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Use of Electroporation To Construct Isogenic Mutants of *Haemophilus ducreyi*

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Little is known about the genetics of *Haemophilus ducreyi*, the etiologic agent of chancroid. To develop a method for constructing isogenic mutants of this organism that could be utilized in pathogenesis-related studies, electroporation techniques were evaluated as a means of introducing DNA into this organism. Electroporation of the plasmid shuttle vector pLS88 into *H. ducreyi* yielded approximately 10^6 antibiotic-resistant transformants per μg of plasmid DNA. Studies of the feasibility of moving mutated genes into *H. ducreyi* were initiated by using *Not*I linker insertion and mini-Tn*10kan* mutagenesis techniques to introduce insertion mutations into cloned *H. ducreyi* genes encoding cell envelope antigens. In the former case, a gene encoding chloramphenicol acetyltransferase was then inserted into the *Not*I linker site created in the cloned *H. ducreyi* gene. The recombinant *Escherichia coli* strains containing these mutated plasmids no longer expressed the homologous *H. ducreyi* cell envelope antigens, as evidenced by their lack of reactivity with monoclonal antibody probes for these *H. ducreyi* proteins. Subsequent electroporation of both circular and linearized forms of plasmids carrying these mutated *H. ducreyi* genes into the homologous wild-type strain of *H. ducreyi* yielded antibiotic-resistant transformants which also lacked reactivity with the cell envelope antigen-specific monoclonal antibodies. Southern blot analysis confirmed that homologous recombination had occurred in these monoclonal antibody-unreactive transformants, resulting in the replacement of the wild-type allele with the mutated allele. Allelic exchange was most efficient when linear DNA molecules were used for electroporation. These results indicate that electroporation methods can be utilized to construct isogenic mutants of *H. ducreyi*.

Haemophilus ducreyi is a gram-negative bacterium that causes the sexually transmitted disease known as chancroid (1, 26). The recently demonstrated association between genital ulcer disease and enhanced transmission of the human immunodeficiency virus (12, 20) has stimulated interest in the virulence mechanisms used by this pathogen in the production of dermal lesions. Unfortunately, the lack of an appropriate genetic exchange system in *H. ducreyi* has precluded development of isogenic mutants to investigate the possible involvement of specific surface antigens and phenotypic characteristics in the expression of virulence by this organism. While conjugative plasmid transfer does occur in *H. ducreyi*, there have been no reports of conjugal transfer of chromosomal markers into this organism (1). Similarly, it is not known whether *H. ducreyi* can be transformed with either linear or chromosomal DNA (1).

The development of a relevant animal model for investigation of dermal lesion production by this pathogen (30), together with the introduction of in vitro tissue culture methods for studying the interaction of *H. ducreyi* with human cells (3), reinforced the need for developing a method for the construction of isogenic mutants of *H. ducreyi*. We have found that electroporation can be used to introduce both circular and linear DNA molecules into *H. ducreyi*. More importantly, we have been able to electroporate linear, recombinant DNA molecules containing mutated genes into *H. ducreyi*, with subsequent homologous recombination of the mutated alleles into the chromosome.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. The four strains of *H. ducreyi* (35000, Hd9, 041, and Cha-1) have all been shown to be virulent in the temperature-dependent rabbit model for chancroidal lesion production by this pathogen (30). *H. ducreyi* strains were grown on chocolate agar supplemented with IsoVitalex (30) (BBL, Becton Dickinson, Cockeysville, Md.) and, where appropriate, with antibiotics at the following concentrations: kanamycin, 20 μg/ml; and chloramphenicol, 1 μg/ml. *H. ducreyi* cultures were grown in a candle extinction jar at 33°C for 48 to 72 h unless otherwise specified. Strains of *Escherichia coli* DH5α containing pUC18-based recombinant plasmids and a derivative of pUC4K (Pharmacia, Inc., Piscataway, N.J.) were grown on Luria-Bertani (LB) medium containing 100 μg of ampicillin per ml unless otherwise specified.

Plasmids. The plasmids used in this study are listed in Table 1. The plasmid shuttle vector pLS88 has been described previously (37). Plasmid pUC4DE*cat* was kindly provided by Bruce Green, Lederle-Praxis Biologics, Rochester, N.Y., and consists of a modified pUC4K plasmid carrying the chloramphenicol acetyltransferase (*cat*) gene from pACYC184 (7) between inverted repeats; the *Eco*RI site within the *cat* gene was removed by site-directed mutagenesis (11a). The recombinant plasmids pHDU100 and pHDU200 were derived from a genomic library of *H. ducreyi* chromosomal DNA constructed in the plasmid vector pUC18; the *H. ducreyi* DNA inserts in these plasmids encode two different cell envelope antigens of *H. ducreyi*. Recombinant plasmids pHDU100 and pHDU200 encode antigens with apparent molecular weights of 49,000 and

TABLE 1. Bacterial strains, plasmids and bacteriophage used in this study

Strain, plasmid or bacteriophage	Relevant characteristics	Source or reference
Strains		
H. ducreyi		
35000	Virulent wild-type strain	30
35000-102	Cm^r mutant of 35000 unable to express the 49-kDa cell envelope antigen	This study
35000-201	Km^r mutant of 35000 unable to express the 23-kDa cell envelope antigen	This study
041	Virulent wild-type strain	30
Cha-1	Virulent wild-type strain	30
Hd9	Virulent wild-type strain	30
E. coli		
DH5α	Host strain for cloning experiments	14
MC1060	Host strain for mini-Tn*10kan* mutagenesis	J.-F. Tomb (6, 35)
Plasmids		
pUC18	Cloning vector, Ap^r	4
pUC4DE*cat*	pUC4K carrying modified *cat* gene	B. Green
pLS88	Shuttle vector capable of replicating in *E. coli*, *H. ducreyi*, and *H. influenzae*	37
pHDU100	49-kDa antigen-positive clone in pUC18	This study
pHDU101	pHDU100 with a *Not*I linker insertion eliminating expression of the 49-kDa antigen	This study
pHDU102	pHDU101 with a *cat* gene inserted into the *Not*I site	This study
pHDU200	23-kDa antigen-positive clone in pUC18	This study
pHDU201	pHDU200 with a mini-Tn*10kan* insertion eliminating expression of the 23-kDa antigen	This study
Bacteriophage λ1105	Vector carrying mini-Tn*10kan*	J.-F. Tomb (35, 36)

23,000, respectively, in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (29a). Plasmids were purified from broth-grown cultures of *E. coli* either by using a standard CsCl density gradient centrifugation method (31) or by means of a mini-prep procedure (31).

MAbs. Lymphocyte hybridomas secreting monoclonal antibodies (MAbs) reactive with *H. ducreyi* cell envelope antigens were obtained from hybridoma fusions involving the use of splenocytes from mice immunized with *H. ducreyi* 35000 cell envelopes (15). MAb 1F8, reactive with the 49-kDa cell envelope antigen, and MAb 2H4, directed against the 23-kDa cell envelope antigen, were identified by their reactivities in Western immunoblot analysis with *H. ducreyi* cell envelopes as antigens (14a). All MAbs were used in the form of culture supernatant fluids.

Mutagenesis protocols. The recombinant plasmid pHDU100 was subjected to linker insertion mutagenesis performed as described by Heffron et al. (16) with some modifications. The circular DNA molecules were randomly linearized by digestion with DNase I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in the presence of 1 mM $MnCl_2$. Phosphorylated *Not*I linkers were added to the linearized molecules by blunt-end ligation (31), after which the ligation reaction was subjected to agarose gel electrophoresis to remove unbound linkers. The linear molecules were eluted from the gel and digested with *Not*I to remove tandem linkers and to create cohesive ends. After extraction with phenol-chloroform and chloroform, the molecules were self-ligated with T4 DNA ligase (Boehringer Mannheim), and the ligation reaction was transformed into *E. coli* DH5α made competent for transformation (14, 31). The resultant transformants were screened for lack of reactivity with MAb 1F8 in the colony blot radioimmunoassay (13). Plasmid DNA was extracted from several of these MAb 1F8-negative transformants by means of the mini-prep technique and the presence of a *Not*I linker in the *H. ducreyi* DNA insert was confirmed by restriction enzyme digests. One of these mutated plasmids, pHDU101, was shown by Western blot analysis to be unable to express the 49-kDa antigen and to possess a *Not*I linker insertion in the *H. ducreyi* DNA insert.

The *cat* gene was excised from pUC4DE*cat* by digestion with *Eco*RI, and the Klenow fill-in procedure (31) was used to create blunt ends onto which were ligated *Not*I linkers. This *cat* gene with *Not*I ends was ligated into the *Not*I site in the mutated plasmid pHDU101, and the ligation mixture was used to transform *E. coli* DH5α, which was then plated on LB containing chloramphenicol (1 μg/ml). A plasmid purified from one of the resultant chloramphenicol-resistant transformants was shown to contain the *cat* gene at the expected position in the *H. ducreyi* DNA insert; this plasmid was designated pHDU102.

Recombinant plasmid pHDU200 was mutagenized in *E. coli* with mini-Tn*10kan* (36) according to the protocol described by de Bruijn and Lupski (9). Briefly, plasmid pHDU200 was transformed into *E. coli* MC1060, which was then infected with λ1105 carrying the mini-Tn*10kan* transposon. These bacteriophage-infected cells were plated on LB agar containing both ampicillin and kanamycin, the resultant colonies were harvested, and the plasmids were extracted by the mini-prep procedure and used to transform *E. coli* MC1060, which was then plated on LB agar containing both ampicillin and kanamycin. These transformants were then screened in the colony blot radioimmunoassay for colonies lacking reactivity with MAb 2H4; plasmids were extracted from these MAb-unreactive strains, and the presence of mini-Tn*10kan* in each *H. ducreyi* DNA insert was confirmed by restriction enzyme digests. One of these mutated plasmids carrying mini-Tn*10kan* in the *H. ducreyi* DNA insert was chosen for further study and designated pHDU201.

Electroporation method. *H. ducreyi* cells were grown on chocolate agar supplemented with Isovitalex for 18 to 24 h immediately prior to use. The cells present on three plates

TABLE 2. Electroporation of plasmid pLS88 into *H. ducreyi* 35000

Expt	CFU			No. of antibiotic-resistant transformants[d] obtained/following amt (μg) of plasmid DNA				
	Before electroporation[a]	After electroporation[b]	6 h after electroporation[c]	0.1	0.5	1	2	5
1	1.2×10^{11}	1.6×10^{10}	1.2×10^{10}	8.9×10^{5}	9.6×10^{6}	5.8×10^{6}	1.6×10^{7}	9.1×10^{6}
2	5.0×10^{10}	1.6×10^{10}	1.7×10^{10}	3.4×10^{5}	3.6×10^{6}	4.4×10^{6}	3.5×10^{6}	3.4×10^{6}

[a] CFU of *H. ducreyi* placed in microelectroporation chamber.
[b] CFU of *H. ducreyi* recovered from microelectroporation chamber immediately after electroporation.
[c] CFU of *H. ducreyi* recovered from chocolate agar plate after 6-h expression period.
[d] Kanamycin-resistant transformants.

with confluent growth were harvested by scraping with a bacteriological loop and suspended in 3 ml of cold 10% (vol/vol) glycerol in distilled water (washing buffer). All subsequent operations were performed in vessels held in crushed ice at all times. This cell suspension was centrifuged for 15 min at 4,300 × *g* at 4°C, and the supernatant fluid was removed. The cell pellet was resuspended in 2 ml of washing buffer and washed two more times with this same buffer. The final supernatant was carefully decanted, and the cell pellet was resuspended in the small amount of liquid remaining in the tube; the volume of the resultant, extremely thick suspension was approximately 150 μl. A 30-μl portion of this suspension was transferred to a 500-μl microcentrifuge tube, to which was then added 10 μl of the DNA solution in water. This mixture was then transferred to the microelectroporation chamber of a Cel-Porator Electroporation System (GIBCO-BRL, Gaithersburg, Md.), where electroporation was performed by using a field strength of 16.2 kV over a 0.15-cm distance. After electroporation, the cell suspension was immediately removed from the chamber and spread onto a single supplemented chocolate agar plate which was then incubated for 6 h as described above. Next, the cells on the surface of the agar plate were scraped into 1.0 ml of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with Levinthal's base (2). The cell suspension was centrifuged as described above, and the supernatant was decanted; the cell pellet was then suspended in 0.5 ml of brain heart infusion broth supplemented with Levinthal's base, and serial dilutions were plated onto appropriate selective media (e.g., supplemented chocolate agar containing kanamycin or chloramphenicol).

SDS-PAGE and immunologic methods. Whole-cell lysates of both *E. coli* and *H. ducreyi* strains were prepared by the method of Patrick et al. (29). Proteins present in these whole-cell lysates were resolved by SDS-PAGE involving a 10% (wt/vol) polyacrylamide separating gel, and Western blot analysis was performed as described elsewhere (18). The colony blot radioimmunoassay procedure was used to screen bacterial colonies for their reactivities with *H. ducreyi*-specific MAbs (13, 15).

Southern blot analysis. Chromosomal DNA was prepared from the wild-type and mutant *H. ducreyi* strains by using the DNA Extraction Kit (Stratagene, La Jolla, Calif.) and protocol supplied by the manufacturer. These chromosomal DNA preparations were digested with appropriate restriction enzymes, resolved by agarose gel electrophoresis with a 0.7% (wt/vol) agarose gel, and transferred to nitrocellulose; Southern blotting was accomplished by published procedures (31). DNA probes were radiolabeled with [^{32}P]dCTP by using a random primed DNA labeling kit (Boehringer Mannheim).

RESULTS

Electroporation of plasmid pLS88 into *H. ducreyi*. Preliminary experiments indicated that electroporation could be used to introduce exogenous DNA, in the form of the plasmid shuttle vector pLS88, into *H. ducreyi* 35000. The efficiency of this transformation did not vary much with DNA amounts ranging from 0.1 to 5 μg, with approximately 10^6 antibiotic-resistant transformants being obtained through the use of the standard electroporation method (Table 2). Use of different field strengths and pulse times did not increase significantly the number of transformants (data not shown). By using the standard conditions described in Materials and Methods, electroporation exerted a small negative effect on the viability of *H. ducreyi*, with the difference in viability before and after electroporation being approximately 1 order of magnitude (Table 2). During the 6-h expression period immediately after electroporation, there was no significant increase in the number of viable cells above the number originally inoculated onto the supplemented chocolate agar plate (Table 2). Control experiments in which electrical current was not delivered to the electroporation chamber consistently failed to yield even a single antibiotic-resistant transformant, confirming that a natural transformation mechanism was not responsible for the entry of this plasmid into the transformed *H. ducreyi* cells.

Construction of mutated *H. ducreyi* genes. The ultimate goal of this investigation was to develop a means for the construction of isogenic mutants of *H. ducreyi* by using cloned *H. ducreyi* genes. We selected two different, cloned genes encoding *H. ducreyi* cell envelope antigens for test purposes. The pUC18-derived recombinant plasmid pHDU100 contains a 4.7-kb insert of *H. ducreyi* DNA and expresses a *H. ducreyi* antigen with an apparent molecular weight of approximately 49,000 in SDS-PAGE that binds MAb 1F8 (Fig. 1, lane A). Recombinant plasmid pHDU200, containing an 8.0-kb DNA insert, expresses a *H. ducreyi* antigen with an apparent molecular weight of approximately 23,000 and which is reactive with MAb 2H4 (Fig. 1, lane E).

Linker insertion mutagenesis was used to inactivate expression of the MAb 1F8-reactive epitope by the recombinant *E. coli* DH5α(pHDU100). Restriction enzyme mapping indicated that the mutated plasmid pHDU101 contained a *Not*I linker insertion in the *H. ducreyi* DNA insert. Next, a *cat* gene was inserted into this *Not*I site, yielding the mutated plasmid pHDU102 (Fig. 2). Western blot analysis confirmed that the recombinant strain DH5α(pHDU102) also did not express an antigen reactive with MAb 1F8 (Fig. 1, lane B).

Transposon insertion mutagenesis was used to mutate the *H. ducreyi* DNA insert in the recombinant plasmid

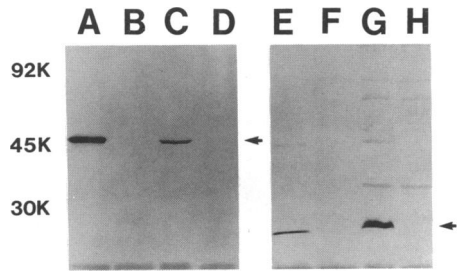

FIG. 1. Western blot analysis of the reactivity of recombinant *E. coli*, wild-type *H. ducreyi*, and *H. ducreyi* mutants with selected MAbs. Whole-cell lysates of these strains were resolved by SDS-PAGE and probed in Western blot analysis with the *H. ducreyi* 49-kDa antigen-specific MAb 1F8 (lanes A to D) or with the *H. ducreyi* 23-kDa antigen-specific MAb 2H4 (lanes E to H). Lane A, *E. coli* DH5α(pHDU100) expressing the 49-kDa antigen; lane B, *E. coli* DH5α(pHDU102) carrying the *cat* gene in the *H. ducreyi* DNA insert; lane C, wild-type *H. ducreyi* 35000 expressing the 49-kDa antigen; lane D, chloramphenicol-resistant *H. ducreyi* mutant strain 35000-102 that lacks reactivity with MAb 1F8; lane E, *E. coli* MC1060(pHDU200) expressing the 23-kDa antigen; lane F, *E. coli* MC1060(pHDU201) carrying mini-Tn*10kan* in the *H. ducreyi* DNA insert; lane G, wild-type *H. ducreyi* 35000 expressing the 23-kDa antigen; lane H, kanamycin-resistant *H. ducreyi* mutant strain 35000-201 that lacks reactivity with MAb 2H4. Molecular weight position markers are present on the left side of this figure. The arrow to the right of lane D indicates the position of the 49-kDa antigen; the arrow to the right of lane H indicates the position of the 23-kDa antigen.

pHDU200. The resultant mutated plasmid pHDU201 was shown to contain a mini-Tn*10kan* insertion in the *H. ducreyi* DNA insert (Fig. 2) and no longer expressed the epitope which bound MAb 2H4 (Fig. 1, lane F).

Electroporation of mutated genes into *H. ducreyi*. Preliminary experiments involving the electroporation of the mutated plasmids pHDU102 and pHDU201 into *H. ducreyi* 35000 indicated that antibiotic-resistant transformants could be obtained at a low frequency (data not shown). These initial experiments utilized plasmid DNA prepared by both the mini-prep and CsCl density gradient methods. Because the results obtained with plasmids prepared by the mini-prep method were inconsistent, all subsequent experiments were performed with plasmid DNA purified by the CsCl density gradient method.

Electroporation of the mutated plasmid pHDU102 into *H. ducreyi* 35000 consistently yielded chloramphenicol-resistant transformants (Table 3). The unlikely possibility that this pUC18-derived plasmid was replicating in *H. ducreyi* was eliminated by the finding that these chloramphenicol-resistant transformants were not resistant to ampicillin. Colony blot radioimmunoassay analysis revealed that approximately one-half of these transformants no longer bound the *H. ducreyi* 49-kDa antigen-specific MAb 1F8 (Table 3). These results suggested that the mutated *H. ducreyi* gene in the plasmid had undergone homologous recombination into the *H. ducreyi* chromosome, thereby eliminating expression of the MAb 1F8-reactive epitope.

We also determined whether electroporation of linearized pHDU102 DNA into the wild-type *H. ducreyi* strain would increase the frequency of homologous recombination. Digestion of pHDU102 with *Ava*I, which cleaves this plasmid only once (i.e., in the vector region), and subsequent electroporation of this linearized molecule into *H. ducreyi* 35000 yielded chloramphenicol-resistant transformants. While the frequency of transformation with these linear DNA molecules was markedly lower than that obtained with the circular form of pHDU102, all of these chloramphenicol-resistant transformants failed to bind the 49-kDa antigen-specific MAb 1F8 (Table 3). Western blot analysis was used to confirm these data derived from colony blot radioimmunoassay analyses; a chloramphenicol-resistant transformant strain (35000-102), derived from the use of the linearized form of pHDU102, did not express the MAb 1F8-reactive

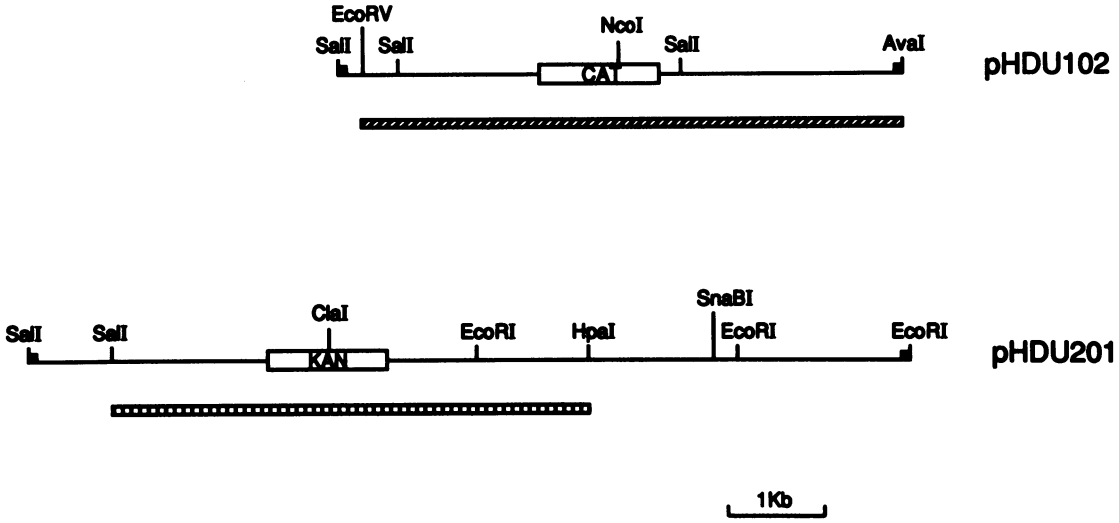

FIG. 2. Partial restriction enzyme maps of the *H. ducreyi* DNA inserts in the mutated plasmids pHDU102 and pHDU201. Only the *H. ducreyi* DNA inserts in these plasmids are shown; the very short, thicker line at the end of each insert indicates a few base pairs of vector DNA containing the polylinker sites *Sal*I, *Ava*I, or *Eco*RI. The position of the *cat* gene in pHDU102 is indicated by the box labeled CAT; the position of mini-Tn*10kan* in pHDU201 is indicated by the box labeled KAN. The *Not*I sites at the ends of the *cat* gene are not shown. The bar with diagonal cross-hatching beneath pHDU102 indicates the extent of the 5.7-kb *Eco*RV-*Ava*I fragment used in the Southern blotting experiments. The bar with the vertical cross-hatching beneath pHDU201 indicates the extent of the 5.1-kb *Sal*I-*Hpa*I fragment used for Southern blotting. A 1-kb distance marker is provided at the bottom of the figure.

TABLE 3. Use of electroporation[a] to introduce mutated genes into the *H. ducreyi* chromosome

Source (type of DNA)	No. of transformants		% Efficiency of elimination of MAb reactivity[d]
	Antibiotic resistant[b]	MAb unreactive[c]	
pHDU102 (circular)			
Expt 1	1,094	419	38
Expt 2	349	223	63
pHDU102 (linear)			
Expt 1	186	186	100
Expt 2	23	23	100
pHDU201 (circular)			
Expt 1	2,764	92	3
Expt 2	2,468	122	5
pHDU201 (linear)			
Expt 1	804	459	57
Expt 2	3,786	3,543	94

[a] Electroporation was performed as described in Materials and Methods by using 5 μg of DNA and 10^{11} CFU of *H. ducreyi* 35000.

[b] Number of colonies which grew on supplemented chocolate agar containing chloramphenicol (experiments involving pHDU102) or kanamycin (experiments involving pHDU201).

[c] Number of colonies unreactive with the 49-kDa antigen-specific MAb 1F8 (experiments involving pHDU102) or the 23-kDa antigen-specific MAb 2H4 (experiments involving pHDU201).

[d] Percentage of antibiotic-resistant colonies that were unreactive with the homologous MAb.

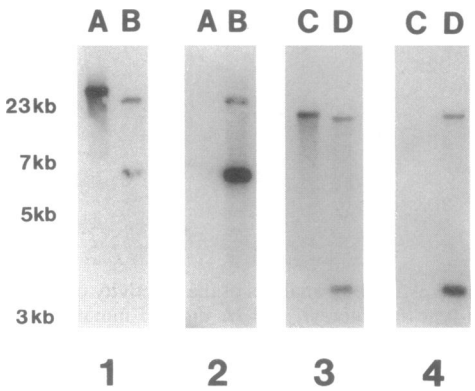

FIG. 3. Southern blot analysis of restriction enzyme digests of chromosomal DNA from wild-type and mutant strains of *H. ducreyi*. The following probes were used in radiolabeled form: panel 1, the 5.7-kb *Eco*RV-*Ava*I fragment from pHDU102 (see Fig. 2); panel 2, the *cat* gene; panel 3, the 5.1-kb *Sal*I-*Hpa*I fragment from pHDU201 (see Fig. 2); panel 4, mini-Tn*10kan*. Lane A, wild-type strain 35000 DNA digested with *Nco*I; lane B, chloramphenicol-resistant mutant strain 35000-102 DNA digested with *Nco*I; lane C, wild-type strain 35000 DNA digested with *Cla*I; lane D, kanamycin-resistant mutant strain 35000-201 DNA digested with *Cla*I. Size markers in kilobase pairs are present on the left side of this figure.

epitope that was present in the wild-type parent strain (Fig. 1, compare lanes C and D).

Additional electroporation experiments were performed by using the mutated plasmid pHDU201 which carries a mini-Tn*10kan* insertion within its *H. ducreyi* DNA insert (Fig. 2). As before, both circular and linearized forms of this plasmid were used; the linearized form was obtained by digesting pHDU201 with *Sca*I, which cleaves this plasmid only once (i.e., in the vector region). Electroporation of *H. ducreyi* 35000 with both forms of this plasmid yielded slightly greater numbers of antibiotic-resistant transformants than were obtained with pHDU102 (Table 3). However, in contrast to the results with pHDU102, the percentage of kanamycin-resistant transformants that were unreactive with the homologous MAb (i.e., the 23-kDa antigen-specific MAb 2H4) was markedly reduced when circular DNA was used for electroporation (Table 3). Even with the linearized form of pHDU201, elimination of expression of the MAb 2H4-reactive epitope was not 100% efficient among the kanamycin-resistant transformants (Table 3). Subsequent Western blot analysis with one of the kanamycin-resistant transformants (strain 35000-201) derived from the use of the linearized form of pHDU201 for electroporation indicated that the MAb 2H4-reactive epitope present in the wild-type parent strain (Fig. 1, lane G) was absent in this transformant (Fig. 1, lane H). Therefore, both this transformant (35000-201) and the chloramphenicol-resistant transformant strain 35000-102 actually represent *H. ducreyi* mutants.

The effect of the quantity of DNA used in these electroporation experiments on the relative efficiency of transformation was shown to be rather minimal. For example, increasing the amount of circular pHDU102 used for electroporation from 0.1 to 5.0 μg resulted in only an eightfold increase in the number of antibiotic-resistant transformants (data not shown). Very similar results were obtained with the linearized form of pHDU102.

Southern blot analysis. Confirmation that these mutated *H. ducreyi* genes carrying antibiotic resistance cartridges actually were inserted into the appropriate sites in the *H. ducreyi* chromosome by homologous recombination was obtained by Southern blot analysis. Chromosomal DNA was purified from the wild-type strain 35000, the chloramphenicol-resistant mutant 35000-102, and the kanamycin-resistant mutant 35000-201. The 35000 and 35000-102 chromosomal DNA preparations were digested with *Nco*I, an enzyme that cleaves a single site within the *cat* gene. In addition, both the 35000 and the 35000-201 chromosomal DNA preparations were digested with *Cla*I, which cleaves the mini-Tn*10kan* transposon only once. All four of these digests were resolved by agarose gel electrophoresis and used for Southern blotting.

Each pair of wild-type and mutant chromosomal DNA digests was probed with the homologous antibiotic resistance genes (i.e., *cat* or mini-Tn*10kan*) and with a probe containing DNA from the homologous cloned *H. ducreyi* insert. Accordingly, the *Nco*I-digested chromosomal DNA preparations were probed with the 1.2-kb *cat* gene and with a 5.7-kb *Eco*RV-*Ava*I fragment from pHDU102 (Fig. 2). This 5.7-kb *Eco*RV-*Ava*I fragment hybridized to a *Nco*I fragment of wild-type DNA with an approximate size of 30 kb (Fig. 3, panel 1, lane A). Probing of the chromosomal DNA from the mutant strain 35000-102 with this same fragment identified two *Nco*I fragments with approximate sizes of 25 and 6 kb (Fig. 3, panel 1, lane B). When the 1.2-kb *cat* gene was used to probe these same two chromosomal DNA digests, there was no detectable hybridization to the wild-type DNA (Fig. 3, panel 2, lane A). In contrast, this *cat* gene bound to 25- and 6-kb *Nco*I fragments from the mutant strain's chromosomal DNA (Fig. 3, panel 2, lane B).

When the 5.1-kb *Sal*I-*Hpa*I fragment from pHDU201 was used to probe *Cla*I-digested chromosomal DNA from both the wild-type and mutant strain 35000-201, a 21-kb band from the wild-type DNA hybridized with this probe (Fig. 3, panel

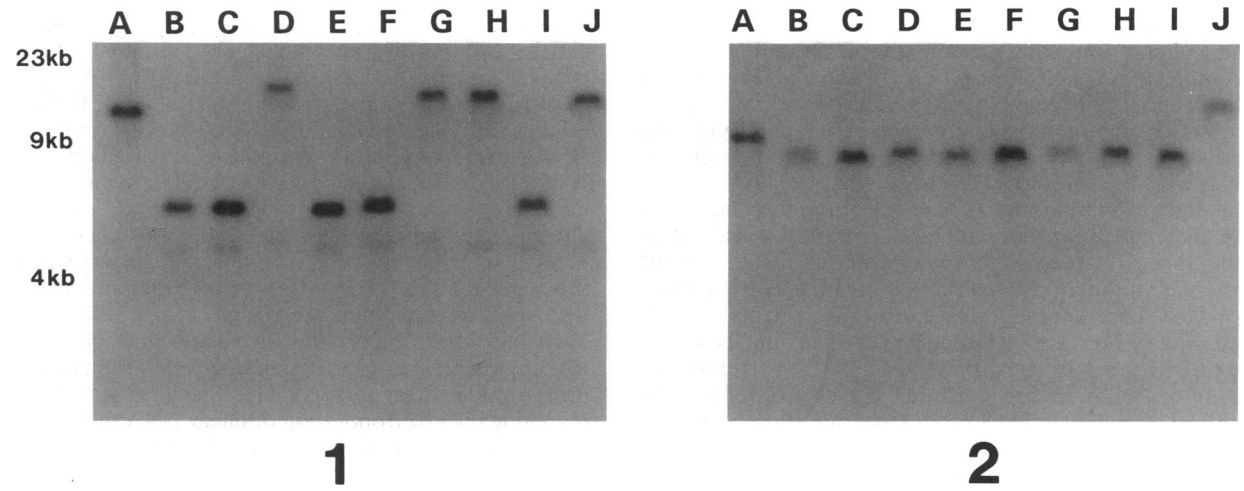

FIG. 4. Southern blot analysis of *Eco*RI digests of chromosomal DNA from the isogenic mutants 35000-102 and 35000-201 and the antibiotic-resistant transformants reactive with MAb probes 1F8 or 2H4. Panel 1: lane A, the isogenic mutant strain 35000-102 which lacks reactivity with MAb 1F8; lanes B through J, nine chloramphenicol-resistant transformants reactive with MAb 1F8. Panel 1 was probed with the *cat* gene. Panel 2: lane A, the isogenic mutant 35000-201 which lacks reactivity with MAb 2H4; lanes B through J, nine kanamycin-resistant transformants reactive with MAb 2H4. Panel 2 was probed with the mini-Tn*10kan* cartridge. Size markers in kilobase pairs are present on the left side of this figure.

3, lane C). The mutant strain's chromosomal DNA digest yielded two fragments of approximately 19 and 3.5 kb that bound this same probe (Fig. 3, panel 3, lane D). Probing of the wild-type chromosomal DNA digest with mini-Tn*10kan* yielded no reactive fragments (Fig. 3, panel 4, lane C), whereas the mutant strain's chromosomal DNA digest contained both a 19- and a 3.5-kb fragment that bound this same probe (Fig. 3, panel 4, lane D).

Additional Southern blot analyses were performed to analyze those antibiotic-resistant *H. ducreyi* transformants, derived from electroporation of pHDU102 and pHDU201 into strain 35000, which retained activity with the homologous MAb probe (i.e., those in which proper allelic exchange did not occur). When chromosomal DNA preparations from nine MAb 1F8-reactive, chloramphenicol-resistant transformants were probed with the *cat* gene, it was found that each strain possessed either a 6- or a 15-kb *Eco*RI fragment that bound this probe, whereas the MAb 1F8-unreactive isogenic mutant strain 35000-102 possessed a 12-kb *Eco*RI fragment which hybridized with the *cat* gene (Fig. 4, panel 1). Apparently, when insertion of the *cat* gene into the *H. ducreyi* chromosome (subsequent to electroporation) was not guided by the flanking sequences of the disrupted *H. ducreyi* gene encoding the 49-kDa antigen, insertion of the *cat* cartridge was not random but appeared to occur at two preferred sites. When *Eco*RI digests of chromosomal DNA from nine MAb 2H4-reactive, kanamycin-resistant transformants were probed with mini-Tn*10kan*, nine of the strains yielded an 8-kb fragment that bound mini-Tn*10kan*, whereas one strain had a 13-kb fragment that hybridized with this same probe (Fig. 4, panel 2). In contrast, a 9.5-kb *Eco*RI fragment from the MAb 2H4-unreactive isogenic mutant 35000-201 hybridized with this cartridge. Therefore, as happened with the *cat* cartridge, when insertion of mini-Tn*10kan* into the *H. ducreyi* chromosome did not occur via allelic exchange involving the gene encoding the 23-kDa antigen, these insertion events occurred at a very limited number of sites.

Electroporation of DNA into other *H. ducreyi* strains. To determine whether electroporation could be used to introduce DNA into other strains of *H. ducreyi*, we electroporated pLS88 into three additional clinical isolates: Cha-1, Hd9, and 041. The efficiencies of transformation of all three strains to kanamycin resistance were equivalent to that obtained with strain 35000 (i.e., approximately 10^6 transformants per μg of DNA) (data not shown).

DISCUSSION

The extremely fastidious nature of *H. ducreyi* together with its propensity to produce cohesive colonies that are very resistant to attempts to produce single-cell suspensions (1, 26) have hampered the development of methods for the genetic analysis of this pathogen. While it has been demonstrated that *H. ducreyi* has the ability to both donate and receive conjugative plasmids (1, 5, 23), to date no other means of genetic exchange has been described for this bacterium.

The application of electroporation techniques to introduce DNA into prokaryotes has permitted the genetic manipulation of bacterial species which previously had been refractory to transformation (22, 34). While most recent studies have focused on electroporation of plasmids carrying various selectable markers into bacteria (8, 10, 21), there have been very few reports of successful electroporation of linear or chromosomal DNA molecules into bacteria (24, 39). Even with *Haemophilus influenzae*, one of the bacteria that can develop a high level of natural competence for transformation with linear DNA, electroporation of chromosomal or linear DNA into this organism has not been convincingly demonstrated, whereas plasmid DNA can be readily electroporated into this bacterium (25, 33).

Both circular and linear DNA molecules were introduced successfully into *H. ducreyi* by electroporation. The efficiency of electroporation of pLS88 plasmid DNA into *H. ducreyi* (i.e., 10^6 CFU/μg of DNA) was similar to or better than that achieved in other types of bacterium-plasmid electroporation systems (8, 10, 21) excluding those involving *E. coli* and other enteric organisms (27, 38). The relatively

modest effect of DNA concentration on electroporation efficiency with both this plasmid (Table 2) and the linear DNA molecules (Table 3) may be attributed, at least in part, to the pronounced tendency of *H. ducreyi* cells to rapidly aggregate or clump immediately upon cessation of vigorous agitation; this clumping phenomenon putatively limits the number of cells exposed to DNA.

The electroporation into *H. ducreyi* of circular and linear DNA molecules carrying antibiotic resistance cartridges within cloned *H. ducreyi* genes consistently resulted in the isolation of antibiotic-resistant transformants. The efficiency with which these antibiotic-resistant transformants was obtained was orders of magnitude lower than that observed with pLS88 (i.e., 10^1 to 10^3 CFU/μg of DNA versus 10^6 CFU/μg of DNA), and this difference probably reflects the added requirement, in the former case, for successful integration of the antibiotic resistance gene into the chromosome. Not all of the antibiotic-resistant transformants obtained from electroporation of the circular forms of the mutated plasmids pHD102 and pHDU201 into *H. ducreyi* 35000 lacked reactivity with the homologous MAb probes (Table 3). This finding indicates that, in these MAb-reactive, antibiotic-resistant transformants, recombination of the antibiotic resistance cartridges into the chromosome did not occur by allelic exchange mediated by the mutated *H. ducreyi* genes. Southern blot analysis revealed that, in these same transformants, insertion of the antibiotic resistance cartridges into the *H. ducreyi* chromosome was not random but occurred at a limited number of sites (Fig. 4).

Using two different *H. ducreyi* genes and two different antibiotic resistance genes, the efficiency of elimination of MAb reactivity in the antibiotic-resistant transformants (i.e., by allelic exchange) was always much higher with the linearized form than with the circular form of the plasmid bearing the respective, mutated *H. ducreyi* genes. In fact, when the linearized form of the mutated plasmid pHDU102 containing the *cat* gene was electroporated into the wild-type strain of *H. ducreyi*, all of the chloramphenicol-resistant transformants lacked reactivity with MAb 1F8, indicating 100% efficiency of replacement of the wild-type allele expressing the 49-kDa antigen with the mutated allele (Table 3). Southern blot analysis confirmed that allelic exchange had taken place in antibiotic-resistant, MAb-unreactive transformants obtained by using linear forms of pHDU102 and pHDU201 (Fig. 3).

The successful use of electroporation to introduce both plasmid and linear DNA molecules into *H. ducreyi* opens new avenues of approach for investigating the genetics of this pathogen. It should be possible to use electroporation to facilitate generalized, transposon-mediated mutagenesis of *H. ducreyi* by introducing into this organism certain plasmids incapable of replication and carrying appropriate transposons; the latter mobile DNA elements would subsequently integrate into the chromosome. This approach has already been used successfully to mutagenize both *H. influenzae* (17) and *Brucella abortus* (19), although in the former case the plasmid carrying the transposon was introduced by transformation. Similarly, the ability to electroporate the shuttle vector pLS88 into *H. ducreyi* makes it feasible to contemplate the cloning of *H. ducreyi* genes in *H. ducreyi* itself. This possibility would allow the use of either wild-type strains or selected mutants of *H. ducreyi* as hosts for cloning genes that are lethal when expressed in *E. coli* or other conventional cloning hosts.

The occurrence of allelic exchange after electroporation of *H. ducreyi* with DNA molecules carrying mutated *H. ducreyi* genes indicates that gene replacement in this pathogen can be successfully accomplished. This ability will make possible, for the first time, the construction of isogenic *H. ducreyi* mutants for use in the investigation of the role(s) of selected surface antigens in the expression of virulence by *H. ducreyi* in appropriate in vivo and in vitro model systems. While this particular approach is limited somewhat by the necessity of first having to clone the *H. ducreyi* gene encoding the antigen of interest, the existence of a large number of MAbs reactive with different surface antigens of this pathogen (11, 15, 28, 32) indicates that antibody probes that will facilitate such molecular cloning efforts are now available. More importantly, it should now be possible to test any putative *H. ducreyi* virulence determinant if probes for the determinant are available. Specifically, this approach involving the electroporation of linear DNA molecules bearing *H. ducreyi* genes containing selectable markers into *H. ducreyi* can be used in conjunction with generalized mutagenesis protocols in which *H. ducreyi* genomic libraries in *E. coli* are mutagenized with transposons (35) and then electroporated, in circular or linearized form, into *H. ducreyi*.

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