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# NOTES

## Influence of Cloned tRNA Genes from a Uropathogenic *Escherichia coli* Strain on Adherence to Primary Human Renal Tubular Epithelial Cells and Nephropathogenicity in Rats†

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**The uropathogenic *Escherichia coli* strain 536 (O6:K15:H31) possesses pathogenicity islands which are incorporated into two tRNA genes, the *selC* and the *leuX* gene. The *leuX* gene influences the expression of different putative virulence factors. We demonstrate an effect of the *leuX*-specific tRNA on adherence and uropathogenicity.**

*Escherichia coli* strains are the main causative agents of urinary tract infections (23). The majority of uropathogenic *E. coli* strains express virulence or pathogenicity factors, such as fimbriae, hemolysin, iron-binding proteins, and O and K antigens, which enable the bacteria to colonize the urinary tract, to adhere to and destroy uroepithelial cells, to avoid activation of serum complement, or to resist activated complement complexes (2, 3, 9, 13).

The genes coding for pathogenicity factors of pathogenic *E. coli* may be organized on so-called pathogenicity islands comprising several virulence determinants in the form of clustered genes. The pathogenicity islands of uropathogenic strain 536 (O6:K15:H31) carry two hemolysin determinants (hemolysins I and II) as well as P-related fimbria-encoding sequences (4, 10). Interestingly, the two pathogenicity islands of uropathogenic strain 536 also carry tRNA-specific loci as flanking sequences. Pathogenicity island I is associated with the *selC* gene, which codes for selenocysteine-specific $tRNA^{Sec}$ (5), while pathogenicity island II is associated with *leuX*, which codes for leucine-specific $tRNA_5^{Leu}$ (15).

Spontaneous deletions of the pathogenicity islands of uropathogenic *E. coli* strains occur frequently and result in nonvirulent mutant strains (3, 11, 14). As a consequence of the pathogenicity island deletions of *E. coli* 536, the tRNA loci *selC* and *leuX* were also destroyed (4). Transcomplementation experiments in which cloned intact tRNA genes were transferred into tRNA-negative mutants revealed an influence of the selenocysteine-specific tRNA ($tRNA^{Sec}$) on anaerobic growth. This is due to the fact that selenocysteine is an essential constituent of formate dehydrogenase, which is involved in mixed acid fermentation (5). The leucine-specific tRNA ($tRNA_5^{Leu}$) was shown to be responsible for the expression of properties such as type I fimbria production, flagellation, production of enterobactin, and serum resistance (25). However, to date, the contribution of tRNA genes to nephropathogenicity in vivo has been unclear.

In this paper we describe the influence of both tRNA genes, *leuX* and *selC*, on adherence to cultured renal tubular epithelial cells (TEC) and the nephropathogenicity of the uropathogenic *E. coli* strain 536 in an ascending rat pyelonephritis model.

The *E. coli* wild-type strain 536 (O6:K15:H31) was isolated from a patient suffering from a urinary tract infection. The avirulent mutant 536-21 was obtained by screening for spontaneous loss of virulence properties, including alpha-hemolysin production (2, 23). The plasmids pGBB50 and pGBB51, which encode *selC* and *leuX*, respectively, were transformed into strain 536-21, delivering the following strains: (i) *E. coli* 536-21 (pGBB50), which codes for the *selC*-specific tRNA ($tRNA^{Sec}$), and (ii) *E. coli* 536-21(pGBB51), which codes for the *leuX*-specific tRNA ($tRNA_5^{Leu}$). Strain 536-21(pSU2716/pBR322) was used as the vector control (23). The relevant properties of strains and clones are given in Table 1.

The nonobstructed pyelonephritis model was used for the in vivo assay as previously described (19). In this model, a suspension of *E. coli* (1.5 ml; $5 \times 10^7$ CFU/ml) is injected into the bladder via the urethra. This results in a vesicoureteral reflux and renal inflammation (12, 19, 26). The rats were sacrificed 7 days following infection, and the number of CFU per gram of kidney were determined as the measure of nephropathogenicity; 16 rats were used per bacterial strain in two tests with eight animals each.

For statistical evaluation the mean number of CFU per gram of kidney for each group of infected rats was calculated. The renal bacterial counts, which were not normally distributed, were compared by the Kruskal-Wallis test (8).

Kidneys of rats killed 7 days after infection were prepared for light microscopic examination by fixation in Bouin's fluid (15 ml of pikric acid, 5 ml of formalin, 1 ml of acetic acid).

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TABLE 1. Properties of *E. coli* 536 and its derivatives[a]

| Property | 536 | 536-21 (pSU2716/ pBR322) | 536-21 (pGBB50) | 536-21 (pGBB51) |
|---|---|---|---|---|
| *selC* | + | − | + | − |
| *leuX* | + | − | − | + |
| Hemolysin production | + | − | − | − |
| Enterobactin | + | − | − | + |
| Serum resistance | + | − | − | + |
| P-related fimbriae | + | − | − | − |
| S fimbriae | + | − | − | − |
| Type I fimbriae | + | − | − | + |
| Flagella | + | − | − | + |
| Motility | + | − | − | + |
| Anaerobic growth | + | − | + | − |
| Formate dehydrogenase production | + | − | + | − |

[a] The *E. coli* strains were grown on Loeb agar (1% tryptone, 0.1% yeast extract, 0.1% glucose, 0.8% NaCl, 1.5% agar). For maintenance of plasmids, chloramphenicol (30 μg/ml) and tetracycline (15 μg/ml) were added as supplements.

Paraffin sections 5 μm thick were stained with hematoxylin and eosin.

For immunohistology, rat kidney tissue that had been fixed, paraffin embedded, and sectioned as described above was rehydrated through descending grades of ethanol to phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked by exposing the sections to 1% hydrogen peroxide in absolute methanol for 30 min, and this was followed by several rinses in PBS. Nonspecific binding of immunoglobulin G was prevented by blocking the sections with 2% native goat serum for 30 min. Thereafter, the sections were exposed to specific polyclonal rabbit anti-*E. coli* 536 serum for 1 h at 25°C. Following several washes in PBS, the bound primary antibodies were visualized with a polyclonal goat anti-rabbit immunoglobulin G coupled to horseradish peroxidase (A-0545; Sigma, Deisenhofen, Germany) as a tracer and diaminobenzidine as the chromogen. The sections were counterstained with hemalaun. Specificity of immunostaining was controlled by incubating with the secondary immunoreagents alone.

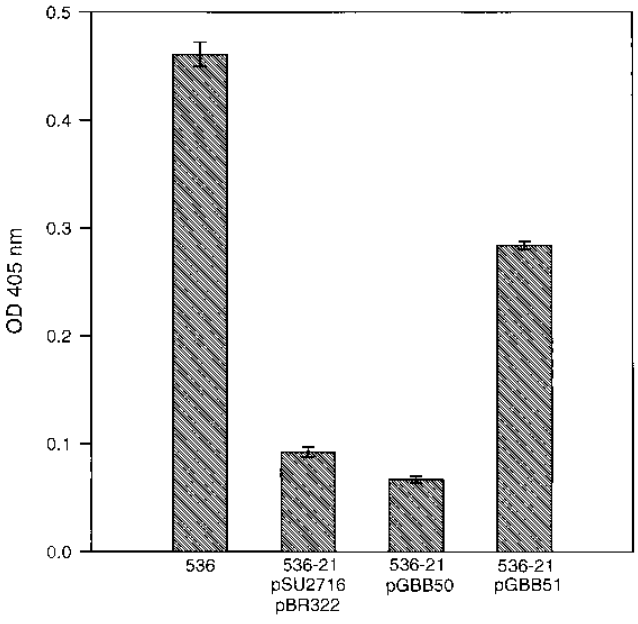


FIG. 1. Adhesion assay of the *E. coli* wild-type strain 536 (O6:K15:H31) and mutant strain 536-21 and its transcomplemented derivatives to human renal TEC, demonstrating the influence of *leuX*-specific tRNA expression ($tRNA^{Leu}_5$) on bacterial adherence. Each strain was tested for adherence on TEC isolated from three different patients. Measurements differed by only 6% and are expressed as mean values. OD, optical density.

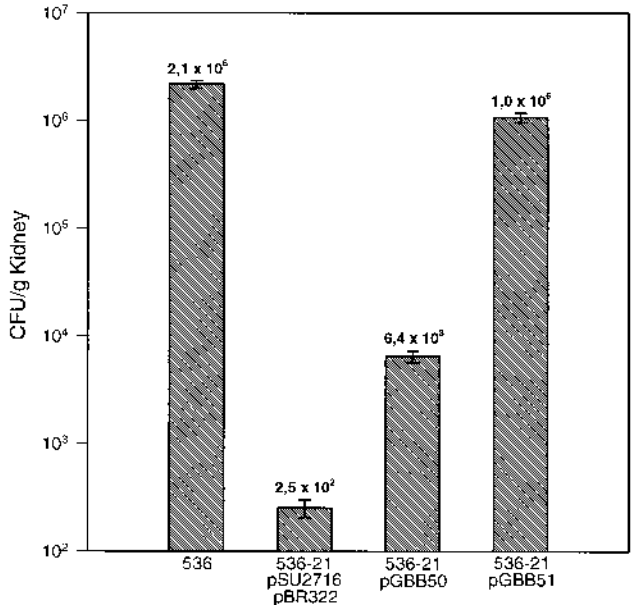


FIG. 2. Nephropathogenicity in a rat pyelonephritis model of wild-type *E. coli* 536 (O6:K15:H31) and mutant 536-21 and its derivatives, showing the influence of tRNA genes *selC* and *leuX* on the CFU per gram of kidney. For statistically significant differences, strain 536 was compared with 536-21(pSU2716/pBR322) and 536-21(pGBB50) or 536-21(pSU2716/pBR322) was compared with 536-21(pGBB51).

Primary human renal proximal TEC were isolated by the method of Detrisac et al. (7) and cultured as described recently (16). Bacterial adherence to TEC was determined by a modified enzyme-linked immunosorbent assay (ELISA) as described previously (20). Each experiment consisted of at least eight parallel measurements and was done in triplicate. The values given are the mean values and standard deviations.

The adherence of the *E. coli* strains described in Table 1 was tested by an adapted ELISA. As indicated in Fig. 1, the wild-type strain, 536, showed strong adherence while the control strain, 536-21(pSU2716/pBR322), showed only weak adherence. This roughly corresponded to a decrease of the mean number of adhering bacteria from 1 to 0.1 per primary tubular cell. In comparison, a relatively low level of cellular adherence was found for strain 536-21(pGBB50), which codes for the *selC*-specific tRNA ($tRNA^{Sec}$). In contrast, strain 536-21 (pGBB51), which codes for the *leuX* tRNA ($tRNA^{Leu}_5$), bound relatively strongly to TEC, reaching 62% of the level of the wild-type (strain 536) signal. The data clearly show a significant influence of the tRNA-specific gene *leuX* on adherence of uropathogenic *E. coli.*

The nephropathogenicity of *E. coli* 536 and its derivatives was tested by the rat pyelonephritis model. After transurethral injection of a bacterial suspension of strain 536, the infected animals developed an acute pyelonephritis persisting for at least 7 days with bacterial cell counts of $2 \times 10^6$/g of kidney (see Fig. 2 and 3). This led to the histological finding of acute pyelonephritis with leukocyte-filled tubules and thickened ep-

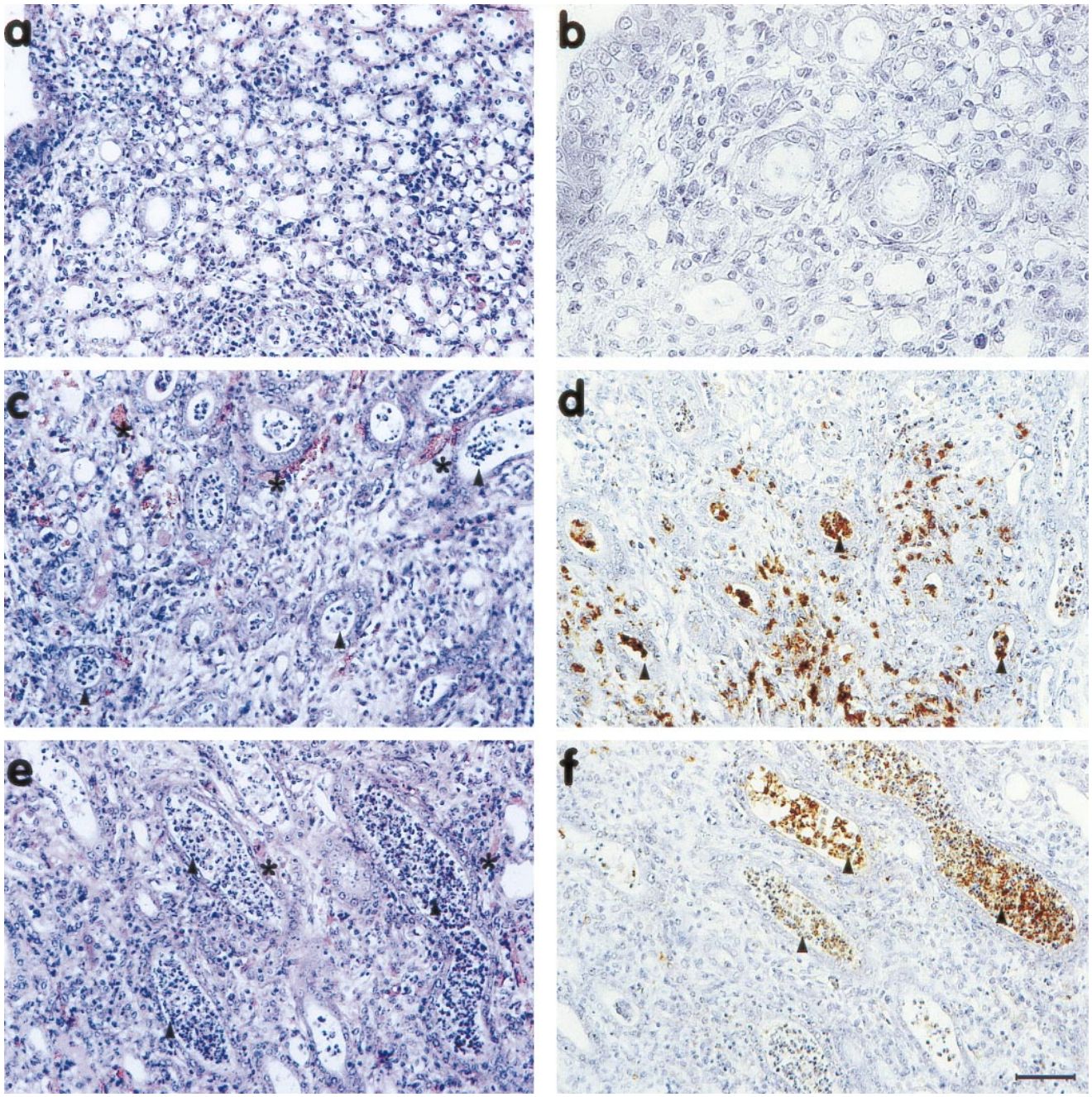


FIG. 3. Renal histology showing rat renal tissue 7 days after infection with *E. coli* 536-21(pGBB51). Immunohistology of renal tissues stained with an anti-*E. coli* antiserum (a, c, and e) and hematoxylin and eosin (b, d, and f) is shown. (a and b) Uninfected kidney; (c and d) kidney from rat infected with *E. coli* 536-21(pGBB51); (e and f) kidney from rat infected with *E. coli* 536-21(pGBB) 50. Bar represents 50 μm. ▲, cell and bacterial infiltration of collecting tubules; ∗, tissue necrosis and hemorrhagy.

ithelial layers of the tubules. The control strain, 536-21(pSU2716/pBR322), showed a dramatic reduction of renal counts compared with those of the wild-type strain. This was accompanied by distinctly less histopathological alterations.

Introduction of the cloned *selC* gene (pGBB50) into strain 536-21 markedly increased its ability to colonize rat kidney cells compared with that of the plasmid control strain 536-21 (pSU2716/pBR322) (Fig. 2). The cloned tRNA gene *leuX*, however, had a much more pronounced effect on nephropathogenicity. Renal histology following infection with strain 536-21(pGBB51) showed severe pyelonephritis which was not distinguishable from the infection with the parental wild-type strain 536 (see Fig. 3). It can be concluded that the tRNA genes *leuX* and, to a minor extent, *selC* influence in vivo nephropathogenicity of uropathogenic *E. coli.*

The histology of the infected kidneys is seen in Fig. 3. Kidneys of rats infected with strain 536-21(pGBB51) or wild-type strain 536 (data not shown) displayed the histological picture of acute pyelonephritis. We found a preferential localization of bacteria in the lumens of the collecting tubules as well as

disseminated bacterial infiltration of the kidney parenchyma. Interestingly, a distinct staining of TEC of some tubules could also be observed, suggesting a tight association of bacteria with this cell type. Experimental infection with strain 536-21 (pGBB50), which codes for the *selC* tRNA, however, resulted in a sparsely distributed immunostaining, indicating the presence of infectious bacteria. The infection with the control strain, 536-21(pSU2716/pBR322), showed little inflammation.

These data confirm our previous observation (see above) that the tRNA genes, especially the more pronounced *leuX* but also *selC*, contribute to the clinical picture of pyelonephritis in an animal model.

In this paper we provide evidence for the fact that expression of two tRNA-specific genes of a uropathogenic *E. coli* strain, *selC* and *leuX*, plays an essential role in the establishment of pyelonephritis in a rat model. It has previously been demonstrated that the tRNA gene *leuX* contributes to virulence in mice following intravenous injection (25). In addition, *leuX* was shown to play an important role in the ability of the *E. coli* strain F-18 to colonize mouse intestines (24). In contrast to the mouse model with intravenous injection, the rat pyelonephritis model closely fulfills the histological and bacteriological criteria of acute pyelonephritis.

The influence of the two tRNA genes *selC* and *leuX* on nephropathogenicity of *E. coli* was tested, because these two loci are part of so-called pathogenicity islands (4). Strain 536 carries two of the pathogenicity islands which carry the genes responsible for hemolysin production and P fimbriae. The *selC* gene is also the target site for a pathogenicity island of an enteropathogenic *E. coli* isolate (21). In addition, the toxin-converting bacteriophage ϕ CTX of *Pseudomonas aeruginosa* as well as virulence-associated genes of *Dichelobacter nodosus* is located next to a serine tRNA gene (for references, see reference 25).

Surprisingly, not only *leuX* but also *selC* contributed to *E. coli* nephrovirulence in a rat model. Despite the fact that the renal bacterial counts per kidney following transfer of *selC* did not occur to the extent it did following transfer of *leuX*, *selC* was shown to significantly contribute to the colonization of the kidneys by uropathogenic *E. coli*. The *selC* product is necessary for anaerobic growth, and its gene product selenocysteine is part of the enzyme formate dehydrogenase, which is involved in mixed acid fermentation (5). The ability to grow under anaerobic conditions via mixed acid fermentation may be important for the colonization of *E. coli* in the kidney, because oxygen-limiting conditions are found in deeper regions of the kidney.

In contrast to the effect of *selC*, the effect of the *leuX* gene on nephrovirulence demonstrated by this study is very strong. *leuX* is important for the expression of a number of properties which obviously influence nephrovirulence (25). One of these properties is the expression of type I fimbriae, which is directly influenced by *leuX*. It has been demonstrated that phase variation which involves the switch of a 314-bp large DNA element located in front of the *fimA* gene is directly influenced by *leuX* (25a). Despite the fact that type I fimbriae are not considered specific virulence factors of uropathogens, this adherence system seems to contribute to general bacterial colonization of certain organs, including the kidney. In addition, the expression of flagella is regulated by *leuX*. Studies with species other than *E. coli* have demonstrated a correlation between motility and pathogenicity (1, 18). However, recent findings indicate the capability of *E. coli* flagella to activate plasminogen (17). This makes it likely that flagella can also contribute to the virulence of certain *E. coli* strains.

The expression of enterobactin is also influenced by *leuX*. Enterobactin is an important compound involved in the uptake of iron from the environment (6). Iron uptake might also be an important factor under iron-limited conditions in the kidney. Another important factor seems to be serum resistance, which is considered a main virulence property of extraintestinal *E. coli* strains. It remains to be elucidated which serum resistance factors are regulated by *leuX*. It has recently been shown that *leuX* is necessary for the expression of three outer membrane proteins of 69, 71, and 74 kDa (24) and additionally seems to be necessary for the expression of more than 20 proteins, analyzed by two-dimensional gel electrophoresis (24a), some of which might also play a role in serum resistance.

In addition to in vivo virulence, *leuX* but not *selC* contributes to binding of *E. coli* strains to primary renal TEC. Strain 536 produces three different adhesins, S fimbriae, P fimbriae, and type I fimbriae. The P fimbriae genes are deleted in strain 536-21, and the S fimbriae genes are not further transcribed (22). The *leuX* gene directly affects expression of type I fimbriae but not that of S fimbriae, which argues for an effect of type I fimbriae on binding of the bacteria to primary human renal epithelial cells.

In view of the presented data, it has clearly been shown for the first time that two pathogenicity island-associated tRNAs, *selC* and *leuX*, are necessary for in vivo virulence of uropathogenic *E. coli* in a rat pyelonephritis model. While the mechanism of the contribution of *selC* seems to be rather clear, the molecular events which lead to the contribution of *leuX* still require further investigation. Studies are under way to elucidate the mechanisms which lead to the pronounced *leuX*-dependent in vivo effects demonstrated in this study.

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## REFERENCES

1. **Allison, C., N. Coleman, P. L. Jones, and C. Hughes.** 1992. Ability of *Proteus mirabilis* to invade human urothelial cells is coupled to motility and swarming differentiation. Infect. Immun. **60:**4740–4746.
2. **Berger, H., J. Hacker, A. Juarez, C. Hughes, and W. Goebel.** 1982. Cloning of the chromosomal determinants encoding hemolysin production and mannose-resistant hemagglutination in *Escherichia coli*. J. Bacteriol. **152:**1241–1247.
3. **Blum, G., V. Falbo, A. Caprioli, and J. Hacker.** 1995. Gene clusters encoding the cytotoxic necrotizing factor 1 (*cnf*1), Prs fimbria (*prs*) and α-hemolysin (*hly*) from the pathogenicity island II (PaiII) of the uropathogenic *Escherichia coli* strain J96. FEMS Microbiol. Lett. **126:**189–196.
4. **Blum, G., M. Ott, A. Lischewski, A. Ritter, H. Imrich, H. Tschäpe, and J. Hacker.** 1994. Excision of large DNA regions termed pathogenicity islands from tRNA-specific loci in the chromosome of an *Escherichia coli* wild-type pathogen. Infect. Immun. **62:**606–614.
5. **Böck, A., K. Forchhammer, J. Heider, W. Leinfelder, G. Sawers, B. Veprek, and F. Zinoni.** 1991. Selenocysteine: the 21st amino acid. Mol. Microbiol. **5:**515–520.
6. **Crosa, J. H.** 1989. Genetics and molecular biology of siderophore-mediated iron transport in bacteria. Microbiol. Rev. **53:**517–530.
7. **Detrisac, C. J., M. A. Sens, A. J. Garvin, S. S. Spicer, and W. Goebel.** 1984. Tissue culture of human kidney epithelial cells of proximal tubule origin. Kidney Int. **25:**383–390.
8. **Diehl, J. M., and D. G. Evans.** 1977. Durchführungsanleitungen für statistische Tests. Verlag Chemie Weinheim, Weinheim, Germany.
9. **Hacker, J.** 1990. Genetic determinants coding for fimbriae and adhesins of extraintestinal *Escherichia coli*. Curr. Top. Microbiol. Immunol. **151:**1–27.
10. **Hacker, J., L. Bender, J. Wingender, B. Lund, R. Marre, and W. Goebel.** 1990. Deletions of chromosomal regions coding for fimbriae and hemolysins occur *in vitro* and *in vivo* in various extraintestinal *Escherichia coli* isolates. Microb. Pathog. **8:**213–225.
11. **Hacker, J., S. Knapp, and W. Goebel.** 1983. Spontaneous deletions and flanking regions of the chromosomally inherited hemolysin determinant of

an *Escherichia coli* O6 strain. J. Bacteriol. **154:**1145–1152.
12. **Henkel, W., and R. Commichau.** 1971. Serumresistenz und "Nephropathogenität" antigenetisch definierter Stämme von *E. coli*. III. Experimentelle Pyelonephritis mit unterschiedlich serumresistenten Keimen. Z. Med. Mikrobiol. Immunol. **156:**297–304.
13. **Hughes, C., J. Hacker, H. Düvel, and W. Goebel.** 1987. Chromosomal deletions and rearrangements cause coordinate loss of hemolysis, fimbriation and serum resistance in a uropathogenic strain of Escherichia coli. Microb. Pathog. **2:**227–230.
14. **Knapp, S., J. Hacker, T. Jarchau, and W. Goebel.** 1986. Large unstable inserts in the chromosome affect virulence properties of uropathogenic *Escherichia coli* O6 strain 536. J. Bacteriol. **168:**22–30.
15. **Komine, Y., T. Adachi, H. Inokuchi, and H. Ozeki.** 1990. Genomic organization and physical mapping of the transfer RNA genes in *Escherichia coli* K12. J. Mol. Biol. **212:**579–598.
16. **Kreft, B., M. Placzek, C. Doehn, J. Hacker, G. Schmidt, G. Wasenauer, M. R. Daha, F. J. van der Woude, and K. Sack.** 1995. S fimbriae of uropathogenic *Escherichia coli* bind to primary human renal proximal tubular epithelial cells but do not induce the expression of intercellular adhesion molecule 1. Infect. Immun. **63:**3235–3238.
17. **Lähteenmaki, K., R. Virkola, R. Pouttu, P. Kuusela, M. Kukkonen, and T. K. Korhonen.** 1995. Bacterial plasminogen receptors: in vitro evidence for a role in degradation of the mammalian extracellular matrix. Infect. Immun. **63:**3659–3664.
18. **Leying, H., S. Suerbaum, G. Geis, and R. Haas.** 1992. Cloning and genetic characterization of a *Helicobacter pylori* flagellin gene. Mol. Microbiol. **6:**2863–2874.
19. **Marre, R., J. Hacker, W. Henkel, and W. Goebel.** 1986. Contribution of cloned virulence factors from uropathogenic *Escherichia coli* strains to nephropathogenicity in an experimental rat pyelonephritis model. Infect. Immun. **54:**761–767.
20. **Marre, R., B. Kreft, and J. Hacker.** 1990. Genetically engineered S and F1C fimbriae differ in their contribution to adherence of *Escherichia coli* to cultured renal tubular cells. Infect. Immun. **58:**3434–3437.
21. **McDaniel, T. K., K. G. Jarvis, M. S. Donnenberg, and J. B. Kaper.** 1995. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. Proc. Natl. Acad. Sci. USA **92:**1664–1668.
22. **Morschhäuser, J., V. Vetter, L. Emödy, and J. Hacker.** 1994. Adhesin regulatory genes as parts of large, unstable DNA regions of pathogenic *Escherichia coli*: cross talk between different adhesin gene clusters. Mol. Microbiol. **11:**555–566.
23. **Mühldorfer, I., and J. Hacker.** 1994. Genetic aspects of *Escherichia coli* virulence. Microb. Pathog. **16:**171–181.
24. **Newman, J. V., R. Kolter, D. C. Lanx, and P. S. Cohen.** 1994. Role of *leuX* in *Escherichia coli* colonization of the streptomycin-treated mouse large intestine. Microb. Pathog. **17:**301–311.
24a. **Olsen, P., and P. Nystrom.** Unpublished data.
25. **Ritter, A., G. Blum, L. Emödy, B. Neuhierl, A. Böck, W. Rabsch, F. Scheutz, and J. Hacker.** 1995. tRNA genes as parts of pathogenicity islands: influence on virulence and metabolic properties of uropathogenic *Escherichia coli*. Mol. Microbiol. **17:**109–121.
25a. **Ritter, A., et al.** Submitted for publication.
26. **Sack, K., H. Freiesleben, W. Wöhrmann, W. Henkel, and R. Commichau.** 1972. Experimenteller Beitrag zum Infektionsweg der Coli-Pyelonephritis. Virchows Arch. Abt. A Pathol. Anat. **355:**323–332.

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