical with regard to susceptibility to seven restriction endonucleases (Table 2). The molecular weights of DNA fragments produced by *Hin*dIII and *Hpa*II were basically in agreement with the values reported by Shafferman et al. (25).

Molecular cloning of the Em resistance determinant from plasmid pI258. In addition to resistance to penicillin, the pI258 plasmid also codes for resistance to Em and the heavy metals cadmium, mercury, arsenite, and arsenate (20). Plasmid pC221 DNA and plasmid pI258 DNA were digested with *Hin*dIII, mixed, and ligated as described above. The ligated mixture of DNA was used in transformation experiments with *S. aureus* 8325-4 as the recipient. Selection was for Em and cadmium-resistant transformants. Only Em-resistant transformants were isolated, all of which were also resistant to Cm.

Plasmid DNA was isolated from a Cm Em transformant and subjected to electrophoresis on agarose gels without prior exposure to *Hin*dIII (Fig. 3A). Slots 1 and 2 of Fig. 3A contained the open circular (nearest the origin [top] of the gel) and supercoiled forms of plasmid pC221 (2.9 Mdaltons) and the Cm Em recombinant plasmid pCW12 (8.7 Mdaltons), respectively. *Hin*dIII-digested pC221, pI258, pCW12,

TABLE 2. *Restriction endonuclease digestion of Tc resistance plasmid DNA*[a]

Restriction endonuclease[b]	Molecular mass of DNA fragments (Mdaltons)
*Hin*dIII	1.6, 1.0, 0.4
*Hpa*II	2.9
*Hpa*II + *Hin*dIII	1.6, 0.5, 0.4, 0.3

[a] Same digestion patterns for pCW2, pCW3, and pCW5 plasmids.

[b] Restriction endonucleases which failed to cut Tc resistance plasmid DNA included *Eco*RI, *Pst*I, *Sal*I, *Bst*EII, and *Bgl*II.

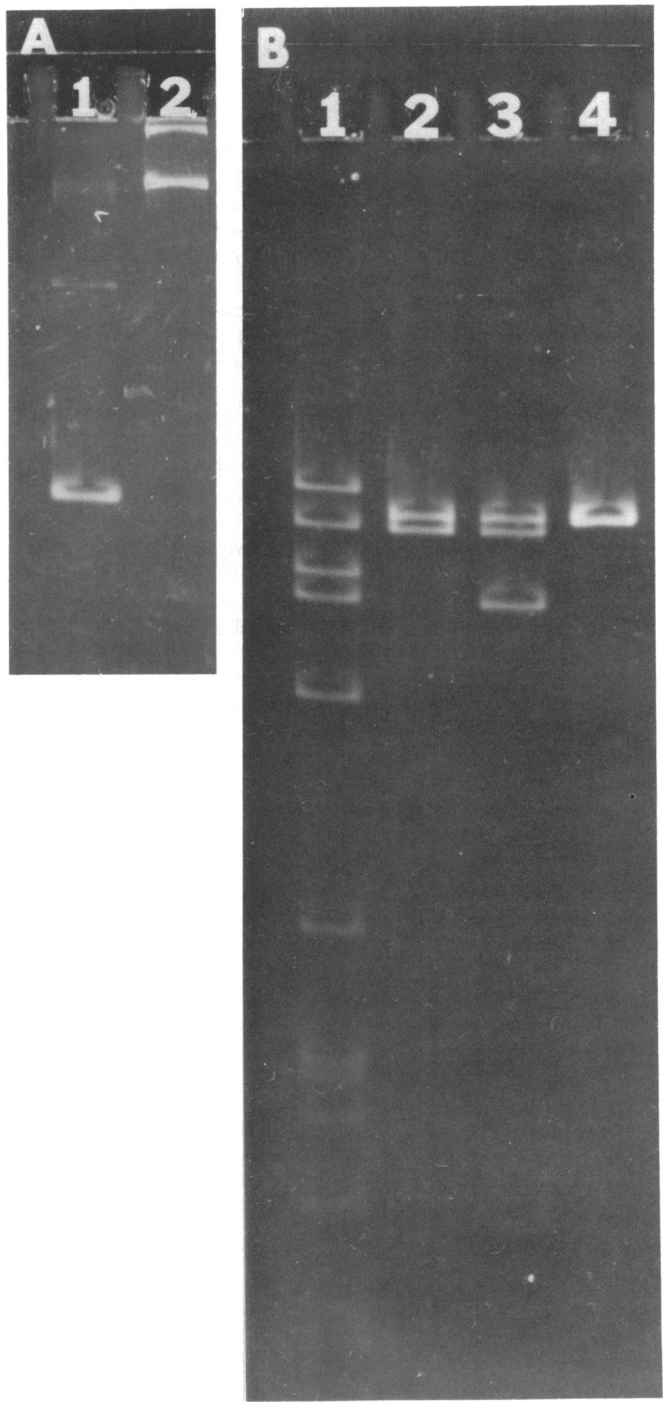

FIG. 3. *Cloning the Em resistance determinant from plasmid pI258 into the HindIII site of plasmid pC221. (A) Undigested plasmid DNA was subjected to electrophoresis on a 1.2% agarose gel at 25 V for 15 h. Slot 1 contained pC221 DNA (2.9 Mdaltons). Slot 2 contained the Cm Em recombinant plasmid pCW12 (8.9 Mdaltons). (B) HindIII-digested plasmid DNAs were subjected to electrophoresis on a 1% agarose gel at 25 V for 16 h. (1) pI258; (2) pCW13; (3) pCW12; (4) pC221.*

and pCW13 DNAs are depicted in Fig. 3B. Restriction endonuclease fragments are identified by lettering consecutively from the origin (top) of the gel. The four DNA fragments (A–D) observed after electrophoresis of *Hin*dIII-digested pCW12 DNA had molecular masses of 2.9, 2.8, 2.2, and 0.6 Mdaltons (slot 3). Fragment A (2.9 Mdaltons) in slot 3 migrated to a position corresponding to intact linear pC221 DNA (slot 4). Fragments B, C, and D in slot 3 corresponded to fragments B, D, and K produced by *Hin*dIII digestion of pI258 (slot 1).

To determine whether *Hin*dIII fragment(s) coded for Em resistance, pCW12 DNA was digested with *Hin*dIII and ligated, and the transformation procedure was repeated. The mini-volume Brij lysate of one chloramphenicol- and Em-resistant transformant contained a plasmid (pCW13) which migrated in the gel ahead of pCW12, indicating a lower molecular weight. Plasmid pCW13 contained *Hin*dIII fragment B (2.8 Mdaltons) from pI258 in addition to pC221 (Fig. 3B, slot 2).

The 2.8-Mdalton *Hin*dIII fragment encoding Em resistance was also cloned into the *Hin*dIII site of Cm resistance plasmid pCW8, and the Cm Em recombinant plasmid was designated pCW14 (Fig. 4A).

Molecular cloning into the *Eco*RI site of pCW13. The recombinant plasmid pCW13 was used to determine the effect of cloning DNA into the *Eco*RI site originally present in plasmid pC221. *S. aureus* φ11 DNA and pCW13 DNA were digested with *Eco*RI, mixed, and ligated. *S. aureus* 8325-4 was transformed with the ligated DNA, and both Cm- and Em-resistant transformants were selected. All transformants were resistant to both antibiotics. The mini-volume Brij-cleared lysate procedure detected one transformant which contained a plasmid (pCW17) which migrated nearer to the gel origin than did plasmid pCW13, indicating an increased molecular weight. Figure 5A depicts *Eco*RI digests of pCW13, pCW17, and φ11 DNAs. Plasmid pCW13 DNA was cleaved once by *Eco*RI producing a linear DNA fragment with a molecular weight of 6.2 Mdaltons (slot 1). The five fragments (A–E) produced by *Eco*RI digestion of pCW17 DNA had molecular masses of 6.2, 3.3, 1.3, 1.1, and 0.7 Mdaltons (slot 2). Fragments B, C, D, and E from pCW17 corresponded to φ11 DNA *Eco*RI fragments C, I, J, and L (slot 3). The largest (6.2 Mdaltons) *Eco*RI fragment from pCW17 migrated to a position in the gel corresponding to both linear pCW13 and φ11 *Eco*RI fragment A. *Hin*dIII + *Eco*RI double digests of pC221, pCW13, pCW17, and φ11 DNAs were performed to determine whether the 6.2-Mdalton fragment from pCW17 represented pCW13 DNA or φ11 DNA (Fig. 5B). Slot 1 of Fig. 5B contained linear pC221 DNA missing the approximately 0.1-Mdalton segment between the *Hin*dIII and *Eco*RI substrate sites (see restriction map, Fig. 2D). Slot 2 contained doubly digested pCW13 DNA. A *Hin*dIII digest of pCW13 would have been expected to produce two DNA fragments with molecular masses of 2.8 and 2.9 Mdaltons. However, the *Eco*RI digest removed sufficient DNA from the 2.9-Mdalton *Hin*dIII fragment (corresponding to pC221) to displace it to a position in the gel corresponding to the 2.8-Mdalton *Hin*dIII fragment originally from pI258. Therefore, only a single DNA band is visible for *Eco*RI + *Hin*dIII doubly digested pCW13. Slot 3 contained a double digest of pCW17 DNA. Again, the DNA band nearest to the gel origin in slot 3 was composed of two DNA fragments representing *Eco*RI + *Hin*dIII

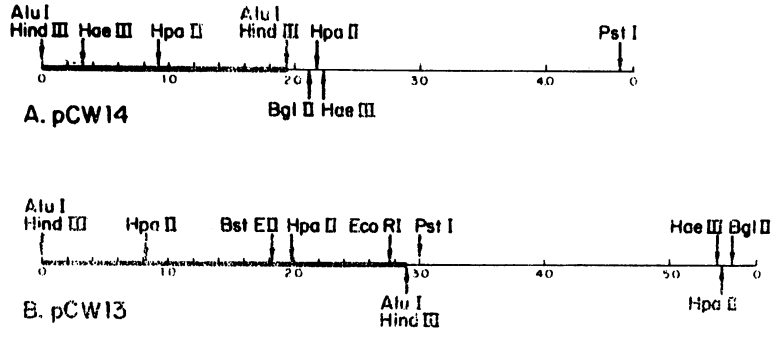

FIG. 4. *Linear restriction maps of two Cm and Em resistance recombinant plasmids. Map units are in megadaltons (Mdaltons). These are linear representations of circular molecules. The two Cm Em recombinant plasmids and their average molecular masses are: (A) pCW14, 4.7 Mdaltons; (B) pCW13, 5.7 Mdaltons. Plasmid pCW14 was constructed by the in vitro insertion of HindIII-B from pI258 into the HindIII site of pCW8. Plasmid pCW13 was constructed by the in vitro insertion of HindIII-B from pI258 into the HindIII site of pC221. The thin-lined portion of each map represents HindIII-B from pI258.*

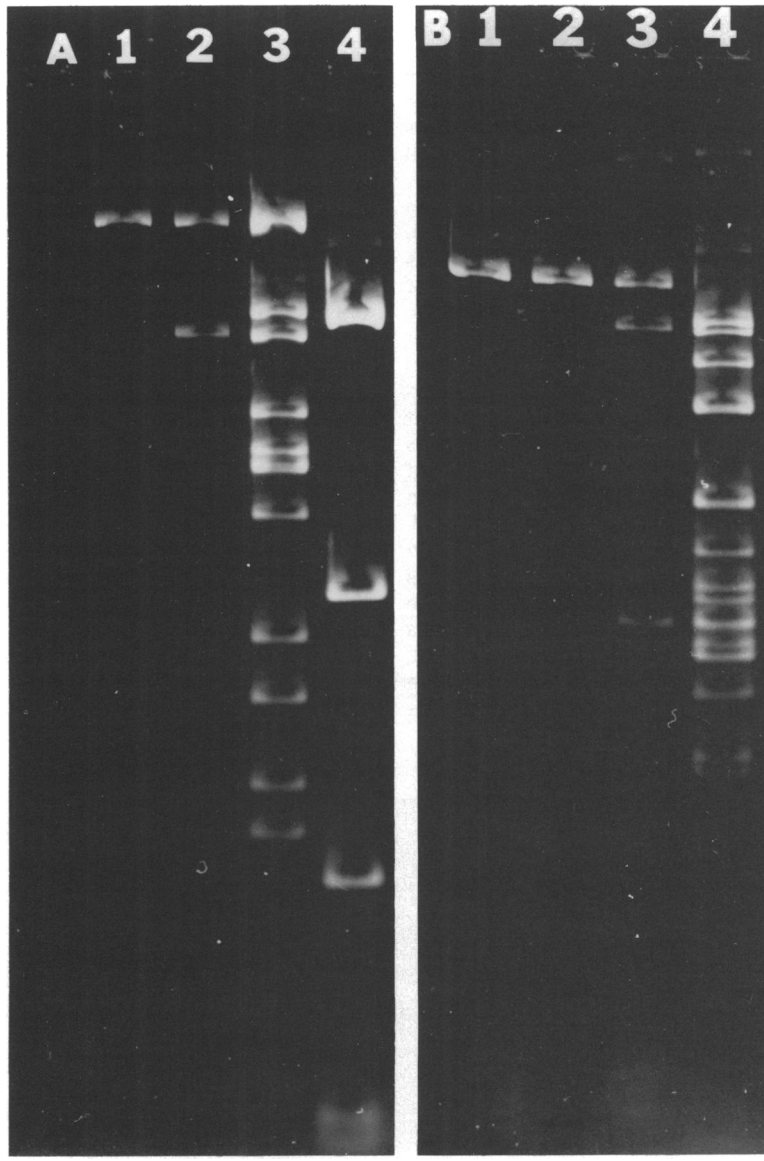

FIG. 5. *Cloning of phage φ11 DNA into the EcoRI site of plasmid pCW13. (A) EcoRI digests of pCW13 DNA, φ11-pCW13 (pCW17) recombinant plasmid DNA, and phage φ11 DNA were subjected to electrophoresis on a 1% agarose gel at 27 V for 14 h. (1) pCW13; (2) pCW17; (3) phage φ11. Slot 4 contained a HindIII digest of phage PM2 DNA. (B) Restriction endonuclease double digests of pC221, pCW13, pCW17, and φ11 DNAs were subjected to electrophoresis at 25 V for 14 h on a 1.2% agarose gel. HindIII + EcoRI double digests contained in slots 1–4 were: (1) pC221; (2) pCW13; (3) pCW17; (4) phage φ11. The HindIII + EcoRI fragment of pC221 DNA visible in slot 1 had a molecular mass of 2.8 Mdaltons.*

digested pCW13. In contrast, the *Hin*dIII +*Eco*RI double digest of φ11 DNA in slot 4 did not contain a DNA band migrating in the gel at a position corresponding to the *Hin*dIII digest of the largest *Eco*RI fragment of pCW17. Therefore, the 6.2-Mdalton fragment produced by *Eco*RI digestion of pCW17 DNA represents pCW13 DNA and not φ11 DNA, confirming that pCW17 is a recombinant plasmid and not a restriction endonuclease-deleted φ11 derivative.

Molecular cloning into the *Bgl*II site of pCW14. An experiment was performed to determine whether DNA cloned into the *Bgl*II site of pCW14 would result in loss of Em resistance. This would be expected if the *Bgl*II site was located within the structural gene for Em resist-

ance. Plasmid pCW14 DNA and ϕ11 DNA were digested to completion with *Bgl*II, mixed, and ligated. The ligated DNA mixture was used in a transformation experiment with *S. aureus* 8325-4 as the recipient. Cm-resistant transformants were selected and checked for Em resistance. All 77 transformants were also resistant to Em. A mini-volume Brij-cleared lysate screen of 12 Cm Em transformants detected five plasmids which migrated closer to the gel origin than did pCW14 (Fig. 6). Agarose gel electrophoretic patterns of *Bgl*II-digested phage ϕ11 and recombinant plasmid DNAs are depicted in Fig. 7. *Bgl*II digestion of pCW14 (slot 2) produced a single DNA fragment which had a molecular mass of 4.7 Mdaltons. Slots 3–5 of Fig. 7 contained digests of plasmid DNA isolated from strains of *S. aureus* whose lysates appeared in slots 5, 11, and 12 of Fig. 6. The ϕ11-pCW14 recombinant plasmids in slots 3–5 of Fig. 7 were designated pCW18, pCW19, and pCW20, respectively. A description of DNA fragments cloned into these plasmids is contained in Table 1. No differences could be detected in the level of Em resistance of strains of *S. aureus* carrying plasmids pCW14, pCW18, pCW19, pCW20, and pI258. In all cases Em resistance was constitutive with a minimum inhibitory concentration of 1,500 μg/ml.

DISCUSSION

Antibiotic resistance plasmids of *S. aureus* have been transformed into *Bacillus subtilis* and used successfully for molecular cloning (9, 10, 12, 13). Recently, Löfdahl et al. (16) reported the establishment of an *Eco*RI cloning vector for *S. aureus*. Fragments of pI258 were cloned into the *Eco*RI site of the naturally occurring Cm and streptomycin-resistance recombinant plasmid pSC194.

This study reports the establishment of *Eco* RI, *Hin*dIII, *Bgl*II, and *Pst*I molecular cloning vectors for *S. aureus*. Table 1 contains a description of the plasmids discussed in the text. Cm and Tc resistance plasmids of *S. aureus* were chosen as potential cloning vehicles because of their presence in the cell in multiple copies and their small size (29). Cm and Tc plasmids from clinical isolates of *S. aureus* were transformed into strain 8325-4 in an effort to obtain plasmids which differed with regard to size and/or restriction endonuclease digestion patterns. Surprisingly, all four Cm plasmids differed both in molecular weight and in the presence of restriction endonuclease cleavage sites (Fig. 1 and 2). This is the first report of Cm plasmids which have molecular masses of 2.6 Mdaltons (pCW6)

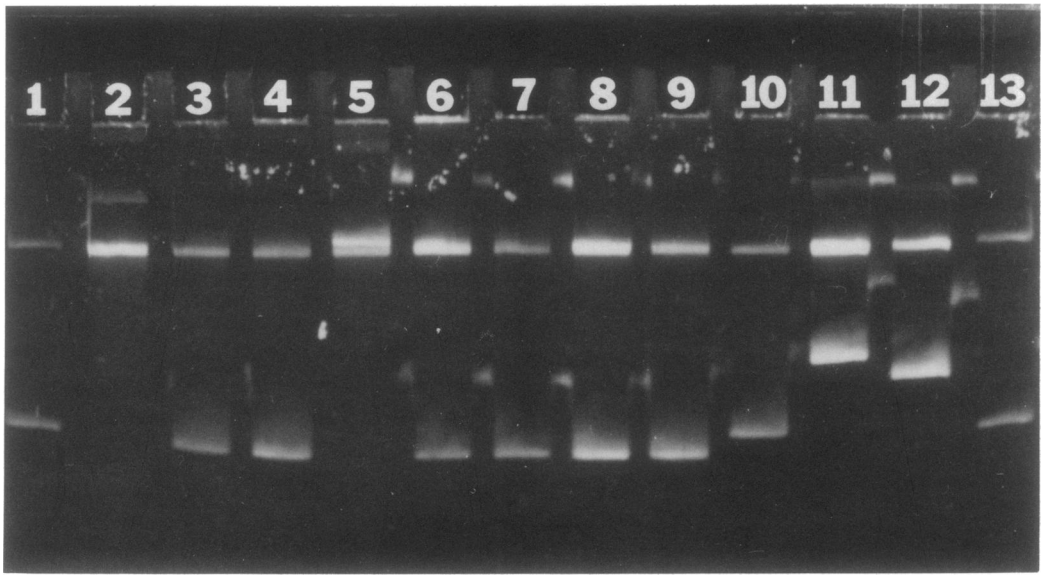

FIG. 6. *Mini-volume Brij lysate screen for plasmids migrating closer to the gel origin than pCW14. Brij-lysates were subjected to electrophoresis at 30 V for 15 h on a 1% agarose gel. The lysate in slot 13 contained pCW14. The chromosomal band (nearest origin [top] of the gel in slot 13) migrated to the same position in all the slots. Slots 1–12 contained lysates of Cm Em transformants. The tranforming DNA used in this experiment was a BglII digested, ligated mixture of ϕ11 and pCW14 DNAs. Slots 2, 5, 10, 11, and 12 appeared to contain plasmids with molecular weights greater than pCW14. The tendency of DNA bands near the edges of the gel to be displaced upward was a characteristic of the electrophoresis apparatus.*

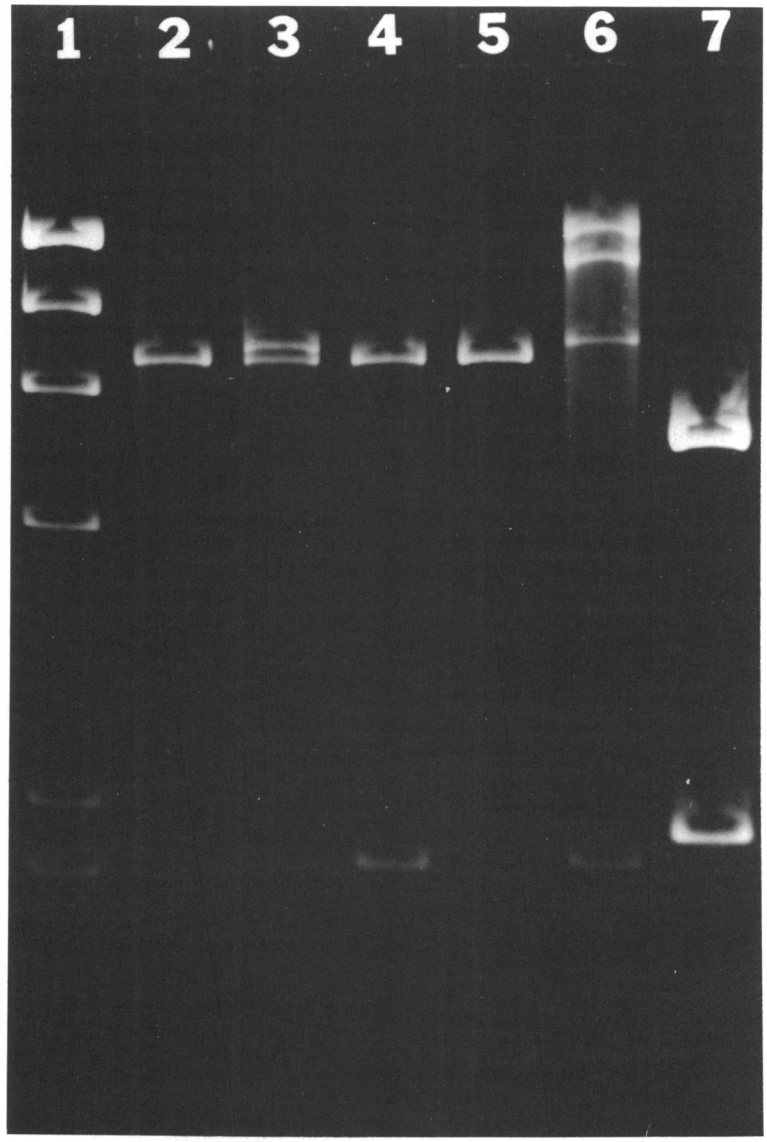

FIG. 7. *Cloning of phage φ11 DNA into the BglII site of plasmid pCW14. DNA samples were subjected to electrophoresis at 27 V for 15 h on a 0.9% agarose gel. Slots 1 and 7 contained HindIII digests of lambda and PM2 DNAs, respectively, which served as molecular weight standards. Fragments are identified by lettering consecutively from the origin of the gel. Slots 2–6 contained the following BglII digests: (2) pCW14; (3) pCW18; (4) pCW19; (5) pCW20; (6) phage φ11. The DNA fragment produced by BglII digestion of pCW14 had a molecular mass of 4.7 Mdaltons. Fragments C, D, and E produced by BglII digestion of φ11 DNA had molecular masses of 5.2, 1.4, and 0.9 Mdaltons, respectively.*

and 2.8 Mdaltons (pCW7). Plasmid pCW8 appears similar to Cm plasmid pC194 (10, 15) with regard to both molecular weight and restriction endonuclease digestion patterns. The four Cm plasmids characterized in this study have been found to comprise three incompatibility groups (manuscript in preparation). The results achieved with the Cm resistance plasmids pCW8 and pC221 and the Cm and Em resistance recombinant plasmids pCW13 and pCW14 demonstrate the capability of these plasmids to serve as cloning vehicles. Insertion of pI258 DNA into the *Hin*dIII sites of pCW8 and pC221 did not disrupt either chloramphenicol resistance or the replicative functions of these plasmids. Transformants resistant to both Cm and Em were

isolated and subsequently shown to contain the recombinant plasmids pCW12, pCW13, and pCW14. The 2.8-Mdalton *Hin*dIII fragment from pI258 which encodes Em resistance contains single substrate sites for restriction endonucleases *Bgl*II, *Pst*I, *Hae*III, and *Hpa*II (Fig. 4). No *Bst*EII or *Eco*RI sites were detected in this fragment. None of the Cm and Em resistance recombinant plasmids encode resistance to cadmium, mercury, or penicillin. The largest *Eco*RI fragment (8 Mdaltons) from pI258 was found to encode the Em resistance determinant intact (data not shown).

Molecular cloning of phage ϕ11 DNA into the *Bgl*II site of plasmid pCW14 did not alter the level of Em resistance, indicating that the *Bgl*II site does not lie within the structural gene(s) coding for Em resistance. In this experiment *S. aureus* phage ϕ11 DNA was used as the source of donor *Bgl*II fragments because a naturally occurring recombinant plasmid, ϕ11de, which contained portions of both the ϕ11 genome and penicillinase plasmid pI258, had been isolated in 1967 by Novick (19). A recombinant plasmid carrying both pCW14 DNA and ϕ11 DNA was, therefore, considered to be an example of an in vitro recombinational event which could and apparently does occur spontaneously in nature.

Phage ϕ11 DNA was also cloned into the *Eco*RI site of the recombinant plasmid pCW13 without disrupting or altering the level of Cm resistance. The pCW13 *Eco*RI site is present within DNA originally from pC221. Therefore, neither the *Eco*RI nor *Hin*dIII sites within pC221 lie within the structural gene coding for Cm acetyl transferase (26).

DNA fragments produced by digestion with *Mbo*I (↓GATC) or *Bam*HI (G↓GATCC) can be cloned into the *Bgl*II (A↓GATCT) site present in pCW13 and pCW14. Excluding the *Bst*EII site, this gives pCW13 the capability to clone DNA fragments from five different cohesive termini-producing endonucleases.

Initially, the aim of this work was to develop doubly selective cloning vehicles similar to those available for *E. coli* (4, 24). Unfortunately, none of the restriction endonuclease sites of pCW8, pC221, pCW13, and pCW14 into which DNA has been cloned appear to lie within a structural gene(s) coding for resistance to either Cm or Em. The development of the cleared lysate procedure has allowed the detection in this lab of recombinant plasmids carrying cloned DNA fragments with no known selectable markers (Fig. 6). It is felt that any deficiency resulting from a lack of a double selective capability for plasmids pCW8, pC221, pCW13, and pCW14 is more than compensated by the availability of the cleared lysate procedure.

A project to clone the competence factor for transformation in *S. aureus* was approved through the National Institute of Allergy and Infectious Diseases in January 1976 and most of the research described above was done prior to the release and implementation of the National Institutes of Health Guidelines for Recombinant DNA Research. In July 1977 the University of Georiga Committee on Biosafety reviewed the project and suggested that we obtain approval to continue the research. The request was denied by the Office of Recombinant DNA Research, and the cloning experiments were immediately discontinued.

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