

## Role of Type 1 Fimbria- and P Fimbria-Specific Adherence in Colonization of the Neurogenic Human Bladder by *Escherichia coli*

Richard A. Hull,<sup>1,2\*</sup> William H. Donovan,<sup>3,4</sup> Michael Del Terzo,<sup>5</sup> Colleen Stewart,<sup>6</sup> Margaret Rogers,<sup>4</sup> and Rabih O. Darouiche<sup>2,6</sup>

Department of Molecular Virology and Microbiology<sup>1</sup> and Center for Prostheses Infections, Department of Physical Medicine and Rehabilitation,<sup>2</sup> Baylor College of Medicine, Department of Physical Medicine and Rehabilitation<sup>3</sup> and Division of Urology, Department of Medicine,<sup>5</sup> University of Texas Medical School, The Institute for Rehabilitation and Research,<sup>4</sup> and Spinal Cord Injury and Medical Services, Infectious Disease Section, Veterans Affairs Medical Center,<sup>6</sup> Houston, Texas

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Recent clinical studies suggest that the deliberate colonization of the human bladder with a prototypic asymptomatic bacteriuria-associated bacterium, *Escherichia coli* 83972, may reduce the frequency of urinary tract infection in individuals with spinal cord injuries. However, the mechanism by which *E. coli* 83972 colonizes the bladder is unknown. We examined the role in bladder colonization of the *E. coli* 83972 genes *papG* and *fimH*, which respectively encode P and type 1 receptor-specific fimbrial adhesins. *E. coli* 83972 and isogenic *papGΔ* and *papGΔ fimHΔ* mutants of *E. coli* 83972 were compared for their capacities to colonize the neurogenic human bladder. Both strains were capable of stable colonization of the bladder. The results indicated that type 1 class-specific adherence and P class-specific adherence, while implicated as significant colonization factors in experiments that employed various animal model systems, were not required for colonization of the neurogenic bladder in human beings. The implications of these results with regard to the selection of potential vaccine antigens for the prevention of urinary tract infection are discussed.

*Escherichia coli* 83972 is a clinical isolate associated with asymptomatic bacteriuria that was first described by Andersson et al. (1), who used the strain to successfully colonize the urine of eight human volunteers. Subsequently, it was suggested that host factors (such as bladder pressure) and bacterial factors (such as the addition of second copies of P fimbria genes) may affect the ability of *E. coli* 83972 to colonize (16, 17). Other studies (4, 6) demonstrated that *E. coli* 83972 could colonize the neurogenic human bladder for extended intervals, with the average being 8 to 12 months (range, 1 to 48 months), and, moreover, that subjects who were colonized with *E. coli* 83972 experienced a significant reduction in the frequency of symptomatic urinary tract infections (UTI).

However, the mechanism of bladder colonization by this strain is not currently known. Abundant experimental data suggest that fimbria-mediated adherence of bacteria to structures in the urinary tract is an important contributor to urinary tract colonization by *E. coli* (9). Genetic analysis of *E. coli* 83972 revealed genes for type 1 (*fim*) and P (*pap*) fimbria synthesis as well as gene sequences homologous with *foc* (type 1C fimbria) and *uca* (*gaf*, G fimbria) genes (7). Recombinant DNA plasmid clones that contained the *fim-83972* or *pap-83972* gene cluster expressed D-mannose-sensitive and D-mannose-resistant (P fimbria class III) hemagglutination, respectively, when they were transferred to an *E. coli* K-12 host.

The expression of adherence phenotypes by *E. coli* 83972

was also examined (7). P fimbrial adherence was expressed in vitro after serial passage on agar plates and was subject to phase variation, just as it is for other *E. coli* strains (10). Type 1 class fimbrial adherence was expressed in vitro after passage in urine. Expression levels of type 1 and P fimbriae by *E. coli* 83972 growing in vivo in the human bladder are not known. In our previous studies, P fimbrial adherence was not detected for *E. coli* 83972 collected from the urine of colonized subjects. However, studies by Hultgren and colleagues (8) demonstrated that the state of fimbrial expression for bacteria collected from urine does not accurately reflect the expression of fimbriae by bacteria in other bladder compartments. Specifically, they found that *E. coli* collected from the bladder lumen, in an experimental murine UTI model, did not express type 1 fimbriae but that bacteria that were attached to the bladder walls did express type 1 fimbriae. The contribution of fimbria-mediated adherence to bladder colonization, therefore, cannot be reliably determined by measuring fimbrial expression by bacteria growing in the lumen of the bladder. We have developed a more direct method to measure the contribution of type 1 and P fimbria-specific adherence in human bladder colonization. In the present study, bladder colonization by *E. coli* 83972 was compared with colonization by isogenic mutants of *E. coli* 83972 that were missing the genes required for P class-specific tissue adherence (*papG*) and type 1 class-specific adherence (*fimH*).

**Construction of a deletion allele of the *papG83972* gene and substitution of the deletion allele into the *E. coli* 83972 chromosome.** An 800-bp deletion mutation was introduced into the *papG* gene cloned from *E. coli* 83972 (7). To accomplish this, an *NcoI* site was introduced into pRHU2006 (a subclone that

\* Corresponding author. Mailing address: Department of Molecular Virology and Microbiology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-4926. Fax: (713) 798-5402. E-mail: rhull@bcm.tmc.edu.

includes *papFG83972*) 6 bp upstream of the start ATG of the *papG* gene. The newly generated 800-bp *NcoI* fragment was then deleted. The final product contained a deletion that prevented the expression of *PapG*. The deletion allele was transferred to the *E. coli* 83972 chromosome by using the marker exchange method described by Link et al. (12). The replacement of the wild-type allele by the *papG83972*Δ allele in the *E. coli* 83972 chromosome was verified by PCR and Southern blot analysis. One *papG* deletion mutant, designated HU2117, was selected for further study. HU2117 contained no additional residual genes, such as antibiotic resistance markers, as a result of the mutagenesis protocol. Since the *papG83972* gene represents the last gene in the *papEFG* operon and is followed by a transcription termination sequence on the chromosome, there are no potential polar effects on the expression of downstream genes. *E. coli* HU2117 no longer exhibited P fimbrial adherence as measured by D-mannose resistant hemagglutination of human erythrocytes.

**Effect of the *papG* deletion upon bladder colonization.** In the experiments discussed below, human bladder colonization by the *E. coli* 83972 wild-type strain was compared with colonization by *E. coli* HU2117. The goal was to determine whether adherence encoded by the *papG83972* gene was required for bladder colonization. Study participants included adult males (mean age, 41 years; range, 25 to 54 years) with a history of spinal cord injury and recurrent episodes of symptomatic UTI. The inoculation protocol used to establish bladder colonization was the same as that described previously (6). Bladder inoculation consisted of instillation, via a sterile bladder catheter, of 30 ml of normal saline containing a mixture of  $5 \times 10^5$  CFU each of the *E. coli* 83972 wild-type strain and HU2117 per ml. One participant (subject E) received two cycles of bladder inoculation on separate occasions. Urine samples were collected 1 week and/or 1 to 2 months postinoculation and assayed to confirm stable colonization with *E. coli* 83972 and/or HU2117. Since both the wild-type and mutant strains appear identical when standard microbiological methods are employed, individual colonies were analyzed by using PCR to distinguish between the two strains. Single colonies ( $n = 50$ ) were picked and suspended directly in a PCR mix. The *papG* region was amplified with the PCR primers Pap147 (5' CCT AAATGAATAAATACTGTAATTACGG 3') and Pap357 (5' TGGCAATATCATGAGCAGCGTTGCTG 3'). These primers flank the *papG* gene, and amplification of wild-type *E. coli* 83972 results in a 1-kb PCR product whereas amplification of HU2117 results in a 225-bp product. The two strains could then be distinguished following agarose gel electrophoresis of PCR fragments. Selected urine *E. coli* isolates were also subjected to DNA fingerprinting analysis by using transverse alternating field electrophoresis-restriction fragment length polymorphism analysis. This procedure was used, in addition to standard clinical laboratory tests, to distinguish the experimental strains from any environmental *E. coli* strains that may have invaded the colonized bladder. Throughout this study, only *E. coli* 83972 or its derivatives were observed in the bladders of colonized subjects.

Results are shown in Table 1. The 1-week urine samples contained both strains on six of nine occasions. After 1 month of bladder colonization, the percentage of *E. coli papG*Δ mutant cells remaining in the urine varied among test subjects.

TABLE 1. Experimental human bladder colonization with a mixture of wild-type *E. coli* 83972 and the *papG* deletion mutant *E. coli* HU2117

Subject	% of wild-type <i>E. coli</i> 83972 in the urine sample <sup>a</sup>		
	Input <sup>b</sup>	1 week after instillation	4 weeks after instillation
A	50	78	NA <sup>c</sup>
B	50	0	0
C	50	18	12
D	50	54	98
E	50	56	58
E'	50	4	0
F	50	100	100
G	50	88	98
H	50	100	100
I	50	NA	0

<sup>a</sup> Test subjects (A to I) were colonized with a mixed inoculum consisting of 50% each ( $10^6$  total CFU) *E. coli* 83972 (*papG*<sup>+</sup>) and HU2117 (*papG*Δ) in a saline solution. Urine cultures were obtained at the intervals indicated. Fifty *E. coli* colonies from each culture were identified as either the wild-type *E. coli* 83972 strain or the *papG*Δ mutant. The titer of *E. coli* growing in subjects' urine varied between  $10^5$  and  $10^6$  per ml of urine.

<sup>b</sup> Calculated value. Cultures of each strain were adjusted to the same optical density at 600 nm and mixed at a 50:50 ratio prior to bladder instillation. The approximate value was confirmed by using PCR in two experiments.

<sup>c</sup> NA, subject not available for culture.

The bladders of the subjects were colonized only with the mutant in three instances and colonized only with wild-type *E. coli* 83972 in two instances. The remaining four subjects were still colonized with a mixture of wild-type and mutant strains. Follow-up data 2 months after bladder inoculation were available for five subjects. In three subjects, only the mutant strain remained, while in the other two subjects, either the wild *E. coli* 83972 strain or a mixture of both strains was found. Two subjects who were colonized with only *E. coli* HU2117 remained colonized >3 months. These data show that the presence of the *papG83972* gene is not absolutely required for bladder colonization. Were *papG* required, only the wild-type *E. coli* 83972 strain would have been found in the bladder urine.

**Construction of a deletion allele of the *fimH83972* gene and substitution of the deletion allele into the *E. coli* 83972 chromosome.** A strategy similar to that used for the construction of *E. coli* HU2117 was used to introduce a 102-bp deletion into the *fimH* gene on the *E. coli* HU2117 chromosome. For *fimH*, *EcoRI* restriction endonuclease sites were created 1 bp upstream and 102 bp downstream of the start ATG. The intervening sequence between the *EcoRI* sites was then deleted. The deletion mutation was transferred to the *E. coli* HU2117 chromosome by using the marker exchange method. The resulting chromosomal deletion extends from, and includes, the start ATG of the *fimH83972* gene, thereby preventing *fimH* expression. Since *fimH* is the last gene in the *fim* operon, there are no potential polar effects upon expression of downstream genes. One *papG*Δ/*fimH*Δ double mutant, designated HU2222, was selected for further study. *E. coli* HU2222 no longer exhibited type 1 class fimbrial adherence as measured by D-mannose-sensitive hemagglutination of guinea pig erythrocytes.

**Effect of the *fimH* deletion upon bladder colonization.** In the following experiments, human bladder colonization by the

TABLE 2. Comparison of levels of human bladder colonization by *E. coli* HU2117 and *E. coli* HU2222

Subject	% of <i>E. coli</i> HU2117 in the urine sample <sup>a</sup>		
	Input <sup>b</sup>	1 week after instillation	4 weeks after instillation
1	50	68	100
2	50	50	8
3	50	100	100
4	50	26	30
5	50	0	0
6	50	28	100

<sup>a</sup> Test subjects (1 to 6) were colonized with a mixed inoculum consisting of 50% each (10<sup>6</sup> total CFU) *E. coli* HU2117 (*fimH*<sup>+</sup>) and *E. coli* HU2222 (*fimHΔ*) in a saline solution. Urine cultures were obtained at the intervals indicated. Fifty *E. coli* colonies from each culture were identified as either the *E. coli* HU2117 strain or the *fimHΔ* mutant. The titer of *E. coli* growing in the urine of the subjects varied between 10<sup>5</sup> and 10<sup>6</sup> per ml of urine.

<sup>b</sup> Calculated value. Cultures of each strain were adjusted to the same optical density at 600 nm and mixed at a 50:50 ratio prior to bladder instillation. The approximate value was confirmed by using PCR in two experiments.

*fimH*<sup>+</sup> *E. coli* HU2117 strain was compared with colonization by *E. coli* HU2222. The goal was to determine whether adherence encoded by the *fimH* gene was required for bladder colonization. The protocol was the same as that described for evaluation of the *papGΔ* single mutant except that the bladders of each of the six study participants were instilled with a mixture of bacteria that was 50% *E. coli* HU2117 and 50% *E. coli* HU2222. *E. coli* bacteria collected from urine cultures were identified as *E. coli* HU2117 or HU2222 by using PCR. The primers used, Pil2ra (5' CCTACAGCTGAACCCGAAGAG ATGATTGTA 3') and Pil9 (5' GTTATAGTTGTTGGTCTG TCGC 3'), flank the *fimH* gene, and amplification of *E. coli* HU2117 resulted in a 507-bp fragment whereas amplification of *E. coli* HU2222 resulted in a 411-bp product.

Results of bladder colonization from six subjects are shown in Table 2. The 1-week urine cultures contained both strains in four of six instances. These data show that there was no absolute requirement for the presence of *fimH* for initial bladder colonization. After 1 month of bladder colonization, three of six subjects were colonized with only *E. coli* HU2117 and one subject was colonized with only *E. coli* HU2222. The remaining two subjects were still colonized with a mixture of the wild type and mutant. Follow-up data were available for one subject (subject 4). This subject exhibited bladder colonization with only *E. coli* HU2222 4 months postinstallation.

**Conclusions.** Isogenic *papG* and *fimH* deletion mutants of *E. coli* 83972 were used in the present study to directly evaluate the role of specific adherence in the colonization of the neurogenic human bladder. Results of our study suggest that P fimbria receptor-specific adherence is not required for human bladder colonization by *E. coli* 83972. This conclusion was consistent with the results of an in vivo study that employed a murine ascending UTI model (13). In that study, *E. coli papEFG* null mutants retained the capacity to colonize the mouse bladder. In contrast, results of other in vivo studies conducted with a murine ascending UTI model support a role for P pili in bladder colonization (5). Also, using a human bladder colonization model, Wullt et al. (16) demonstrated that a second copy of P fimbria genes added to *E. coli* 83972 enhanced early establishment of *E. coli* in the human urinary tract. Perhaps P

fimbriae have a role in an early stage of infection, such as colonization of the urethra, that is precluded by the direct bladder instillation protocol. Finally, numerous clinical correlation studies suggested that P fimbriae were associated with symptomatic bladder infection (9).

Although our results show that PapG-specific adherence is not required, other parts of the P pilus may still contribute to colonization. For example, Westerlund et al. (15) discovered that other structural components of P fimbriae, specifically the PapE and PapF proteins, may promote attachment to fibronectin. The possible contribution made by other structural components of P fimbriae to human bladder colonization is not currently known. *E. coli* 83972 also possesses genes associated with additional adherence fimbria types. It remains possible that P-specific adherence contributes to colonization but is not required in the context of the other adhesins. Such redundancy in adherence mechanisms seems beneficial to the bacteria for efficient colonization of the urinary tract. The bacteria may employ different mechanisms to maintain colonization in response to changing environmental conditions in the bladder. We are currently evaluating the contribution of other adhesins and other P fimbria components.

This study represents the first attempt to directly evaluate the requirement of type 1 fimbria-specific adherence for human bladder colonization. Results of the present study reveal that type 1 fimbria receptor-specific adherence, as directed by the *fimH* gene, is not required for *E. coli* to establish colonization in the neurogenic bladder. Furthermore, some subjects who were colonized with *fimH* null mutant *E. coli* retained bladder colonization for up to 4 months, indicating that *fimH* is also not required for the maintenance of *E. coli* in the bladder. This was a surprising result and was in sharp contrast to results of numerous previous studies that implicated *fimH* as a gene that is absolutely required for bladder colonization. For example, Aronson et al. (2) demonstrated that the addition of a soluble receptor analog that blocked type 1 fimbria-mediated attachment to tissue prevented experimental UTI in the mouse. More recently, Langermann et al. (11) revealed that mice that were immunized with the *fimH* gene product were protected from subsequent challenge with *E. coli* by the murine UTI model. Thankavel et al. (14) also demonstrated that antibody directed specifically at the putative receptor binding region of *fimH* significantly reduced *E. coli* bladder infection in the experimental mouse model. Finally, Connell et al. (3) found that an *E. coli fimH* null mutant exhibited reduced survival in the murine UTI model.

The human subjects in our study all possessed neurogenic bladders as a result of prior spinal cord injury. As a result, and regardless of the method employed for bladder drainage, all subjects may have had retained residual urine in the bladder after voiding. It is possible that bacteria residing in the postvoid residual urine provided a seed for subsequent bladder recolonization and eliminated any requirement for specific attachment to the bladder epithelium. This observation may have important implications with regard to the development of strategies for preventing UTI. For example, antiadherence vaccines, such as those based upon the *fimH* antigen, may be ineffective for preventing UTI in patients with the highest risk for UTI, such as those with neurogenic bladders or the institutionalized elderly, who often rely upon bladder instrumen-

tation for voiding urine. For these significant and growing patient groups, other strategies for UTI prophylaxis, such as the development of effective bacterial interference protocols, may be required.

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