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Characterization and Sequence Analysis of Pilin from F-Like Plasmids

L. S. FROST,[1] B. B. FINLAY,[1] A. OPGENORTH,[1] W. PARANCHYCH,[1]* AND J. S. LEE[2]

Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7,[1] and Department of Biochemistry, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 0W0[2]

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Conjugative pili are expressed by derepressed plasmids and initiate cell-to-cell contact during bacterial conjugation. They are also the site of attachment for pilus-specific phages (f1, f2, and QB). In this study, the number of pili per cell and their ability to retract in the presence of cyanide was estimated for 13 derepressed plasmids. Selected pilus types were further characterized for reactivity with anti-F and anti-ColB2 pilus antisera as well as two F pilus-specific monoclonal antibodies, one of which is specific for a sequence common to most F-like pilin types (JEL92) and one which is specific for the amino terminus of F pilin (JEL93). The pilin genes from eight of these plasmids were cloned and sequenced, and the results were compared with information on F, ColB2, and pED208 pilin. Six pilus groups were defined: I, was F-like [F, pED202(R386), ColV2-K94, and ColVBtrp]; IIA was ColB2-like in sequence but had a lowered sensitivity to f1 phage due to its decreased ability for pilus retraction [pED236(ColB2) and pED203(ColB4)]; IIB was ColB2-like but retained f1 sensitivity [pED200(R124) and pED207(R538-1)]; III contained R1-19, which had a ColB2-like amino terminus but had an additional lysine residue at its carboxy terminus which may affect its phage sensitivity pattern and its antigenicity; IV was R100-1-like [R100-1 and presumably pED241(R136) and pED204(R6)] which had a unique amino-terminal sequence combined with a carboxy terminus similar to that of F. pED208(Folac) formed group V, which was multipiliated and exhibited poor pilus retraction although it retained full sensitivity to f1 phage. The pED208 pilin gene could not be cloned at this time since it shared no homology with the pilin gene of the F plasmid.

Conjugative pili are filaments found on the surface of bacteria which promote cell-to-cell contact during bacterial conjugation (2). They facilitate recognition of a competent recipient cell and also interact with the surface exclusion system of donor cells carrying pili of the same type to block redundant conjugation (41). In addition, they act as the site of attachment for F-specific bacteriophages, spherical RNA phages (R17 and QB) which attach to the sides of the pilus (11) and filamentous DNA phages (f1 and M13) which attach to the tip of the pilus (10). The role of the pilus during conjugation (32) and phage infection (23) may depend on its ability to retract into the donor cell (12, 26), that is, disassemble into the membrane, bringing the recipient cell or phage particle to the donor cell surface. Retraction can be demonstrated by the addition of cyanide (30, 31, 40) to the cells, which results in the disappearance of the pili from the cell surface.

The prototype conjugative plasmid is the F plasmid, and many related F-like plasmids (9) have been described. These plasmids carry conjugation systems or transfer regions that are closely related to F both on the basis of DNA homology (35) and genetic criteria (39). These plasmids elaborate F-like pili which allow infection by F-specific bacteriophages (33) and can complement mutations in the genes of the F plasmid essential for pilus formation (N. S. Willetts and J. Maule, Genet. Res., in press). A number of plasmid-specific functions have been determined, however, which allow a plasmid to identify its own origin of transfer during conjugation, to regulate the expression of its transfer region, and to generate a pilus which interacts specifically with its own surface exclusion system (Willetts and Maule, in press). Furthermore, the F-like pili of these plasmids can be differentiated serologically (25) and through variations in phage sensitivity (3, 33) which are thought to be based on differences in the primary sequence of the pilus subunit. The overall similarity of F-like pili is emphasized by the formation of mixed pili by cells carrying two different F-like plasmids (24).

F-like pili are composed of single repeating subunits, pilin (8, 28), of molecular weight 7,000 to 7,200 (14, 20, 29), which are arranged in a helical manner into a tubular filament of 8-nm outer diameter and 2-nm inner diameter and several micrometers in length. There are 3.6 subunits per turn and a pitch within the helix of 12.8 nm (17). Studies by circular dichroism to estimate the alpha-helical content in the subunit have placed it at 65 to 70% (4, 13).

The gene for pilin, *traA*, has been sequenced for F and ColB2 (14, 20), and an homologous leader peptide of 51 amino acids was revealed. The pilin molecules were identical except for a small alteration in sequence at the amino terminus. Both these pilin types have been shown to have N-acetylated amino termini. It has also been demonstrated that the amino-terminal regions of these pilin molecules make up the major antigenic determinant for these pili and provide the basis of the antigenic diversity between these serogroups (15). The pilin genes from eight different F-like plasmids were cloned, sequenced, and compared with the sequences previously reported for F and ColB2 pilin genes to further elucidate the molecular basis of diversity among these F-like pili with respect to antigenicity and phage sensitivity. A ninth plasmid, pED208, the derepressed mutant of Folac, which has no DNA homology within its transfer region with the F plasmid (14) and is serologically distinct with respect to its pilus type (7), expresses a pilus of

* Corresponding author.

TABLE 1. Pilus types of F-like derepressed plasmids

FinOP$^+$ plasmid[a]	Transfer-derepressed plasmid[a]	Revised pilus type[b]	Sfx type[a]	Serological group[a,c]	Pilus type[d]	Amino terminus	Carboxy terminus	traA$^+$ *Pst*I fragment (kb)
	F(JCFLO)	I	I	A	I	F	F	1.4
R386	pED202	I	I	A[e]	I	F	F	1.4
	ColV2-K94	I	II	A	I	F	F	1.4
	ColVBtrp	I	III	A	I	F	F	1.4
ColB2-K77	pED236	IIA	II	C[e]	II	ColB2	F	1.4
ColB4-K98	pED203	IIA	II	C[e]	II	ColB2	F	2.1
R538-1	pED207	IIB	II	C	I	ColB2	F	2.1
R124	pED200	IIB	II	C[e]	I	ColB2	F	1.4
R1	R1-19	III	III	B	III	ColB2	R1	2.1
R100	R100-1	IV	IV	D	IV	R100	F[f]	15
R136	pED241	IV	IV	D	IV	R100[c]	ND[g]	ND
R6	pED204	IV	IV		IV	ND	ND	ND
Folac	pED208	V	V	E[e]	V	pED208[h]	ND	ND

[a] N. S. Willetts and J. Maule, in press.
[b] The pilus types summarize both the previous data based on patterns of phage sensitivity (column 6) and serological differences (column 4) as well as DNA sequence information (columns 7, 8, and 9). Type IIB is identical to IIA but retains its sensitivity to f1 phage.
[c] A. M. Lawn and E. E. Meynell (25).
[d] Type I plated f1, f2, and QB with EOP similar to that of F; type II gave a 50- to 100-fold decrease in the EOP of f1; type III showed a 10- and 30-fold reduction in the EOP of f1 and QB, respectively; type IV showed a 50- to >100-fold reduction in the EOP of f1 and QB, respectively, and a 20-fold reduction in the EOP of f2. Type V was insensitive to f2 and QB but gave a normal EOP with f1. Results are from N. S. Willetts and J. Maule, in press.
[e] Determined by whole-cell ELISA in this lab.
[f] F carboxy terminus with Ala_{117}-Ser transition.
[g] ND, Not determined.
[h] L. S. Frost et al. (19).

nearly identical dimensions (W. Folkhard, K. R. Leonard, J. Dubochet, D. A. Marvin, and W. Paranchych, Abstr. 11th Int. Congr. Biochem., abstr. no. 03-3-5116.10, 1976) to the F pilus and shares complete sensitivity to the filamentous DNA phages, f1 and M13 (7, 27). The protein sequence of its amino-terminal tryptic peptide reveals that it also has an N-acetylated amino terminus (20). It has also been shown that the first five amino acid residues which differ from those of other pilins sequenced make up the major antigenic determinant of pED208 pili (43).

MATERIALS AND METHODS

Bacterial strains and plasmids. The derepressed mutants of wild-type F-like plasmids are listed in Table 1, which is a précis of the table presented by Willetts and Maule (Willetts and Maule, in press). The plasmids were carried in the host strain *Escherichia coli* JC6256 (F$^-$ *lac trp*). Cells were assayed for pili in the background strain ED2601 (F$^-$ *lac his trp lys* T6 Str *fla*). All pilin genes were cloned into the positive insertion vector pUC8 and grown on *E. coli* JM83 (36).

Pilus retraction assay. Piliated cells (2×10^8 to 5×10^8 cells per ml) were assayed by electron microscopy for the number of pili per 100 cells before and after (5 and 20 min) the addition of 0.01 M sodium cyanide as described previously (40). The pili were labeled with R17 phage particles for easier visibility.

Plasmid purification. Both the derepressed F-like plasmids and the chimeras of pUC8 containing the various pilin genes were isolated and purified by the method of Humphries et al. (22). Purification of small amounts of chimeric plasmid DNA was by the method of Birnboim and Doly (6).

Cloning and sequencing of pilin genes. The procedures used to clone the various pilin genes have been described previously (14, 20). All DNA sequence analysis was done with chimeric DNA which had been purified by cesium chloride centrifugation and linearized by *Eco*RI or *Hin*dIII digestion. The pilin gene sequence was obtained with a synthetic single-stranded 16-base pair (bp) primer which is complementary to the sense strand of the F pilin gene near the carboxy terminus of the leader peptide (5'-ATGATG TTCTTCCCGC-3') (20). The annealing reactions were carried out by the method of Wallace et al. (38) with the following modifications. The linearized DNA (200 ng in 12 μl) was hybridized in an Eppendorf tube with 1 μl (0.5 ng) of primer and 1 μl of 10× sequencing buffer in a boiling water bath for 4 min and quick cooled on ice. The sequencing reactions were carried out by the method of Sanger et al. (34) with reagents for dideoxy sequencing as supplied by New England BioLabs, Inc., Beverly, Mass. The DNA sequence at the 5' and 3' ends of the insert was obtained with linearized plasmid and single-stranded primers which anneal to regions in the pUC8 vector immediately adjacent to the cloning site (15-bp primer, Bethesda Research Laboratories, Inc., Gaithersburg, Md.; reverse primer, New England BioLabs) (21).

Competitive ELISA with whole cells. Competitive ELISAs (enzyme-linked immunosorbent assays) were performed as previously described (37, 42). Cells were grown to late-log phase (10^9 cells per ml), and 40 ml was pelleted by centrifugation at 12,000 × *g* and suspended in 1 ml of phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 1% bovine serum albumin. Cells (0.3 ml) were placed in an Eppendorf tube, and serial dilutions (1/2) were made to 1/128 with PBS containing 0.05% Tween 20 and 1% bovine serum albumin. The antisera were diluted to the appropriate concentration, an equal volume was added to each cell sample, and the cell-antibody mixture was incubated overnight at 4°C. The cells were pelleted for 5 min, and the supernatant was assayed by an ELISA with a microtiter plate that had previously been incubated at 4°C with 2 μg of F or ColB2 pili per ml and drained and washed with PBS containing 0.05% Tween 20. The ELISA procedure used disodium *p*-nitrophenyl-phosphate (1 mg/ml; Sigma Chemical Co., St. Louis, Mo.) as a substrate and the appropriate conjugate, either goat anti-rabbit or goat anti-mouse immunoglobulin G conjugated to alkaline phosphatase (Sigma Chemical Co.)

diluted 1/1000 with PBS containing 0.05% Tween 20. The A_{450} was determined with a Titertek Multiscan ELISA plate reader (Flow Laboratories, Inc., McLean, Va.). The results are expressed as percent inhibition, where the percent ratio of the absorbance of the sample to the absorbance of a control containing no competitor (maximum absorbance) was subtracted from 100%.

Polyclonal and monoclonal antisera. Rabbit polyclonal antisera raised against F or ColB2 pili has been described previously (15) and was used at a 1/1000 dilution. Monoclonal antibodies to F pili have been described elsewhere (manuscript in preparation). JEL92 and JEL93 monoclonal antibodies are specific for the amino-terminal region of F pilin. Purified monoclonal immunoglobulin G was prepared by affinity chromatography on a protein A-Sepharose column (Pharmacia, Uppsala, Sweden) by the method of Worobec et al. (43). The immunoglobulin G was eluted with 1 M acetic acid and dialyzed against PBS. The monoclonal antibodies had A_{280}s of 0.31 per ml (JEL92) and 0.36 per ml (JEL93). Molar concentrations of immunoglobulin G were calculated by the relationship A_{280} of 1.5 = 1 mg/ml, with molecular weight 150,000, i.e., 1.4 nmol/ml for JEL92 and 1.6 nmol/ml for JEL93. JEL92 was used at a dilution of 1/500, and JEL93 was used at a dilution of 1/1,000 in the ELISA.

RESULTS

Characterizing the pili of derepressed plasmids. *E. coli* ED2601 carrying the derepressed plasmids in Table 2 were characterized with respect to the number of pili per 100 cells before and after the addition of 0.01 M NaCN. The presence of cyanide led to the loss of pili from the cell surface without an increase in the number of free pili in the medium. This result is thought to be a demonstration of pilus retraction or dissociation of the pilus into pilin subunits in the membrane, which balances pilus outgrowth, an energy-requiring process (30). ColB2 and pED208 show a marked insensitivity to the presence of cyanide, which may be reflected in the lowered sensitivity to f1 phage exhibited by ColB2. However, this is not the case for pED208, which retains full sensitivity to this phage.

At least one pilin gene from each pilus group previously described (Table 1) (Willetts and Maule, in press) was characterized with respect to its pilin sequence and its reactivity by a whole-cell ELISA with various antisera. Group I, which exhibited the same phage sensitivity pattern, had previously been subdivided into I, (ColVBtrp), Ia (F), and Ib (R124 and R538-1) (41) on the basis of serological differences. Group II pili were thought to be very similar to Group I except for their lowered sensitivity to f1 phage as mentioned above (ColB2 and ColB4) (41). Group III pili (R1-19) and Group IV pili (R100-1) have an altered susceptibility to all three phages tested (f1, f2, and QB) and are serologically distinct from F and R538-1 pili and each other (25, 41).

TABLE 2. Effect of cyanide on piliation

Transfer-derepressed plasmid	No. of pili/100 cells		
	Without CN^-	Plus CN^- for 5 min	Plus CN^- for 20 min
F(JCFLO)	143	25	14
pED202	206	29	49
ColV2-K94	394	147	88
ColVBtrp	182	86	75
pED236	360	352	374
pED203	164	94	80
pED207	212	90	37
pED200	214	76	36
R1-19	180	71	40
R100-1	108	26	22
pED241	138	33	29
pED204	14	<1	<1
pED208	1,700	1,200	1,100

Cloning the pilin gene of F-like derepressed plasmids. Purified plasmid DNA was digested with *Pst*I and electrophoresed on a 1% agarose flat-bed gel with Tris-acetate buffer (15, 17). The DNA in the gel was transferred to nitrocellulose and probed with a 1.4-kilobase (kb) *Pst*I fragment containing the *traALE* genes of the F transfer region (20) labeled with [α-^{32}P]dATP by nick translation as previously described (14) (Fig. 1). ColVBtrp, ColV2-K94, pED200, and pED202 contained 1.4-kb *Pst*I fragments which hybridized with the *traALE* probe and were identical in size to the 1.4-kb fragments found in F and pED236 (14, 20). The R1-19, pED203, and pED207 pilin genes were on equivalent 2.1-kb *Pst*I fragments. R100-1-*Pst*I was further digested with *Bam*HI or *Hin*dIII to decrease the size of the *Pst*I fragment (15 kb) containing the pilin gene. The *traALE* probe annealed to a 4.3-kb *Bam*HI-*Pst*I fragment and also to 14- and 1.3-kb *Hin*dIII-*Pst*I fragments (Fig. 1). Digestion of R100-1 with *Pst*I, *Bam*HI, and *Hin*dIII together resulted in two bands annealing to the *traALE* probe, a 3.0-kb *Bam*HI-*Hin*dIII fragment and a 1.3-kb *Hin*dIII-*Pst*I fragment (data not shown). The pilin gene was found to be on the 3.0-kb fragment, while a sequence equivalent to the carboxy-terminal region of *traE* was found on the 1.3-kb fragment (see below). The pED208 pilin gene could not be cloned with the F pilin gene sequence as a probe (14, 16).

The orientation of the insert in the chimera and the sequence at the 3′ and 5′ ends were obtained with synthetic primers which anneal to sequences immediately neighboring the cloning site in pUC8 (see Materials and Methods). The sequence at the 3′ *Pst*I site of *traALE* and upstream into *traE* was present in all the F-like pilin chimeras. The sequence at the 5′ *Pst*I site which contains the carboxy-terminal sequence of *traY* and the leader sequence for *traA* (18, 20) is conserved in F, ColB2, R124, ColV2-K94, ColVBtrp, and R386. The sequence at the 5′ end of the 2.1-kb inserts in R1-19, ColB4, and R538-1 were identical for all three chimeras and different from any known sequences in F. This unique sequence was found to start immediately upstream of the ribosome-binding site for *traA* (18, 20) and, presumably, to affect the sequence of *traY*. This area corresponds, in part, to the 1.2-kb substitution found in the heteroduplex map of F and R1-19 that occurs at the head of the transfer region (35; A. J. Clark, *Symposium on Origin and Evolution of Sex*, in press). The R100-1 pilin gene was carried on a 4.3-kb *Bam*HI-*Pst*I fragment in which the 3′ sequence was analogous to the 3′ end of the F *traALE Pst*I fragment, and the 5′ end was a sequence unique to R100-1. Sequence analysis of the 3′ and 5′ ends of the two smaller subclones of the 4.3-kb *Bam*HI-*Pst*I fragment (namely, the 3.0-kb *Bam*HI-*Hin*dIII fragment and the 1.3-kb *Hin*dIII-*Pst*I fragment) revealed that the pilin gene was carried on the 3.0-kb *Bam*HI-*Hin*dIII fragment, with a unique sequence both upstream and downstream from the gene. Thus, in addition to differences at the head of the transfer region involving

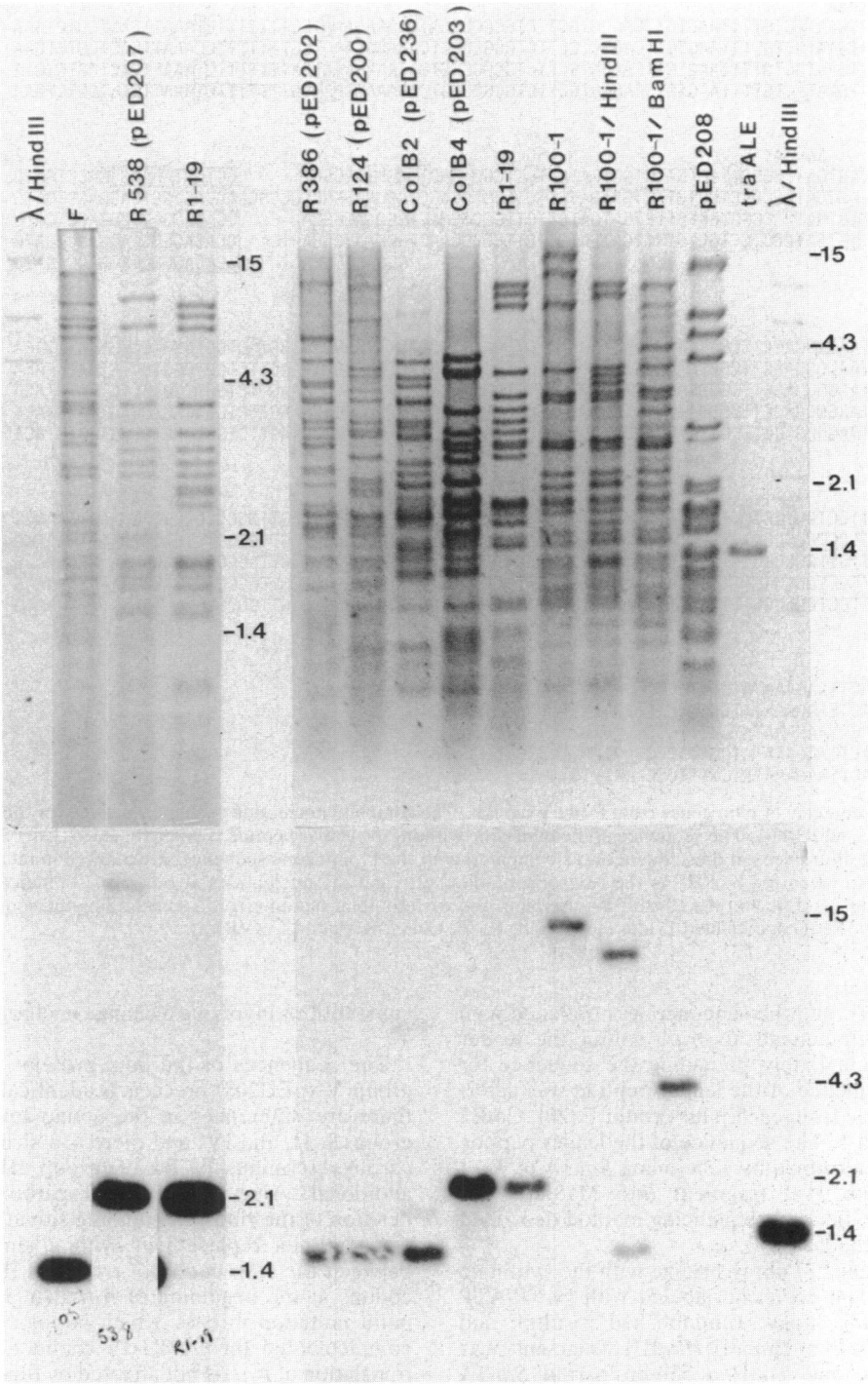

FIG. 1. Restriction digests of derepressed plasmids probed with α-^{32}P-labeled 1.4-kb DNA fragment containing F *traALE*. The figure is a composite of two blots run under similar conditions at different times. R1-19 is included in both blots as an internal reference. All plasmids were digested with *Pst*I, and R100-1 was further digested with *Bam*HI or *Hin*dIII. The digests were electrophoresed on 1% agarose gel in Tris-acetate buffer (14), transferred to nitrocellulose, and hybridized to the probe at 37°C in the presence of 0.1 mg of calf thymus DNA per ml.

genes specific for conjugal DNA metabolism and operon control, there is an insert in the region of *traL* and *traE* which may affect pilus synthesis or function or both. These discontinuities in the sequence have been noted previously (35; Clark, in press) with heteroduplex mapping between F and R100-1. The organization and sequence of DNA in R1-19 and R100-1 involving the transfer region from *traM* to *traY* will be the subject of a separate communication.

The sequence of F-like pilin genes. Purified DNA of a chimera containing a particular pilin gene was linearized

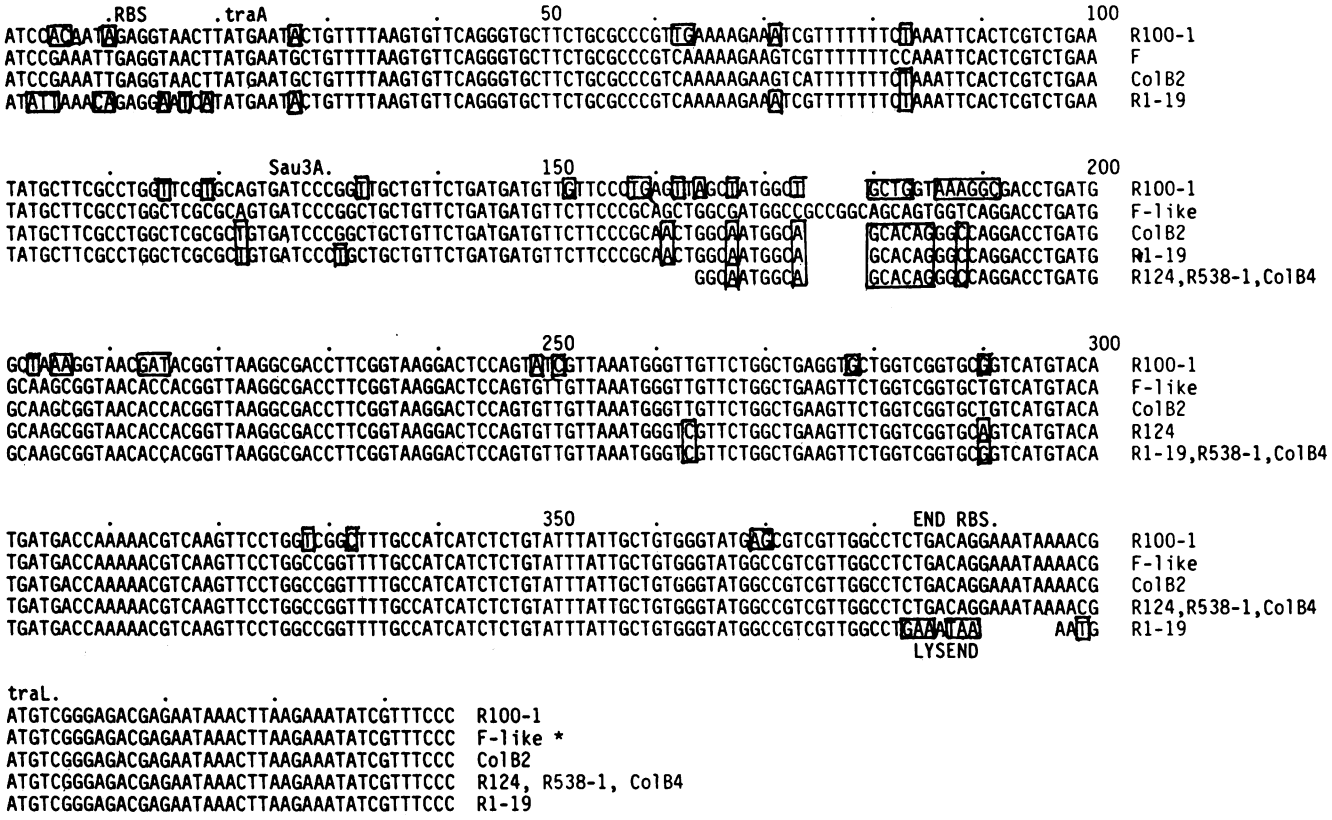

FIG. 2. Nucleotide sequence of pilin genes from F-like plasmids. The complete nucleotide sequence is given for the prototype of each pilus group, F, ColB2, R1-19, and R100-1. The sequence of the pilin gene without the leader peptide is given for R386, ColV2-K94, ColVBtrp, R124, R538-1, and ColB4. The differences in the sequences, as compared with the F pilin gene sequence, are marked in rectangles. The numbering of the nucleotides is also given for F. RBS is the ribosome-binding site, and the nucleotides at position 173 indicate the sequence at the cleavage site of the leader peptide and the start of mature pilin. We wish to point out an error in transcribing the sequence of *traA* (22): $T_{364} \rightarrow G$, i.e., $Val_{115} \rightarrow Gly$. Sequence labeled F-like includes F, R386, ColV2-K94, and ColVBtrp.

with *Eco*RI or *Hin*dIII, and the sequence was obtained with a 16-bp primer which anneals to *traA* within the leader peptide sequence immediately preceding the sequence for mature pilin. The sequence of the leader peptide was determined for one example from each pilus group; F (20), ColB2 (14), R1-19, and R100-1. The sequence of the leader peptide of R1-19 pilin was determined by subcloning *Sau*3A or *Taq*I digests of the 2.1-kb *Pst*I fragment into M13mp18 or M13mp19 and by the dideoxy sequencing method described in Materials and Methods.

The R100-1 pilin gene did not hybridize with the synthetic 16-bp primer which had been end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (unpublished results), and consequently, the 3.0-kb *Bam*HI-*Hin*dIII fragment was mapped with *Sau*3A and *Taq*I; a 550-bp partial *Sau*3A fragment, containing the entire pilin gene, was cloned into pUC8. This chimera was subsequently digested with *Eco*RI and *Hin*dIII, inserted into M13mp18 or M13mp19, and sequenced as above in both orientations to obtain the entire sequence of R100-1 propilin.

The DNA sequence of the various pilin genes is given in Fig. 2 and includes the DNA sequence for the leader peptide in propilin for the prototype in each pilus group (F, ColB2, R1-19, and R100-1). The translated protein sequence for the pilin genes is given in Fig. 3. The leader sequences for all four pilus types are nearly identical except for conservative amino acid substitutions at positions 3, 38, and 47 and two substitutions involving a change in charge at positions 15 and 47.

The sequences of the pilin proteins in all groups except group V (pED208) are nearly identical (Fig. 3). However, there are differences in the amino termini of the pilin of groups I, II, and IV, and there is a striking alteration at the carboxy terminus of R1-19 (group III). This involves an additional lysine residue at the carboxy terminus and the deletion of the ribosome-binding site at the head of the *traL* gene and its replacement with a single-base-pair spacer between the stop codon of *traA* and the double ATG start codons at the beginning of *traL*. An F plasmid carrying a point mutation in *traA* which is polar on *traL* (40) can be complemented by the R1-19 chimera, suggesting that the translation of *traL* is not affected by this sequence alteration.

R100-1 pilin has five other amino acid substitutions besides an altered amino terminus; two of these involve a change in the charge of the residue ($Ser_{62} \rightarrow$ Lys and $Thr_{65} \rightarrow$ Asp). There is some evidence based on sequence studies of point mutants in F pilin (unpublished results) that this region is involved in RNA phage binding and that these amino acid substitutions may define the characteristically reduced RNA phage-plating efficiency of R100-1. The other three substitutions, at positions 77, 103, and 117, are fairly conservative, and their effect on pilus assembly and function is not immediately obvious.

Assaying pilus type on whole cells by the competitive

PROPILIN LEADER SEQUENCE

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                    1                                   10                                  20
F-like (I)          MET ASN ALA VAL LEU SER VAL GLN GLY ALA SER ALA PRO VAL LYS LYS LYS SER PHE PHE SER LYS PHE THR ARG LEU
ColB2-like (II)     MET ASN ALA VAL LEU SER VAL GLN GLY ALA SER ALA PRO VAL LYS LYS LYS SER PHE PHE SER LYS PHE THR ARG LEU
R1-19 (III)         MET ASN [THR] VAL LEU SER VAL GLN GLY ALA SER ALA PRO VAL LYS LYS LYS SER PHE PHE SER LYS PHE THR ARG LEU
R100-1 (IV)         MET ASN [THR] VAL LEU SER VAL GLN GLY ALA SER ALA PRO VAL [GLU] LYS LYS SER PHE PHE SER LYS PHE THR ARG LEU
pED208 (V)          NOT DETERMINED

                              30                                  40                                  50
F-like (I)          ASN MET LEU ARG LEU ALA ARG ALA VAL ILE PRO ALA ALA VAL LEU MET MET PHE PHE PRO GLN LEU ALA MET ALA-
ColB2-like (II)     ASN MET LEU ARG LEU ALA ARG ALA VAL ILE PRO ALA ALA VAL LEU MET MET PHE PHE PRO GLN LEU ALA MET ALA-
R1-19 (III)         ASN MET LEU ARG LEU ALA ARG ALA VAL ILE PRO ALA ALA VAL LEU MET MET PHE PHE PRO GLN LEU ALA MET ALA-
R100-1 (IV)         ASN MET LEU ARG LEU [VAL] ARG ALA VAL ILE PRO [VAL] ALA VAL LEU MET MET [LEU] PHE PRO [GLU] LEU ALA MET ALA-
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PILIN PROTEIN

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                      52                                  60                                  70
F-like (I)          Ac ALA [GLY SER SER] GLY GLN ASP LEU MET ALA SER GLY ASN THR THR VAL LYS ALA THR PHE GLY LYS ASP SER
ColB2-like (II)        Ac ALA [GLN] GLY GLN ASP LEU MET ALA SER GLY ASN THR THR VAL LYS ALA THR PHE GLY LYS ASP SER
R1-19 (III)             * ALA [GLN] GLY GLN ASP LEU MET ALA SER GLY ASN THR THR VAL LYS ALA THR PHE GLY LYS ASP SER
R100-1 (IV)             * ALA [GLY LYS GLY] ASP LEU MET ALA [LYS] GLY ASN [ASP] THR VAL LYS ALA THR PHE GLY LYS ASP SER
pED208 (V)              Ac [THR ASP LEU LEU ALA GLY GLY LYS ASP VAL ASP LYS] ...

                                  80                                  90                                 100
F-like (I)          SER VAL VAL LYS TRP VAL VAL LEU ALA GLU VAL LEU VAL GLY ALA VAL MET TYR MET MET THR LYS ASN VAL LYS
ColB2-like (II)     SER VAL VAL LYS TRP VAL VAL LEU ALA GLU VAL LEU VAL GLY ALA VAL MET TYR MET MET THR LYS ASN VAL LYS
R1-19 (III)         SER VAL VAL LYS TRP VAL VAL LEU ALA GLU VAL LEU VAL GLY ALA VAL MET TYR MET MET THR LYS ASN VAL LYS
R100-1 (IV)         SER [ILE] VAL LYS TRP VAL VAL LEU ALA GLU VAL LEU VAL GLY ALA VAL MET TYR MET MET THR LYS ASN VAL LYS

                                                    110                                 120
F-like (I)          PHE LEU ALA GLY PHE ALA ILE ILE SER VAL PHE ILE ALA VAL GLY MET ALA VAL VAL GLY LEU
ColB2-like (II)     PHE LEU ALA GLY PHE ALA ILE ILE SER VAL PHE ILE ALA VAL GLY MET ALA VAL VAL GLY LEU
R1-19 (III)         PHE LEU ALA GLY PHE ALA ILE ILE SER VAL PHE ILE ALA VAL GLY MET ALA VAL VAL GLY LEU [LYS]
R100-1 (IV)         PHE LEU [VAL] GLY PHE ALA ILE ILE SER VAL PHE ILE ALA VAL GLY MET [SER] VAL VAL GLY LEU
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* Presence of a blocking group has not been determined.

FIG. 3. Amino acid sequence of propilin for each of the pilus groups. A partial sequence for pED208, based on the sequence of tryptic peptides (19), is also given. The leader peptide is assumed to be identical for all members of a pilus group, based on restriction analysis of the chimeras containing the pilin genes. Type I is F, R386, ColV2-K94, and ColVBtrp; type II is ColB2, ColB4, R124, and R538-1; type III is R1-19; type IV is R100-1; type V is pED208. The rectangles indicate differences in the amino acid sequence. The presence of an N-acetyl group has been demonstrated for F, ColB2, and pED208 pilin and is inferred for other members of type I and II (see the text).

ELISA. Whole cells carrying various derepressed plasmids were tested by a competitive ELISA (described in Materials and Methods) with polyclonal rabbit antisera raised against F or ColB2 pili (Fig. 4A and B). These results corroborate the experiments done by Lawn and Meynell (25), who were able to discern four serological groups among the F-like pili tested, namely F, R538-1, R1-19, and R100-1. They concluded that F, R538-1, and R100-1 had unique antigenic determinants and were only weakly cross-reactive with each other. R1-19 was strongly cross-reactive with R538-1 but lacked the weak cross-reactivity shared by the other three serogroups.

Anti-F pilus antiserum reacted strongly with cells carrying F, ColVBtrp, and R386 pili (80 to 100%) which have the F

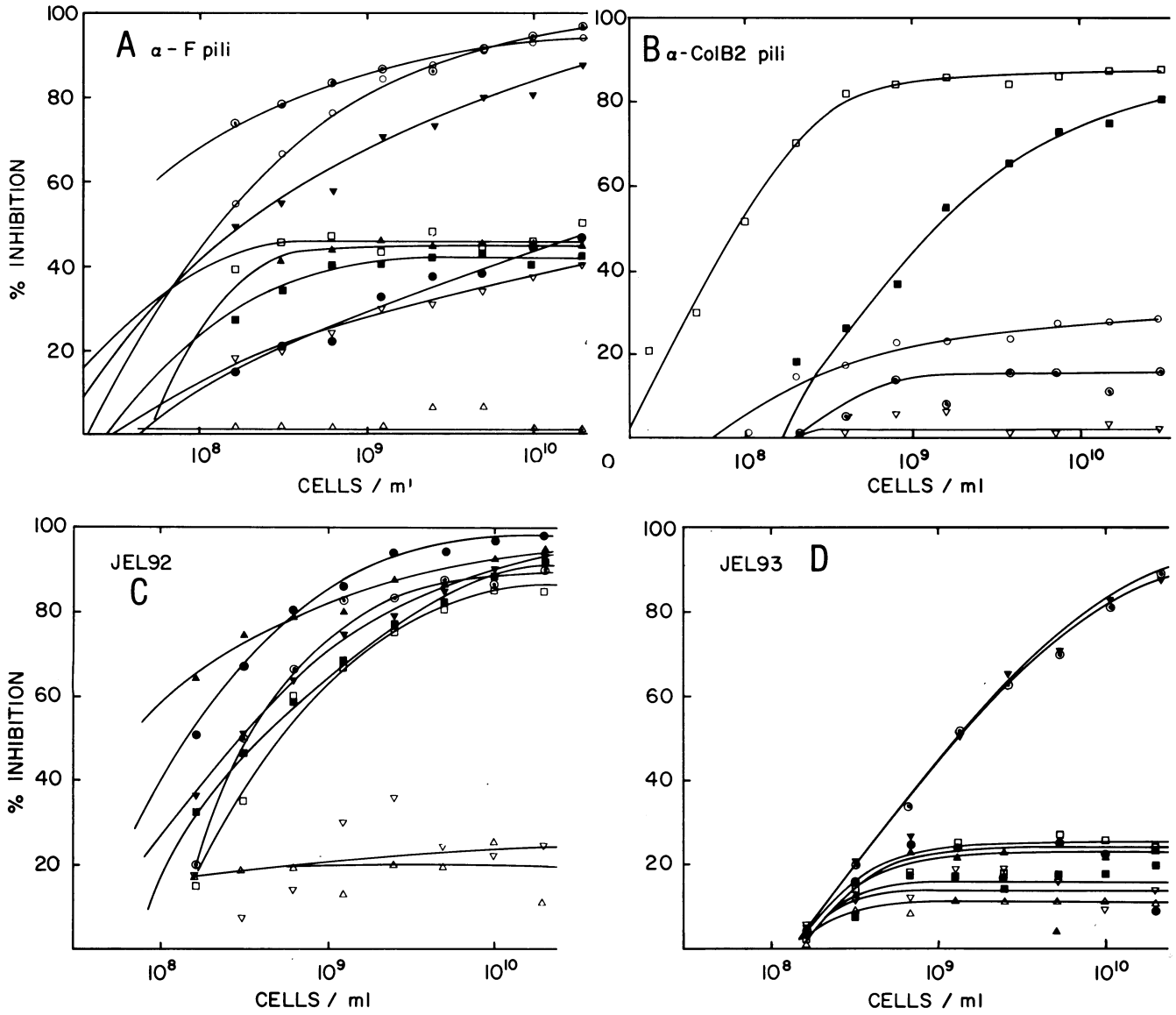

FIG. 4. Results of competitive ELISAs with whole cells carrying derepressed plasmids and four types of antisera directed against F or ColB2 pili. Cells (2×10^{10}/ml) and subsequent 1/2 dilutions were incubated with an equal volume of diluted antiserum as described in Materials and Methods. The cells were pelleted by centrifugation, and the supernatant was used in an ELISA in which the microtiter plate had been preincubated with 2 μg of homologous pili per ml. The results are expressed as percent inhibition of antisera as compared with that of an uncompeted control (100%). (A) Anti-F pilus rabbit polyclonal antiserum (1/1,000); (B) anti-ColB2 pilus rabbit polyclonal antiserum (1/1,000); (C) JEL92 mouse monoclonal antiserum (region near Met_{60}) (1/1,000); (D) JEL93 mouse monoclonal antiserum (F amino terminus) (1/500 dilution). Symbols: ⊙, F(ED2602); △, F⁻(ED2601); ▼, R386(pED202); ▲, R124(pED200); ●, R538-1(pED207); ■, R1-19; ▽, R100-1; □, ColB2(pED236); ○, ColVBtrp.

pilin subunit. Anti-F pilus antiserum reacted moderately with cells carrying ColB2-like pili (ColB2, R124, and R538-1), R100-1 cells and R1-19 cells (40 to 45%). This finding agrees with the results of Lawn and Meynell (25), in which R1-19 cells showed moderate cross-reactivity with 2/4 antisera raised against F piliated cells and no cross-reactivity with the two other anti-F pilus sera.

Anti-ColB2 pilus antiserum reacted less strongly with cells carrying R1-19 pili than with those carrying ColB2 pili (Fig. 4B), although the maximum inhibition for R1-19 cells approached that for ColB2 cells at high cell concentrations ($>10^{10}$ cells per ml). It reacted weakly with cells carrying F pili (15 to 25%) and did not react at all with R100-1 cells.

These results confirm our previous findings that the amino terminus constitutes the major epitope in conjugative pili (15, 43) and that F, ColB2(pED236) or R538-1(pED207), R100-1, and pED208 each define a serogroup which contains a pilus with a subunit bearing a unique amino terminus. R1-19 pilin has the same amino terminus as ColB2 but has an altered carboxy terminus, which may affect its antigenicity and allow it to be differentiated by some anti-F pilus antisera from ColB2-like cells, as was shown by Lawn and Meynell (25).

Whole-cell ELISAs were also performed with monoclonal antibodies (JEL92 and JEL93) which were purified and quantitated as described in Materials and Methods. JEL93 is specific for the amino terminus of F pilin and reactive with an octapeptide representing the first eight amino acids of F

pilin (manuscript in preparation). It reacted strongly with F or R386 cells and very weakly with cells carrying pili from other serogroups (Fig. 4D). JEL92 is specific for the predicted beta turn centered at Met_{60} in pilin (Fig. 3) and reacted with all piliated cells tested except R100-1 (Fig. 4C). The nonreactivity of R100-1 cells could be explained by the presence of Lys_{62} and Asp_{65}, which would be expected to alter the antigenicity of that part of the pilin subunit.

It is worth noting that whole-cell ELISAs with JEL92 were very useful in ascertaining the presence of pili(n) on the cell surface and confirming that nonreactivity with a given antiserum was not due to a lack of pili on the cell surface. The presence of pili on R100-1 cells was confirmed by electron microscopy after labeling the cells with R17 phage particles for easier visibility.

DISCUSSION

The sequences of the pilin genes of F-like plasmids are very similar and differ significantly only at the amino terminus of the mature pilin subunits. The leader sequence in propilin of all the conjugative plasmids presented here contain 51 amino acids with few substitutions. These pilin genes, when cloned into pUC8-derived chimeras and introduced into a strain carrying an F plasmid with a point mutation in *traA* are able to complement the mutation and restore pilus synthesis and conjugal transfer (unpublished results). Thus, the mechanism for cleavage of the leader sequence, N-acetylation of the amino terminus and pilus assembly-retraction, is conserved for all these F-like plasmids, which is as expected since the degree of DNA homology is very high (35).

One-pilus-specific function is the recognition of the *traT* surface exclusion protein in the outer membrane of cells carrying a plasmid specifying the same surface exclusion system (Sfx) (1). Comparing the results presented in this paper with those of Willetts and Maule (Willetts and Maule, in press) and Willetts and Skurray (41), it can be seen that the pilus type matches the surface exclusion system, in which F-like pili interact with SfxI, ColB2-like pili interact with SfxII, R1-19 pili interact with SfxIII, and R100-1-like pili interact with SfxIV. Presumably, pED208 pili are specific for the Folac Sfx system. ColV2 and ColVBtrp (F-like or group I pili) recognize SfxII and SfxIII systems, respectively, and are exceptions to this rule.

Since the only major difference in the proteins of the pilin genes sequenced resides in the amino terminus, it seems plausible that the *traT* protein of a particular Sfx system can interact with the correct amino-terminal sequence and block mating pair formation. However, surface exclusion is obviously more complex since the amino-terminal sequence is not exposed on the pilus filament (see below), and R1-19 pili, which have a ColB2-like amino terminus, do not interact with SfxII. It would be interesting to obtain the sequence of the *traT* proteins for the five Sfx systems and deduce whether they have vastly different protein sequences or whether the changes in the *traT* protein are subtle and localized in a single domain.

Another pilus-specific function is the sensitivity to the three major types of bacteriophages (f1, R17 or f2, and QB), in which the pilus acts as the site of attachment (33). Altered sensitivity to these phages could be due to changes in the pilin sequence which affect the attachment step in the infectious process or it could be due to differences in other *tra* proteins which affect phage penetration into the cell or phage multiplication within the cell. For instance, f2 is known to require a functional *traD* gene product (41) for infection, and cells carrying ColB2-like pili (Table 2) have a variable efficiency of plating (EOP) for f1, suggesting there is an alteration in a function unrelated to the primary sequence of pilin which affects f1 infection. Cells carrying plasmids specifying ColB2-like pili (R124 and R538-1) which are capable of retraction have a 10% decrease in f1 sensitivity as compared with that in F piliated cells (Willetts and Maule, in press), and this may be due to the altered amino terminus. Cells carrying R1-19 pili have a 10% EOP for f1 and a lowered sensitivity to f2 (60%) and QB (3%) (Willetts and Maule, in press). The carboxy-terminal lysine may be sufficient to explain this pattern of phage sensitivity, as compared with that of ColB2-like pili which have identical amino termini to R1-19. This would suggest that the carboxy terminus and amino terminus of the same or neighboring subunits are in close apposition. A point mutant in the F pilin gene (WPFL44) which carries a $Gly_{120} \rightarrow Asp$ substitution at the penultimate residue (unpublished results) has the phage sensitivity pattern f1, 79; f2, 6; and QB, 0% (40). Thus, it seems that a charged residue at the carboxy terminus affects f2 and QB attachment and, to a lesser extent, f1 attachment and suggests that the carboxy terminus is exposed on the sides of the pilus where these phage are known to bind.

There have been reports that F-like pili are glycosylated, phosphorylated, and associated with phospholipids (5, 8, 13). In addition, while it is known that the amino termini of F, ColB2, and pED208 pilin are blocked with an N-acetyl group (14, 19, 20) and that the amino termini of R386, ColB4, and R124 pilin are blocked (unpublished results), it has not been shown that R1-19 and R100-1 pilin subunits are similarly blocked. It is not clear whether these modifications of the pilin subunit play an important role in phage attachment or other pilus-related functions.

The N-acetyl group has been shown to contribute an important part in defining the amino-terminal epitope of pED208 pilin (43) and F pilin (unpublished results). Thus, it seems highly likely that various pili of a particular type in which an N-acetylated amino terminus has been demonstrated for one member of that type (I and II) also contain an N-acetylated pilin subunit to be serologically related.

The serological differences between the various pilus types are clearly due to the alterations in sequence at the amino terminus of the pilin subunit. Electron microscopy studies with the monoclonal antibodies JEL92 and JEL93 indicate that the amino-terminal region is not exposed laterally on the pilus but is exposed on unassembled pilin subunits in terminal knobs (25) at one end of the pilus (unpublished results). This result has been confirmed with polyclonal antisera raised against a synthetic peptide derived from the amino-terminal sequence of pED208 which also binds to the knob at one end of the pilus (unpublished results).

While the amino-terminal region is the major epitope in conjugative pili studied to date, at least one other epitope is present and may involve the carboxy terminus. This region has been implicated in QB and to a lesser extent, f2 attachment, which would suggest that it is exposed laterally on the pilus (see above). It has been shown by electron microscopy that F-specific antibodies bind to the sides of ColB2 pili but not to the sides of R1-19 pili (unpublished results). This would suggest that the epitope involving the carboxy terminus of F is exposed on the sides of the pilus and is destroyed or altered by the presence of a carboxy-lysine residue in R1-19 pilin.

It can be argued that factors other than pilin protein sequence are responsible for the different phenotypes found in cells carrying derepressed F-like plasmids, but this difficulty should be overcome by sequencing defined point mutations in the F pilin gene (40). These studies on the pilin genes of derepressed plasmids may provide a method for probing other pilus-related phenomena, such as retraction and pilus modification and assembly.

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