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Characterization of Transfer Regions within the HII Incompatibility Group Plasmid pHH1508a

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A circular map of the 208-kilobase IncHII plasmid pHH1508a was constructed by using the restriction endonucleases *Xba*I, *Xho*I, and *Not*I. Tn*5* insertion mutagenesis showed that transfer genes were distributed widely over the plasmid map. Analysis of the deletion mutant pDT1178 indicated that genes required for conjugative pilus production are located in a 96.0-kilobase region of the plasmid.

Plasmids belonging to the H incompatibility group have been shown to encode multiple drug resistance in members of the family *Enterobacteriaceae* (1). Two major H incompatibility groups, IncHI and IncHII, are now recognized. All plasmids of the HI incompatibility group are thermosensitive for transfer (8, 13). Recent work has shown that transfer genes of the IncHI prototype plasmid R27 are located in at least two widely separated regions (12). The IncHII prototype plasmid, pHH1508a, was originally isolated from *Klebsiella aerogenes*; this plasmid specifies H-type pili and determines resistance to streptomycin, trimethoprim, and potassium tellurite (3). In the present study, a restriction map of pHH1508a was constructed, and the nonthermosensitive transfer system of this plasmid was characterized by Tn*5* insertion mutagenesis, Southern blot hybridization, and in vitro deletion mutant analysis.

Plasmid DNA was isolated by the Sarkosyl-lysate method of Bazaral and Helinski (2) as modified by Whiteley and Taylor (14). Restriction enzymes *Apa*I, *Acc*I, *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Pst*I, *Sal*I, *Xba*I, and *Xho*I were obtained from Boehringer Mannheim Canada, Ltd., Dorval, Quebec, Canada, and *Not*I was obtained from New England BioLabs, Inc., Beverly, Mass. Conditions for digestion were as recommended by the suppliers. Digested DNA samples were subjected to electrophoresis with 0.4 and 0.6% agarose gels in Tris-acetate buffer (7). Sizes of restriction digest fragments were determined with reference to bacteriophage lambda DNA digested with *Hin*dIII and *Eco*RI (5). Plasmid DNA was extracted from agarose gels by running the DNA fragments into a DEAE membrane and then eluting the DNA by immersing the membrane in a concentrated salt solution (1 M NaCl, 1 mM EDTA, 20 mM Tris [pH 8.0]); this was followed by incubation at 65°C for 45 min. DNA was precipitated with ethanol, dried, and suspended in TE buffer (50 mM Tris, 5 mM EDTA [pH 8.0]) before phenol extraction. DNA was transferred onto nitrocellulose filters (BA85; Schleicher & Schuell, Inc., Keene, N.H.) by the method of Southern (9). Extracted DNA fragments were labeled with [α-^{32}P]dCTP (New England Nuclear Corp., Boston, Mass.) by nick translation (6). Hybridizations were carried out as previously described (7). X-ray films (Xar; Kodak) were then exposed to nitrocellulose filters at −70°C for various time periods before they were developed. Matings and screenings for potential transfer-deficient mutants were performed as described previously (10), except that kanamycin (16 μg/ml) was used to select Tn*5* insertions into pHH1508a. Rifampicin (100 μg/ml) or nalidixic acid (24 μg/ml) was used for counterselection against donor strains. Preparation of an in vitro deletion mutant was performed as described previously (11), except that Mueller-Hinton agar (Oxoid Canada, Ltd.) containing trimethoprim (10 μg/ml) was used to select for transformants. In phage pilHα sensitivity testing (4), Tra$^-$ mutants, the deletion mutant, and a control strain carrying pHH1508a were grown in brain heart infusion broth. A sample (0.1 ml) of each culture was added to 1 ml of soft agar and poured on a brain heart infusion plate. pilHα suspension (100 μl; 10^{10} PFU/ml) was spotted onto the middle of the plate, which was incubated at 30°C overnight, and then examined for signs of lysis. Antiserum against H pili was prepared by subscapular injection of rabbits with *Escherichia coli* K-12 J53-1 (pHH1508a) mixed with Freund incomplete adjuvant. Antiserum was absorbed with *E. coli* J53-1 cells (two plates of heavily inoculated cells suspended in 0.1 M phosphate-buffered saline) sonicated for five 30-s cycles. Immunogold labeling experiments were carried out as previously described (15) before electron microscopic examination with a Phillips 300 transmission electron microscope.

Of the 10 restriction enzymes surveyed that had a 6-base-pair recognition sequence, only 2 were found to cut pHH1508a into a reasonable number of fragments. The enzymes *Xba*I and *Xho*I cut pHH1508a into 11 and 12 fragments, respectively. The sizes of these restriction fragments are shown in Table 1. From the sizes of these restriction fragments, the plasmid pHH1508a was determined to be approximately 208 kilobases (kb) in size. The restriction enzyme *Not*I, which has an 8-base-pair recognition sequence, cut pHH1508a into two large fragments similar in size. The three enzymes *Xba*I, *Xho*I, and *Not*I were used in the construction of the pHH1508a restriction map. The transposon Tn*7*, which is responsible for the resistance to streptomycin and trimethoprim in pHH1508a, was determined to be flanked by pHH1508a *Xba*I fragments C and E and was contained within *Xho*I fragment C. pHH1508a *Xba*I fragments I, J, and K correspond to the internal fragments of Tn*7* generated by *Xba*I digestion. The deletion mutant pDT1178, which was 96 kb in size and transfer deficient, retained the resistance to trimethoprim, streptomycin, and potassium tellurite. Restriction enzyme digestion of pDT1178 DNA showed that the deletion mutant contained *Xba*I fragments C, D, E, F, I, J, and K of pHH1508a. Similarly, *Xho*I fragments C, F, and K, as well

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TABLE 1. Sizes of *Xba*I and *Xho*I restriction fragments of pHH1508a[a]

Fragments[a]	Size (kb)[b]	
	*Xba*I	*Xho*I
A	54.0	38.0
B	48.0	37.0
C	38.0	34.0
D	25.0	19.0
E	12.7	17.5
F	12.4	17.0
G	7.5	13.0
H	4.4	12.4
I	3.4	7.2
J	2.6	6.5
K	0.8	4.7
L		2.0

[a] Fragments are designated alphabetically in order of size with A being the largest.

[b] Sizes determined with reference to lambda phage DNA digested with various restriction enzymes.

as portions of *Xho*I-B and *Xho*I-D of pHH1508a, were contained in pDT1178.

Information from Tn*7* hybridization studies and knowledge of pDT1178 digestion patterns were used to map the region of pHH1508a DNA which corresponded to the 96-kb region of the deletion mutant. Nick translation and hybridization of extracted DNA fragments were then used for mapping the remainder of the plasmid. The circular restriction map of pHH1508a is shown in Fig. 1. It should be noted that although *Xho*I fragments I, J, and L were located within *Xba*I fragment A, the exact orientation of these three small fragments could not be determined. Similarly, the orientation of *Xho*I fragments E, G, and H within *Xba*I fragment B was not clearly established. After 600 Tn*5* insertion mutants were screened, 7 transfer-deficient mutants of pHH1508a were obtained; all had at least a 10^5-fold reduction in transfer frequency. The frequency of loss of conjugative transfer was 7 of 600, or approximately 1% of all Tn*5* insertions. Hybridization studies showed that insertions into *Xba*I fragments A, B, C, E, and G completely abolished conjugative transfer. The location of these insertions sites (Fig. 1) demonstrated that the genes required for conjugative transfer of pHH1508a are widely distributed throughout the plasmid.

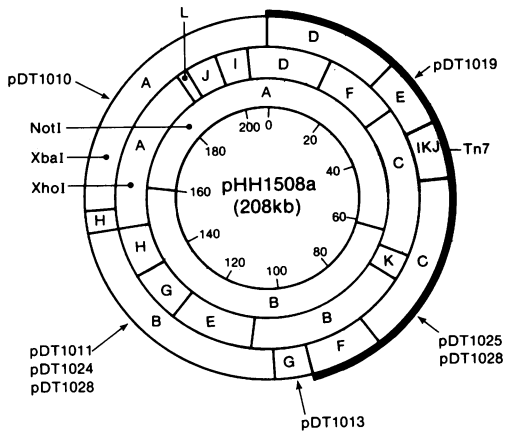

FIG. 1. Physical and genetic map of pHH1508a. Restriction endonuclease cleavage sites and fragments are indicated in the outer circles. These sites were oriented with respect to the *Xba*I site at coordinate 0, and the map is numbered by distance (in kilobases) from that site. The restriction fragments are labeled alphabetically in decreasing order of size, with A being the largest fragment. Locations of Tn*5* insertions are shown by the position of the insertion mutant designations. The location of transposon Tn*7* is indicated by the shaded area. The solid area on the outermost circle represents the 96-kb region of deletion mutant pDT1178.

Although the RNA bacteriophage pilHα produced a clear zone of lysis on brain heart infusion plates of the *E. coli* K-12 strain carrying pHH1508a, no lysis was observed with *E. coli* carrying either the seven Tra$^-$ mutants or the deletion mutant pDT1178. The immunogold labeling experiment, on the other hand, showed the presence of gold-labeled H pili in both pHH1508a and pDT1178 preparations. The number of pili coded for by pDT1178 was, however, much less than the number coded for by the original plasmid pHH1508a. With pHH1508a, gold-labeled H pili were readily observed within any given field. On the other hand, pDT1178 showed the presence of H pili only if many fields were carefully screened. These results indicate that the 96.0-kb region of pHH1508a DNA corresponding to pDT1178 contained the genes required for H pilus production and assembly and that immunogold labeling was a more sensitive technique than pilHα lysis for detection of H pili. From these studies, a circular restriction map of the 208-kb IncHII plasmid pHH1508a was constructed. Like those of the IncHI plasmid R27 (12), the transfer genes of pHH1508a are widely distributed over the entire plasmid. The genes coding for the H pilus, which is an essential component of the overall transfer process, were determined to be located in a 96.0-kb region of pHH1508a. Furthermore, since the deletion mutant pDT1178 is self-replicating, an origin of replication must also be located within this region. Other genes outside this region, however, appeared to be involved in the control of the number of H pili produced, as well as in other plasmid functions essential for conjugative transfer.

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