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Construction and Expression of Recombinant Plasmids Encoding Type 1 Fimbriae of a Urinary *Klebsiella pneumoniae* Isolate

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The type 1 fimbriae of *Klebsiella pneumoniae* have been implicated as important virulence factors in mediating *Klebsiella* urinary infections. The chromosomally encoded fimbrial genes were cloned by a cosmid cloning technique. Further subcloning was performed with the cloning vehicles pBR322 and pACYC184, and a recombinant plasmid containing the fimbrial genes was constructed. After transformation by this plasmid, both *Escherichia coli* and *Salmonella typhimurium* were shown to express fimbriae which reacted with *Klebsiella* fimbrial antiserum. The approximate location of the relevant genes on the chimeric plasmid was determined by insertion of the transposable element Tn*5*. Hemagglutination-negative phenotypes were used to estimate the minimum size of the DNA fragment necessary to encode fimbrial biosynthesis and expression. The size of the coding region of this fragment was found to be 5.5 kilobase pairs.

Many strains of *Klebsiella pneumoniae* produce type 1 fimbriae (pili) which are responsible for mannose-sensitive hemagglutination (MSHA) of guinea pig erythrocytes. Some reports have suggested that the type 1 fimbriae of *K. pneumoniae* mediate attachment to uroepithelial cells and, therefore, facilitate colonization of the urinary tract (9–11). In contrast to uropathogenic strains of *Escherichia coli* which possess fimbriae responsible for mannose-resistant hemagglutination (mannose-resistant HA) of human erythrocytes (13, 17), no such mannose-resistant fimbrial adhesins have been observed in strains of *K. pneumoniae*.

Type 1 fimbriae are produced by many strains of enterobacteria. Recently, Hull et al. constructed a recombinant plasmid possessing the genes coding for type 1 fimbriae of *E. coli* (15). A comparison, at the molecular level, of the genetic elements encoding type 1 fimbriae in enteric bacteria would prove useful in determining the degree of homology between the DNAs of genera which synthesize fimbriae. Antiserum prepared against purified pilin of *K. pneumoniae* was found not to cross-react with *E. coli* type 1 fimbriae (11). This difference in antigenicity may reflect a difference at the genetic level. Therefore, unique sequences of DNA involved in fimbrial expression may be found within different members of the enterobacteria.

To examine the regulatory mechanisms involved in the expression of type 1 fimbriae by *K. pneumoniae*, we decided to use the techniques of recombinant DNA technology to isolate the relevant genes on chimeric plasmids. This report describes the cloning of the type 1 fimbrial genes. The size of the DNA fragment necessary for phenotypic expression of fimbriae was estimated by insertion of the transposable element, Tn*5*, into a recombinant plasmid.

MATERIALS AND METHODS

Bacterial cultures, growth conditions, and HA tests. *K. pneumoniae* strain IA551 was isolated by the bacteriology laboratory in the University of Iowa Medical School, Iowa City. The strain was isolated from the urine of a patient presenting with symptoms of a urinary tract infection. The fimbriate phase of the organisms was maintained by serial passage in broth medium incubated at 37°C for 48-h periods.

Unless otherwise stated, *E. coli* HB101 (3) was used in all transformation experiments, competent cells being prepared by the method of Morrison (21). Competent cells of *Salmonella typhimurium* JL781 (24) were used to detect expression of fimbriae by a *Salmonella* strain.

Bacteria possessing recombinant plasmids were grown in L-broth or on L-agar plates (19) for 18 to 24 h at 37°C. Media were supplemented with antibiotics at the following concentrations (μg/ml): ampicillin, 100; tetracycline, 20; kanamycin, 25; chloramphenicol, 25 (200 for plasmid amplification).

HA tests were performed on glass microscope slides with a 3% suspension of guinea pig erythrocytes (13). One drop of erythrocytes was mixed with a small amount of bacteria, and the slide was gently rocked for

approximately 30 s. HA-positive cultures agglutinated the erythrocytes within 5 to 10 s. To test for MSHA, the erythrocytes were resuspended in phosphate-buffered saline containing 2% (wt/vol) D-mannose.

Bacterial agglutination tests were performed by the method described by Duguid and Campbell (7).

Bacterial suspensions were prepared for electron microscopy after being washed in sterile distilled water. One drop of the suspension was placed on a carbon-coated copper grid, and excess fluid was removed with filter paper strips. The bacteria were negatively stained for 30 s with phosphotungstic acid and examined with a JOEL transmission electron microscope.

Buffers and reagents. TE buffer consisted of 10 mM Tris (pH 8.0) and 1 mM EDTA. Restriction endonuclease digests were analyzed after electrophoresis through horizontal 0.8% agarose gels in 40 mM Tris–5 mM sodium acetate (pH 7.9)–1 mM EDTA buffer.

Restriction endonucleases used in this study were obtained from commercial sources, and digestions were performed under conditions recommended by the manufacturer. Ligation of enzyme digests was carried out in ligase buffer containing 50 mM Tris-hydrochloride (pH 7.8), 10 mM $MgCl_2$, 20 mM dithiothreitol, 1 mM ATP, and 50 μg of bovine serum albumin per ml. Ligations were performed in 100-μl volumes with T4 DNA ligase (New England Biolabs).

Preparation of DNA. The chromosomal DNA of *K. pneumoniae* IA551 was prepared by the method of Stauffer et al. (25).

Hybrid plasmid DNA was isolated from transformed cells after sodium dodecyl sulfate lysis and ethidium bromide-cesium chloride equilibrium density gradient centrifugation (14, 23). The procedure of Cameron et al. (4) as modified by Williams et al. (27) was used to examine transformants for the presence of plasmid DNA. DNA samples were stored in TE buffer at 4°C.

Cloning of the *fim* genes by cosmid pMF7. The initial cloning of *K. pneumoniae* chromosomal DNA fragment coding for fimbriae was performed by the cosmid cloning technique of Meyerowitz and co-workers (18). Cosmid pMF7 was kindly supplied by M. G. Feiss and has been described in detail elsewhere (12). Partial *Eco*RI digests of *Klebsiella* chromosomal DNA (6.5-μg samples) were ligated into *Eco*RI-restricted pMF7 (7.2 μg). After packaging into λ phage particles, ampicillin-resistant (Ap^r) transductants of *E. coli* HB101 were selected and screened for MSHA activity. Clones exhibiting the desired phenotype were examined by electron microscopy to confirm the presence of fimbriae.

Isolation of Tn*5* insertions. Phage λ*b*221 *c*I857 carrying the transposable kanamycin resistance (Kn^r)-determining element Tn*5* (1) was used to insert Tn*5* into plasmid pBP7. Bacteria possessing pBP7 were incubated at 37°C in tryptone broth (10 ml) containing 0.2% maltose until the cell density was approximately 5×10^8 bacteria per ml. After centrifugation, the bacteria were resuspended in 2 ml of 0.01 M $MgSO_4$, and 0.1-ml aliquots were infected with λ:Tn*5* phage at a multiplicity of infection slightly greater than 1. After incubation for 1 h at 30°C, the mixtures were spread on L-agar plates (19) containing kanamycin plus chloramphenicol and incubated at 30°C for 18 to 24 h. Kn^r Cm^r bacteria (Cm^r is chloramphenicol resistant) were harvested from the plates, and plasmid DNA was prepared and used to transform the nonfimbriate *E. coli* HB101 strain. The Kn^r Cm^r transformants were tested for HA activity, and the insertion site of Tn*5* into pBP7 of HA^+ and HA^- clones was determined by restriction enzyme digestion.

RESULTS

Cloning and subcloning of the type 1 fimbrial genes. The in vitro λ packaging procedure was used to obtain Ap^r transductants possessing pMF7 with inserted *Klebsiella* chromosomal DNA fragments. Of 291 Ap^r transductants tested, 3 were found to be $MSHA^+$, and 1 of these was used for the preparation of plasmid DNA. Plasmid pBP1 (Fig. 1) consisted of approximately 52 kilobase pairs (kb) and transformed *E. coli* HB101 to Ap^r and HA activity, with 100% cotransfer of the two properties.

The gene(s) encoding fimbrial production and expression was subcloned with pBR322 (2) and pACYC184 (6) as vehicles (Fig. 1). Briefly, after digestion with a suitable restriction endonuclease and ligation into the appropriate vector, those transformants possessing recombinant plasmids were detected as a result of insertional inactivation of an antibiotic resistance determinant. Plasmids pBP4, pBP5, pBP6, and pBP7 were isolated from $MSHA^+$ clones.

The smallest plasmid isolated, pBP7, which coded for the mannose-sensitive hemagglutinin was found to be 14.5 kb in size. A physical map of this plasmid is shown in Fig. 2. The size of the inserted DNA was calculated to be 11.1 kb, and all Cm^r transformants containing pBP7 were $MSHA^+$. Further subcloning experiments were unsuccessful in producing strains which possessed recombinant plasmids and which were phenotypically HA^+. For example, removal of the 2.3-kb *Eco*RI fragment or the large 8.4-kb *Pst*I fragment from pBP7 and subsequent ligation into the *Eco*RI and *Pst*I sites of pBR322 failed to produce chimeric plasmids which, after transformation of *E. coli* HB101, enabled the bacteria to express functional fimbriae.

Identification of the *fim* gene(s) within the 11.1-kb fragment of pBP7. To map the *fim* genes within pBP7, we made use of the transposon Tn*5*. A number of plasmids were isolated with insertions of Tn*5* and used to transform the nonfimbriate HB101 strain. Cm^r Kn^r transformants were isolated and tested for their ability to agglutinate guinea pig erythrocytes in the presence and absence of mannose. Those transformants which had lost the ability to cause MSHA of erythrocytes have the Tn*5* inserted into the structural or regulatory DNA elements required for fimbrial expression. The sites of Tn*5* insertions within $MSHA^+$ and $MSHA^-$ transfor-

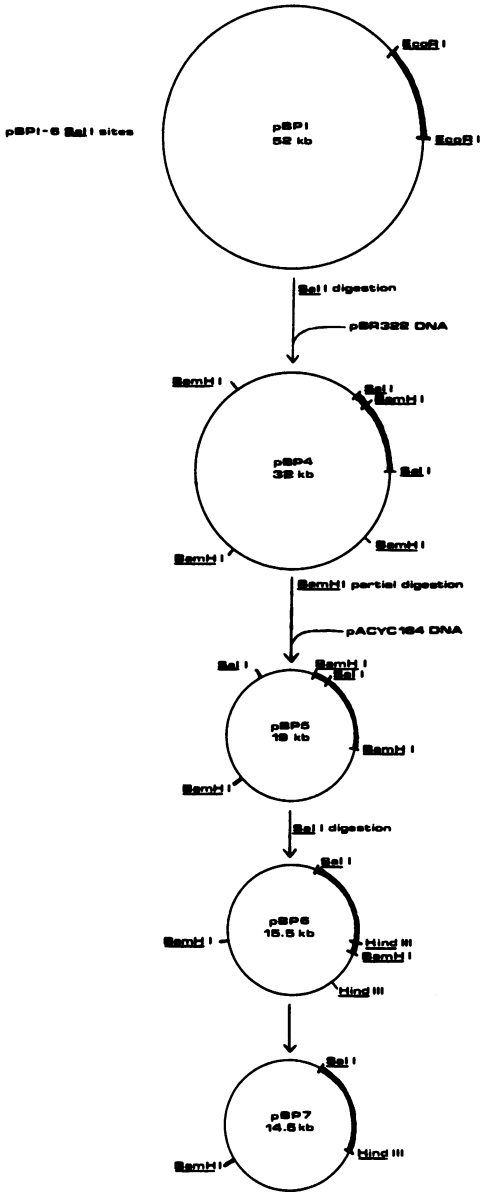

FIG. 1. Derivation of plasmid pBP7 from the original plasmid, pBP1, isolated by the cosmid cloning technique. Only those restriction enzyme sites used in the subcloning experiments are shown. The thick black lines denote cloning vehicle DNA.

mants were determined by analyses of restriction endonuclease fragments after digestion with two or three different enzymes. The procedure for mapping the sites of Tn*5* insertions has been described in detail by Stauffer et al. (25).

A total of 18 independent Tn*5* insertions into pBP7 were mapped. Of these, 7 resulted in loss of MSHA activity by Cm^r Kn^r transformants, and the remaining 11 insertions did not affect HA activity. Figure 3 shows the locations of the Tn*5* insertions in pBP7, demonstrating that the genes necessary for fimbrial production and expression span a minimum of 5.5 kb.

Production of fimbriae by transformants possessing recombinant plasmids. *E. coli* HB101 transformed with pBP7 consistently agglutinated guinea pig erythrocytes, whereas cultures of HB101 which possessed no plasmid did not cause MSHA of erythrocytes. The presence or absence of fimbriae was confirmed by electron microscopy. The transformed bacteria were observed to be strongly fimbriate in contrast to the nontransformed HB101 strain which was never observed to produce fimbriae. Similarly, antiserum raised against the type 1 fimbriae of *K. pneumoniae* IA551 agglutinated fimbriate cultures of both IA551 and HB101 containing the recombinant plasmid, but was never observed to agglutinate the original *E. coli* strain (Table 1). To demonstrate that the structural gene encoding fimbriae was present in pBP7, this plasmid was used to transform *S. typhimurium* JL781. The fimbriae of *S. typhimurium* have been shown to be serologically distinct from those of *K. pneumoniae* (7, 22). As shown in Table 1, $MSHA^+$ bacteria possessing pBP7 were agglutinated at high titer by the antifimbrial serum, while no such agglutination was observed with the original *Salmonella* strain. This would indicate that the transformed bacteria were expressing *Klebsiella* fimbriae as opposed to *Salmonella* fimbriae whose expression might have been switched on by pBP7.

DISCUSSION

To examine the genetic regulation of fimbrial production in *K. pneumoniae* at the molecular level, the genes encoding fimbriae were cloned into an *E. coli* plasmid vector. After preparation of a chromosomal library, those strains of *E. coli* possessing the relevant genetic element were selected by their ability to cause MSHA of guinea pig erythrocytes. By conventional subcloning techniques, the plasmid pBP7, which contained the fimbrial genes, was constructed. After insertion of the transposable element, Tn*5*, the size of the coding region of the DNA fragment required for expression of type 1 fimbriae was shown to be approximately 5.5 kb. The molecular weight of the subunit pilin of *K. pneumoniae* has been shown to be 21,500 (11); therefore, it is reasonable to assume that this DNA fragment contains accessory genes along with the structural gene necessary for the synthesis of fimbriae. Indeed, recent information concerning the molecular biology of other fimbrial antigens has suggested that multicistronic DNA regions control the expression of these

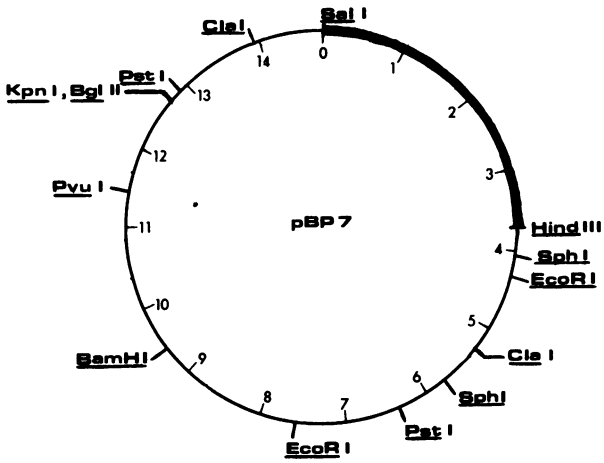

FIG. 2. Physical map of plasmid pBP7.

adhesins (16, 20). Presently, we are using a minicell-producing *E. coli* strain to determine the number and size of the gene products encoded by pBP7. Further analysis with insertion and deletion mutants should provide information concerning the orientation of specific genes, including the structural gene, in this plasmid.

The cosmid cloning technique has been used to isolate recombinant plasmids encoding type 1 fimbriae of *E. coli* (15). Fader et al. (11) have reported a similarity in chemical composition between purified fimbriae of *E. coli* and *K. pneumoniae* although a distinct lack of immunological cross-reactivity was reported. Therefore, within the enterobacteria, it is possible that distinct DNA sequences have evolved in those genetic elements determining the structure of morphologically and functionally related adhesins. A comparison of these sequences should prove useful in elucidating the evolution and mechanism of possible virulence factors of pathogenic bacteria. Also, the similarity of regulatory mechanisms involved in the biosynthesis of fimbriae may be explored with these cloned DNA fragments.

Unlike the wild-type *K. pneumoniae* IA551 strain from which the fimbrial genes were derived, the transformed *E. coli* caused MSHA of

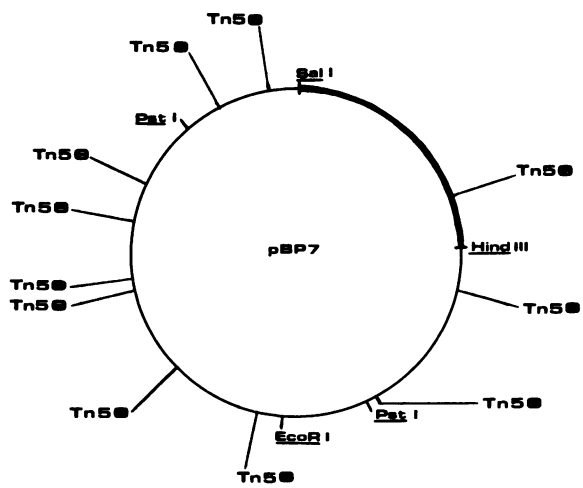

FIG. 3. Location of Tn*5* insertions into pBP7 after endonuclease mapping. The locations were determined as described in the text. The symbol + or − after Tn*5* indicates the phenotypic expression of fimbriae as determined by MSHA and electron microscopy.

TABLE 1. Expression of mannose-sensitive fimbriae by the wild-type and plasmid-containing bacterial strains

Strain (plasmid)	MSHA	MSHA titer[a]	Agglutination titer vs antifimbrial serum
K. pneumoniae IA551	+	2.2×10^8	128
E. coli HB101	−		<2
E. coli HB101 (pBP7)	+	6.1×10^7	128
S. typhimurium JL781	+	ND[b]	<2
S. typhimurium JL781 (pBP7)	+	ND	64

[a] Number of bacteria per milliliter required to produce visible HA.
[b] ND, Not determined.

erythrocytes after growth in broth or on agar. This was also observed in the transformed *Salmonella* strain. Therefore, unlike the original IA551 strain which expressed fimbriae only as broth-grown cultures, the recombinants did not exhibit type 1 fimbrial phase variation (26). This result may be explained by the fact that plasmid pBP7 does not possess the regulatory elements which are responsible for transition from the fimbriate to the nonfimbriate state. Alternatively, since the recombinant plasmid exists as multiple copies within each bacterial cell, excess fimbrial protein may be synthesized and expressed by the cell at all times. We propose to investigate phase variation by cloning the fimbrial genes into a λ phage vector, preparing lysogens, and examining regulation with the Mud*lac* phage (5). This should enable us to determine whether *K. pneumoniae* type 1 fimbrial phase variation is under transcriptional control as has been previously reported for *E. coli* (8).

Finally, cloning of the *K. pneumoniae* fimbrial gene should facilitate understanding the relevance of the antigen as a possible virulence factor in urinary tract infections caused by this species. Both in vivo and in vitro adherence studies with the genetically defined strain, *E. coli* HB101, will enable us to determine the role of fimbriae as adhesins in the absence of *Klebsiella* capsular material and other surface antigens.

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