

# Gene 19 of Plasmid R1 Is Required for Both Efficient Conjugative DNA Transfer and Bacteriophage R17 Infection

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**F-like plasmids require a number of genes for conjugation, including *tra* operon genes and genes *traM* and *traJ*, which lie outside the *tra* operon. We now establish that a gene in the “leading region,” gene 19, provides an important function during conjugation and RNA phage infection. Mutational inactivation of gene 19 on plasmid R1-16 by introduction of two nonpolar stop codons results in a 10-fold decrease in the conjugation frequency. Furthermore, infection studies with the male-specific bacteriophage R17 revealed that the phage is not able to form clear plaques in *Escherichia coli* cells carrying an R1-16 plasmid with the defective copy of gene 19. The total number of cells infected by phage R17 is reduced by a factor of 10. Both the conjugation- and infection-attenuated phenotypes caused by the defective gene 19 can be complemented in *trans* by introducing gene 19 alleles encoding the wild-type protein. Restoration of the normal phenotypes is also possible by introduction of the *pilT* gene encoded by the unrelated IncI plasmid R64. Our functional studies and similarities of protein 19 to proteins encoded by other DNA transfer systems, as well as the presence of a conserved motif in all of these proteins (indicative for a putative muramidase activity) suggest that protein 19 of plasmid R1 facilitates the passage of DNA during conjugation and entry of RNA during phage infection.**

Bacterial conjugation is one of the major processes by which genetic material is transferred between bacteria. This type of horizontal gene transfer provides the basis for the rapid spread of antibiotic resistance genes among bacterial populations. The importance of gene transmission within the microbial population is becoming increasingly apparent as multidrug-resistant bacteria are emerging at an alarming rate (7). Plasmid R1 is a conjugative resistance plasmid belonging to the incompatibility class IncFII (19). Genes required for conjugation are organized in the so-called resistance transfer region. This region is very similar to the transfer region of the related F plasmid, for which complete sequence information is available (12). On the basis of their functions, the transfer genes can be classified as genes required for (i) pilus synthesis and assembly, (ii) control of transfer gene expression, (iii) surface exclusion, (iv) aggregate stability, or (v) signal, origin nicking, unwinding, and transport. Extensive sequence comparisons suggest relationships between F transfer proteins and proteins from other DNA transfer systems, such as the *Agrobacterium tumefaciens* Ti system (21).

The current definition of the transfer region is that it comprises the large transfer (*tra*) operon and the genes *traM*, *traJ*, *finP*, and *finO*, as well as the origin of conjugative transfer (*oriT*). In this study we focused on the function of another gene which maps outside the *tra* operon. This gene is encoded by plasmid R1 and was previously sequenced in this laboratory and designated gene 19 (17). It has also been designated gene X in plasmids R100 and F (11, 39) and ORF169 in plasmid F (28). Gene 19 is transcribed in the direction opposite from that of *traM* and is separated from *traM* by *oriT* (Fig. 1). It encodes

a polypeptide of 169 amino acids. Expression of gene 19 is controlled posttranscriptionally by RNase III (25). As part of the so-called “leading region” (35), gene 19 is the first gene to be transmitted into the recipient cell during the process of conjugal DNA transfer. Its location and high degree of conservation among F-like plasmids (18, 28, 43) suggest an important function of this gene in conjugation, but such a function could not be demonstrated in earlier experiments (28).

Here, for the first time, we establish that gene 19 is involved in conjugation as well as bacteriophage infection. On the basis of our observations, we propose to classify gene 19 as a transfer gene, although its exact role in the DNA transfer process still remains to be established. A possible function suggested by the presence of a conserved motif in protein 19—the temporally and spatially controlled opening of the peptidoglycan during phage infection and conjugal DNA transfer—is discussed.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, phages, and growth conditions.** The bacterial strains, plasmids, and phages used in this work are shown in Table 1. *Escherichia coli* cells were grown in 2× TY medium (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter). The following antibiotics, when needed, were added at the indicated final concentrations: epicillin (dihydroampicillin), 100 μg ml<sup>-1</sup>; chloramphenicol, 30 μg ml<sup>-1</sup>; and kanamycin, 40 μg ml<sup>-1</sup>.

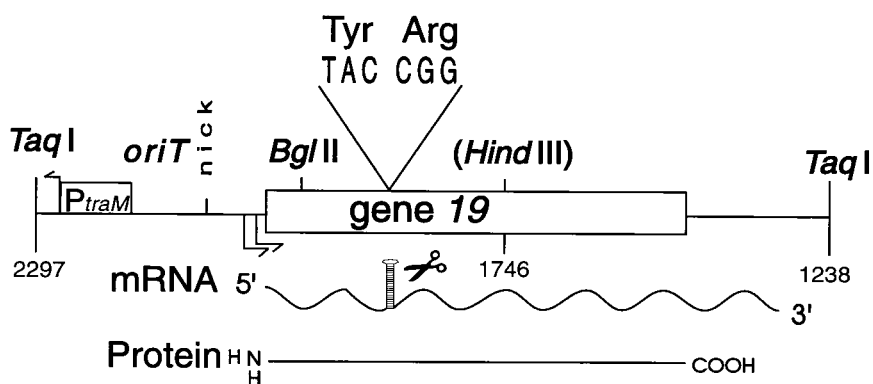
**Enzymes, chemicals, and oligonucleotides.** Restriction endonucleases and other enzymes used for standard cloning and DNA modification procedures were purchased from Boehringer GmbH (Mannheim, Federal Republic of Germany) and New England Biolabs Inc. (Beverly, Mass.). All radioactively labeled compounds were purchased from Amersham International (Amersham, United Kingdom) or from Dupont, NEN Research Products (Frankfurt, Federal Republic of Germany). Oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer.

**DNA manipulations.** Recombinant DNA techniques were performed as described by Sambrook et al. (36) or Ausubel et al. (2) or according to the manufacturers' protocols.

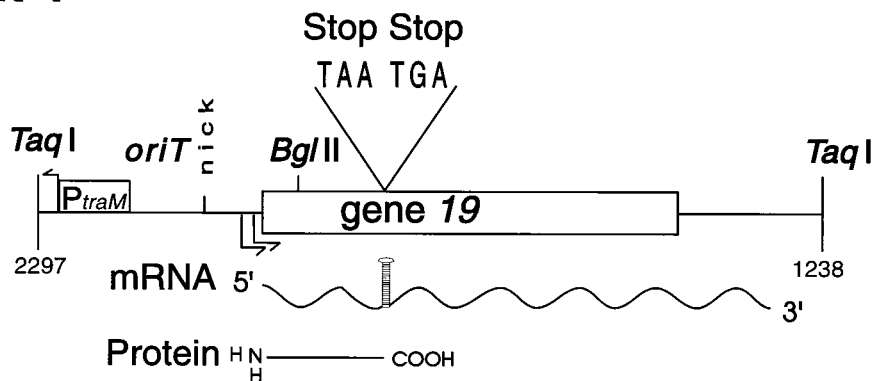
**Site-specific mutagenesis.** Oligonucleotide-directed site-specific mutagenesis of gene 19 was carried out by the protocol of Kunkel (26). The mutagenic oligonucleotide for the construction of pDA1 (mut 2) had the sequence 5'-

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## wild-type



## mut 1



## mut 2

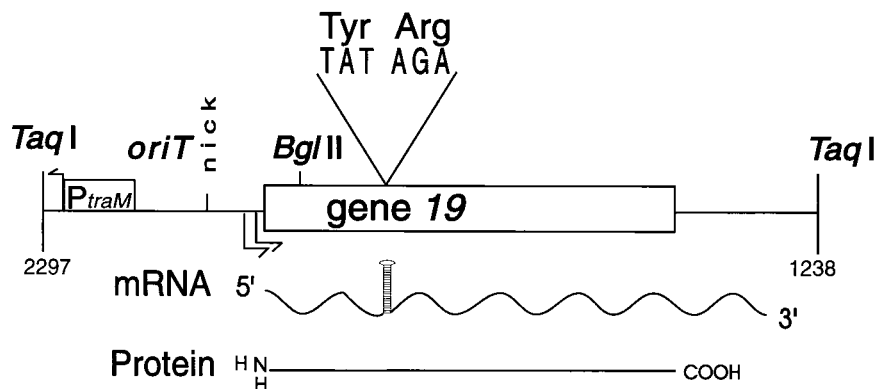


FIG. 1. Map of gene 19 and surrounding DNA. The *TaqI* fragment of R1-16 containing gene 19 and *oriT* is shown, as well as the derivatives used in this work. The base pairs of interest and the deduced amino acid sequence resulting in the differences between the various plasmids are shown above the gene 19 bar. The two main transcription start sites are indicated by arrows. The transcripts containing the hairpin, which is a substrate for RNase III, are drawn as wavy lines below the bar. In vitro cleavage by RNase III, marked by scissors, occurs only in the case of the wild-type mRNA (25). The line below the transcript indicates the product of translation, which is limited to an N-terminal fragment in mut 1 and is wild type in the case of mut 2. The position of the nick site was taken from the experimentally determined nick site of plasmid F (40). The numbering starts from the *EcoRI* site beyond gene 19 (17).

TABLE 1. Bacterial strains, plasmids, and phages

Strain, plasmids, or phage	Genotype or descriptions <sup>a</sup>	Source or reference
<i>E. coli</i> strains		
J5	F <sup>-</sup> <i>pro met lac</i> <sup>+</sup> λ <sup>+</sup>	Sandoz Forschungsinstitut, Vienna, Austria
MC1061	<i>hsdR2 hsdM</i> <sup>+</sup> <i>hsdS</i> <sup>+</sup> <i>araD139</i> Δ( <i>ara-leu</i> )7697 Δ( <i>lac</i> )X74, <i>galE15 galK16 rpsL mcrA mcrB1</i>	31
Phages		
M13mp10::CAT	M13 phage-derived cloning vector, Cm <sup>r</sup>	6
M13mp10::CAT/mut1	M13mp10::CAT, <i>EcoRI-HindIII</i> fragment from pCK260 (mut 1 of Fig. 1), Cm <sup>r</sup>	This work
M13mp10::CAT/mut2	M13mp10::CAT, <i>EcoRI-HindIII</i> fragment from pDA1(mut 2 of Fig. 1), Cm <sup>r</sup>	This work
M13mp18	M13 phage-derived cloning vector	32
R17	Male-specific RNA phage	Olke Uhlenbeck, University of Colorado
Plasmids		
pBluescript SK+ pCK217	pUC19-derived, phage f1 <i>ori</i> , COIE1 <i>ori</i> , <i>lac'IPOZ'</i> , T7/T3 promoters, Ap <sup>r</sup> pBluescript SK+, <i>EcoRI-HindIII</i> fragment from phCS100 (pos. 1238–2297 <sup>wt</sup> of R1), Ap <sup>r</sup>	Stratagene, La Jolla, Calif. 25
pCK217/H	pBluescript SK+, pCK217 with a <i>HindIII</i> site (at pos. 1746) created by oligo-nucleotide-directed mutagenesis, Ap <sup>r</sup>	25
pCK217/HK	pCK217/H; the synthetic <i>HindIII</i> site of pCK217/H was filled in by Klenow fragment and religated, creating a frameshift mutation in gene <i>I9</i> ; Ap <sup>r</sup>	This work
pCK260	pBluescript SK+, <i>EcoRI-HindIII</i> fragment from phCS141 (pos. 1238–2297 <sup>mut1</sup> of R1), Ap <sup>r</sup>	25
pCS751	pT7/6, gene <i>I9</i> with a synthetic promoterless upstream nontranslated sequence; upstream of the ATG start codon the synthetic sequence 5'-gtgattgagaggtgatcgcgac-3', which ends with an artificial <i>PstI</i> site, was inserted; Ap <sup>r</sup>	This work
pDA1	pCK260; contains a gene <i>I9</i> allele with a three-nucleotide exchange, yet coding for a wild-type protein (pos. 1238–2297 <sup>mut2</sup> of R1); Ap <sup>r</sup>	This work
pGX	pMMB67/HE, <i>EcoRI-PstI</i> fragment from pCS751, Ap <sup>r</sup>	This work
pGX/mut1	pGX, <i>EcoRI-BglII</i> fragment from pCK260, Ap <sup>r</sup>	This work
pGX/mut2	pGX, <i>EcoRI-BglII</i> fragment from pDA1, Ap <sup>r</sup>	This work
pGX217/HK	pGX, <i>EcoRI-BglII</i> fragment from pCK217/HK, Ap <sup>r</sup>	This work
pMMB67/HE	Broad-host-range, low-copy-number expression vector, RSF1010 replicon, contains a <i>tac</i> promoter in front of an M13mp18 polylinker and a <i>lacI</i> <sup>q</sup> gene, Ap <sup>r</sup>	14
R1-19	Derepressed variant of IncFII plasmid R1, Ap <sup>r</sup> Cm <sup>r</sup> Km <sup>r</sup> Sm <sup>r</sup> Su <sup>r</sup>	T. Leisinger, ETH Zürich, Zürich, Switzerland
R1-16	Derepressed and deleted variant of IncFII plasmid R1, Km <sup>r</sup>	W. Goebel, Würzburg, Germany
R1-16/mut1	R1-16 with mutation mut1 of Fig. 1, Km <sup>r</sup>	This work
R1-16/mut2	R1-16 with mutation mut2 of Fig. 1, Km <sup>r</sup>	This work

<sup>a</sup> Pos., position; wt, wild type.

ggaagaatcccgttATAgAgtaatgccatcggg-3', where the mutagenic bases are in uppercase letters. The sequence of the oligonucleotide used for the construction of pCK260 (mut 1) was described by Koraimann et al. (25).

**Homologous recombination with R1-16 for generation of R1-16/mut1 and R1-16/mut2.** The allelic exchanges by homologous recombination were performed by the method of Blum et al. (6), with the variation that a plasmid allele was replaced instead of a chromosomal one. Recombinant M13mp10::CAT phages carrying the mutated gene *I9* alleles were constructed and named M13mp10::CAT/mut1 and M13mp10::CAT/mut2 (Table 1). Plasmid R1-16 harboring cells were then infected with the recombinant phages (defective for replication in these host cells) and selected for chloramphenicol resistance. Resistant cells carried prophages in their plasmids. Subsequent growth in the absence of chloramphenicol and exposure to sodium desoxycholate led to the excision of the prophage. These cells were identified by their loss of chloramphenicol resistance. The phage excision resulted in the substitution of the wild-type gene by the mutant in a certain proportion of the segregants. Recombinants were identified by the PCR technique. The two resulting mutant R1-16 derivatives (Fig. 1) were tested in conjugation assays.

**Screening for mutants by the PCR technique.** Mutant R1-16 plasmids were identified by screening by the PCR technique. Single colonies were grown in small volumes, and plasmid DNA was prepared as described by Birnboim and Doly (5), with the deviation that DNA was precipitated with 2.0 M ammonium acetate. One hundred nanograms of each DNA sample was mixed with 50 pmol of each oligonucleotide primer and 2.5 U of *Taq* polymerase and amplified as described by Ausubel et al. (2). The oligonucleotides for amplification had the sequences 5'-ggtataaatgttcacgaggg-3' (positions 1665 to 1685 in Fig. 1) and 5'-cggataaagtcaccagaggtgg-3' (positions 1954 to 1975 in Fig. 1).

The products were analyzed by gel electrophoresis. Screening for the two point mutants was based on the disappearance of an *NciI* cleavage site. These proce-

dures produced two R1-16 mutants, R1-16/mut1 (derived from pCK260) and R1-16/mut2 (derived from pDA1).

**Conjugation assays.** Overnight cultures from single colonies grown in 2 ml of 2× TY, supplemented with kanamycin in the case of the donors, were used for mating. Forty microliters of the donor cells, i.e., *E. coli* MC1061 harboring either wild-type or mutated R1-16 plasmids, was pipetted into each well of a 24-well microtiter plate containing 0.9 ml of 2× TY and incubated for 30 min at 37°C. One hundred microliters of an overnight culture of recipient cells, i.e., *E. coli* J5, was added to each well, and the mixture was incubated for 30 min at 37°C without shaking. Subsequently, an aliquot of each mating mixture was transferred to a test tube, and conjugation was interrupted by vigorously vortexing and placing the tubes on ice. Dilutions of 10<sup>-3</sup> to 10<sup>-6</sup> in 0.9% NaCl were plated on lactose-MacConkey agar plates containing kanamycin. The conjugation frequency was expressed as the number of transconjugants per input donor cell.

**Complementation of gene *I9* defect.** Complementation of the gene *I9* defect was performed with donor strains carrying both the resistance plasmid and a complementation plasmid. The latter plasmids, which are based on pMMB67/HE (14), are called pGX, pGX/mut1, pGX/mut2, and pGX217/HK and are described in Table 1. The mating procedure described in the previous section was used, but the overnight cultures of the donors were supplemented with kanamycin and epipicillin. Induction of transcription from the *tac* promoter with IPTG (isopropyl-β-D-thiogalactopyranoside) was found not to be necessary for complementation.

**S1 nuclease protection assays.** Quantitation of several mRNA species was performed as described by O'Donovan et al. (33). Two oligonucleotides were used. The first one was complementary to the *traA* mRNA within the underlined portion and had the sequence 5'-ggaactgacgtttttggtcatcatgatcaccgaccgacca gaacaagttagtgattgatt-3'. The other oligonucleotide was used for measuring the amount of the *lpp* mRNA, which codes for lipoprotein, and functioned as an

internal standard. The sequence of this oligonucleotide was 5'-ggttagcagagctgcgtactcttagcagcctgtatagttt-3', where complementarity to the *lpp* mRNA is indicated by underlining. RNA was isolated from strains containing R1-16 or one of its derivatives as described by Miller (30). The polyacrylamide gel-purified oligonucleotides were radioactively labeled as described by Sambrook et al. (36) and purified with Nensorb 20 columns as described by the manufacturer (DuPont, NEN Research Products). For hybridization of RNA to the oligonucleotides, 10 µg of dried RNA was dissolved in 30 µl of hybridization buffer {40 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] [pH 7.4], 1 mM EDTA, 0.4 M NaCl, 0.1% sodium dodecyl sulfate [SDS], and 50% deionized formamide} and mixed with 0.1 pmol of each labeled oligonucleotide. Hybridization was allowed to take place at 44°C for 6 h following a denaturation step at 68°C for 5 min. After hybridization, 200 µl of S1 nuclease buffer (0.28 M NaCl, 50 mM Na-acetate [pH 4.5], and 4.5 mM ZnCl<sub>2</sub>) containing 200 U of S1 nuclease per ml was added. Digestion was for 30 min at 37°C. The hybrids were subsequently extracted first with phenol-chloroform-isoamyl alcohol (25:24:1) and then with chloroform-isoamyl alcohol (24:1). The hybrids were precipitated with ethanol, dissolved in water, and analyzed by gel electrophoresis on denaturing 8% polyacrylamide gels. Following autoradiography of the gels, the bands on the X-ray films were quantitated by densitometry.

**Surface exclusion assays.** Surface exclusion assays were done in the same way as conjugation assays with MC1061(R1-19) as the donor strain. J5 containing R1-16 or one of its derivatives was used as a recipient strain. Mating mixtures were diluted 10<sup>-2</sup> to 10<sup>-4</sup> in 0.9% NaCl before aliquots were plated on lactose-MacConkey agar plates containing kanamycin and epicillin (50 µg ml<sup>-1</sup>).

**Infection studies with the RNA phage R17.** R17 phages were propagated on the host strains, i.e., MC1061 with R1-16 or one of its derivatives, as described by Sambrook et al. (36). The top agar was supplemented with CaCl<sub>2</sub> to a final concentration of 10 mM. Alternatively, infection studies were also performed in liquid 2× TY medium supplemented with 2 mM CaCl<sub>2</sub> and kanamycin. Growth rates with and without phages were compared. Phages were added to 20 ml of a bacterial suspension at a multiplicity of 10 after the optical density at 600 nm had reached 0.6. Sixty minutes after phage infection, sodium citrate was added to a final concentration of 20 mM to avoid reinfection, and the CFU were determined immediately as well as 60 min later. The increase of R17 RNA during phage maturation was measured by using a 40-ml culture of cells treated in the same way as described above but without the addition of sodium citrate. Aliquots were taken 30, 40, 50, 60, and 70 min after phage infection, and RNA was isolated by the hot-phenol method described by Miller (30) and analyzed by ethidium bromide gel electrophoresis. The relative amount of R17 RNA was quantitated by densitometry and related to the amount of 23S rRNA.

**Electron microscopy of sex pili.** Phage R17 attachment to sensitive cells was observed by infecting an exponentially growing culture of *E. coli* MC1061 harboring one of the R1-16 plasmids. Cells were grown in M9 minimal medium supplemented with 0.5% Casamino Acids to approximately 2 × 10<sup>8</sup> to 4 × 10<sup>8</sup> cells per ml. After addition of 10<sup>11</sup> PFU of phage per ml, the mixture was placed on ice for 30 min. Drops of the suspension were placed on electron microscope specimen screens coated with a Formvar film backed with a thin carbon layer. Five minutes was allowed for adsorption of the bacteria to the film, and then the drops were sponged off with filter paper and replaced by drops of a 1% phosphotungstic acid solution at pH 7.4. The phosphotungstic acid solution was removed immediately, and the specimens were examined in a Philips CM10 transmission electron microscope.

**Electron microscopy of the phages.** For ultrastructural investigations, agar blocks were cut with a Pasteur pipette peripherally to the plaques. The samples were fixed in 3% glutaraldehyde (in 60 mM phosphate buffer, pH 7.2) for 2 h at room temperature. After the samples were rinsed several times in the buffer, they were postfixed for 2 h with 1% osmium tetroxide buffered with 100 mM phosphate buffer, pH 7.2. Subsequently, the material was rinsed twice in the buffer, dehydrated in a graded ethanol series, and embedded in Agar 100 epoxy resin. Ultrathin sections were stained with lead citrate followed by uranyl acetate and viewed with a Philips CM10 electron microscope.

## RESULTS

### Construction of plasmid R1-16 with mutations in gene 19.

To investigate its function, gene 19 was subjected to site-specific mutagenesis and tested for a conjugation phenotype. Two different alleles were created by nucleotide exchanges in which three bases, at positions 1806, 1808 and 1809, were altered (25), giving rise to two new codons in each allele. Two translational stop codons were introduced in the case of mut 1 (pCK260) (see Fig. 1 and Table 1 for details about this and other plasmids used in this study). Expression of this allele gave rise to a shortened protein 19 of 46 amino acids with a C-terminal deletion. Since this mutation also interfered with RNase III cleavage of the encoded transcript, we employed an additional mutation strategy. In the resulting mutant, mut 2,

TABLE 2. Average conjugation frequency of *E. coli* MC1061 harboring R1-16 or one of its mutant derivatives<sup>a</sup>

Plasmid	Conjugation frequency <sup>b</sup>	
	Avg <sup>c</sup>	Relative (%)
R1-16	2.22 × 10 <sup>-1</sup>	100
R1-16/mut1	2.00 × 10 <sup>-2</sup>	9 ± 5.6
R1-16/mut2	1.85 × 10 <sup>-1</sup>	83 ± 23.4

<sup>a</sup> The recipient was *E. coli* J5. Matings were performed as described in Materials and Methods.

<sup>b</sup> Values represent combined results of at least nine independent experiments.

<sup>c</sup> The conjugation frequency is expressed as the number of transconjugants per input donor cell.

the nucleotide sequence of the same two codons was changed without altering the coding information (pDA1) (mut 2 in Fig. 1). In mut 2 transcripts, RNase III cleavage is also blocked (unpublished observations). The region including the mutations was sequenced, confirming the presence of the exchanged nucleotides. The expression of both proteins was verified by separating labeled translation products on SDS-polyacrylamide gels (data not shown).

In order to test the mutant forms of gene 19 for phenotypic effects in a natural genetic background, we transferred the mutant forms of the gene onto plasmid R1-16. R1-16 is a deletion mutant of R1-19, which itself is a derepressed mutant of R1 (29) with a high frequency of conjugation. Plasmid R1-16 was chosen because it lacks all of the resistance determinants except kanamycin (16), a feature that permits positive selection for phage DNA integration during the allele exchange experiment. The exchange of the wild-type gene 19 encoded by plasmid R1-16 with the mutant forms described above was performed by using the strategy developed for chromosomal genes by Blum et al. (6). The details of the method are described in Materials and Methods. The resulting R1-16 mutants were identified by PCR amplification of the mutated DNA region followed by restriction with *Nci*I and analysis of the products by agarose gel electrophoresis. The overall integrity of the R1-16-derived mutant plasmids was verified by comparing restriction patterns of plasmid DNA preparations of the mutant plasmids with those of the wild-type plasmid. No differences between the isogenic wild-type and mutant plasmids were detectable. The resulting mutant plasmids were designated R1-16/mut1 and R1-16/mut2.

**Characterization of the conjugation-attenuated phenotype of R1-16/mut1.** The mutant plasmid R1-16/mut1 consistently showed a significant decrease in its conjugation frequency, to only 9% of that of the wild-type plasmid. Mutant R1-16/mut2 with the wild-type amino acid sequence, however, conjugated with the frequency of the wild-type plasmid (Table 2). In order to further characterize the 10-fold reduction in the conjugation frequency of the mutant R1-16/mut1 plasmid, we utilized assays which enabled us to dissect the numerous steps of conjugation.

(i) **Pilus synthesis and assembly.** First, we addressed the question of whether pilus synthesis and assembly were affected in cells harboring a defective gene 19. The morphology of the pili present on cells harboring one of the mutated plasmids was assayed by electron microscopy. Sex pili and other types of appendages of the *E. coli* cell could be differentiated because of the specific adsorption of the RNA phage R17 laterally along the sex pili. Figure 2A shows *E. coli* MC1061 harboring R1-16/mut1. No difference in the number (30 to 40 pili per 100 cells) or the morphology of the pili in the attachment by R17 phage could be observed between the cells harboring wild-type

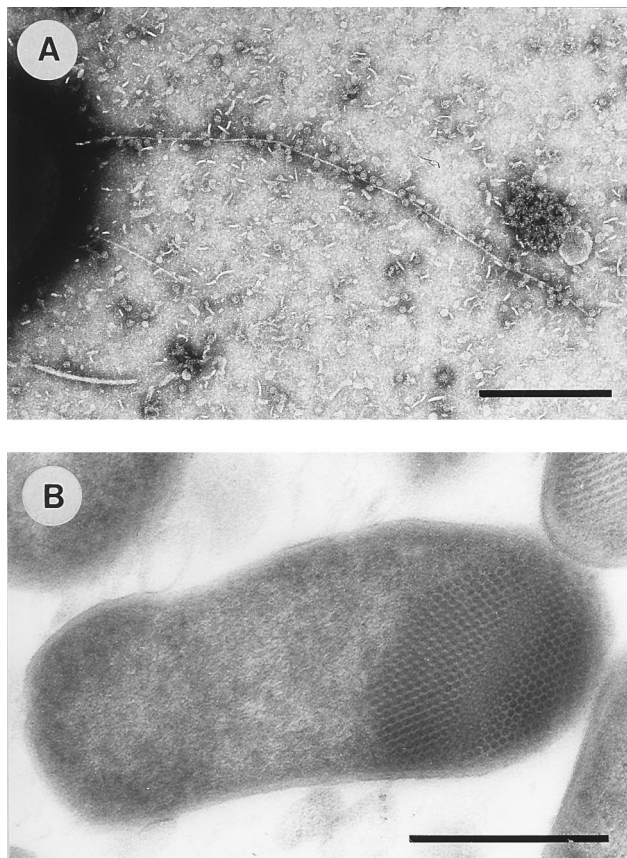


FIG. 2. Morphological studies by electron microscopy of *E. coli* cells harboring wild-type R1-16 or its derivatives. (A) Sex pili and other types of appendages of the *E. coli* cell could be discerned because of the specific adsorption of the RNA phage R17 laterally along the sex pili. The binding of phage to pili is the same with *E. coli* cells harboring wild-type R1-16 or one of its derivatives (R1-16/mut1 is shown). (B) The male-specific RNA bacteriophage R17 was found in *E. coli* cells harboring wild-type R1-16 or one of its derivatives (R1-16/mut1 is shown). Bars, 0.5  $\mu\text{m}$ .

plasmid R1-16 and those harboring its mutant derivative R1-16/mut1 or R1-16/mut2.

(ii) **TraA mRNA steady-state level.** Involvement of gene 19 in regulation of expression of the transfer operon was assayed by measuring the steady-state level of the *traA* mRNA. Since expression of the *tra* operon is required prior to the onset of conjugation, a possible effect of protein p19 on the amount of the *tra* operon mRNA was examined. Because *traA* is the second gene in the *tra* operon (13) and encodes an mRNA which can easily be measured because of its enhanced stability and high steady-state level (24), we determined the steady-state level of its mRNA relative to that of the lipoprotein-specific *lpp* mRNA as an internal standard. The *traA* and *lpp* mRNA levels were measured by hybridization of labeled complementary oligonucleotides to total RNA followed by S1 nuclease digestion. The protected oligonucleotides were resolved on polyacrylamide gels (Fig. 3) and quantitated by densitometry. The measurements revealed that cells harboring R1-16/mut1 (Fig. 3, lane C) contain amounts of *traA* mRNA indistinguishable from those of cells harboring the wild-type R1-16 plasmid (Fig. 3, lane B), indicating that gene 19 exerts no influence on the steady-state level of the *traA* mRNA.

(iii) **Kinetics of the DNA transfer.** In order to test whether the mutant gene 19 causes any changes in the kinetics of the

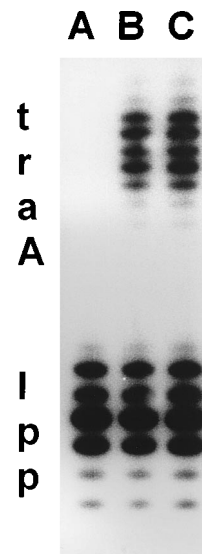


FIG. 3. Steady-state abundance of *traA* mRNA. RNA prepared from the following strains was used for S1 nuclease protection assays as described in Materials and Methods: *E. coli* MC1061, which is devoid of *traA* transcript because of the absence of an R1-16 plasmid (control) (lane A); *E. coli* MC1061(R1-16) (lane B); and *E. coli* MC1061(R1-16/mut1) (lane C). An autoradiogram of a denaturing 8% polyacrylamide gel is shown. The appearance of more than one band per protected oligonucleotide is due to partial degradation of double-stranded nucleic acids by S1 nuclease. *lpp* mRNA was used as an internal standard.

DNA transfer, we performed experiments in which matings were interrupted at different time points after the onset of conjugation. Mating was initiated by mixing donor and recipient cells. The first transconjugants could be selected after a mating period of 7 min in each case. The mating frequency was determined to be 0.02 for the wild-type plasmid and 0.002 for the mutant (averages of two independent experiments). The number of transconjugants increased in both cases with prolonged incubation, but the conjugation frequency of the mutant plasmid was about 10-fold lower at all times.

(iv) **Surface exclusion.** Surface exclusion functions are provided by transfer genes *traS* and *traT* (20). Since Loh et al. (28) discussed a requirement for the leading region in efficient surface exclusion, we investigated the involvement of gene 19. Mating assays with *E. coli* MC1061(R1-19) as the donor strain and *E. coli* J5 containing R1-16, R1-16/mut1, or R1-16/mut2 as the recipient strain revealed no significant differences in conjugation frequencies (The numbers of transconjugants per donor cell with these plasmids in the recipient strain were as follows [combined results of four independent experiments]: no plasmid,  $5.8 \times 10^{-1} \pm 3.1 \times 10^{-1}$ ; R1-16,  $1.8 \times 10^{-2} \pm 1.2 \times 10^{-2}$ ; R1-16/mut1,  $3.5 \times 10^{-2} \pm 1.2 \times 10^{-2}$ ; and R1-16/mut2,  $1.6 \times 10^{-2} \pm 6.0 \times 10^{-3}$ ). Therefore, we conclude that gene 19 is not required to mediate surface exclusion.

**Characterization of the infection-attenuated phenotype of R1-16/mut1.** Growth of the male-specific RNA bacteriophage R17 on lawns of R1-16-containing cells resulted in clear plaques. However, lawns of bacteria harboring R1-16 with the defective gene 19/mut1 allele developed only very turbid plaques. Additionally, the number of plaques was reduced approximately 12-fold compared with that of the wild-type. This experiment suggested that some step in phage infection or maturation is impaired by the lack of a functional gene 19. In order to investigate this effect further, we grew bacteriophage R17 on cells carrying the plasmid R1-16 or R1-16/mut1 in

liquid culture and measured the effect of phage infection on bacterial growth. As shown in Fig. 4A, the optical density declined after addition of a phage lysate to the cells containing the wild-type plasmid. In the case of cells harboring the mutant plasmid with the defective gene *19*, growth was almost unaffected. Light microscopy revealed that lysis occurs at the same time following phage addition in both the wild-type cells and the cells carrying mutant gene *19*, although the number of cells that undergo lysis is much lower for the mutant (data not shown). These results, together with the fact that R17 forms plaques on cells harboring the plasmid R1-16/mut1, indicate that the mutation in gene *19* does not interfere with cell lysis at the end of the life cycle of the phage.

Another possible explanation for the fact that the mutant developed turbid and fewer plaques can be that an early step of phage development is affected by the gene *19* mutation. Therefore, we investigated separately adsorption of the phage, efficiency of infection by phage particles, replication of phage RNA, and maturation of the phage particles within infected cells. The attachment of R17 phage laterally to the pili encoded by the plasmids tested is not affected by the defective gene *19* as demonstrated by electron microscopy studies (Fig. 2A). No difference in the number of phage particles attached to each pilus could be seen for the wild-type and mutant cells.

(i) **Number of viable cells.** In order to compare the number of viable cells in a culture infected with phage R17 with that in a noninfected culture, we determined the CFU at 60 and 120 min after phage addition. As shown in Table 3, the reduction of CFU after addition of phage R17 is much more efficient with cells carrying the wild-type plasmid than with cells harboring R1-16/mut1: three to four times more cells are killed.

(ii) **Phage RNA replication.** To address the question of whether mutant gene *19* affects replication of phage RNA, we determined the concentration of RNA plus strands in infected cell cultures prior to cell lysis (Fig. 4B). Quantitation of plus-strand phage RNA revealed that ninefold less R17 RNA was present in R1-16/mut1 cells than in wild-type cells at time points ranging from 30 to 70 min following addition of phage to the growing cultures (Fig. 4C). A Northern blot was prepared from a gel similar to that shown in Fig. 4B and probed with a labeled oligonucleotide complementary to the coat protein gene of bacteriophage R17 in order to confirm the identity of the measured band as plus-strand phage RNA. No such band was observed in a control experiment in which the same *E. coli* strain without a plasmid present was used (data not shown). Although the abundance of phage RNA is much diminished in R-16/mut1-carrying cells, the rates of increase in the amount of R17 RNA are the same in both cases (Fig. 4C). The finding that the kinetics of RNA replication in the mutant and wild-type cells are the same indicates that RNA replication, once established, occurs normally in the mutant. The finding that ninefold less phage RNA is present suggests that a step prior to RNA replication is affected by a mutant gene *19*.

(iii) **Maturation of R17 phage particles within infected cells.** As can be seen on the electron micrograph in Fig. 2B, phage particles also appear within infected cells harboring the mutant plasmid R1-16/mut1. Thus, maturation of the phage within the infected cells is also not affected by the mutation.

**Complementation of the attenuated phenotypes associated with a defective gene *19*.** We tested whether the wild-type and mutant gene *19* alleles under the control of the *tac* promoter on the low-copy-number plasmid pMMB67/HE can complement the conjugation deficiency of R1-16/mut1 in *trans* (Table

4). Both the wild-type (pGX) allele and the gene *19*/mut2 allele (pGX/mut2) complement the conjugation defect caused by gene *19*/mut1. Complementation is observed only when the donor strain carries one of these two plasmids. The presence of one of these plasmids in the recipient strain does not restore the wild-type conjugation frequency (data not shown). Likewise, bacteriophage development can be rescued when R1-16 with a defective gene *19* is complemented with either the wild-type or mut 2 allele of gene *19* in *trans*. Figure 5 shows that phage R17 forms clear plaques on the complemented strains. Neither the translational stop allele mut 1 (pGX/mut1) nor a translational frameshift allele (pGX217/HK) can complement the R1-16/mut1 phenotypes.

Because sequence similarities between protein 19 and PilT encoded by plasmid R64 suggested that *pilT* is the R64 homolog of gene *19*, we investigated whether *pilT* can also complement the phenotypes produced by a defective gene *19*. Both the reduction of conjugation frequency and the reduced R17 phage infection could be complemented by introduction of the *pilT* gene on a multicopy plasmid (data not shown).

## DISCUSSION

The DNA region of plasmid R1 that is transferred first into the recipient cell during bacterial conjugation contains gene *19*. Since the function of gene *19*, despite its prominent location and high level of conservation, was still unknown, we set out to investigate it by a reverse-genetics approach. A plasmid R1 derivative, R1-16/mut1, carrying a gene *19* allele inactivated by two nonsense mutations was created and tested in conjugation and phage R17 infection studies. The results obtained in these experiments clearly demonstrate that the expression of a functional protein 19 is necessary for both efficient conjugation and bacteriophage R17 infection.

A more detailed characterization of the conjugative phenotype produced by gene *19*/mut1 showed that the 10-fold-lower conjugation frequency neither was due to a reduced transfer operon expression nor resulted from abnormal piliation of the donor cells. Other functions that we can exclude on the basis of our experimental results are surface exclusion and control of transfer gene expression. We also propose that aggregate stability is not affected by the inactivation of gene *19*, since mating aggregates which were resistant to rigorous shaking were formed. Since there is only one other generally recognized class of proteins that have a function in conjugation, we speculated that protein 19 plays a role in signaling, origin nicking, unwinding, and transport of DNA. However, DNA transfer of R1-16/mut1 occurred with the same kinetics as in the case of the wild-type plasmid, indicating that the functions that promote the above-mentioned processes are present.

Previously, Loh et al. (28) suggested that ORF169 of the F plasmid, which is 97% identical to protein 19 of plasmid R1, could play a role in conjugal DNA transfer. However, experimental data obtained from their studies with a multicopy F-plasmid derivative containing a deletion in the leading region spanning from *oriT* to *ssb* did not indicate that this region contributes functions essential for conjugal DNA transfer. We explain the failure of Loh et al. (28) to detect an altered conjugation phenotype by the different plasmid constructs used in the experiments (their high-copy cosmid replicon versus our low-copy natural replicon) and the different types of mutations that were tested (their deletion of kilobases of DNA versus our point mutation). Yet, it remains to be investigated whether a

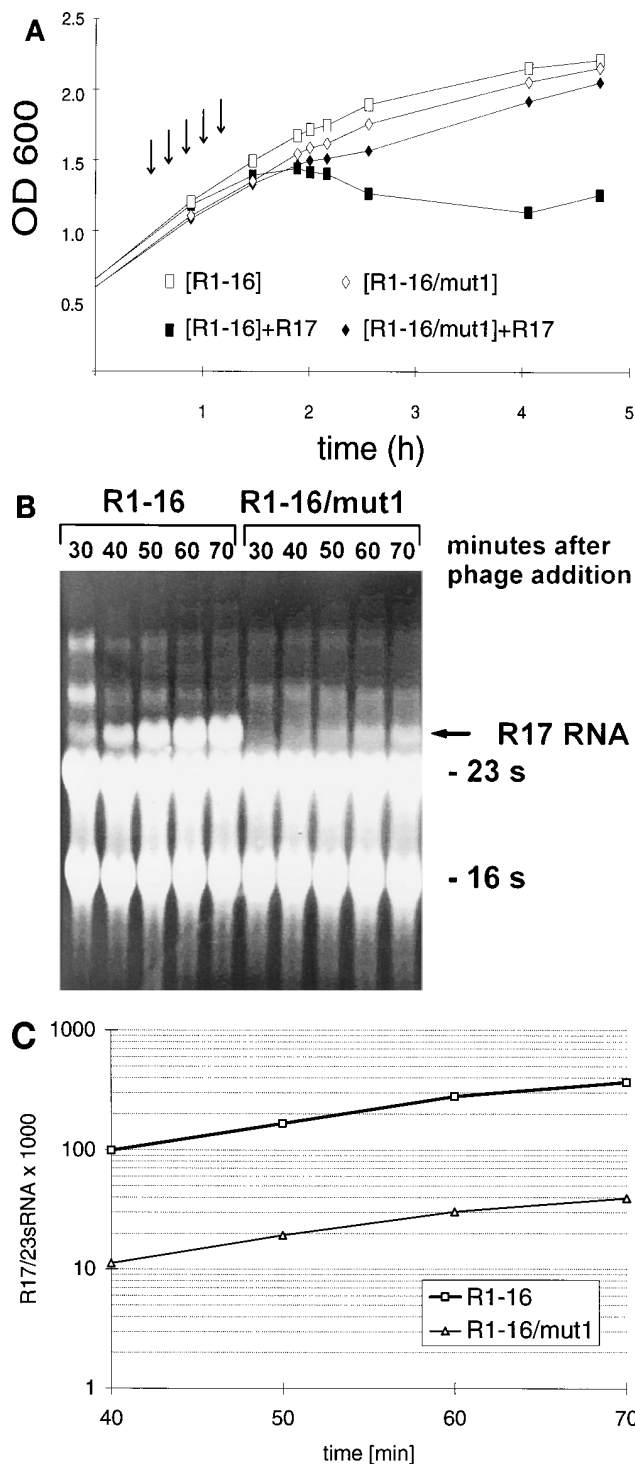


FIG. 4. (A) Bacterial growth and phage R17 infection. Growth curves for *E. coli* MC1061(R1-16) and MC1061(R1-16/mut1) in the absence and presence of bacteriophage R17 are shown. Phage R17 was added when an optical density at 600 nm of 0.6 was reached (time zero). The arrows indicate the time points when aliquots were taken for the RNA isolation. (B) Replication of bacteriophage R17 RNA in *E. coli* MC1061(R1-16) and MC1061(R1-16/mut1). RNA was isolated as described in Materials and Methods and analyzed by neutral agarose gel electrophoresis. The bands were made visible by ethidium bromide staining and UV irradiation. (C) Kinetics of phage R17 RNA replication. A neutral agarose gel as shown in panel B was used for quantitation of phage R17 RNA and 23S rRNA by densitometry. R1-16- and R1-16/mut1-containing cells were used as hosts for phage R17 infection. The amount of phage R17 RNA was normalized by the amount of ribosomal 23S rRNA present.

TABLE 3. Reduction of viable *E. coli* cells by phage R17<sup>a</sup>

Plasmid	CFU/ml (% of uninfected-control value) <sup>b</sup> at:	
	60 min	120 min
None	109 ± 4.9	110 ± 1.4
R1-16	19 ± 6.3	11 ± 3.8
R1-16/mut1	70 ± 23.2	49 ± 19.7

<sup>a</sup> The number of viable *E. coli* cells harboring either R1-16 or R1-16/mut1 was determined. Aliquots of the cultures were taken 60 and 120 min after addition of R17 phage, and CFU were determined.

<sup>b</sup> Values represent combined results of five independent experiments.

similar genetic approach with the F plasmid will lead to the same result that we obtained with plasmid R1.

Infection studies with cells containing R1-16/mut1 and bacteriophage R17 suggest that the efficiency of an early step in the development of the bacteriophage is greatly reduced. The results of our experiments exclude the possibilities that gene 19 is involved in the attachment of bacteriophage particles laterally to the R1-16/mut1-encoded conjugative pili, replication of the viral RNA, assembly of the bacteriophage within infected cells, and lysis. Although the viability assays performed provide only indirect evidence, we propose that the step which is affected by a defective gene 19 is the entry of the bacteriophage R17 RNA into the cells.

Gene 19 increases the efficiency of conjugal DNA transfer and the efficiency of infection by bacteriophage R17, although it is not essential. Both attenuated phenotypes caused by a defective gene 19 can be complemented *in trans* by a wild-type gene 19 present on a coresident low-copy plasmid. On the basis of these findings, we propose that gene 19 provides an accessory function that greatly enhances conjugal DNA transport out of the cells and entry of bacteriophage RNA into the cells. Such a function would require that the encoded protein is located in the envelope of the bacterium. A hydrophobic sequence is present at the N terminus of the polypeptide, making a membrane location very likely. Furthermore, a membrane location of protein 19 was demonstrated by fractionation studies (3).

The nonessentiality feature of gene 19 strikingly resembles the virulence-attenuated phenotype which was found with a *virB1* mutant of the *A. tumefaciens* Ti plasmid (4). *VirB1* has approximately 50% sequence similarity to protein 19 (Fig. 6 and Table 5) and has been proposed to be located at the periplasmic face of the inner membrane of *A. tumefaciens* (41). These similarities suggest that the function provided by gene 19 is conserved in DNA transfer systems distantly related to IncF plasmids. Supporting evidence for this hypothesis comes

TABLE 4. Complementation of the conjugation defect

Coresident plasmid	Conjugation frequency <sup>a</sup>	
	Avg	Relative (%)
pMMB67/HE	$3.35 \times 10^{-2}$	11.8 ± 2.6
pGX	$2.02 \times 10^{-1}$	75.8 ± 36.4
pGX/mut1	$3.05 \times 10^{-2}$	11.2 ± 6.0
pGX/mut2	$2.22 \times 10^{-1}$	84.8 ± 44.9
pGX217/HK	$2.88 \times 10^{-2}$	10.5 ± 4.2

<sup>a</sup> Conjugation frequencies of R1-16/mut1 with various coresident plasmids are given. The relative conjugation frequency was based on the conjugation frequency of the wild-type plasmid R1-16 in the presence of the plasmid pMMB67/HE, which was taken as 100%. Values represent combined results of four independent experiments.

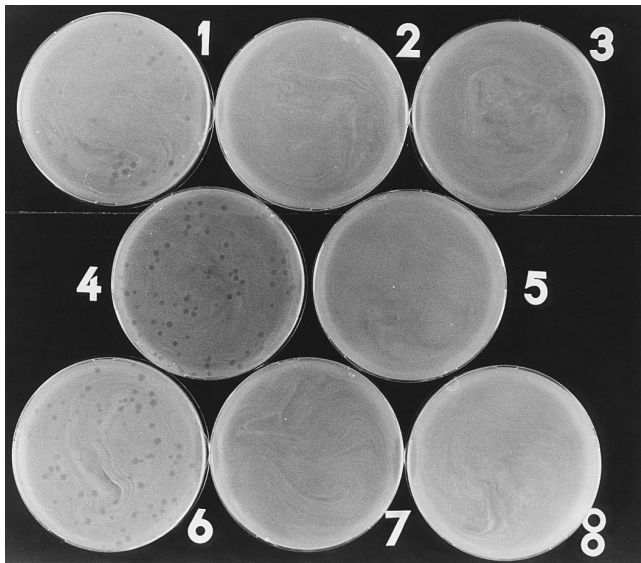


FIG. 5. Complementation of the infection defect. The male-specific RNA bacteriophage R17 was propagated on the host strains as described by Sambrook et al. (36). Plates: 8, *E. coli* MC1061 as a negative control; 1, MC1061(R1-16) as a positive control; 2, MC1061(R1-16/mut1); 3 to 7, MC1061(R1-16/mut1) with the coresident plasmids pMMB67/HE, pGX, pGX/mut1, pGX/mut2, and pGX217/HK, respectively.

from *PilT*, which is encoded by the *IncI* plasmid R64. This protein has extensive sequence homology (62% similarity and 45% identity) with protein 19 (22) (Fig. 6 and Table 5). We could demonstrate that *pilT* can complement a defective gene 19 in *trans*. The ability of *pilT* to restore the wild-type phenotype of R1-16/mut1 provides evidence for a functional relationship between these two genes. Furthermore, a *pilT* mutant shows the same conjugation-attenuated phenotype (22). These previously unknown relationships between different DNA transfer systems are extended by the finding that the *IncN* plasmid pKM101-encoded *TraL* protein has extensive sequence similarity to *VirB1* (34) (Fig. 6 and Table 5). It is important to note that inactivation of *traL* reduced the conjugation frequency 10- to 100-fold (42); furthermore, *TraL* was found to be conditionally lethal (34). On the basis of sequence comparisons, we also propose that *TrbN* encoded by the *IncP* plasmid RP4 belongs to this group of proteins (Fig. 6 and Table 5). Similarly, the *IpgF* protein, encoded by a virulence plasmid of *Shigella flexneri*, has been described as having a high degree of sequence similarity to protein 19 (1) (Fig. 6 and Table 5). The *ipgF* gene is part of a gene cluster located between the *ipa* and *mxi* gene loci of the 220-kb pWR100 plasmid, which confers virulence properties to *S. flexneri*. The function of the *IpgF* protein, which is very likely located in the periplasm, is unknown (1).

The attenuated phenotypes observed with the gene 19/mut1 allele as well as with the nonfunctional *virB1*, *traL*, and *pilT* genes could be due to a host cell function which might partially compensate for the defective genes. Such a function could be provided by a host-encoded muramidase, since all of these proteins show the presence of a conserved amino acid sequence motif that was first identified by Koonin and Rudd (23). Later the conserved sequence motif was redefined on the basis of the structure of the soluble lytic transglycosylase of *E. coli*, and it has been suggested that this motif is indicative of a lysozyme-like activity (8, 9).

We therefore propose that one function of gene 19 during

TABLE 5. Similarity scores<sup>a</sup>

Protein (source)	Similarity <sup>b</sup> with:						
	p19 <sup>31-126</sup> (R1)	PilT <sup>50-152</sup> (R64)	VirB1 <sup>40-159</sup> (T1)	TraL <sup>40-161</sup> (pKM101)	TtbN <sup>56-150</sup> (RP4)	IpgF <sup>29-123</sup> ( <i>S. flexneri</i> )	Slt70 <sup>492-587</sup> ( <i>E. coli</i> )
p19 <sup>31-126</sup> (R1)							
PilT <sup>50-152</sup> (R64)	62/50/3 (74/29 ± 3)						
VirB1 <sup>40-159</sup> (T1)	54/34/4 (46/32 ± 2)	62/50/3 (74/29 ± 3)					
TraL <sup>40-161</sup> (pKM101)	47/27/4 (43/35 ± 3)	47/27/4 (43/35 ± 3)	54/34/4 (46/32 ± 2)				
TtbN <sup>56-150</sup> (RP4)	58/38/5 (52/36 ± 2)	58/38/5 (52/36 ± 2)	55/38/3 (79/38 ± 2)	57/24/6 (41/33 ± 3)			
IpgF <sup>29-123</sup> ( <i>S. flexneri</i> )	44/21/4 (37/29 ± 2)	44/21/4 (37/29 ± 2)	60/40/6 (50/33 ± 2)	58/38/5 (52/36 ± 2)	51/26/4 (51/26 ± 2)		
Slt70 <sup>465-560</sup> ( <i>E. coli</i> )	60/39/2 (69/30 ± 3)	60/39/2 (69/30 ± 3)	58/34/7 (37/33 ± 2)	55/38/3 (79/38 ± 2)	44/21/4 (37/29 ± 2)	49/35/0 (63/29 ± 3)	53/33/6 (47/30 ± 3)
	46/28/3 (49/32 ± 2)	46/28/3 (49/32 ± 2)	58/34/7 (37/33 ± 2)	43/20/4 (36/32 ± 2)	60/39/2 (69/30 ± 3)	60/39/2 (69/30 ± 3)	46/28/3 (49/32 ± 2)
			39/22/4 (36/34 ± 2)	47/33/4 (46/30 ± 2)	47/33/4 (46/30 ± 2)	47/33/4 (46/30 ± 2)	58/34/7 (37/33 ± 2)
				47/33/4 (46/30 ± 2)	49/32/4 (46/30 ± 2)	49/32/4 (46/30 ± 2)	49/32/4 (46/30 ± 2)
				45/19/4 (34/29 ± 2)	49/32/4 (46/30 ± 2)	49/32/4 (46/30 ± 2)	45/19/4 (34/29 ± 2)
							49/32/4 (46/30 ± 3)

<sup>a</sup> The parts of the proteins that were compared are indicated and include the three conserved sequence motifs that characterize the catalytic and substrate binding sites of Slt70. These motifs are present in a broad family of muramidases and chitinases (9). For references and GenBank numbers, see the legend to Fig. 6.

<sup>b</sup> The Best fit algorithm (gap weight, 2.5; length weight, 0.1) of the Wisconsin package (15) was used. Data are reported as percent similarity/percent identity/number of gaps (quality score/average quality ± standard deviation [based on 10 randomizations]).



Block A			
P19	30	YKIDPDLRL <b>RA</b> ISW <b>KE</b> SRVYRVNAIGIN	55 (169)
IpgF	28	YNI <b>P</b> SSLL <b>KA</b> IA <b>ES</b> GFNKSAVNVN	53 (152)
PilT	49	YQIEPLLL <b>KA</b> IS <b>ES</b> SLRPGAININ	74 (186)
TrbN	55	YEIPANILL <b>LA</b> IRE <b>EG</b> GKPGQVWKNT	80 (234)
VirB1	39	PSVATST <b>LA</b> IA <b>ES</b> RFDPLAIHND	64 (239)
TraL	39	PDV <b>S</b> PLT <b>MA</b> IV <b>GH</b> ES <b>NG</b> PYRININ	64 (244)
Slt70	464	KEIPQSY <b>MA</b> IA <b>ES</b> AWNPVKVSPV	489 (645)
		**	
Block B		Block C	
P19	61	GS <b>GL</b> MQVDSQHF	72 111 WEAVG <b>AY</b> NA <b>AG</b> 120
IpgF	59	DY <b>GI</b> MQINDFHS	70 109 WEAVG <b>AY</b> NA <b>AG</b> 118
PilT	86	DY <b>GL</b> MPINSTHI	97 138 W <b>N</b> CLGS <b>Y</b> NA <b>AG</b> 147
TrbN	85	DV <b>GE</b> LQFNTAYL	96 136 W <b>T</b> RAAN <b>Y</b> H <b>S</b> R 145
VirB1	93	DV <b>GL</b> MQINSRNF	104 146 R <b>RA</b> IS <b>AY</b> NT <b>G</b> 155
TraL	94	DM <b>GL</b> AQINSN <b>N</b> L	105 148 R <b>H</b> AL <b>S</b> C <b>Y</b> NT <b>G</b> 157
Slt70	491	AS <b>GL</b> MQIMP <b>G</b> TA 502	546 I <b>F</b> SS <b>AA</b> Y <b>NA</b> <b>G</b> 555
		*****	*** *

FIG. 6. Multiple sequence alignments. Multiple sequence alignments around the conserved sequence motifs (asterisks), including the catalytic (Glu at position 478 in Slt70) and substrate binding (Ala-551-Tyr-552-Asn-553 in Slt70) sites of the 70-kDa soluble lytic transglycosylase of *E. coli* (9), are shown. The three blocks are also included in the previously identified conserved domain in putative bacterial and bacteriophage transglycosylases (23). By using the MACAW program (37), the statistical probability of finding one of the three blocks of the given length and composition by chance alone was computed to be below  $10^{-13}$ ,  $10^{-10}$ , and  $10^{-10}$  for blocks A, B, and C, respectively. Numbers of the first and last residues of the aligned regions and the total lengths of the sequences (in parentheses) are given. Residues identical or functionally similar in all sequences are shaded. Residues identical in at least six sequences are shown in boldface. Proteins (with references and GenBank accession numbers) are as follows: P19 of plasmid R1 (17) (X15279); IpgF of *S. flexneri* (1) (L04309); PilT of plasmid R64 (22); TrbN of plasmid RP4 (27) (M93696); VirB1 of Ti plasmid pTI15955 (38) (X06826); TraL of plasmid pKM101 (34) (U09868); and Slt70 of *E. coli* (10) (M69185).

the DNA transfer process could be to facilitate the passage of the single-stranded DNA strand through the peptidoglycan layer. Likewise, gene 19 could promote phage R17 RNA entry into the cells during the infection step. The sequence homologies and the experimental data obtained in our laboratory and other laboratories (see above) indicate that localized peptidoglycan hydrolysis may be required in order for processes as diverse as DNA transfer and virulence factor export to take place.

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