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Expression of Type 1 Fimbriae and Mannose-Sensitive Hemagglutinin by Recombinant Plasmids

GERALD-F. GERLACH, STEVEN CLEGG,* NANCY J. NESS, DANA L. SWENSON, BRADLEY L. ALLEN, AND WADE A. NICHOLS

Department of Microbiology, University of Iowa, Iowa City, Iowa 52242

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Deletions within the cloned genes (*fimA*) encoding the type 1 major fimbrial subunits of two isolates of *Klebsiella pneumoniae* resulted in a nonfimbriate but hemagglutinating phenotype after transformation of *Escherichia coli* HB101 or ORN103. Phenotypic expression of type 1 fimbriae could be restored by transformation with plasmids containing the *fimA* genes of the fimbrial gene clusters from different strains. The surface fimbriae expressed were serologically identical to those of the polymerized product of the introduced *fimA* gene. The *fimA* gene products of *Salmonella typhimurium* and *Serratia marcescens* could utilize the accessory fimbrial genes of *K. pneumoniae* to produce surface-associated, hemagglutinating fimbriae. The relatedness of the type 1 fimbrial gene clusters from multiple isolates of members of the family *Enterobacteriaceae* was examined by DNA hybridization techniques. These analyses demonstrated little nucleotide sequence agreement among distinct genera of the enteric bacteria.

Type 1 fimbriae are filamentous appendages that are expressed by a variety of members of the family *Enterobacteriaceae* (4) and have been shown to facilitate bacterial adherence to mannose-containing residues of eucaryotic cells (27). Their role as possible virulence determinants has stimulated an interest in the genetic apparatus involved in the regulation, assembly, and expression of these appendages (1, 8, 10, 18, 23, 29). Consequently, gene clusters encoding type 1 fimbriae have been cloned from *Escherichia coli* (16), *Klebsiella pneumoniae* (13, 30), *Salmonella typhimurium*, *Serratia marcescens*, and *Enterobacter cloacae* (5). Studies of the *E. coli* type 1 fimbrial gene cluster have revealed that the major fimbrial subunit, which composes more than 90% of the fimbrial protein, is distinct from the functional adhesin (26). The fimbria-associated polypeptides are assembled only in the presence of a relatively large gene product that is present in all fimbrial gene clusters identified to date (7, 28). Besides the major fimbrial subunit and the receptor-binding moiety, other minor fimbrial proteins also appear to be necessary for the expression of functional type 1 fimbrial filaments (19).

By using the *E. coli* gene cluster, it has been possible to construct mutants that are fimbriate but nonhemagglutinating (Fim^+ HA^- [26]). In this paper we describe the construction of mutants, from two distinct *K. pneumoniae* type 1 fimbrial gene clusters, that are nonfimbriate but retain hemagglutinating specificity (Fim^- HA^+). Because the type 1 fimbriae from different genera within the family *Enterobacteriaceae*, and occasionally even within the same genus, are antigenically distinct (13, 20), it would be interesting to determine whether the fimbrial subunits of one strain can utilize the accessory genes from a second strain. The construction of a Fim^- HA^+ mutant of *K. pneumoniae* and the molecular cloning of fimbrial genes from *S. typhimurium* and *S. marcescens* have enabled us to perform such studies. The results of these experiments are reported herein.

Initial work by Buchanan and co-workers (3) suggested that the gene encoding the major fimbrial subunit of *E. coli* was conserved among many isolates of this species but showed little sequence agreement with genes from other species. However, the relatedness of other genes within the gene cluster was not examined. Considering that the type 1 fimbriae of all enteric bacteria share the property of mannose sensitivity, it may be possible that the gene(s) encoding receptor-binding specificity may be more highly conserved. Consequently, gene probes, derived from different regions of the fimbrial gene cluster, in three genera of the family *Enterobacteriaceae*, were used to determine the relatedness of nucleotide sequences within the enteric bacteria.

MATERIALS AND METHODS

Bacterial strains and media. All isolates were obtained from the Special Microbiology Laboratory of the University of Iowa Hospitals and Clinics or the Iowa State Hygienic Laboratory. The isolates were maintained on blood agar, and the number of strains and species used are shown in Table 2. *E. coli* HB101 (24) and ORN103 (28) were used in all transformation experiments and were maintained on Luria agar supplemented with the appropriate antibiotics (24). All incubations were performed at 37°C for 18 to 24 h unless otherwise stated.

Hemagglutination and serological assays. Hemagglutination tests were performed using 3% (vol/vol) suspensions of fresh guinea pig erythrocytes to detect the type 1 fimbria-associated adhesin (13). Serological identification of fimbriae on the surface of bacteria was performed using a microagglutination assay, and antisera were raised against fimbriae purified as described previously (14).

Recombinant plasmids. The construction and characterization of plasmids encoding the expression of type 1 fimbriae of *K. pneumoniae* IA565 (pGG101), *K. pneumoniae* IA551 (pBP7), *S. typhimurium* 6704 (pISF101), and *S. marcescens* IA506 (pMH2) have been described elsewhere (5, 7, 13). For *trans* complementation analyses the plasmids used contained compatible replicons. Double transformants were screened for the presence of two distinct plasmid molecules as previously described (6).

DNA colony blot. The DNA colony blot was performed on nitrocellulose disks (Schleicher & Schuell, Inc., Keene, N.H.), using standard techniques (24). DNA probes were

* Corresponding author.

prepared by radiolabeling appropriate DNA fragments (see Fig. 1), using the random priming procedure (12). The washes established high-stringency conditions.

Nucleotide sequence analysis. DNA sequencing was performed by the method of Maxam and Gilbert (25). The complete nucleotide sequences of the *fimA* genes of *K. pneumoniae* IA565, *K. pneumoniae* IA551, and *S. typhimurium* 6704 have been described previously (13, 31).

Electron microscopy. Bacterial suspensions, in phosphate-buffered saline, were applied to carbon-coated grids, stained with phosphotungstic acid, and examined with a Hitachi H-600 electron microscope.

RESULTS

Construction of Fim$^-$ HA$^+$ strains. Recombinant plasmids pGG101 and pBP7, encoding the expression of *K. pneumoniae* type 1 fimbriae, were used to construct deletion derivatives (Fig. 1). Plasmid pGG135 was constructed after a partial *ClaI* digest to remove the C-terminal 390 base pairs of the *fimA* gene of pGG101 (13), and removal of this fragment was confirmed by restriction enzyme digestion. The deletion derivative pGG133 was prepared by subcloning the 7.0-kilobase-pair *ClaI* fragment of pGG101. This plasmid does not contain the three genes of the left-hand end of the gene cluster including the *fimA* gene (Fig. 1). Plasmid pBP750 was constructed by linearization of pBP7 at the *PstI* site, within the *fimA* gene, removal of nucleotides by using high concentrations (10 U/μg of DNA) of S1 nuclease, and religation. Transformants were screened for the Fim$^-$ HA$^+$ phenotype, and the size of the deletion was determined by nucleotide sequencing, which demonstrated the loss of 270 bases solely within the fimbrial subunit gene. After transformation of either *E. coli* HB101 or ORN103 with any of the three deletions (pGG135, pGG133, or pBP750), transformants exhibited mannose-sensitive hemagglutinating activity but were found to be nonfimbriate when observed by electron microscopy (Fig. 2a). In addition, transformants did not react with serum raised against hemagglutinating fimbriae prepared from the homologous fimbriate bacteria. The hemagglutinating activity was strongest when the high-copy-number vector, pIBI30 (International Biotechnologies, Inc., New Haven, Conn.), was used as the cloning vehicle.

Complementation analyses. The Fim$^-$ HA$^+$ transformants were subsequently transformed with a second plasmid containing the gene encoding the fimbrial subunit and compatible cloning vectors. The plasmid profiles of double transformants were verified by restriction enzyme digestion, and the results of the complementation studies, using pGG135, are summarized in Table 1. Identical results were obtained with either pGG133 or pBP750. In the presence of a gene encoding the major fimbrial subunit, all transformants were phenotypically Fim$^+$ HA$^+$, regardless of the source of the fimbrial subunit gene (Fig. 2b and d). In addition, the fimbriae expressed on the surface of such transformants were serologically identical to those on transformants from which the subunit gene had been cloned. For example, pGG135-pISF141 transformants produced fimbriae that are serologically identical to those of *S. typhimurium*, and pBP750-pGG161 transformants express *K. pneumoniae* IA565 type 1 fimbriae and not *K. pneumoniae* IA551 fimbriae. The transformation of *fimA* deletions with a plasmid (pSHA25) containing the *S. marcescens fimA* gene (Fig. 1) likewise resulted in the expression of fimbriae (Fig. 2d). These fimbriae were thin (2 to 3 nm) and are identical to those found on the surface of pMH2 transformants (Fig. 2c). *E. coli* transformants containing only the cloning vectors (pACYC184 or pBR322) or the recombinant plasmids possessing just the *fimA* genes were phenotypically Fim$^-$. Because transformants containing pGG135, pGG133, or pBP750 agglutinate erythrocytes, it was possible that double transformants that are Fim$^+$ HA$^+$ possess the fimbrial appendage and the hemagglutinin as separate structures on the bacterial surface. Therefore, fimbriae were purified from representative strains and, in all cases, these cell free preparations agglutinated guinea pig erythrocytes only in the absence of D-mannose.

Frequency of type 1 fimbrial gene sequences among enteric bacteria. The DNA probes used in the colony blot assays are shown in Fig. 1. Table 2 summarizes the hybridization analyses and demonstrates that, in most cases, significant nucleotide sequence agreement was found only within genera. Furthermore, all HA$^+$ strains within each genus are recognized by the specific probe. However, probes derived from *K. pneumoniae* IA551 reacted with genomic DNA from all 5 isolates of *E. coli* tested and only 5 of 20 *K. pneumoniae* strains.

Regardless of the origin of the location from within the fimbrial gene cluster, the DNA probes demonstrated consistent hybridization characteristics. For example, when pISF101 was used as a source of DNA for the probes, nucleotide sequences from within the fimbrial subunit gene only (PI), from the right-hand end of the gene cluster (PII), or comprising the entire cluster (PIII) hybridized to *Salmonella* genomic DNA only. Identical results were obtained for DNA probes derived from *K. pneumoniae* IA565. The probes derived from the cloned gene cluster of *S. marcescens* hybridized to genomic DNA from 10 of 11 strains of *Serratia* species but did not hybridize to 1 nonfimbriate strain of *Serratia liquefaciens* (Table 2). In addition, a weak hybridization to *S. marcescens* probe PI was observed when genomic DNA from three *Enterobacter* isolates (two MSHA$^+$ and one MSHA$^-$) was used.

DISCUSSION

The differentiation between fimbrial filament and adhesive moiety is now well established for the P pili (21, 22) and the type 1 fimbriae of *E. coli* (15, 26). In the former system, recombinant plasmids conferring the Fim$^+$ HA$^-$ or the Fim$^-$ HA$^+$ phenotype have been constructed (21). In the *E. coli* type 1 fimbrial system, however, only the Fim$^+$ HA$^-$ phenotype has been described (19, 26), leading to the speculation that the *fimA* gene product is necessary for the expression of the adhesive moiety of this fimbrial type (19). On the other hand, the early work of Eshdat and co-workers (9) and the recent report by Abraham and co-workers (2) indicated that mannose-sensitive adhesion can occur in the absence of the type 1 fimbrial filaments. Our observations of the Fim$^-$ HA$^+$ phenotype by transformants lacking a functional *fimA* gene support these results and, in addition, show that the *fimA* gene product is not required for the expression of mannose-sensitive receptor-binding ability in *K. pneumoniae*. Indeed, one of the deletion derivatives used in these studies (pGG133) contains no nucleotides of the *fimA* structural gene. Therefore, a functional mannose-sensitive adhesin can be synthesized by the type 1 fimbrial gene cluster, in *K. pneumoniae*, even in the absence of the major fimbrial subunit.

The strongest hemagglutination reaction was observed when the appropriate genes were carried on high-copy-number cloning vectors, indicating that relatively large

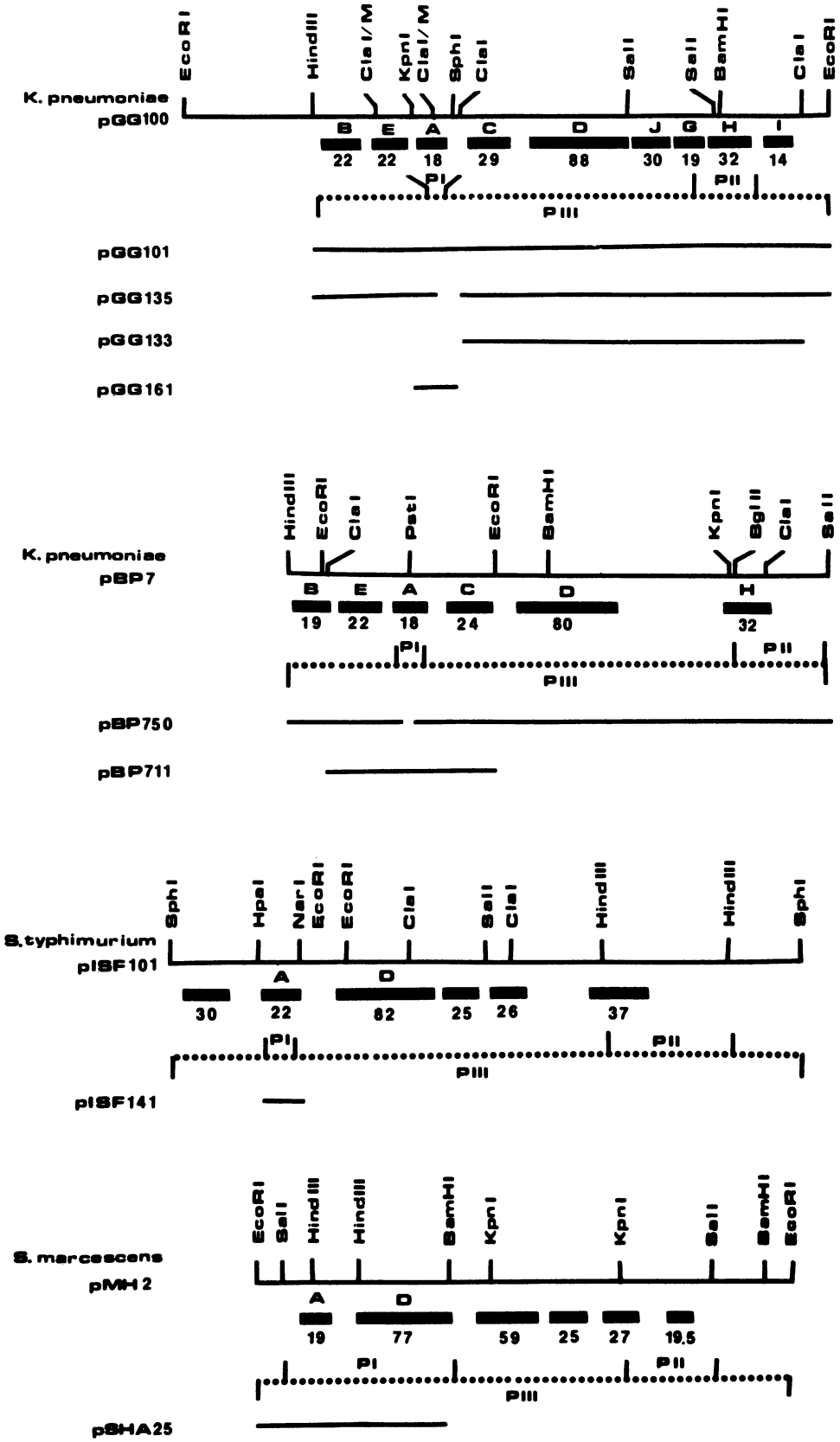

FIG. 1. Physical maps of recombinant plasmids pGG100, pBP7, pISF101 and pMH2 and their deletion derivatives. The location of the fimbria-associated genes are shown by the thick black lines, and the sizes of the gene products are in kilodaltons. The gene designed *fimA* encodes the major fimbrial subunit, and *fimD* encodes the high-molecular-weight polypeptide so far observed in all type 1 fimbrial gene clusters. The genes for the *K. pneumoniae* systems have been designed by analogy to those of the *E. coli* system (19). The black lines represent the nucleotide sequences retained by the deletions. Plasmids pGG161, pBP711, pISF141, and pSHA25 possess the *fimA* gene of *K. pneumoniae* IA565, *K. pneumoniae* IA551, *S. typhimurium* 6704, and *S. marcescens* IA506, respectively. The sizes of DNA probes PI, PII, and PIII are shown by the dotted lines.

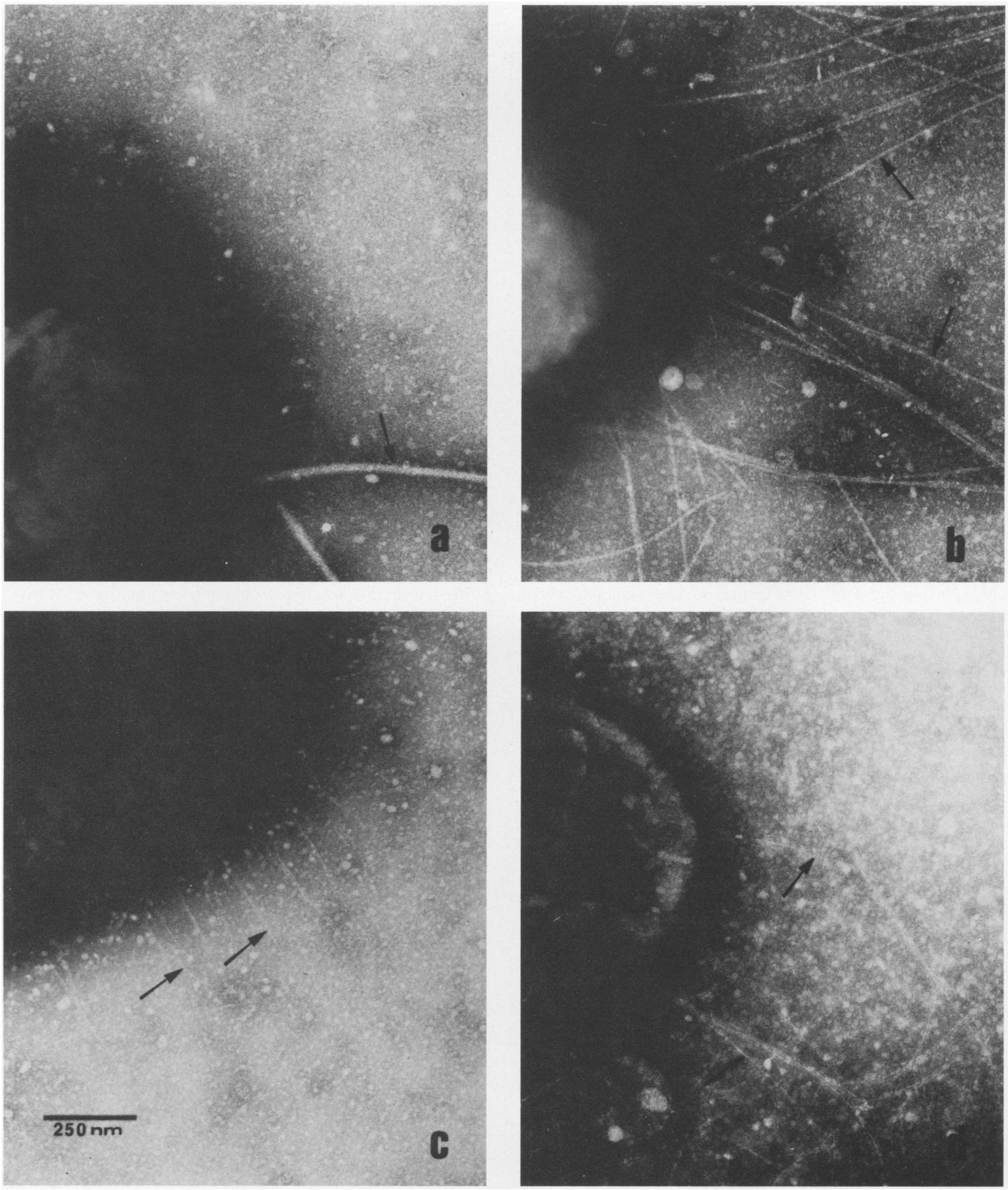

FIG. 2. Electron micrographs of *E. coli* ORN103 transformed with pGG133 (Fim^- HA^+) (a), *E. coli* ORN103 transformed with pGG135 plus pISF141 (Fim^+ HA^+) (b), *E. coli* HB101 transformed with pMH2 (Fim^+ HA^+) (c), and *E. coli* ORN103 transformed with pGG135 plus pSHA25 (Fim^+ HA^+) (d). The arrows indicate the following: in panel a, flagella; in panel b, the thick type 1 fimbriae that react with *S. typhimurium* fimbrial antiserum; in panels c and d, the thin type 1 fimbriae of *S. marcescens*.

TABLE 1. Agglutination and hemagglutination reactions of *E. coli* transformants, using fimbrial type-specific sera

E. coli ORN103 transformed by:	Reaction[a] with antifimbrial sera raised against fimbriae expressed by:				Mannose-sensitive hemagglutination
	K. pneumoniae IA565	*S. typhimurium* 6704	*K. pneumoniae* IA551	*S. marcescens* IA506	
pGG135	−	−	−	−	+
pGG135 + pGG161[b]	+++	−	−	−	+++
pGG135 + pISF141	−	+++	−	−	+++
pGG135 + pBP711	−	−	+++	−	+++
pGG135 + pSHA25	−	−	−	+++	+++

[a] −, No reactivity; +++, clumping of bacterial suspension within 15 s.
[b] *E. coli* ORN103 containing only pGG161, pISF141, pBP711, or pSHA25 was Fim$^-$.

amounts of the adhesin need to be present, in the absence of the fimbrial filament, to cause hemagglutination.

In the *fimA* deletion derivatives the hemagglutinin must be transported to the surface of the bacterial cell in order to mediate hemagglutination, but need not exist in the same conformation as when it is associated with the polymerized fimbrial subunit. It was interesting that none of the Fim$^-$ HA$^+$ strains reacted with fimbria-specific antisera that had been raised against hemagglutinating fimbriae. These results confirm observations by Hanson and Brinton (15) indicating that intact fimbrial appendages possess the adhesin structures, but when associated with the fimbrial filament, these receptor-binding components are poor immunogens.

Because functional adhesive fimbriae are composed of more than one gene product, the ability of fimbrial subunits to assemble polypeptides from different systems was analyzed using the Fim$^-$ HA$^+$ mutants. The mutants, when complemented in *trans* by plasmids containing the homologous *fimA* gene, produced functional fimbriae on the surface of the bacteria. In addition, complementation was found to occur when the fimbrial subunit genes from different genera were used (Fig. 1). In all cases the bacteria produced adhesive type 1 fimbriae, as evidenced by seroreactivity, and the fimbriae were morphologically intact when observed by electron microscopy (Fig. 2b and d). Also, the fimbriae produced were antigenically identical to those from which the subunit gene was derived, confirming that the fimbrial filament is the immunodominant component of the appendage. Hemagglutinating activity was associated with the fimbriae because purified fimbrial protein retained receptor-binding specificity. Therefore, the fimbrial filament from one genus of enteric bacteria may associate with secondary fimbrial polypeptides from a second genus. From our data it might be inferred that the association between adhesin and fimbrial filament is nonspecific since hemagglutinating fimbriae could be isolated from the double transformants. In addition, there appeared to be little sequence agreement, as evidenced by the hybridization data, among regions containing the adhesin genes. However, our results do not differentiate between a loose association of the adhesins from one genus and the fimbrial shaft of a second genus compared with a specific protein-protein interaction that may occur in the wild-type strains. This problem could be further explored using immunogold labeling of adhesin-specific antibodies to detect the location of the adhesin in the hybrid fimbriae. This association is especially interesting for the FimA protein of *S. marcescens* since these fimbriae differ morphologically from those of *K. pneumoniae* and *S. typhimurium* (Fig. 2c). Furthermore, previous data from our laboratory have shown that the primary amino acid sequences of the *K. pneumoniae* and *S. typhimurium* fimbrial subunits share an amino acid identity of only 50%. Both variable and invariable regions could be identified by comparing these proteins (31). One of the functions of the invariable regions may be to facilitate interaction with other fimbria-associated proteins, such as the adhesin.

The results of the complementations shown in Table 1 are different from those previously described using transposon insertion mutants (6). However, these two experimental designs are not identical in that only one, two, or three gene products from the heterologous cluster are present in the complementation experiments (Fig. 1) described herein. In contrast, in the complementation studies with Tn*5*-induced mutations, most fimbria-associated polypeptides from two

TABLE 2. Colony blot DNA hybridization, using probes derived from cloned fimbrial gene clusters

Bacteria	Total no. of isolates	No. exhibiting MSHA[a]	No. of isolates exhibiting hybridization to DNA probes of[b]:			
			K. pneumoniae		*S. typhimurium* 6704	*S. marcescens* IA506
			IA565	IA551		
Klebsiella pneumoniae	20	9	17	5	0	0
Klebsiella oxytoca	7	0	0	0	0	0
Escherichia coli	5	5	0	5	0	0
Salmonella typhimurium	9	5	0	0	9	0
Serratia marcescens	9	8	0	0	0	9
Serratia liquefaciens	2	1	0	0	0	1
Enterobacter cloacae	5	3	0	0	0	3[c]
Morganella morganii	5	2	0	0	0	0
Providencia spp.	5	2	0	0	0	0
Yersinia spp.	5	2	0	0	0	0

[a] MSHA, Mannose-sensitive hemagglutination.
[b] DNA probes PI, PII, and PIII used in these assays are shown in Fig. 1.
[c] Two MSHA$^+$ isolates and one MSHA$^-$ isolate hybridize weakly only to the PI DNA probe of *S. marcescens*.

distinct gene clusters are present. This may lead to protein-protein interactions between gene products of the incomplete homologous systems, resulting in no expression of functional appendages.

The work of Buchanan and co-workers (3) has demonstrated that nucleotide sequences of the *E. coli* fimbrial subunit gene are conserved among isolates of *E. coli* and *Shigella* species but not within other members of the *Enterobacteriaceae*. Our results are consistent with this observation and further indicate that nucleotide sequences of the remaining accessory genes are also conserved within species. Interestingly, the fimbrial gene cluster, cloned from *K. pneumoniae* IA551, appears to be more frequently found among isolates of *E. coli*. We have previously reported that this gene cluster is similar to that of *E. coli* (5, 31) but, for a number of reasons, is not identical. First, the predicted amino acid sequence of the fimbrial subunit is not that reported for the *E. coli* subunit (17). Second, the predicted N-terminal amino acid sequence is identical to that reported for the fimbriae purified from a second strain of *K. pneumoniae* (11) and again different from that of *E. coli* (17). Third, specific antiserum raised against the fimbriae of *K. pneumoniae* IA551 does not react with *E. coli* fimbriae, nor does *E. coli* antiserum recognize epitopes of the *Klebsiella* fimbriae (B. K. Purcell, Ph.D. thesis, University of Iowa, Iowa City, Iowa, 1987). It is possible that the gene cluster from strain IA551 was originally derived from *E. coli* and established itself in *K. pneumoniae* at a later time than the fimbrial cluster isolated from strain IA565. Consequently, the IA551 gene cluster is more closely related to that of *E. coli* and is present in a relatively small number of *K. pneumoniae* strains.

Our results indicate that the mechanism of synthesizing type 1 fimbriae within members of the *Enterobacteriaceae* appears to be highly conserved, as evidenced by complementation to produce functional fimbriae. From our studies it is not possible to state whether, in the presence of both homologous and heterologous *fimA* gene products, there is a preference for the minor fimbrial components to assemble with the homologous fimbrial subunit. Currently, we are determining the specificity of the association between fimbrial polypeptides.

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