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Role of Sigma E-Regulated Genes in *Escherichia coli* Uropathogenesis

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The sigma E regulon encodes proteins for maintenance and repair of the *Escherichia coli* cell envelope. Previously, we observed that an antirepressor of sigma E, DegS, is essential for uropathogenic *E. coli* virulence. Here we use a mouse urinary tract infection model to assay the virulence of mutants of *E. coli* genes described as sigma E dependent. Deletion mutants of candidate genes were made in the uropathogenic *E. coli* strain CFT073. Swiss Webster female mice were inoculated with a mixture of mutant and wild-type strains. Bladder and kidney homogenates were cultured 2 days after infection, and CFU of the wild type and mutant were compared. Eleven mutants were assayed, and two, CFT073 *degP* and CFT073 *skp*, showed significantly diminished survival compared to wild type. DegP is a chaperone and degradase active in the periplasm. Skp is also a periplasmic chaperone. The virulence of the *skp* deletion mutant could not be restored by complementation with *skp*. The virulence of the *degP* deletion mutant, in contrast, could be restored. However, complementation with a *degP* allele encoding a serine-to-alanine (S210A) mutation at the protease active site fails to restore virulence. Unlike *degP* mutants in other bacteria, the *E. coli degP* mutant is tolerant of oxidative stress. It disappears abruptly from bladder and kidney cultures between 6 and 12 hours after inoculation. A mutant of *degQ*, a close homolog of *degP*, was not attenuated in mice. This is the first report that the DegP degradase is an *E. coli* virulence factor in an animal infection model.

A subset of *Escherichia coli* strains has the ability to leave their niche in the gut and infect extraintestinal regions of the host. Among these are strains that cause urinary tract infections (UTIs). Uropathogenic *E. coli* strains carry adhesins and toxins, such as P pili and hemolysin, which differentiate them from other *E. coli* strains. This laboratory seeks to uncover other virulence factors. We have used a mouse model of UTI both as a screening tool for genes specifically expressed during infection and for assessment of the relative virulence of mutants compared to wild-type *E. coli* (32, 37, 38).

Previously, we found that *degS* is essential for infection by the uropathogenic *E. coli* strain CFT073 (32). DegS is an indirect regulator of the alternate sigma factor, sigma E. DegS is an inner membrane protein that degrades RseA, a repressor of sigma E. Freed from RseA repression, sigma E mediates the recognition by RNA polymerase of the promoters of the genes in its regulon (1).

The sigma E regulon has been characterized in *E. coli* K-12 (10, 33). It consists of at least 40 genes, and those with known function can be divided into categories of periplasmic folding factors and degradases, lipopolysaccharide (LPS) biogenesis, sensor proteins, and transcriptional regulation. These functions are centered on envelope assembly and maintenance, prompting the name sigma E, for *e*xtracytoplasmic.

The discovery that *degS* is necessary for infection implicates sigma E in uropathogenesis (32). The host environment triggers a signal that leads to DegS proteolysis of RseA and, in turn, sigma E activation. This cascade suggests that at least one or more genes with sigma E promoters play a role in infection.

In this study we chose genes from the sigma E regulon for deletion in strain CFT073. Candidates were selected from genes described experimentally to be sigma E dependent in *E. coli* (10). Among these, we avoided those known to be essential or regulated by other sigma factors in addition to sigma E. Two mutants, CFT073 *degP* and CFT073 *skp*, demonstrate significantly diminished survival in a mouse model of urinary tract infection.

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MATERIALS AND METHODS

Bacterial strains and growth media. Table 1 shows *Escherichia coli* strains used in this study. MG1655 (ATCC 47076) is a laboratory K-12 strain. CFT073 (ATCC 700928) is a clinical strain isolated from a woman with pyelonephritis (28). The genomic sequences of both MG1655 and CFT073 have been published (5, 45). Cultures were grown at 37°C in Luria-Bertani (LB) broth or 1.5% LB agar unless otherwise noted. The following antibiotics (Sigma, St. Louis, MO) were added, as appropriate: ampicillin at 100 μg/ml, carbenicillin at 250 μg/ml, chloramphenicol at 30 μg/ml, kanamycin at 50 μg/ml, and erythromycin at 50 μg/ml.

Construction of deletion mutants and gene complementations. Genes in strains MG1655 and CFT073 were deleted and replaced by a chloramphenicol selection marker (*cat*). The marker was inserted by electroporation and lambda *red*-directed recombination of PCR products containing *cat* flanked by 40-bp sequences homologous to the target locus (11).

Selected deletion mutants were complemented by single-copy gene insertion into the chromosomal *att*Tn*7* locus. A modification of the method of Bao et al. (3) was used. Plasmid pCIITn7K-a was a gift of Eric Stabb. It features a mini-Tn*7* transposon with a kanamycin resistance marker. For complementation, the full-length gene and its promoter were amplified from wild-type CFT073 genomic DNA by PCR and then ligated into the pCIITn7K-a multiple cloning site after AvrII and KpnI digestion. The ligation product was electroporated into *E. coli* strain CC118. The plasmid was transferred to the mating *E. coli* strain S17-1λ*pir*, again by electroporation. Triparental mating was carried out with this strain as donor, the deletion mutant as recipient, and a third strain with the helper plasmid pUX-BF13 contributing the Tn*7* transposase. Transconjugants were selected on LB agar containing chloramphenicol, kanamycin, and erythromycin.

The *degP* S210A allele was created by overlap and extension during PCR. Two complementary primers were synthesized from the sequence adjacent to and including the serine codon at position 210 of *degP* in CFT073. Instead of TCT, however, the coding strand was changed to GCT for alanine. The primers were paired with outer primers to create two *degP* fragments in separate reactions.

TABLE 1. *Escherichia coli* strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
WAM2625	MG1655 laboratory K-12	ATCC 700926
WAM2267	CFT073 uropathogenic, blood isolate	ATCC 700928
WAM3174	UPEC76 CFT073 *pap prs*	29
WAM591	CC118 λ *pir* (plasmid maintenance)	14
WAM1301	S17-1 λ *pir* (conjugative donor)	14
WAM2871	Tn*7 tsp* (pUX-BF13) conjugative donor	3
Plasmids		
pACYC177	Low-copy, p15A origin of replication	6
pKD3	Template for λ Red *cat* cassette	11
pKD46	Red recombinase expression	11
pCIITn*7*k-a	Conjugative mini-Tn*7* delivery	Gift of Eric Stabb
pUX-BF13	Tn*7* transposase (in *trans*) helper	3

The overlapping fragments were annealed to generate full-length template in a third PCR using only the outer two primers. The *degP* S210A product was inserted into the *att*Tn*7* locus of the CFT073 *degP* deletion mutant by triparental mating, as described above.

Confirmation of deletion mutations was done by PCR using primers directed across the target locus (Table 2). Insertion of the cloned allele into the *att*Tn*7* locus was confirmed by sequencing of PCR products amplified from flanking chromosomal DNA.

Plasmid complementation of the CFT073 *skp* deletion mutant was done using pACYC177. Primers containing restriction sites for AvrII and BamHI were used to amplify *skp* by PCR. The PCR product and pACYC177 were digested with AvrII and BamHI and ligated together. The construct was electroporated into both wild-type CFT073 (WAM2267) and CFT073 *skp* (WAM3170) to create WAM3561 and WAM3562, respectively.

Immunoblotting was performed on whole cells grown to the mid-logarithmic growth phase and then boiled for 5 minutes and separated by sodium dodecyl sulfate (SDS)–polyacrylamide (10%) gel electrophoresis. After transfer to nitrocellulose, blots were exposed to anti-DegP/MBP antibody, a gift of Jon Beckwith (40). Anti-rabbit immunoglobulin G–horseradish peroxidase was used as secondary antibody, and its binding was visualized by using the LumiGLO chemiluminescence kit (Kierkegaard & Perry Laboratories, Gaithersburg, MD).

Mouse model of urinary tract infection. Relative survival of mutants was compared to the wild type as previously described (32). Briefly, the wild type and mutant were grown in broth culture without shaking and then were mixed 1:1 for a total of 1×10^9 CFU in 50 μl of phosphate-buffered saline (PBS). The mixture was inoculated into the bladders of Swiss Webster female mice (Harlan, Madison, WI) by urethral catheterization. Two days later, mice were sacrificed and bladders and kidneys were excised, homogenized, and quantitatively cultured.

A deletion mutation of *lacZYA* was created in *E. coli* CFT073 by the lambda *red* technique. This mutant, WAM2880, was compared to wild-type CFT073 (WAM2267) in a 1:1 competitive infection in eight mice. After 2 days, bladders and kidneys were excised and cultured onto LB agar. Mutants were identified by replica plating of colonies onto selective (chloramphenicol) agar. No difference was seen between survival of the wild type and *lacZYA* mutant (data not shown). The WAM2880 strain was then used as "wild type" in subsequent experiments, and Lac^+ mutants were differentiated from Lac^- "wild-type" ones on MacConkey

TABLE 2. Oligonucleotide primers used to make deletion mutants

Gene deletion	Strain (WAM)	Primer
degP	3155	Fwd, ACAGCAATTTTGCGTTATCTGTTAATCGAGACTGAAATACTGTGTAGGCTGGAGCTGCTTC Rev, GGAGAACCCCTTCCCGTTTTCAGGAAGGGGTTGAGGGAAACATATGAATATCCTCCTTAG
skp	3170	Fwd, AACTGCACCCTCCGGTGCAAATGGGATGGTAAGGAGTTTATGTGTAGGCTGGAGCTGCTTC Rev, ACTGCTGCGCTAAATCAGCCAGTCGAATTGAAGGCATTACCATATGAATATCCTCCTTAG
mdoG	3172	Fwd, ATGAAACATAAACTACAAATGATGAAAATGCGTTGGTTGATGTGTAGGCTGGAGCTGCTTC Rev, TCAACGTCTGATCGGCATTCACCAGCGCAGCACGCATTTCCATATGAATATCCTCCTTAG
surA	3179	Fwd, ATTAGTTGCTCAGGATTTTAACGTAGGCGCTGGCACGTTGTGTGTAGGCTGGAGCTGCTTC Rev, CCACGTAATCCGCAGTGCGGTTAATTGAAATGGAAAAAGTCATATGAATATCCTCCTTAG
cutC	3380	Fwd, GGTACGAGCAAGCATCATATTGGGCGACATGATGCAACGGTGTGTAGGCTGGAGCTGCTTC Rev, ACTGGTGACGGGCGTTCTCGATGTTGACGGGAATGTCGATCATATGAATATCCTCCTTAG
dsbC	3379	Fwd, ACTTCTCGGCGACGAAGTTGTATCTGTTGTTTCACGCGAATGTGTAGGCTGGAGCTGCTTC Rev, TATGTTATTTACCTTGTTGGCAGCGTTTTCAGGCTTTGTTCATATGAATATCCTCCTTAG
yggN	3384	Fwd, CGGCAATGACCGCTCACGCCGACTACCAGTGCAGCGTCACTGTGTAGGCTGGAGCTGCTTC Rev, GAGTCACAACGCGGCTACAAACATCTTTGCCAAACTGCTGCATATGAATATCCTCCTTAG
ytfJ	3394	Fwd, TGCCGATGATGGCTTCCGCACATCAGTTCGAAACCGGTCATGTGTAGGCTGGAGCTGCTTC Rev, TGCACCTCTTCCTGAGTAAGTGCCCCGTCTTTGGCCCATCATATGAATATCCTCCTTAG
ygiM	3395	Fwd, TAGCGCGACTGCCGTCTCACACGCTGAAGAAACGCGCTATTGTGTAGGCTGGAGCTGCTTC Rev, GGTGCGGCAGTACCAGACCGAGCAGCAAGCCAAGCCCCAGCATATGAATATCCTCCTTAG
yraP	3368	Fwd, AACACGCTTTTCCCTCACCAGGATGTTTAAGGAGAATACTGTGTAGGCTGGAGCTGCTTC Rev, ATAAGACGCTGCAAGCGTCGCATCAGGCATTACAAGGGCCATATGAATATCCTCCTTAG
degQ	3144	Fwd, AATCTCTTTTCTTATCATTCAGGTACGAGAGCAGGAATATGTGTAGGCTGGAGCTGCTTC Rev, ACCGGACATCACACGTCAGCCTGATGCCCGGTTTACGACACATATGAATATCCTCCTTAG
fkpA	3392	Fwd, GTTAACCCTGGGGTGAGATGCCCCGATCCTGGAGATATGGATGAGTAAAGGAGAAGAAC Rev, TAAAAAAACCGCCGCCTGGTCAGGCGGCGGTTCTTAGTGCCATATGAATATCCTCCTTAG
yidQ	3393	Fwd, CGAAACGTTAAAGTGCGATAGCCGCCACAGTGCGACGTATGTGTAGGCTGGAGCTGCTTC Rev, TCAGTTATCAGGCATTCTGGCCGGTGGAATAACCGCATTCATATGAATATCCTCCTTAG

TABLE 3. *Escherichia coli* strains constructed for this study

Strain (WAM)	Parent (WAM)	Deleted locus[a]	Gene	Gene or construct description
3340	2625	b0161	*degP*	(K-12), periplasmic chaperone, degradase
3155	2267	c0197	*degP*	(CFT073), periplasmic chaperone, degradase
3170	2267	c0215	*skp*	Outer membrane protein folding, chaperone
3172	2267	c1313	*mdoG*	Periplasmic, glucan biosynthesis
3179	2267	c0066	*surA*	Peptidyl-prolyl *cis-trans* isomerase
3144	2267	c3989	*degQ*	Periplasmic serine protease
3368	2267	c3904	*yraP*	Putative lipoprotein
3379	2267	c3473	*dsbC*	Disulfide isomerase II
3380	2267	c2289	*cutC*	Copper homeostasis
3394	2267	c3718	*ytfJ*	Hypothetical protein
3395	2267	c3805	*ygiM*	Hypothetical protein
3398	2267	c3544	*yggN*	Hypothetical protein
3392	2267	c4121	*fkpA*	Peptidyl-prolyl *cis-trans* isomerase
3393	2267	c4608	*yidQ*	Hypothetical protein
2880	2267	c0457-9	*lacZYA*	Lactose utilization
3156	3155	c0197	*degP*	*att*Tn*7*-*degP*$^+$ complementation
3157	3155	c0197	*degP*	*att*Tn*7*-*degP*$_{S210A}$ complementation
3341	3170	c0215	*skp*	*att*Tn*7*-*skp*$^+$ complementation
3501	2267			Wild-type CFT073 pACYC177-*skp*$^+$
3502	3170	c0215	*skp*	CFT073 *skp* pACYC177-*skp*$^+$

[a] Locus designations are open reading frames in the MG1655 or CFT073 sequenced genomes.

agar. The *skp*, *mdoG*, and *yraP* mutants show impaired growth on MacConkey medium. These were therefore cultured initially on LB agar and then replica plated onto chloramphenicol-containing LB agar, which revealed which colonies were mutant and which were wild type (WAM2267) (Table 3).

In vitro phenotype testing. Growth curves were initiated with a 1:100 dilution of overnight cultures into 4 ml of fresh medium. Broth cultures were grown in glass tubes shaken at 200 rpm. The optical density at 600 nm (OD_{600}) was measured at 20-min intervals, and quantitative cultures were compared at time zero and mid-logarithmic and late stationary phases.

Agglutination was assessed by mixing suspensions of bacteria in PBS with 3% (vol/vol) guinea pig erythrocytes (Hema Resource & Supply, Aurora, OR) or human erythrocytes (University of Wisconsin Hospital) on a glass slide (23). Mannose was added to agglutinated cells to a final concentration of 50 mM (29). Hemolysis was assessed by measurement of zones of clearing around colonies grown overnight on 5% sheep blood agar supplemented with 10 mM calcium chloride. Motility was evaluated by inoculation into 0.3% LB agar and incubation overnight at 37°C.

A disk-diffusion assay (Kirby-Bauer testing) was performed by diluting overnight broth cultures with PBS to an OD_{600} of 0.13, or approximately 0.5 McFarland standard (4). A cotton swab was used to spread the bacteria onto an LB agar plate. Paper disks 6 mm in diameter (0.25 in.; Schleicher & Schuell, Keene, NH) were saturated with 20 μl test reagent and placed atop the swabbed plate. After overnight incubation, zones of growth inhibition around the disks were measured. Reagents tested were hydrogen peroxide, paraquat (methyl viologen), and polymyxin B (all from Sigma, St. Louis, MO). All were used at a concentration of 300 mM. Bile salt tolerance was assessed by quantitative culture on MacConkey agar (0.15% sodium cholate/deoxycholate; Difco, Sparks, MD).

Complement resistance was observed by survival of bacteria in 50% serum. Normal or heat-inactivated human serum was added to bacteria suspended in Veronal-buffered saline (43). Tubes were incubated at 37°C, and quantitative cultures were performed at 0, 1, 2, and 3 h.

Lipopolysaccharide was examined by silver staining. Bacteria were grown to mid- logarithmic phase, pelleted, and resuspended in a lysis buffer containing 2 M Tris pH 6.8, 20% glycerol, 10% SDS, and 5% 2-β-mercaptoethanol. Proteinase K was added (0.2 mg), and samples were incubated overnight at 37°C. Samples were boiled for 5 min and run on a 10% SDS-polyacrylamide gel (15). A silver stain kit was used for visualization (Bio-Rad, Hercules, CA).

Statistical analysis. Raw colony counts were adjusted to CFU per gram of kidney or bladder. The minimum threshold of detection of infection was therefore 33 CFU/g for kidney and 200 CFU/g for bladder. Cultures with no growth were tallied at these thresholds, which are shown in Fig. 1 and 2, below. The wild type and mutant were compared by the Mann-Whitney test, with significance set at $P \leq 0.05$. Analysis was performed using Prism 4.0 (GraphPad Software, Inc., San Diego, CA).

TABLE 4. Recovery at 48 h of mutants and wild type after competitive infection of the urinary tract[a]

CFT073 gene deletion	Strain (WAM)	Kidney			Bladder		
		Ratio	*n*	*P*	Ratio	*n*	*P*
degP	3155	0.00	14	0.0003	0.00	13	0.0009
skp	3170	0.01	10	0.0052	0.23	12	0.035
mdoG	3172	0.89	8	0.87	0.65	8	0.44
cutC	3380	1.06	8	0.38	0.46	7	0.80
dsbC	3379	0.44	8	0.51	0.55	8	0.13
yggN	3384	1.13	5	0.69	2.36	5	0.10
ytfJ	3394	0.94	5	1.0	2.00	5	0.42
ygiM	3395	5.50	4	0.68	0.57	5	0.84
yraP	3368	0.25	8	0.51	0.38	8	0.19
degQ	3144	0.66	8	0.80	0.25	8	0.51
fkpA	3392	0.82	7	0.90	0.96	8	0.96
yidQ	3393	1.20	9	0.66	0.50	9	0.14

[a] Data shown are the ratio of mutant versus wild-type CFU (median); *n*, number of mice with infection; *P*, comparison by Mann-Whitney U test.

RESULTS

Deletion mutation of sigma E-dependent genes. Dartigalongue et al. observed 24 promoters in the *E. coli* K-12 strain MC4100 that responded to induction of sigma E activity (10). The promoter sequences were compared to those in the genome of the uropathogenic *E. coli* strain CFT073 by nucleotide-nucleotide BLAST. All 24 promoters are present in CFT073 except 1. The exception is associated with *yghF* (*ecfA*) in MC4100, which is absent in CFT073.

From the remaining promoters, 15 associated genes were chosen as candidates for deletion mutation in strain CFT073. Seven genes (*rpoD*, *rpoH*, *rpoE*, *lpxD*, *rfaD*, *yaeL*, and *yqjA*) were excluded because they have other promoters besides the one for sigma E and/or they are known to be essential for viability (10). Two sigma E-responsive promoters appear to be associated with *yggN* (10).

Deletion and replacement of the targeted genes with a chloramphenicol resistance gene were done by electroporation and lambda *red*-directed linear homologous recombination, as described above. The mutation method was unsuccessful with three, *yaeT*, *nlpB*, and *yfiO*, despite repeated attempts. Of the 12 successful deletion mutations, one, CFT073 *surA* (WAM3179), showed no agglutination of guinea pig red blood cells, suggesting defective type 1 pilus expression. The other 11 were assayed for virulence in mice.

Mutant survival in mouse infection. Eleven mutants of sigma E-dependent genes were each tested for survival in the urinary tracts of 8 to 12 mice in a mixed infection with the wild-type CFT073 parent strain (Table 4). The mutant and wild type were combined at a 1:1 ratio and inoculated into mouse bladders via urethral catheterization. After 2 days, homogenates of bladders and kidneys were cultured. The mutant was distinguished from the wild type by chloramphenicol resistance (CFT073 *skp*, CFT073 *mdoG*, and CFT073 *yraP*) or by its appearance as a red colony on MacConkey plates compared to white colonies of CFT073 *lacZYA* (WAM2880). Enumeration determined the relative proportions of each strain. Of the 11 mutants tested, 2, CFT073 *degP* and CFT073 *skp*, showed sig-

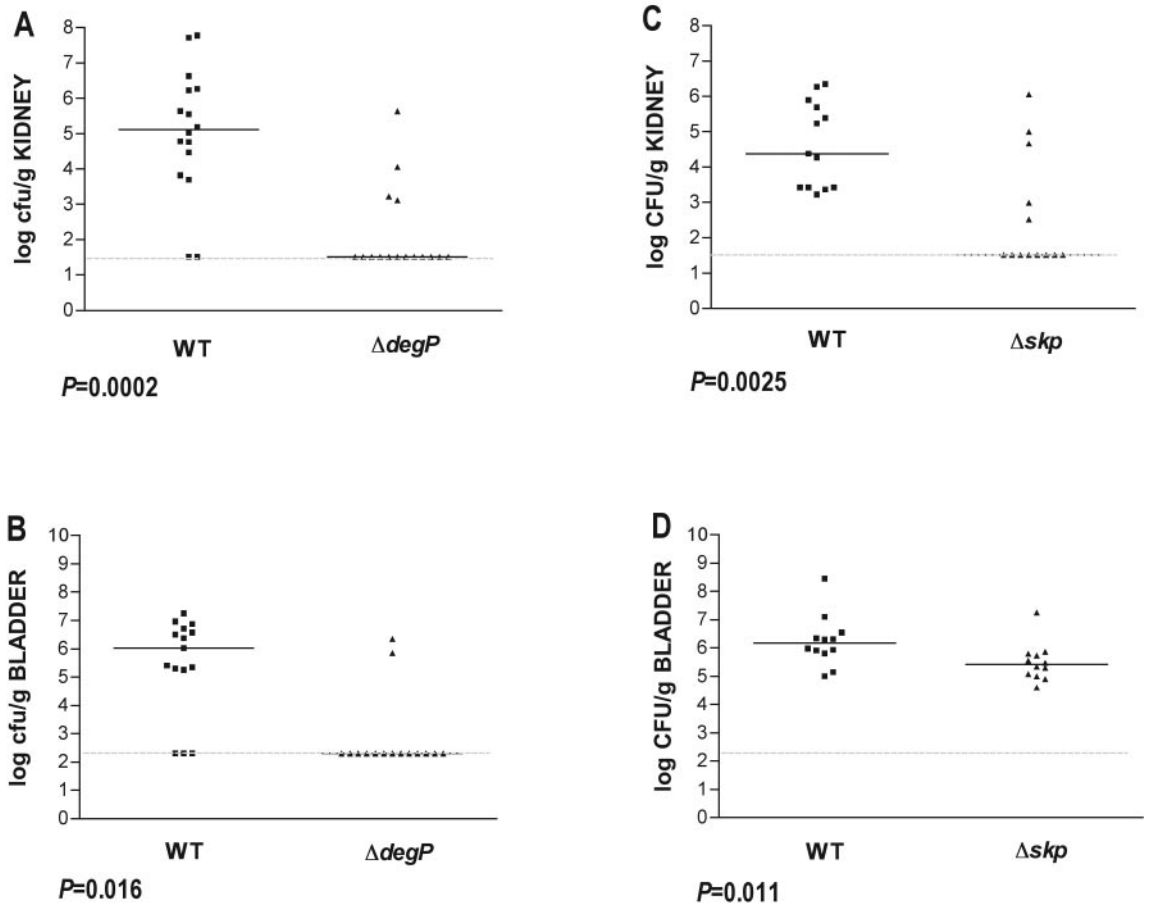

FIG. 1. Mutant recovery relative to wild type from mouse UTI. Kidney (A and C) and bladder (B and D) cultures 2 days after transurethral inoculation of a 1:1 mixture of wild-type (WT) or mutant (designated by deleted gene) *E. coli* strain CFT073. (A and B) WAM2880 versus WAM3155; (C and D) WAM2267 versus WAM3170.

nificantly lower recovery 2 days after inoculation compared to the wild type (Fig. 1).

CFT073 *degP* disappears abruptly. A time course study was performed, with mice sacrificed and kidneys and bladders cultured at 6, 12, and 24 h (Table 5). The CFT073 *skp* mutant appears to be at a significant disadvantage compared to the wild type from the earliest time point, and its proportionate recovery rate stays low at subsequent 12- and 24-h culture times, though not as low as at 6 hours.

TABLE 5. Recovery at 6, 12, and 24 h of CFT073 *degP* and CFT073 *skp* mutants compared to wild type after mixed infection of the urinary tract[a]

CFT073 gene deletion	Time (h)	Kidney			Bladder		
		Ratio	*n*	*P*	Ratio	*n*	*P*
degP	6	1.10	7	0.90	0.34	10	0.25
degP	12	0.00	8	NC[b]	0.00	8	0.0003
degP	24	0.00	11	<0.0001	0.00	10	<0.0001
skp	6	0.10	6	0.03	0.10	6	0.03
skp	12	0.05	5	0.10	0.07	5	0.15
skp	24	0.09	6	0.09	0.11	8	0.01

[a] CFT073 *degP*, WAM3155; CFT073 *skp*, WAM3170; wild type is WAM2267. Data shown are the ratio of mutant versus wild-type CFU (median); *n*, number of mice with infection; *P*, comparison by Mann-Whitney U test.
[b] NC, not calculable (no recovered mutant).

The CFT073 *degP* mutant is recovered in numbers comparable to the wild type at 6 h, especially in the kidney. In striking contrast, however, at 12 hours the *degP* mutant was not recovered from any of the mouse kidneys. Bladder cultures at 12 hours likewise grew almost entirely wild-type CFT073. The pattern of minimal recovery of the *degP* mutant continued at 24 h (Table 5) and at 48 h (Fig. 1).

CFT073 *degP* survives poorly upon single strain inoculation. The WAM3155 CFT073 *degP* mutant was inoculated into the bladders of eight mice in a single strain preparation. Nine other mice were inoculated with WAM2267 (wild-type CFT073) alone. Two days later the mice were sacrificed, and kidneys and bladders were cultured. All nine mice inoculated with the wild type showed bladder and kidney infections when cultured at 2 days. In contrast, four of the eight CFT073 *degP*-infected mice showed kidney infection, and six of the eight showed bladder infection. In both organs, infection with the *degP* mutant was much less intense (data not shown). Statistical comparison (Mann-Whitney test) of the two groups showed a significant difference ($P < 0.0001$).

DegQ differs from DegP. DegQ is a periplasmic serine protease with homology to DegP. A *degQ* deletion of CFT073 was

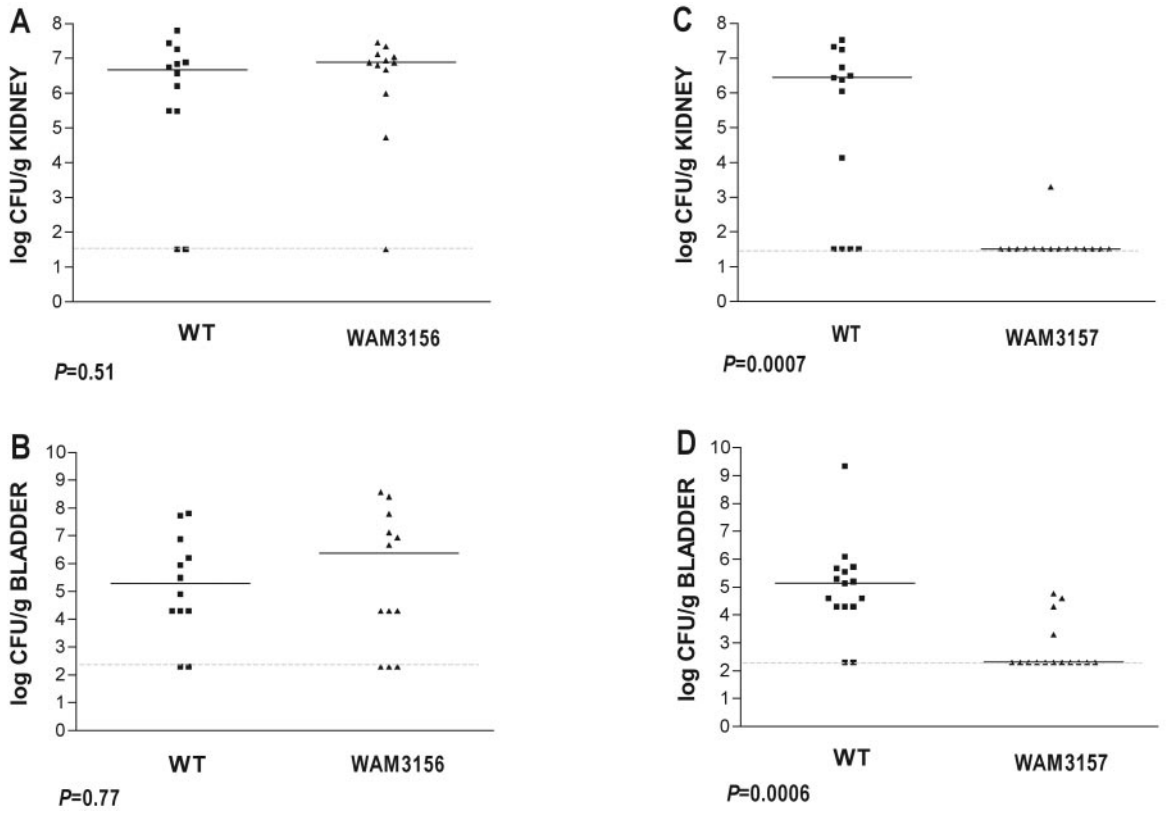

FIG. 2. Recovery of complementation mutants in mouse UTI. Kidney and bladder cultures 2 days after inoculation of a 1:1 mixture of the wild type (WAM2267) and CFT073 *degP* complemented with full-length *degP* inserted at the *att*Tn*7* locus (WAM3156) (A and B) or wild type (WAM2267) and CFT073 *degP* complemented with the $degP_{S210A}$ allele (WAM3157) (C and D).

made and assayed in the mouse UTI model. Eight mice were inoculated with the wild type and *degQ* at a 1:1 ratio. Two days later bladders and kidneys were cultured. In contrast to the *degP* mutant, no significant difference was seen between wild type and CFT073 *degQ* recovery from kidneys ($P = 0.95$) or bladders ($P = 0.65$).

Complementation of mutants. Complementation derivatives of the *skp* and *degP* CFT073 mutants were created by insertion of the full-length genes of each, respectively, in single copy at the *att*Tn*7* locus in the chromosome. The inserted sequence includes the sigma E-dependent promoter from the native locus. The complemented mutants were mixed 1:1 with wild-type CFT073, inoculated transurethrally, and then cultured quantitatively 2 days later.

Unlike the deletion mutant, cultures showed that the *degP* complementation strain, WAM3157, was recovered in numbers that did not differ significantly from wild type (Fig. 2A and B). However, the *skp* complementation strain, WAM3341, like its parent CFT073 *skp* deletion mutant, was significantly outcompeted at the 2-day time point by the wild type (data not shown).

The plasmid pACYC177 was used to make an alternative complementation strain of the CFT073 *skp* mutant. Full-length *skp* was cloned into pACYC177, and the construct was electroporated into wild-type CFT073 and CFT073 *skp*. The two plasmid-carrying strains were mixed 1:1 and inoculated transurethrally into 13 mice. After 2 days, the mice were sacrificed, and bladder and kidney homogenates were cultured onto LB agar containing kanamycin. Replica plating onto chloramphenicol agar revealed no growth. Only the "wild type" (WAM3251), therefore, persisted in the mouse urinary tract.

Role of the DegP protease domain in infection. The GCT codon encoding serine at residue 210 of DegP was altered by PCR overlap and extension to TCT for alanine. The S210A substitution abolishes DegP protease activity in *E. coli* strain K-12 (36). The *degP* S210A allele was delivered by a mini-Tn*7* transposon to the *att*Tn*7* locus in the chromosome of CFT073 *degP*. Immunoblotting demonstrated that both Tn*7* *degP* and Tn*7* *degP* S210A complementation of the CFT073 *degP* mutant restored expression of a 48-kDa band detected by anti-DegP antibody (not shown). Unlike the Tn*7* *degP* complementation, however, complementation of CFT073 *degP* with Tn*7* *degP* S210A failed to restore virulence in the mouse UTI model (Fig. 2C and D).

In vitro phenotype testing. The CFT073 *degP* and CFT073 *skp* mutants were compared to the wild type in vitro. Both mutants show growth curves indistinguishable from wild type at 37°C in LB broth (not shown). At 44°C the CFT073 *skp* mutant continues to match the wild type in growth (not shown). The CFT073 *degP* mutant, however, plateaus at an optical density slightly below that of the wild type and of CFT073 *degP* mutants complemented with $degP^+$ or with the *degP* S210A allele (Fig. 3). The K-12 *degP* mutant showed marked impairment in growth rate relative to its K-12 parent

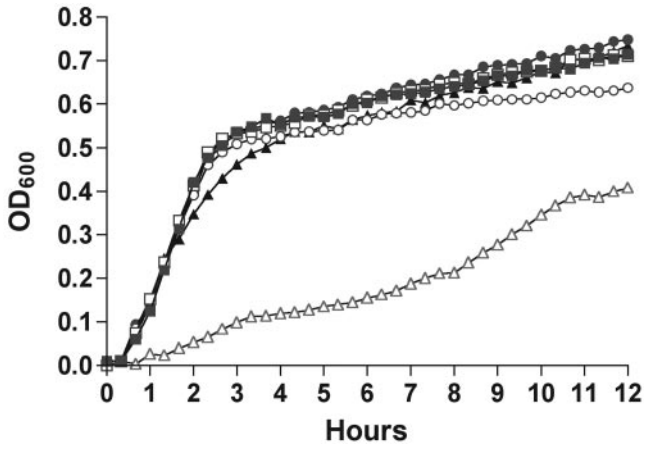

FIG. 3. Growth curves in LB broth at 44°C. ●, wild-type CFT073 (WAM2267); ○, CFT073 *degP* (WAM3155); ▲, MG1655 (WAM2625); △, MG1655 *degP* (WAM3340); ■, CFT073 *degP* Tn*7* *degP*$^+$ (WAM3156); □, CFT073 *degP* Tn*7* *degP*$_{S210A}$ (WAM3157).

strain and to the CFT073 *degP* mutant (Fig. 3). In human urine at 37°C, growth curves of the wild type, CFT073 *degP*, and CFT073 *skp* are indistinguishable, with the same final OD_{600} of approximately 0.50 at 24 h. The wild type, CFT073 *degP*, and CFT073 *skp* all show equal salt tolerance, with growth inhibition occurring between 0.5 and 1.0 M added sodium chloride as seen by serial dilutions of salt and LB broth in microtiter plates.

On LB agar the colony appearance and morphology of all mutants tested in mice are similar to those of the wild type. The CFT073 *surA* colonies are smaller and more translucent. The *surA* deletion mutant also shows no agglutination of guinea pig red blood cells, suggesting poor type 1 pilus expression. It was not tested in the mouse UTI model. Other mutants show mannose-sensitive, guinea pig red blood cell agglutination comparable to the wild type. The CFT073 *degP* and CFT073 *skp* mutants were mixed with human red blood cells and found to agglutinate them. The agglutination is mannose resistant, consistent with P pilus expression. The two mutants show zones of hemolysis on sheep blood agar the same size as wild type. They show similar-sized zones of growth inhibition in Kirby-Bauer testing with 300 mM hydrogen peroxide, 300 mM paraquat, and 300 U polymyxin B.

Quantitative cultures of the CFT073 *skp* mutant revealed that CFU counts on MacConkey agar averaged 30% of counts on LB agar. CFT073 *yraP* and CFT073 *mdoG* also showed sensitivity to MacConkey medium, with mean counts 70% and 50%, respectively, of paired cultures on LB plates. None of the other mutants tested prior to mouse infection, including CFT073 *degP*, demonstrated consistent differences between colony counts on paired LB and MacConkey plates.

CFT073 wild type is highly resistant to killing by nonspecific human serum, with continued growth in the presence of 66% serum, as demonstrated by increasing CFU in culture. *E. coli* K-12, in contrast, cannot be cultured after exposure for the same amount of time to 1% serum. The K-12 strain can be recovered if the serum is heat inactivated. The CFT073 *degP* and CFT073 *skp* mutants show the same high complement resistance of the parent strain (data not shown). Silver stains of gel-separated LPS preparations of CFT073 *degP* and CFT073 *skp* mutants show a banding pattern identical to that of wild-type CFT073 (not shown).

DISCUSSION

This study demonstrates that *degP* plays a significant role in *E. coli* infection of the urinary tract. Another gene, *skp*, may also be important, but complementation studies did not restore virulence to its mutant, unlike complementation of the Δ*degP* strain. Both genes were found by constructing deletion mutants from a list of genes described as sigma E regulated and screening them for loss of virulence in a mouse UTI model.

This laboratory became interested in sigma E and its potential role in virulence after noting that a partial deletion mutant of *degS* in CFT073, a uropathogenic *E. coli* strain, shows colony morphology changes and is attenuated in the mouse UTI model (32). DegS is an indirect activator of sigma E. It acts as a degradase which frees sigma E from RseA repression. Inactivation of *degS* would be expected to diminish sigma E activity via unremitting RseA repression. The lack of virulence seen in the *degS* mutant suggests that sigma E activity is necessary for infection. This observation led us to look for virulence genes among members of the *E. coli* sigma E regulon.

The sigma E regulon has been characterized in a laboratory strain, *E. coli* K-12 (10). We chose candidates from this list for deletion mutation and then assayed each mutant for survival in a mouse urinary tract infection model. As noted above, exclusion criteria included genes known to be essential and genes regulated by other sigma factors in addition to sigma E. Additional exclusions were necessary. For example, one candidate, *yghF*, is present in the K-12 MC4100 strain but absent in CFT073, and three candidates, *yaeT*, *yfiO*, and *nlpB*, could not be deleted. The latter two have subsequently been described as encoding lipoproteins, and *yfiO* was found to be essential in *E. coli* K-12 (30). Also, in *E. coli* K-12 the products of these three genes, YaeT, YfiO, and NlpB, have been shown to associate, along with YfgL, to form a multicomponent complex important in the biogenesis of the outer membrane (47).

One mutant, CFT073 *surA*, shows minimal guinea pig red blood cell agglutination. This suggests diminished type 1 pilus expression. The association of SurA and type 1 pilus expression has recently been reported (22). The type 1 pilus adhesin is important for *E. coli* persistence in the mouse bladder (2). Therefore, the nonagglutinating CFT073 *surA* mutant was not assayed for virulence in the mouse UTI model.

Of the 11 mutants inoculated into mice, 5 have deletions of genes mostly uncharacterized (genes starting with a *y* in Table 2). None of these show significantly diminished survival in the mouse urinary tract. They therefore remain without a virulence or other discernible phenotype, though *yraP* has recently been described as encoding a lipoprotein (30).

Other deletion mutants also have no apparent in vivo survival defect, though they appear important in other pathogens. For example, *fkpA*, like *surA*, encodes a periplasmic prolyl isomerase, although it is in a different family (27). FkpA also has chaperone activity. In *Salmonella enterica* serovar Typhimurium, an *fkpA* inactivation mutant shows decreased intracellular survival in a macrophage-like cell line (16). Mutants of *fkpA* in *Legionella pneumophila* and *Chlamydia trachomatis*

also show impaired survival in host cells (12, 26). It is notable that disease with these three pathogens features a well-characterized and necessary intracellular replication phase, while *E. coli* uropathogenesis does not.

This is the first report of *degP* as necessary for *E. coli* virulence in an animal infection model, though it has been well-studied as a virulence gene in other pathogens. It was described as "*htrA*" for high temperature resistance in *E. coli* K-12 in 1988 (25). Johnson et al. found it during a screen of transposon-generated mutants of *Salmonella enterica* serovar Typhimurium for avirulence after oral inoculation of mice (19). *Klebsiella pneumoniae*, *Brucella abortus*, *Brucella melitensis*, and *Yersinia enterocolitica* mutants of *degP* (*htrA*) were subsequently also found to have reduced virulence in infection models, and a live *Salmonella enterica* serovar Typhi vaccine strain with inactivation mutations in *aroC* and *aroD* was associated with no bacteremia in human volunteers when a third mutation in *degP* was added (8, 24, 31, 41, 42). More recently, *degP* orthologs have been shown to contribute to the virulence of the gram-positive pathogens *Streptococcus pyogenes* and *Streptococcus pneumoniae* (18, 20).

Interestingly, the uropathogenic *E. coli* CFT073 mutant shows few of the defects described for *degP* mutants in other pathogens. Most of the above-named bacteria, including the streptococci, have been found to be more sensitive to oxidative stress when *degP* is inactivated. However, CFT073 *degP* is no less tolerant of hydrogen peroxide or paraquat stress than its parent strain. DegP has been described as involved in P pilus assembly (21), yet P pilus expression is qualitatively normal in CFT073 *degP*, as assessed by mannose-resistant agglutination of human red cells. Its envelope remains intact, at least when stressed with bile salts and polymyxin B, and it retains the very high complement resistance of wild-type CFT073. Colonies of the *degP* mutant are indistinguishable from those of the wild type on LB or blood agar, and silver staining of a gel-separated LPS preparation shows a "ladder" pattern identical to that of the parent strain.

The CFT073 *degP* mutant is remarkably attenuated in the mouse UTI model. We have seen other *E. coli* mutants with diminished virulence, and they behave like the CFT073 *skp* in this study, with a steady decrease over time in recovered CFU when serial cultures are made of bladders and kidneys. The *degP* mutant, in contrast, is present in approximately equal numbers as wild type 6 hours after inoculation, but at 12 hours it is almost completely absent from both bladders and kidneys. Clearly, this unusual pattern of sudden eradication merits further study, with special attention to the early host response during UTI.

degP has a homolog, *degQ*, with a predicted product that is 60% identical and 75% similar at the amino acid sequence level to DegP, which is encoded immediately upstream of *degS* on the CFT073 chromosome (45). An inactivation mutant of *degQ* has no apparent phenotype in *E. coli* K-12, but when overexpressed it can rescue the temperature-sensitive phenotype of a K-12 *degP* mutant (44). We wondered if *degQ* plays a role in *E. coli* virulence and made a deletion mutant of it in CFT073. In competition with the wild type in the mouse UTI model, no significant difference was seen in their relative recovery. This is similar to results recently reported for a *degQ* mutant of *S. enterica* serovar Typhimurium which was not attenuated in mice either by oral or intraperitoneal inoculation (13). Compensation by DegQ may be responsible for the relative resilience to stress seen here of the CFT073 *degP* mutant in vitro, but DegQ does not appear to make up for the loss of DegP function inside the mouse, nor does it seem to have an independent role in infection.

DegP is at present the only known periplasmic protein that functions as both a protease and a chaperone (39). We modified the genetic complementation method used in this study to see if the protease function is necessary for virulence. Full-length *degP* with its sigma E promoter was inserted into the CFT073 chromosome by mini-Tn*7*. This restores recovery of the CFT073 *degP* mutant in kidney and bladder cultures 2 days after inoculation, making it comparable to wild type. Next, a *degP* allele was created which encodes alanine instead of serine at the active site of the protease domain. The *degP* S210A allele failed to restore virulence to the CFT073 *degP* mutant in the mouse UTI model, though it did express DegP, as visualized by immunoblotting. The failure of *degP* S210A complementation in the mouse UTI model suggests that the degradase function of DegP is necessary for virulence.

As noted, among the sigma E-dependent genes tested, CFT073 *skp* is the only mutant with significant attenuation besides CFT073 *degP*. As shown in Fig. 1, the two mutants differ in their recovery patterns. Whereas CFT073 *degP* is attenuated both in bladder and kidney, CFT073 *skp* is attenuated in the kidney but survives better in the bladder (though not as well as wild type). Tropism for kidney or bladder infection has been observed in uropathogenic *E. coli* (pyelonephritis versus cystitis strains), and this tropism has been associated with differences in adhesin expression (35). It may be that the *skp* mutant has an adhesin expression defect. P pilus and type 1 fimbria expression did appear normal in agglutination testing of CFT073 *skp* prior to infection. However, in vivo expression of these or other adhesins may differ from that of the wild-type strain.

In contrast to the *degP* mutant, the CFT073 *skp* mutant could not be complemented to restore virulence in the mouse UTI model, either by chromosomal insertion of *skp* at *att*Tn*7* or by plasmid complementation. Unlike *degP*, *skp* appears to be part of an operon. Downstream are *lpxD*, *fabZ*, and *lpxA* (10). Sequencing of the mutant at the *skp* deletion locus reveals no additional mutations other than the intended, in-frame deletion, which should not cause a polar effect on genes downstream. There may yet be such an effect, or there may be inadequate or inappropriate *skp* expression in the complementation constructs. Whatever the case, it is unclear whether *skp* per se is involved in virulence. Skp has been described as a periplasmic chaperone important in the maturation of outer membrane porins, such as OmpA, OmpC, OmpF, and LamB (7). It may have functional redundance with DegP and SurA, but these "parallel pathway" chaperones (34) may not be able to make up for its loss during infection.

The members of the sigma E regulon play important roles in periplasmic and outer membrane homeostasis. It is notable, therefore, that so few are essential for pathogenesis. Sigma E (*rpoE*) has been noted to be involved in pathogenesis in other bacteria, but the described mutants show defects in oxidative sensitivity (*Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis*) or intracel-

lular survival (*Haemophilus influenzae*) (9, 17, 46, 48). It may be that some sigma E-regulated genes important for virulence in other bacteria are less necessary in *E. coli* uropathogenesis. Furthermore, the in vitro phenotype of the *degP* mutant of *E. coli* CFT073 differs from that described in other bacteria. These disparities likely arise from different mechanisms of survival and spread within a host. The findings here suggest that the roles of DegP and other members of the sigma E regulon are not the same in different pathogens and in different infection pathways.

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