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Genetic and Nucleotide Sequence Analysis of the Gene *htdA*, Which Regulates Conjugal Transfer of IncHI Plasmids

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IncHI plasmids are naturally repressed for conjugative transfer and do not allow efficient propagation of the IncH pilus-specific phage Hgal. Transposons Tn*7*, Tn*5*, and Tn*lacZ* were inserted into IncHI plasmids R478, R477-1, and R27, respectively, leading to the isolation of several plasmid mutants which exhibited increased levels of transfer and also permitted good lysis with phage Hgal. A 4.3-kb *Hin*dIII fragment from R478 reversed both phenotypic effects of derepression for the R477-1::Tn*5* and the R478::Tn*7* derivatives, pKFW99 and pKFW100, respectively. Exonuclease III deletions of this fragment and nucleotide sequence analysis indicated that the gene responsible for transfer repression, named here *htdA*, encoded a polypeptide of 150 amino acids. Cloning and sequence analysis of pDT2454 (R27::Tn*lacZ*) revealed that the transposon had inserted into an open reading frame (ORF) which had an 83% amino acid identity with the R478 *htdA* gene. Maxicell analysis showed both the R27 and R478 HtdA products had molecular masses of 19.9 kDa. Conjugation experiments showed that the cloned *htdA* determinants caused a significant reduction of the transfer frequencies of wild-type R478 and R27 plasmids. Examination of both R478 derepressed mutants, pKFW100 and pKFW101, indicated that both transposon insertions occurred upstream of the *htdA* ORF. The results suggest that HtdA is a regulatory component of IncH plasmid transfer and also show that the region upstream of the *htdA* ORF is involved in transfer repression. The locations of the *htdA* determinants were identified on the plasmid maps of R27 and R478.

H incompatibility plasmids are divided into subgroups, HI and HII (8). IncHI plasmids are thermosensitive for transfer, having an optimum rate of transfer between 26 and 30°C (25, 35). Pilus production is naturally repressed in IncHI plasmids, though derepressed mutants have been isolated by transposon mutagenesis (7, 32). In contrast, IncHII plasmids are nonthermosensitive for conjugative transfer but are constitutive for pilus synthesis (8). The IncHI group is further subdivided into subgroups IncHI1, IncHI2, and IncHI3 (26, 42). This subdivision is based mainly on DNA homology studies, but the IncHI2 subgroup also encodes several characteristics, not found on IncHI1 plasmids, such as resistance to potassium tellurite and inhibition of certain types of DNA phages (31, 36). The majority of IncH isolates reported belong to either the IncHI1 or IncHI2 subgroup. Restriction and partial genetic maps have been constructed for the IncHI1 plasmid R27 (22, 33), the IncHI2 plasmid R478 (41), and the IncHII plasmid pHH1508a (43).

Analysis of the IncHI1 plasmid R27, using transposons Tn*7* and Tn*5*, has shown that there are two distinct regions, Tra 1 and Tra 2, involved in the control of conjugative transfer of this plasmid (22, 34). Similar analysis of the IncHII plasmid pHH1508a suggests that the transfer genes are spread widely over the plasmid genome (43). Complementation experiments between R27 and pHH1508a have shown that there is some relatedness between the transfer systems of these plasmids (22). The conjugative pili produced by IncHI1, IncHI2, and IncHII plasmids are referred to as H pili (6, 20). Pili produced by the sole member of the IncHI3 group, MIP233, are dissimilar in morphology to the pili produced by the other IncH groups (7). Recent studies on H-pilus formation suggest that H pili are assembled from the base and that full-length pili are formed after 20 min during conditions which favor the production of these pili (20).

Two single-stranded RNA phages, pilHα and Hgal, have been reported to be specific for H pili (10, 19, 23). Both phages are thermosensitive for propagation and have been shown to adsorb to the shafts of these pili. This study describes the isolation, nucleotide sequence, and partial characterization of a transfer gene present in plasmids R478 and R27. Using phage Hgal and derepressed plasmid derivatives, we show here that this gene, *htdA* (H transfer determinant), is involved in the regulation of pilus production in IncHI plasmids.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All the bacterial strains and plasmids used in this study are listed in Table 1 or illustrated in Fig. 1. For plasmid cloning experiments involving the vectors pUC13 and pUC119, transformants were plated on LB medium containing 20 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml, 20 μg of isopropyl-β-D-thiogalactopyranoside (IPTG) per ml, and 100 μg of ampicillin per ml. For cloning experiments using the vector pOK12, transformants were plated on 30 μg of X-Gal, 20 μg of IPTG, and 100 μg of kanamycin per ml. A higher concentration of X-Gal was used for pOK12, as this vector has a modified *lacZ* promoter structure which results in a fainter blue color on X-Gal medium than that produced by strains containing the vector pUC119 or pUC13 (39). Antibiotics were used at the following concentrations in other experiments: kanamycin, 100 μg/ml; tetracycline, 20 μg/ml; nalidixic acid, 30 μg/ml; rifampin, 100 μg/ml; trimethoprim, 50 μg/ml; and chloramphenicol, 30 μg/ml. All antibiotics were obtained from Sigma Chemical Co. *Escherichia coli* strains were grown in tryptone

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference
E. coli K-12 strains		
DH5α	Plasmid host for *lacZ* expression; F^- λ^- Δ(*lacZYA-argF*)U169 ϕ80d*lacZ*ΔM15 *hsdR17 recA1 endA1 gyrA96 thi-1 relA1 supE44*	4
CC170	Donor for Tn*lacZ*; Δ(*ara leu*)*7697* Δ*lacX74* Δ*phoA20 galE galK thi rpsE rpoB argE*(Am) *recA1* Tn*lacZ*(Km)	21
J62::Tn*7*	Donor for Tn*7*; *lac pro his trp* Tn*7*(Sm Sp Tp)	3
DT967::Tn*5*	Donor for Tn*5*; *trp ilv* Tn*5*(Km)	7
J53-1	Donor host strain for conjugations; *proA metF* Nal	2
RG192	Recipient host strain for conjugations; Δ*lac* Δ*ara-leu* Rif	35
CSR603	Maxicell strain; *uvrA uvrB recA*	27
IncH plasmids		
R478	IncHI2 plasmid; Asr, Km, Tc, Cm, Te, Phi, PacB	41
R477-1	IncHI2 plasmid; Sm, Sp, Su, Tc, Asr, Hg, Te, Phi, PacB	35
R27	IncHI1 plasmid; Tc, Cit	22
pKFW99	R477-1::Tn*5*; derepressed for transfer	This study
pKFW100	R478::Tn*7*; derepressed for transfer	This study
pKFW101	R478::Tn*7*; derepressed for transfer	This study
pDT2454	R27::Tn*lacZ*, derepressed for transfer	This study
Vectors		
pUC13	Cloning vector with *lacZ* reporter gene; Ap	38
pOK12	Cloning vector with modified *lacZ* promoter; Km	39
pUC119	Phagemid vector with *lacZ* reporter gene; Ap	29
pUCKW	Cosmid vector; Ap	41
Miniplasmids		
pKFW72	Derived from R478 by using *Sal*I; Cm	41
pKFW107	65- and 4.5-kb *Sal*I fragments of pKFW101; Tp, Sm, Cm	This study
R478 constructs		
pKFW65	pUC13 *Bam*HI clone spanning coordinates 32.6–53.5 of R478[b]	41
pKFW11	pUC13 clone of 4.3-kb *Hin*dIII fragment from R478	This study
pDT2562	pUC119 clone with 6.6-kb *Eco*RI insert from pKFW100; Tp	This study
pDT2563	pUC119 clone with 9.8-kb *Eco*RI insert from pKFW107; Tp	This study
pDT2564	6.2-kb *Sal*I-EcoRI subclone of pDT2462	This study
pDT2565	6.2-kb *Sal*I-*Eco*RI subclone of pDT2463	This study
R27 constructs		
pDT2415	pUC119 clone with 8.5-kb *Sal*I fragment from pDT2454; Km	This study
pDT2416	7.8- and 8.5-kb *Sal*I inserts in pUC119 from pDT2454; Km	This study
pDT2501	6.0-kb *Hin*dIII subclone from pDT2416	This study
pDT2503	pUCKW clone with the 24-kb *Xba*I-D fragment of R27[b]	This study
pDT2504	pUCKW clone of *Xba*I-D::Tn*lacZ* from pDT2454; Km	This study
pDT2505	Opposite orientation of pDT2504; Km	This study
pDT2506	*Bam*HI deletion of pDT2504	This study
pDT2507	*Bam*HI deletion of pDT2505	This study
pDT2508	pUC119 clone containing 8.9-kb *Sal*I from pDT2503	This study

[a] Antibiotic resistances: Nal, nalidixic acid; Rif, rifampin; Tp, trimethoprim; Sm, streptomycin; Ap, ampicillin; Su, sulfonamides. For explanation of others, see the legend to Fig. 6.
[b] R478 and R27 map coordinates as shown in Fig. 6.

soya broth or brain heart infusion (BHI) broth, both obtained from Oxoid.

Propagation of bacteriophage Hgal. The IncH-specific phage, Hgal, was propagated in strain J53-1 harboring a transfer-derepressed IncHI plasmid. The incubation temperature for growth of the host strain and for propagation of Hgal was 22 to 26°C throughout. Two cycles of propagation were found to be required to generate a suitable high-titer stock. Initially, the host strain was grown overnight at 22 to 26°C and then used to seed a standard agar overlay plate. A 15-μl drop of phage suspension was placed on the overlay and incubated overnight. Several agar plugs from the zone of lysis were then placed in a microcentrifuge tube containing 1 ml of BHI broth. The suspension was vortexed for 15 s and then centrifuged at 7,000 rpm for 5 min. The supernatant was removed, and the titer of phage was calculated by standard agar overlay techniques. This temporary phage stock was then immediately used to generate agar overlays exhibiting semiconfluent or complete Hgal lysis. Each plate (three or four were used for one stock) was then flooded with 3 ml of BHI broth and placed at 4°C for 4 h with constant gentle agitation. The resulting supernatant was removed, centrifuged at 7,000 rpm for 10 min, and transferred to a fresh sterile container, and chloroform was added at a final concentration of 2% (vol/vol). Phage stocks produced by this procedure were stable for 1 year when stored at 4°C. We have found that SM buffer, which is the normal buffer for λ (29) and many other bacteriophage, was not suitable for even very short term storage of Hgal.

DNA manipulations. All plasmid DNA was isolated by the Birnboim and Doly procedure (5) followed by CsCl-ethidium bromide density gradient ultracentrifugation. Restriction enzymes were obtained from Boehringer Mannheim Biochemicals or GIBCO/BRL. T4 DNA ligase was purchased from GIBCO/BRL. Restriction digestions and DNA ligations were performed as recommended by the manufacturers. DNA manipulations, transformations, and agarose gel electrophoresis

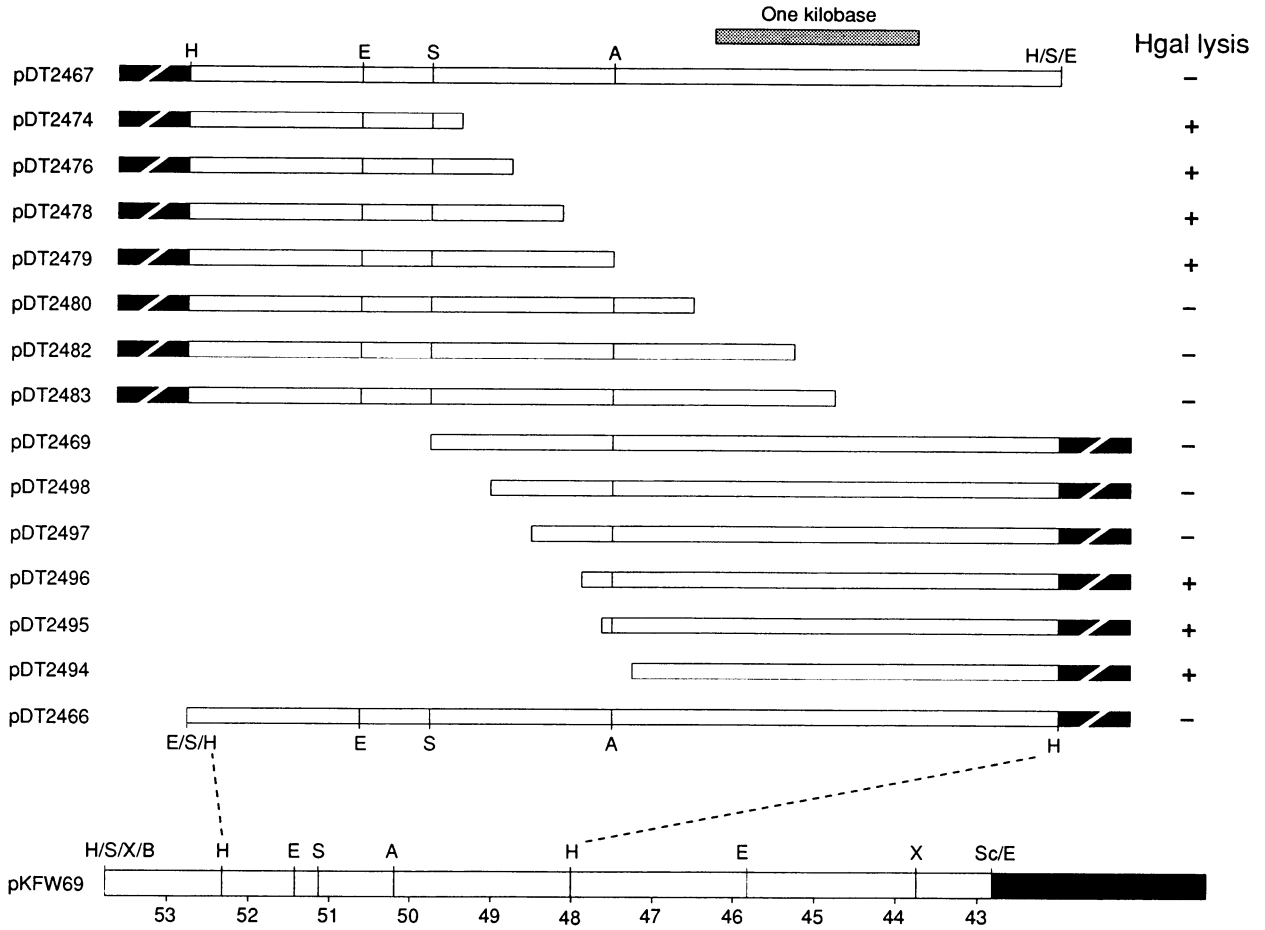

FIG. 1. Exonuclease deletion clones of the 4.3-kb R478 fragment used in nucleotide sequencing and complementation analysis. Exonuclease deletions were generated in clones pDT2466 and pDT2467 as described in Materials and Methods. The symbols + and − at the right refer to the ability of phage Hgal to lyse strain J53-1(pKFW99) containing each derivative (parent clone or exonuclease III deletion thereof). The shaded section is the scale for all plasmids except pKFW69, and maps are drawn to this scale except for the pUC119 sections, which are shown as truncated black regions. The position of the 4.3-kb *Hin*dIII fragment in clone pKFW69 is indicated. pKFW69 is in vector pUC13 (black region), and the scale shown underneath the plasmid map is coordinated according to the R478 restriction map (Fig. 6). E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; S, *Sal*I; Sc, *Sac*I; X, *Xba*I; A, *Apa*I.

were carried out by standard procedures (29). IncH miniplasmids were generated as described previously (41) and were electroporated into strain DH5α, using a Bio-Rad gene pulser (11).

Transposon mutagenesis and conjugation techniques. The procedure used for transposon mutagenesis in this study was based on that described previously by Bradley (7), except that transfer derepression was evaluated by using the pilus-specific phage Hgal. The IncH plasmids selected (Table 1) were first conjugated into the strains containing a transposon inserted into the bacterial chromosome. The plasmids were then conjugated from these transposon-containing strains into strain J53-1. Transconjugants containing Tn*5* or Tn*lacZ* plasmid inserts were selected by expression of kanamycin resistance, while Tn*7* insertions were isolated by expression of trimethoprim resistance. These transconjugants were then screened for increased frequency of transfer and lysis with phage Hgal.

Conjugation frequencies were calculated by using J53-1 as a donor host strain and RG192 as a recipient. All plates were made in triplicate, and the average frequency obtained from at least two experiments is reported. Donor and recipient strains were grown in BHI broth for 6 h at 28°C and 37°C, respectively; 100 μl of each donor culture was then mixed with 400 μl of recipient and 500 μl of prewarmed BHI broth. The conjugation mixtures were incubated at 28°C for 2 h. Frequencies of transfer were calculated relative to the number of viable donor cells in each conjugation.

Generation of deletions with exonuclease III. The procedure used for production of deletions by using exonuclease III was a modified version of that described by Sambrook et al. (29). S1 nuclease and exonuclease III were obtained from GIBCO/BRL. The 4.3-kb *Hin*dIII fragment from pKFW11 was first transferred to the vector pOK12. The fragment was then inserted in pUC119, and exonuclease III deletions were made for both cloned orientations. Ten micrograms of plasmid DNA in a total volume of 80 μl was digested overnight with restriction enzymes *Sac*I and *Bam*HI. When complete digestion was verified, 400 U of exonuclease III was added directly to the digested DNA. Five-microliter samples were removed at intervals over a total time period of 50 min and mixed with 15 μl of S1 reaction mixture on ice. S1 reaction mixture consists of (per 15 μl) 0.3 M NaCl, 40 mM potassium acetate (pH 4.5), 2.5 mM $ZnSO_4$, 6% glycerol, and 0.5 U of S1 nuclease. Samples

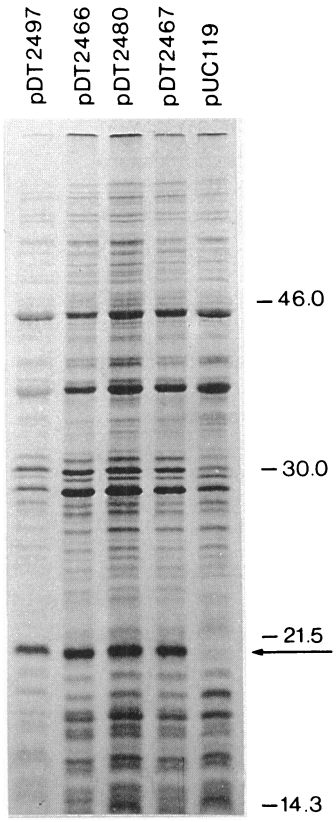

FIG. 2. Detection of gene products by the maxicell analysis. *E. coli* cells harboring each plasmid were pulse-labeled with [^{35}S]methionine, using the maxicell procedure (Materials and Methods). The arrow shows the product of *htdA* from both R478 4.3-kb parent clones and the two smallest deletion plasmids defined by complementation experiments as retaining the functional repressor phenotype (Fig. 1). Plasmid pUC119 was used as a control. Molecular masses are indicated by the markers (in kilodaltons) on the right.

were then incubated at 30°C for 30 min. After addition of 2 μl of S1 stop buffer (0.3M Tris base, 50 mM EDTA [pH 8.0]), the samples were incubated at 65°C for 10 min. Following the S1 nuclease step, each three successive samples were pooled and ethanol precipitated. The dried pellets were each resuspended in 80 μl of sterile H_2O, ligated, and finally transformed into strain DH5α.

DNA sequencing and analysis. Nucleotide sequences for both strands were determined by the Sanger chain termination method (28) on denatured double-stranded plasmid DNA, using a Sequenase 2.0 kit from United States Biochemical. [α-^{35}S]dATP was purchased from NEN/Dupont. Sequencing involving the exonuclease deletion plasmids (Fig. 1) was carried out with the standard pUC/M13 forward primer. Sequencing from the ends of transposon Tn*5* was done with the primer 5′GGTTCCGTTCAGGACGCTAC3′, using clones pDT2415 and pDT2501. This primer is complementary to part of the inverted repeat sequence of Tn*5*. The reverse-strand sequences were obtained with primers 5′ATCTCTATGCTT TACTG3′ and 5′CGCCACTGTTAAACCAA3′. DNA sequence analysis was performed with the Beckman Microgenie program (24). Protein analysis, nucleic acid homologies, and protein homologies were determined with GCG software (Genetics Computer Group Inc., University of Wisconsin, Madison).

Primer extension. RNA isolation was carried out as described previously (1). The synthetic oligonucleotide used, 5′TGGTCTCGGCGTAAACCATGTATTAGTGAG3′, was complementary to residues +14 to +43 relative to the ATG start codon. Labeling of the primer and primer extension analysis were performed as described previously (1). Avian myeloblastosis virus reverse transcriptase was obtained from Promega. The extended products were electrophoresed next to the corresponding Sanger sequencing reactions on a 7% sequencing gel.

Plasmid protein identification. Plasmid proteins were identified by the *E. coli* maxicell procedure (27). The [^{35}S]methionine-labeled proteins were analyzed on 15% polyacrylamide gels containing 0.1% sodium dodecyl sulfate as described by Laemmli (18).

Nucleotide sequence accession numbers. The sequences of the R478 and R27 *htdA* genes have been assigned Genbank accession numbers L20341 and L20342, respectively.

RESULTS

Transfer-derepressed IncHI plasmids. Bacteriophage Hgal plates poorly on cells carrying repressed IncHI plasmids. A number of such plasmids were subjected to transposon mutagenesis, and the plasmids containing these insertions were screened for those which allowed good lysis with Hgal. R477-1 was mutagenized with Tn*5*, as this IncHI2 plasmid carries many antibiotic resistance markers (Table 1) but does not encode kanamycin resistance. Several tranconjugants were obtained from DT967::Tn*5*(R477-1), one of which, J53-1 (pKFW99), was selected for further work. R478, also IncHI2, carries a kanamycin resistance marker (Table 1) and hence was mutagenized with Tn*7*. Six tranconjugants were isolated from J62::Tn*7*(R478), which displayed good to excellent lysis with Hgal. Two of these, pKFW100 and pKFW101, which came from separate experiments, were chosen for further study since they exhibited the best levels of transfer. R27, an IncHI1 plasmid, had previously been mutagenized with Tn*7* and Tn*5* (32, 34), yet no suitable derepressed mutants were obtained. Hence, in the current study, Tn*lacZ* was used for R27 mutagenesis. Several R27::Tn*lacZ* isolates which showed increased levels of transfer were obtained, and one of these, pDT2454, which allows a clear zone of lysis with Hgal, was chosen for further analysis.

Some of the subsequent experiments in this work involved the R477-1::Tn*5* mutant, pKFW99, since this was the only isolate available for some time. When R478 was found to be derepressable, and suitable R478::Tn*7* derivatives were selected, the usefulness of pKFW99 diminished. However, as will become evident later, the choice of pKFW99 was critical to the initial identification of the cloned repression determinant.

Cloning of R478 DNA fragments which repressed Hgal lysis. DNA banks of plasmid R478 were generated to test whether there was a wild-type function which repressed transfer and Hgal growth. The bank of clones was screened for those which reversed the ability of a host carrying the derepressed mutant derivative of R477-1, pKFW99, to serve as a good host for Hgal. Two clones isolated by this screen shared a common 4.3-kb *Hin*dIII fragment, the map of which is shown in Fig. 1. One of these clones, pKFW11, contained just this insert and hence was chosen for further study. An initial experiment indicated that the transfer of pKFW99 decreased by a factor of 10^3 when pKFW11 was present in the host strain. Therefore, both the increased plating of Hgal and the high transfer frequency of pKFW99 (in J53-1) were suppressed by an element within this 4.3-kb fragment. We named this repression

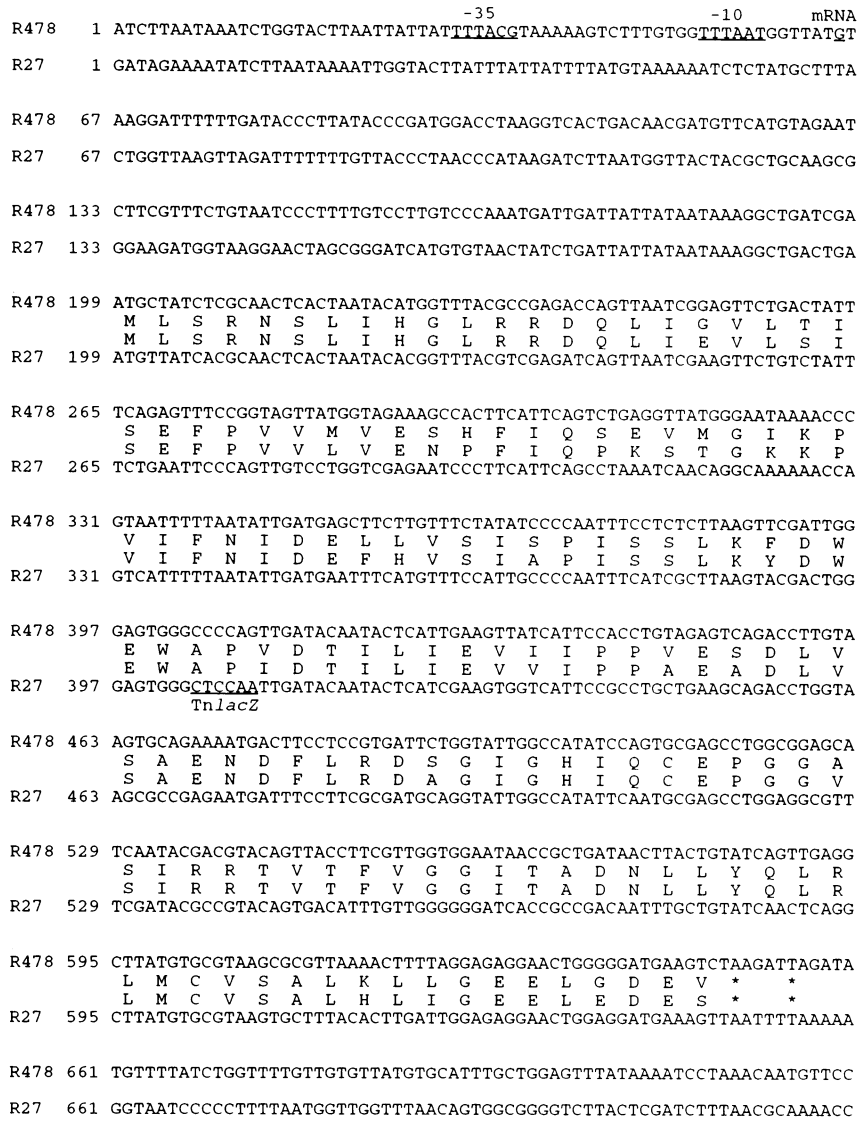

FIG. 3. Nucleotide sequences of the R478 and R27 *htdA* genes. The predicted amino acid sequences start at nt 199 for both plasmids. Asterisks indicate stop codons. The transcription start point and putative −10 and −35 promoter regions are underlined for the R478 sequence. The promoter for the R27 sequence was not identified. The site of insertion of Tn*lacZ* in pDT2454 is underlined in the R27 sequence.

phenotype HtdA. The fragment was transferred to the vector pUC119 in both orientations and deleted with exonuclease III (Fig. 1). Analysis of these deletions by using HgaI indicated that the minimal region which repressed HgaI was an 800-bp stretch of DNA common to the opposing deletion clones pDT2480 and pDT2497 (Fig. 1).

Nucleotide sequence and protein analysis of R478 *htdA*. The minimal region identified as containing the R478 *htdA* was examined by maxicell and nucleotide sequence analysis. The maxicell procedure detected a single protein of 19.9 kDa which was common to both deletion clones, pDT2497 and pDT2480 (Fig. 2). The nucleotide sequence of this region revealed one open reading frame (ORF), from nucleotides (nt) 199 to 648 (Fig. 3), which could encode a 150-amino-acid polypeptide with a predicted molecular mass of 16.6 kDa. This ORF was the only one large enough to reasonably agree with the molecular mass estimated by gel electrophoresis. The methionine residue located at nt 199 appears to be the most suitable candidate for initiation of translation of a protein of this size, although there are other methionine residues located further downstream. By using primer extension analysis and an oligonucleotide which was complementary to a region of DNA just downstream of the putative initiation codon, an extension product which ended at nt 65 was produced (Fig. 4). Seven nucleotides upstream of nt 65 (Fig. 3), there is a region which agrees with 5 of 6 nt of the consensus −10 sequence (15). A further 17 nt upstream of this putative −10 element is a possible −35 sequence which agrees with 4 of 6 nt of the consensus −35 motif (15).

Complementation analysis. Six clones derived from R478 (both orientations of the 4.3-kb *Hin*dIII fragment, the two largest deletions which contained the complete *htdA* ORF [*htdA*$^+$], and the two opposite deletions which disrupted *htdA* [Fig. 1]) were transformed into strains harboring IncHI plas-

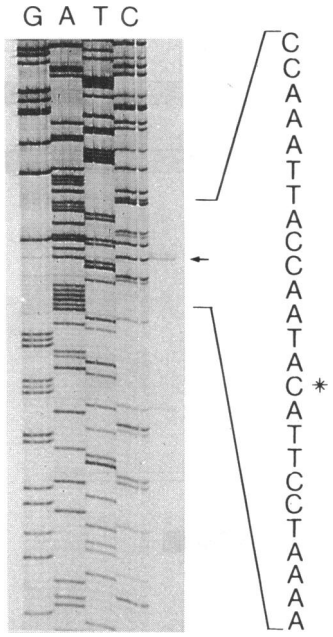

FIG. 4. Primer extension analysis. The primer extension shown was performed on RNA isolated from DH5α(pDT2480). The antisense primer used corresponded to nt 213 to 242 of the R478 *htdA* sequence (Fig. 3). The right lanes contain the DNA sequence ladder produced by the primer and pDT2480 plasmid DNA. The arrow indicates the 5′ end of the *htdA* mRNA. An asterisk is placed beside the end point in the corresponding DNA sequence.

mids or the phage-sensitive (transfer-derepressed) mutant derivatives. The objective of this experiment was to determine whether the region defined as suppressing Hgal plating (Fig. 1) would also reduce transfer of these plasmids in 2-h matings. It also allowed us to ascertain whether the IncHI2 gene from R478 could complement the derepressed phenotypes of pKFW101 and pKFW100, the R478::Tn*7* derivatives, and pDT2454, the R27::Tn*lacZ* derivative. When the plasmid clones were introduced into cells containing pKFW99, all those carrying the intact *htdA* ORF decreased transfer of pKFW99 significantly (Table 2). In particular, the *htdA*$^+$ clones, pDT2480 and pDT2497, which are deleted for neighboring material on either the 5′ or 3′ end of the gene (Fig. 1), reduced transfer of pKFW99 by more than a factor of 10^3. Table 2 shows that while transfer frequencies of pKFW101 and pDT2454 were decreased by all R478 clones containing the complete ORF, the reductions were not as great as seen with pKFW99 and were not similar to the transfer rates of the wild-type plasmids (in the absence of any *htdA*$^+$ clone). Complementation experiments with the other R478 derepressed mutant, pKFW100, showed that transfer of this derivative was decreased by a factor of 10^2 by the *htdA*$^+$ clones and thus was phenotypically like pKFW99. Finally, introduction of the *htdA*$^+$ clones into hosts carrying plasmid R478 or R27 resulted in an even further repression of transfer of these wild-type plasmids (Table 2).

Cloning and sequence analysis of the Tn*lacZ* insertion in the R27 derivative pDT2454. Restriction analysis of pDT2454 indicated that Tn*lacZ* had inserted into an 8.9-kb *Sal*I fragment of R27. As there is only one *Sal*I site in Tn*lacZ*, we decided to use this restriction enzyme to subclone all or part of the transposon and the surrounding regions of R27. The DNA bank generated was transformed into DH5α with selection for kanamycin-resistant transformants. Two selected clones, pDT2415 and pDT2416, had common 8.5-kb inserts (Fig. 5). Also, pDT2416 contained the complete transposon, but the orientations of the *Sal*I fragments were not contiguous (Fig. 5).

Nucleotide sequencing indicated that the transposition in pDT2454 occurred in a region encoding an ORF which closely resembled the R478 ORF (Fig. 3). The R478 and the predicted R27 ORFs are identical in length (450 bp), and 79.1% of the nucleotides and 83.3% of the amino acid residues are identical. At the ends of both ORFs are double stop codons separated by 3 nt. There is 96% homology between the 25 nt preceding nt 199 for the R478 and R27 determinants but not for the region specifying the putative R478 promoter. Primer extension experiments with the R27 determinant were not successful. The R478 and R27 sequences were used to search recent data banks, but no significant homologies with any previously known DNA or protein sequences were detected.

Mapping of the transposon insertion in R27. It was shown previously (22) that there are two 8.9-kb *Sal*I fragments in R27 and only one, *Sal*I-H1, is mapped to the *Xba*I-D fragment (Fig. 6). In pDT2454, Tn*lacZ* was therefore in the *Sal*I-H1 fragment of R27, since the *Xba*I-D fragment was also disrupted by the transposon. However, there are no internal restriction sites mapped in this *Sal*I fragment, so further cloning was required to determine the exact location of Tn*lacZ*. Cosmid banks of R27 and pDT2454 were made by using the enzyme *Xba*I. We isolated from R27 one clone, pDT2503, which contained only the *Xba*I-D fragment of R27 together with an unknown multiple of the vector section. Two other kanamycin-resistant

TABLE 2. Complementation of IncH plasmids and derepressed derivatives by various repression clones and deletions

Plasmid	Description[a]	Transfer frequency[b]					
		R478	pKFW101 (R478::Tn*7*)	pKFW100 (R478::Tn*7*)	R27	pDT2454 (R27::Tn*lacZ*)	pKFW99 (R477-1::Tn*5*)
None	No introduced clone or deletion	2.4×10^{-3}	3.0×10^{-2}	1.1×10^{-2}	7.7×10^{-5}	5.0×10^{-1}	3.4×10^{-2}
pDT2467	Complete 4.3-kb R478 clone	2.3×10^{-5}	5.3×10^{-3}	2.7×10^{-4}	1.0×10^{-6}	1.9×10^{-2}	5.6×10^{-3}
pDT2480	1.8-kb deletion of pDT2467 (intact ORF)	7.5×10^{-7}	1.3×10^{-3}	2.8×10^{-5}	$<6.0 \times 10^{-7}$	4.4×10^{-2}	3.8×10^{-6}
pDT2479	2.2-kb deletion of pDT2467 (ΔORF)	3.1×10^{-4}	4.5×10^{-2}	1.8×10^{-2}	3.0×10^{-5}	7.6×10^{-1}	1.7×10^{-3}
pDT2466	Reverse clone of pDT2467	6.0×10^{-5}	3.2×10^{-3}	1.6×10^{-4}	1.1×10^{-6}	1.5×10^{-2}	3.0×10^{-3}
pDT2497	1.7-kb deletion of pDT2466 (intact ORF)	5.1×10^{-7}	4.8×10^{-3}	8.3×10^{-5}	$<4.4 \times 10^{-7}$	8.9×10^{-3}	4.9×10^{-5}
pDT2496	1.95-kb deletion of pDT2466 (ΔORF)	8.3×10^{-3}	3.5×10^{-1}	6.1×10^{-2}	4.6×10^{-4}	8.4×10^{-1}	2.9×10^{-2}
pDT2508	8.9-kb *Sal*I clone from R27	2.1×10^{-5}	7.0×10^{-4}	1.8×10^{-2}	1.3×10^{-5}	1.8×10^{-2}	3.5×10^{-5}
pDT2416	Tn*lacZ* clone from pDT2454	8.4×10^{-3}	ND	ND	2.3×10^{-3}	ND	ND

[a] ORF and ΔORF refers to the 450-nt open reading frame depicted in Fig. 3. Restriction maps of clones pDT2508 and pDT2416 are shown in Fig. 5.
[b] Calculated per donor cell after a 2-h mating at 28°C; average of at least two separate matings.

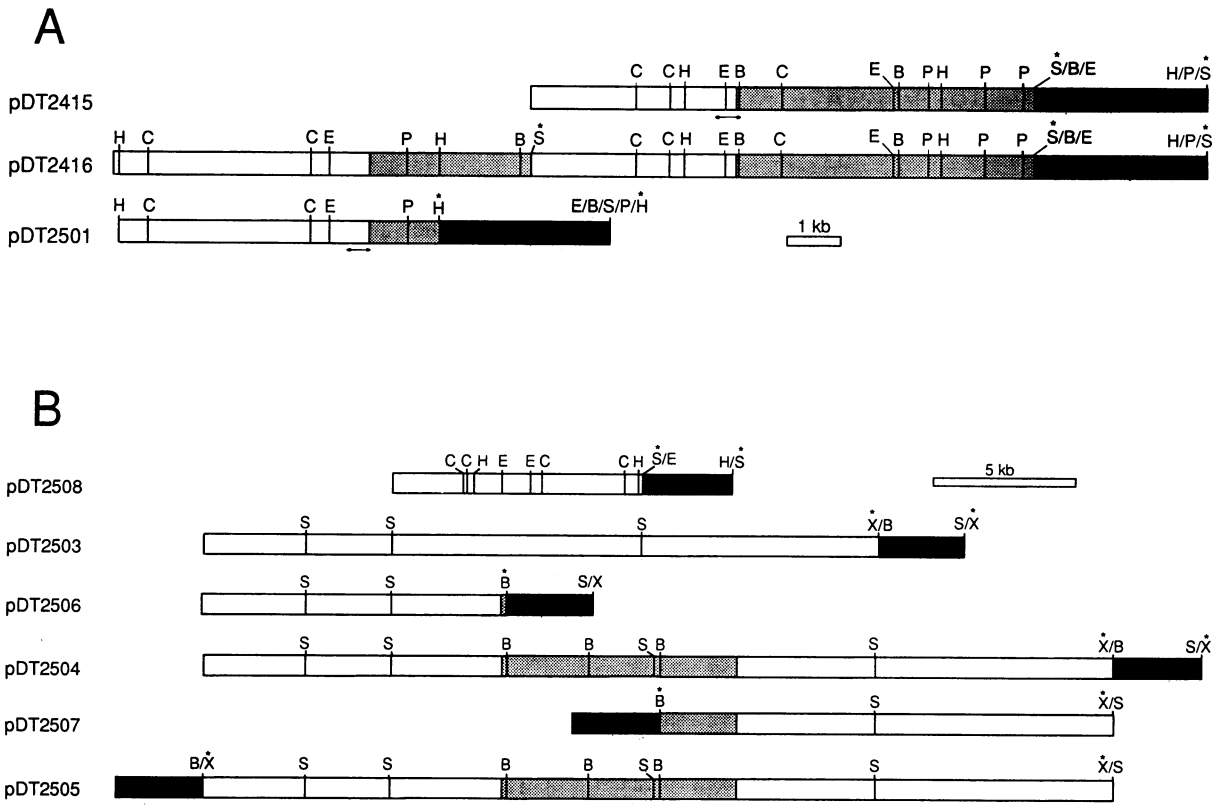

FIG. 5. Restriction maps of pDT2454 plasmid clones used for DNA sequencing of the R27 *htdA* gene (A) and maps of R27 and pDT2454 cosmid clones and derivatives used for isolating and more detailed mapping of the gene (B). The shaded areas show regions which were Tn*lacZ* derived. Derivatives pDT2503 to -7 were cloned into the cosmid vector pUCKW (black area) and were mapped only with the enzymes *Sal*I, *Xba*I, *Apa*I, and *Bam*HI. Plasmid clones were further mapped with *Cla*I, *Eco*RI, and *Hin*dIII. The vector for all plasmid clones in the diagram was pUC119 (black area). pDT2508 contains the 8.9-kb *Sal*I fragment subcloned from pDT2503. Asterisks indicate enzyme sites used in construction of clones. Arrows show the regions that were sequenced. B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; X, *Xba*I.

colonies, isolated from pDT2454, were found to contain the same insert cloned in opposite directions, as well as multiples of vector fragments. Comparison with pDT2416 and pDT2503 showed that both latter cosmid clones, designated pDT2504 and pDT2505, had the *Xba*I-D fragment containing a Tn*lacZ* insertion (Fig. 5). No internal *Bam*HI sites were detected in pDT2503, but there are mapped sites in Tn*lacZ* (Fig. 5). Deletions were generated by using *Bam*HI in both pDT2504 and pDT2505. Comparisons between these deletions and the map of pDT2416 identified the exact position of the transposon insertion into R27 (Fig. 5), thus indicating the location and orientation of the R27 *htdA* gene (Fig. 6).

The *Sal*I-H1 fragment from pDT2503 was subcloned into pUC119 (Fig. 5), and both this clone, pDT2508, and the corresponding clone with the Tn*lacZ* insertion, pDT2416, were used in transfer experiments with the plasmids shown in Table 2. Clone pDT2508 repressed transfer of several plasmids (in particular R478 and pKFW99), with transfer frequencies reduced by factors of up to 10^3. Clone pDT2416 increased transfer of R27 by a factor of 10^2 but had no effect on the transfer of R478 (Table 2). Both clones were subjected to maxicell analysis (Fig. 7). Clone pDT2508 produced a protein of 19.9 kDa. A similar-size protein was not observed in the maxicell profile of pDT2416 (Fig. 7).

Mapping of transposon insertions and *htdA* in R478. Restriction analysis of plasmids pKFW101 and pKFW100 showed that Tn*7* had transposed into the 11.3-kb *Apa*I-H fragment of R478 (Fig. 6). This fragment is present within a 51-kb *Sal*I fragment of R478, which has been shown to contain an origin of replication (41). Using this information, we constructed trimethoprim-resistant (mediated by Tn*7*) miniplasmids from pKFW101, using *Sal*I. The most useful isolate was pKFW107 (Table 1), since it was identical (except for the Tn*7* insertion) to the previously mapped R478 miniplasmid, pKFW72 (41). Comparison between pKFW107 and pKFW72 showed that the location of the transposon corresponded approximately with coordinate 49.5 in the map of R478 (Fig. 6). Since plasmid pKFW65 spans this region of R478 (41), it was digested with *Sac*I and religated to obtain the smaller derivative, pKFW69. Analysis of pKFW69 (Fig. 2) confirmed the position of the same 4.3-kb *Hin*dIII fragment that we had cloned in pKFW11 (the original R478 clone) and thus located *htdA* in R478. Therefore, the insertion site in pKFW101 was close to or within the *htdA* ORF. On the basis of this mapping and the published map of Tn*7* (14), two trimethoprim-resistant 6.2-kb *Sal*I-*Eco*RI constructs, pDT2564 and pDT2565 (Fig. 8 and Table 1), were then obtained from pKFW100 and pKFW101, respectively. Maxicell analysis showed that both clones still produced the complete 19.9-kDa protein (Fig. 7). Sequence analysis of the two clones revealed that the Tn*7* insertion in pKFW100 was 290 bp upstream of the initiation methionine residue of *htdA* (Fig. 8) and that the insertion in pKFW101 was 248 bp upstream of the same residue (Fig. 8).

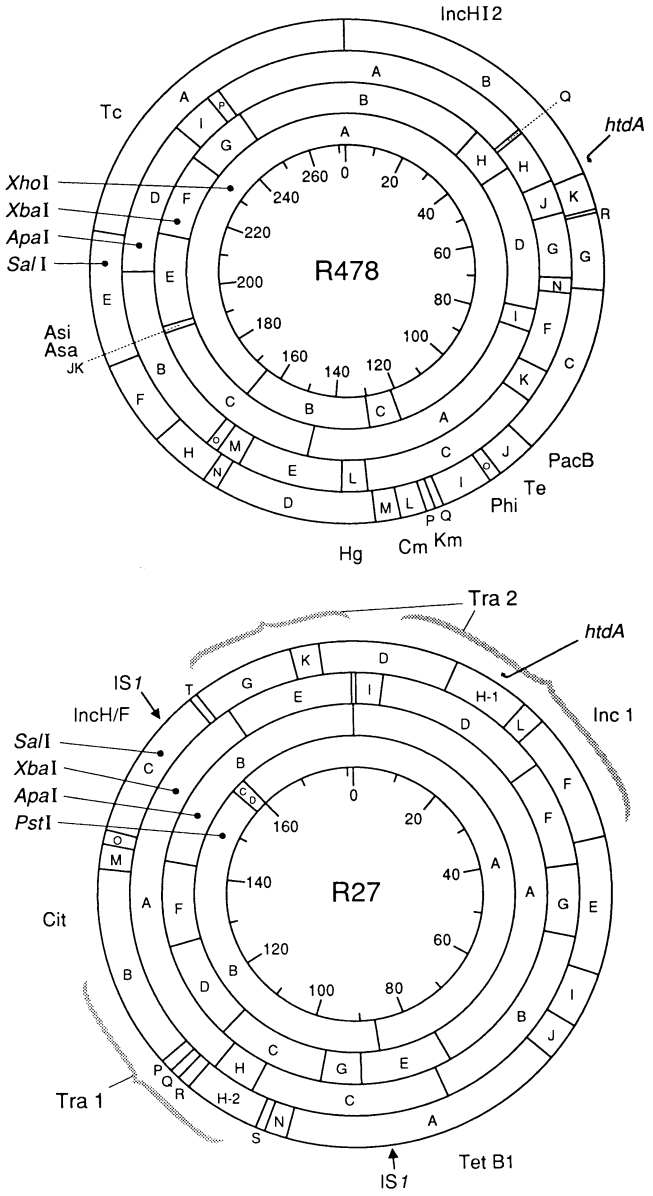

FIG. 6. Genetic maps of plasmids R478 and R27 showing the location of the transfer repression determinant, *htdA*. Each restriction enzyme circle is divided into alphabetically labeled fragments, in decreasing order of size, with A being the largest fragment. The R478 map is oriented with respect to the *Sal*I site at coordinate 0. The R27 map is oriented from the *Apa*I site at coordinate 0. Both maps are scaled in kilobases. The R27 *Sal*I map has been modified as a result of finer mapping of *Xba*I-D in cosmid pDT2503. The shaded sections outside the R27 map show areas of transposon mutagenesis which resulted in a transfer-defective phenotype. The direction of transcription of *htdA* is shown by arrowheads. Abbreviations for resistance determinants: Tc and Tet B1, tetracycline; Cm, chloramphenicol; Te, potassium tellurite; Km, kanamycin; Asi, arsenite; Asa, arsenate; Hg, mercuric chloride; PacB, colicin B. Other abbreviations: IS*1*, insertion element; Phi, bacteriophage inhibition; Cit, citrate utilization; IncH/F, IncHI2 and Inc1 are incompatibility regions.

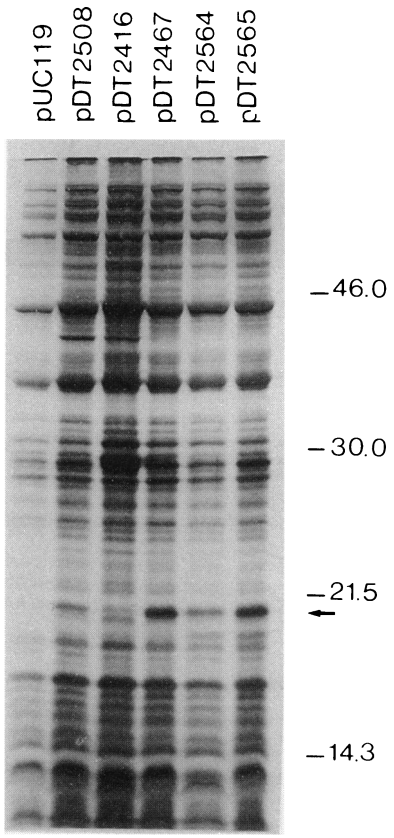

FIG. 7. Maxicell analysis of gene products from transposon insertion clones. *E. coli* cells harboring each plasmid were pulse-labeled with [^{35}S]methionine, using the maxicell procedure (Materials and Methods). pDT2508 consists of the 8.9-kb *Sal*I fragment from R27, while pDT2416 has the identical fragment with Tn*lacZ* inserted. pDT2467 has the 4.3-kb fragment from R478 (which produces HtdA [Fig. 2]). Both pDT2564 and pDT2565 contain the left end of Tn*7* fused with a region (1.3 kb) surrounding *htdA*, which were cloned from pKFW100 and pKFW101, respectively. The arrow indicates the position of the 19.9-kDa product of *htdA*. All clones are in the vector pUC119, which was included as a control. Molecular masses are indicated by the markers (in kilodaltons) on the right.

DISCUSSION

This work describes the isolation and partial characterization of the gene, *htdA*, found on the IncHI2 plasmid R478 and the IncHI1 plasmid R27. R27 and R478 are the prototype plasmid members of their respective subgroups and have been previously shown to have little DNA homology (42). The *htdA* determinant is shown in this study to encode a protein which is involved in the regulation of conjugative transfer of these plasmids. This represents the first genetic analysis of a transfer-related gene from this group of plasmids.

Previous work has shown that derepression of conjugal transfer in R27 is due to increased pilus production (19). The present study shows that derepressed IncH plasmids can be quickly detected by using the pilus-specific phage Hgal rather than time-consuming transfer frequency experiments. R478 HtdA was initially identified by reversal of derepression in the R477-1::Tn*5* mutant, pKFW99. Conjugation studies indicated that R478 *htdA* repressed in *trans* the transfer of the wild-type R478 plasmid and its derepressed derivative pKFW100, as well as pKFW99, with transfer frequencies decreasing by up to a

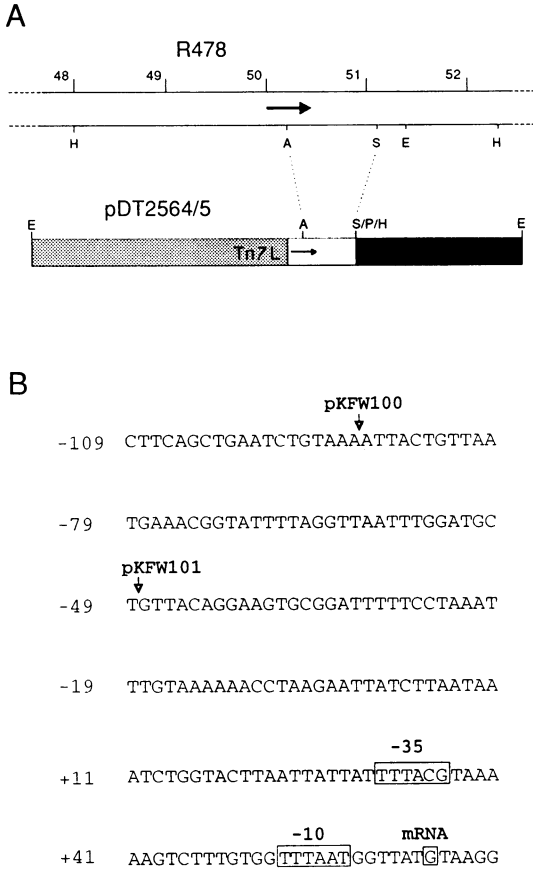

FIG. 8. Maps of constructs pDT2464 and pDT2465 cloned from the derepressed R478 derivatives. (A) pKFW100 and pKFW101; (B) insertion sites of Tn7 in the corresponding R478 nucleotide sequence which generated the derepressed phenotypes. The section of the R478 map shown is scaled in kilobases. The two horizontal arrows show the size and the direction of transcription of *htdA*. The maps of both clones pDT2564 and pDT2565 are represented by one illustration, as they are indistinguishable by agarose gel electrophoresis. The left arm of Tn7 (Tn7L) is displayed by a shaded box. The cloning vector pUC119 is shown by the black box. Restriction sites: E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; A, *Apa*I. The R478 sequence shown is upstream of that depicted in Fig. 3, and the nucleotides are numbered (on the left) relative to the start point previously defined (Fig. 3). The putative promoter regions (boxed) are indicated. Vertical arrows show the sites of transposon insertion.

factor of 4×10^4 (Table 2). Transfer of the wild-type R27 was also reduced by these clones, but since this plasmid transferred at a low frequency after 2-h matings, the maximum levels of repression of R27 by some derivatives could not be ascertained. However, the presence of a plasmid carrying the *htdA* gene in hosts with the R478 and R27 derepressed derivatives, pKFW101 and pDT2454, had only a minor decrease on the transfer rate of these plasmids (Table 2), and thus the selection of pKFW99 for the original identification of HtdA was fortunate. In pDT2454, *htdA* was shown to be interrupted by the insertion of Tn*lacZ* (Fig. 3). Analysis of the transpositions in pKFW101 and pKFW100 indicated that both derivatives still produced HtdA (Fig. 7). Therefore, disruption of the gene in R27 leads to transfer derepression, but transpositions in the region 250 to 290 nt upstream of the *htdA* ORF in R478 (Fig. 8) also cause derepression. This re-repression of certain derivatives by the cloned determinant shows that HtdA is involved in IncH plasmid transfer regulation. Given that the transposition in pKFW100 is further upstream than in pKFW101 and the reintroduced HtdA efficiently represses transfer of pKFW100 but not pKFW101 (Table 2), then the region between 250 and 290 nt upstream of the ORF is also involved in transfer repression. It may be that a second transcript originates from this region, which is necessary for the other IncH transfer components. The methodology used in this study selected only for derepressed derivatives, and therefore the possibility exists that other transpositions in this region can abolish transfer. In the IncF plasmid system, most of the transfer genes are encoded by one transcript, whose transcription is both positively and negatively regulated by several components (12, 16, 17, 30). One of these genes, *finO*, produces a 22-kDa protein which is required for full repression of IncF transfer (13, 37). Insertion of an IS*3* element into *finO* in the F plasmid results in transfer derepression (9, 44). Both the largest R478 clones, pDT2466 and pDT2467, were less effective at repressing transfer than the deleted derivatives which still produced HtdA (Table 2). The deletion pDT2480 contains *htdA* and downstream sequence but not any of the region transposed into the pKFW100 or pKFW101. Additional transfer genes may be present in the larger clones (but absent in pDT2480), which negated repression in the complementation experiments. The R27 clone pDT2416 (containing *htdA*::Tn*lacZ*) increased transfer of R27 significantly, which supports the possibility of other transfer genes close to *htdA*. Maxicell analysis showed that both the R27 and R478 HtdAs were 19.9-kDa proteins. Comparison of the R478 and R27 *htdA* sequences revealed a high degree of amino acid homology, having 126 of 150 of the same amino acid residues (Fig. 3). In the region from nt 493 to 611, 23 of the nucleotides are nonidentical, yet all but one of the amino acid residues are identical. This suggests that the region near the C terminus may be important to the function of both plasmid proteins. Both determinants were mapped on the restriction maps of R27 and R478. The R27 gene is located at coordinate 17.5 (Fig. 6), which is in the previously identified Tra 1 region of this plasmid. Analysis of a previously reported R27::Tn*7* derivative (34) suggested that there was a 1-kb insertion into the 24-kb *Xba*I-D fragment of R27 which caused transfer derepression, though the exact location of this insertion was not ascertained. In the present study, the site of Tn*lacZ* which resulted in derepression in this study is also in the *Xba*I-D fragment. The R478 gene is located at coordinate 50 (Fig. 6) within a large transfer operon, the extent of which has not yet been fully defined (40).

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