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Virulence Properties of *Escherichia coli* 83972, a Prototype Strain Associated with Asymptomatic Bacteriuria

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Little is known about bacteria associated with asymptomatic bacteriuria (ABU) with regard to urinary tract colonization mechanisms. In this study, virulence properties of *Escherichia coli* 83972, a strain that was isolated from a clinical ABU episode, were examined. The genetic potential for expression of P and type 1 pili was demonstrated, and DNA sequences related to type 1C and G (UCA) pilus genes were also detected. However, *E. coli* 83972 did not express D-mannose-resistant or D-mannose-sensitive hemagglutination after growth under standard conditions in vitro or upon isolation from the urine of colonized test subjects. Limited uroepithelial cell adherence was observed in vivo, and weak D-mannose-sensitive hemagglutination was detected after extended growth in urine in vitro.

Urine of people who have structural or functional abnormalities of the urinary tract is likely to contain bacteria (38). Urine colonization often occurs in the absence of clinical symptoms and is called asymptomatic bacteriuria (ABU). In some patient groups, treatment of ABU is often not warranted and the benign bacteria causing asymptomatic colonization may even be beneficial in preventing infection by more antibiotic-resistant or virulent organisms (8–10, 22, 26–29, 34, 37, 38).

Unfortunately, the ABU-associated bacterial strain that colonizes the bladder is self-selecting, and physicians have little knowledge of the potential for urovirulence of the organism. Usually the strain is allowed to persist until it, or another invading strain, produces symptoms of urinary tract infection (UTI). The inevitable variation in virulence among such strains may account for the disparate reports regarding the outcomes of treatment and nontreatment of ABU. A better understanding of the virulence potential of the ABU-associated organism and of its potential for long-term asymptomatic bladder colonization would reduce the uncertainty involved in treatment decisions.

Studies by Andersson et al. (1) have identified an *Escherichia coli* strain capable of long-term asymptomatic bladder colonization. These researchers used *E. coli* 83972 to colonize eight volunteers who had a variety of underlying illnesses. *E. coli* 83972 persisted in the urine of the volunteers between 1 and 232 days (mean, 88 days). None of the volunteers developed fever or any other symptom of systemic illness. A recent study by Wullt et al. (40) confirmed this observation.

We have examined the prototypic ABU-associated bacterium, *E. coli* 83972, with respect to genetic and phenotypic properties that may contribute to bacterial persistence in the urinary tract. *E. coli* 83972 was selected for study because of its demonstrated capacity for long-term asymptomatic human bladder colonization.

Adherence genotype of *E. coli* 83972. *E. coli* 83972, serotype O nt/K5 (nt, nontypeable), is a wild-type ABU-associated isolate that colonized the urinary tract of a girl in Gothenburg, Sweden, for 3 years. Colony blot and Southern blot hybridization analyses were used to identify adherence gene sequences in *E. coli* 83972 that are associated with extraintestinal colonizations (23). Specific hybridization probes used for detection of *draABC*, *papEFG*, and *pilA* to *-G* genes have been described elsewhere (4, 12, 30). Probes specific for *focH* and *sfaS* were prepared by PCR amplification of recombinant-DNA-containing strains HB101(pPIL110-54) and HB101(pANN801-13) kindly provided by J. Hacker. The sequences of PCR primers used were 5′ GACGTGGATACGACGATTACTG 3′ and 5′ TACGCATAGGTATAGGTGAC 3′. The probe for *ucaA* was prepared by PCR amplification of *Proteus mirabilis* HU1069 (6). Primer sequences were 5′ CTCATAAGCGATGGTGTA ATGAACTGTAGC 3′ and 5′ TATGACGGTACAATTACT TTTACTGGAAA 3′. The *ucaA* probe was used for detection of genetically related *E. coli* G pilus genes. Hybridizations were conducted at high stringency (1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 68°C). The results are shown in Table 1. DNA sequences related to four of six adherence gene families tested, including *pap*, *pil*, *foc*, and *uca*, were present.

In vitro adherence phenotype of *E. coli* 83972. *E. coli* 83972 was tested for expression of adherence by standard hemagglutination (HA) assays following growth in vitro. Prior to testing, strains were grown under conditions thought to optimize expression of the specific adhesion to be tested; i.e., bacteria were passaged on L agar plates prior to assay of P or G pilus adherence or were passaged as 48-h static cultures of broth or urine up to 10 times prior to the assay of type 1 pilus adherence (15). *E. coli* G pili are genetically related to *P. mirabilis* Uca pili but are also hemagglutinins (6, 32). Bacteria were collected from agar plates or liquid cultures and suspended in buffered saline-gelatin (BSG) (8.5 g of NaCl, 0.3 g of KH_2PO_4, 0.6 g of Na_2HPO_4, 10 ml of 1% gelatin, 990 ml of distilled water [pH 7.0]) at a concentration of 10^9 to 10^{10} per ml. Thirty-microliter

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TABLE 1. Hybridization of *E. coli* 83972 with probes for adhesin genes associated with *E. coli* causing extraintestinal infections

Adhesin gene	Pilus	Hybridization	HA[a]	Infection associated with adhesin
pil	Type 1	+[b]	−/weak	UTI (43)
pap	P	+[b]	−/−	Pyelonephritis (38)
uca	G	+	−/−	UTI (6, 39)
foc	Type 1C	+	NT	UTI (37)
dra	DR	−	−	Cystitis (34)
sfa	S	−	−	Meningitis (36)

[a] Results are shown for bacteria passaged in broth and urine (broth/urine); HA was observed only after 10 passes. NT, not tested (this adhesin does not mediate HA).

[b] DNA sequences homologous with *pap* and *pil* were present as single copies on the *E. coli* 83972 chromosome.

TABLE 2. Hemagglutination phenotype of recombinant-DNA-containing *E. coli* carrying cloned adherence genes from *E. coli* 83972

E. coli strain[a]	Cloned *E. coli* 83972 gene	HA phenotype		
		Sheep	Human	Guinea pig
HU1958	*pap*	+	+	−
HU1886	*pil*	−	−	+
HU1903	*uca*	−	−	−
P678-54	None	−	−	−

[a] Strains designated HU are recombinant plasmid containing derivatives of the laboratory *E. coli* strain P678-54. Our previous studies have shown that strain P678-54 does not contain its own genes for *pap*, *pil*, or *uca* (G) pili.

samples of bacteria were mixed with 30 μl of washed erythrocytes in BSG on a chilled glass plate. The plate was incubated over ice with occasional rocking for 10 min or until HA was observed. Bacteria were tested for HA of sheep and human erythrocytes in the presence of D-mannose for P and G pili or HA of guinea pig erythrocytes in the absence of D-mannose (MSHA) for type 1 pili. The results are also shown in Table 1. MSHA of guinea pig erythrocytes was observed after bacteria were passaged extensively in urine. No other HA phenotype was detected.

In vivo adherence phenotype of *E. coli* 83972. As part of an ongoing human bladder colonization study, the urine of seven male volunteers who had neurogenic bladders subsequent to spinal cord injury, and who had a history of recurrent UTI, was deliberately colonized with *E. coli* 83972. The inoculation protocol has been described previously (1). Study participants remained bacteriuric with *E. coli* 83972 as the only colonizing bacterium for extended periods (>6 months). *E. coli* 83972 bacteria collected from the urine of volunteers were tested directly for adherence phenotype, and exfoliated uroepithelial cells of colonized volunteers were observed for attached bacteria. Urine (200 to 400 ml) was collected from test subjects who had ABU with *E. coli* 83972 for a minimum of 1 month. An additional urine sample collected at the same time was sent to the clinical microbiology laboratory for culture and sensitivity. If the results from the clinical workup indicated that *E. coli* 83972 was present in a mixed culture with any other bacteria, results from that experiment were discarded. Within 1 h of collection, urine specimens were centrifuged at 5,000 × *g* to collect suspended bacteria and uroepithelial cells. The urine was discarded, and the pellet was resuspended in 30 ml of BSG. The suspension was centrifuged at 1,500 × *g* for 10 min, and the supernatant containing bacteria was transferred to a fresh tube. The pellet containing uroepithelial cells was resuspended in 30 ml of BSG and centrifuged again at 1,500 × *g* for 10 min (first wash). The supernatant was discarded, and the pellet was washed three more times with BSG. Washed cells were then examined for attached bacteria in a phase-contrast microscope.

Bacteria in the first supernatant were collected by centrifugation at 5,000 × *g* and resuspended in BSG at a concentration of 10^9 to 10^{10} per ml. A 10-μl sample was removed for determination of bacterial titer and to confirm that *E. coli* 83972 was the only organism present. *E. coli* 83972 was differentiated from other potentially contaminating *E. coli* strains based upon its unique colony morphology on MacConkey agar. A minimum of 100 colonies were visually observed for each experiment. In addition, whole-cell DNA samples obtained from five typical colonies were compared with *E. coli* 83972 DNA by restriction fragment length polymorphism analysis. The remainder of the sample was used for HA tests by using the method described for in vitro HA assays. Six separate experiments typically yielded negative results for HA phenotypes for sheep, humans, and guinea pigs. Moderate uroepithelial cell adherence was observed (4 of 40 cells had 1 to 10 bacteria attached). These data suggest that although *E. coli* 83972 possesses genes associated with three different hemagglutinins, the phenotypes associated with these agglutinins are expressed weakly or not at all. The negative adherence phenotypes may result from incomplete or mutant adhesin genes in *E. coli* 83972 or from down-regulation of adhesin gene expression. The following experiments were done to determine if *E. coli* 83972 adherence genes are potentially functional.

Adherence characteristics of cloned adherence genes. We have prepared recombinant DNA cosmid clones that contain the entire genetic region representing each of the four *E. coli* 83972 adhesin gene clusters (*pil*, *pap*, *uca*, and *foc*) (13). The recombinant molecules were transferred to a laboratory *E. coli* strain that does not itself possess any of the adherence genes tested. Clones encoding *pap*, *pil*, or *uca* were then tested for function by using a standard HA assay. As shown in Table 2, the recombinant DNA strains containing *pap* or *pil* genes expressed adherence (HA) in vitro. The *uca* clone did not express G pilus adherence. These results show that *E. coli* 83972 possesses functional copies of both P and type 1 pilus genes.

DNA sequence analysis of the *papG* allele of strain 83972. Several reports have suggested that different alleles of the gene encoding the P adhesin, *papG*, may be associated with different clinical syndromes (14, 16). We used DNA sequence analysis of the *papG83972* allele to determine its similarity with the *pap-1* (class I), *pap-2* (*prs*) (class III), and *pap-3* (class II) families of alleles. The results presented in Fig. 1 show that *papG* 83972 has 97% DNA sequence homology with the *pap-2* allele, and the products exhibit 95% protein sequence homology.

E. coli 83972 is a bacterial isolate that has been shown clinically and experimentally to persist in the human bladder for extended periods without producing overt symptoms of infection. In 1991, *E. coli* 83972 was used to evaluate the contribution of bacterial adherence to colonization of the human urinary tract (1). Initial characterization suggested that *E. coli* 83972 did not express P or type 1 pili in vitro and did not adhere in vitro to human uroepithelial cells. It was thought to contain DNA sequences related to *pil* but not *pap* genes. Moreover, the investigators found that upon introduction into *E. coli* 83972 of functional *pil* or *pap* adherence gene clusters as recombinant plasmids, the recombinant plasmid-containing derivatives exhibited reduced persistence in the bladder. They

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   1  atgaaaaaatggttccctgctttttattttatccctgtcaggctgtaatgatgctttg   60
                c                                                 c

  61  gct...atccagagtacaatgttttactcgtttaatgataacatttatcgtcctcgactt   120
        gca a                                                    a

 121  agtgttaaagtaaccgatgttattcaaattatagtggatataaactctgcatcaagtacg   180
                        a  g     t c                c

 181  gcaactttaagctatgtggactgcaatggatttacatggtctcatggtatttactggtct   240
                        c              c   a        c

 241  gagtattttgcatggctggttgttcctaaacatgtttcctataatggatatgatatatat   300
                                                              a

 301  cttgaacttcagtccagaggaagtttttcacttgatgcagaagataatgataattactat   360

 361  cttaccaagggatttgcatgggatgaagcaaacacatctggacggacatgtttcaatatc   420
                                                       a

 421  ggagaaaaaagaagtctggcatggtcatttggtggtgttaccctgaacgccagatttcct   480
                                                                 g

 481  gttgaccttcctaaggggattatacgtttccagtcaagttcttacgtggcattcagcgt   540
                                          t

 541  aataattatgattatattggtggacgctacaaaattccttcctcgttaatgaaaacattt   600
                                          c     t

 601  ccttttaatggtacattgaatttctcaattaagaataccggaggatgccgtccttctgca   660
                                       a

 661  cagtctctggaaataaatcatggtgatctgtcgattaatagcgctaataatcattatgcg   720

 721  gctcagactctttctgtgtcttgcgatgtgcctacaaatattcgtttttttcctgttaagc   780

 781  aatacagctccggcatacagccatggtcagcaattttcggttggtctgggtcatggctgg   840
            aa

 841  gactccattatttcggttaatggcgtggacaaaggagagacaacgatgagatggtacaga   900
                    a                  c

 901  gcaggtacacaaaacctgaccatcggcagtcgcctctatggtgaatcttcaaagatacaa   960

 961  ccaggagtactatctggttcagcaacgctgctcatgatattgccataa  1008
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FIG. 1. DNA sequence of the *pap*G allele of strain 83972. Differences in the sequence of *papG2* (*prs*) are shown below the sequence.

concluded that persistence of *E. coli* bacteriuria was not determined by bacterial adherence. Results of work presented here, and of other published studies demonstrating reduced in vivo colonization by *E. coli* strains that overexpress adhesin genes (11, 25), suggest that other conclusions are possible.

Upon reexamination of the genetic potential for adherence of *E. coli* 83972, we found that it possesses DNA sequences related to four bacterial adhesins associated with extraintestinal colonization. Genes for P and type 1 pili were expressed poorly or not at all when tested in the *E. coli* 83972 genetic background but were fully functional when tested as recombinant clones in a different host. It is possible that genetically unlinked regulatory elements that reduce expression of these adhesins under the in vitro and in vivo test conditions used exist in *E. coli* 83972. Several regulatory mechanisms that may control expression of either P or type 1 pili in *E. coli* 83972 have been described (2, 3, 7, 20, 24, 31, 33, 39). Additional studies will be needed to identify specific mechanisms regulating in vitro and in vivo pilus adherence gene expression by *E. coli* 83972 and of the role of these regulatory systems in promoting long-term asymptomatic bladder colonization.

The *pap*-83972 genes are most related to the *pap* allele that encodes class III specificity group P adhesin. P pilus alleles have been divided into three groups based upon receptor specificity of the adhesin they encode. Class I P adhesins use the Pk antigen as a human tissue receptor while class III P adhesins bind to the LKE (Luke) antigen (17, 18). Alleles encoding class III pili are most often associated with *E. coli* from cystitis or ABU (14, 16), suggesting a potential role for class III pili in bladder colonization.

Type 1 pili also promote bladder colonization in animal model studies. In a murine model of ascending UTI, *E. coli* mutants defective in synthesis of type 1 pili exhibited significantly reduced persistence in the bladder and agents that prevent type 1 pilus specific attachment also reduced colonization (5, 19, 21, 35, 36). Based upon these observations and upon the genetic potential of *E. coli* 83972 for adhesin synthesis, it is possible that P pili, type 1 pili, or both are required for persistence of *E. coli* 83972 in the human bladder. We are currently preparing mutant derivatives of *E. coli* 83972 that lack the genetic capacity for expression of either pilus type so that the role of each in ABU may be determined in direct in vivo human colonization studies.

Nucleotide sequence accession number. The sequence of *papG* of *E. coli* 83972 has been deposited in GenBank under accession no. AF097355.

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