degS Is Necessary for Virulence and Is among Extraintestinal *Escherichia coli* Genes Induced in Murine Peritonitis

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Extraintestinal *Escherichia coli* strains cause meningitis, sepsis, urinary tract infection, and other infections outside the bowel. We examined here extraintestinal *E. coli* strain CFT073 by differential fluorescence induction. Pools of CFT073 clones carrying a CFT073 genomic fragment library in a promoterless *gfp* vector were inoculated intraperitoneally into mice; bacteria were recovered by lavage 6 h later and then subjected to fluorescence-activated cell sorting. Eleven promoters were found to be active in the mouse but not in Luria-Bertani (LB) broth culture. Three are linked to genes for enterobactin, aerobactin, and yersiniabactin. Three others are linked to the metabolic genes *metA*, *gltB*, and *sucA*, and another was linked to *iha*, a possible adhesin. Three lie before open reading frames of unknown function. One promoter is associated with *degS*, an inner membrane protease. Mutants of the in vivo-induced loci were tested in competition with the wild type in mouse peritonitis. Of the mutants tested, only CFT073 *degS* was found to be attenuated in peritoneal and in urinary tract infection, with virulence restored by complementation. CFT073 *degS* shows growth similar to that of the wild type at 37°C but is impaired at 43°C or in 3% ethanol LB broth at 37°C. Compared to the wild type, the mutant shows similar serum survival, motility, hemolysis, erythrocyte agglutination, and tolerance to oxidative stress. It also has the same lipopolysaccharide appearance on a silver-stained gel. The basis for the virulence attenuation is unclear, but because DegS is needed for σ^E activity, our findings implicate σ^E and its regulon in *E. coli* extraintestinal pathogenesis.

Extraintestinal *Escherichia coli* strains cause meningitis, sepsis, and infections of the urinary tract. These strains are the primary cause of the latter, being responsible for 80% of community-acquired cases, and cause an estimated 30% of all cases of neonatal meningitis (52). The toll of these infections is high: one in five neonates with meningitis dies despite treatment, and urinary tract infection leads to 7.3 million physician office visits and $1.6 billion in medical costs annually (23).

Extraintestinal *E. coli* differ from fecal strains phenotypically, an observation first made more than 80 years ago when Lyon reported that *E. coli* strains isolated from urine were more often hemolytic (34). More recently, a study found 58% of pyelonephritis isolates carried hemolysin versus 9% of fecal *E. coli* from healthy women (40). Disparities are also seen for toxins such as cytotoxic necrotizing factor 1, the siderophore aerobactin, and adhesins such as P pili (20).

Recently, the genomic sequence of the uropathogenic *E. coli* strain CFT073 was reported (53). A three-way comparison of its genome with those of the laboratory strain MG1655 and the enterohemorrhagic strain EDL933 found that, although all three are *E. coli*, they have in common only 39% of their combined (i.e., nonredundant) set of proteins. Examination of the large pathogenicity island in CFT073 shows marked differences between it and islands described in two other uropathogenic strains, J96 and 536. The hypothesis was made that these

and perhaps other extraintestinal *E. coli* strains arose independently from multiple clonal lineages (53).

The differences among *E. coli* strains indicate that there exist forces that drive not only the evolutionary divergence of strains in different niches (laboratory, bowel, urinary tract) but also drive the convergence of disparate lineages within the same niche. Certainly, the physical conditions of the environment, as well as the nature and extent of the host immune response, rank as some of the most potent of these forces, for these would be quite different in different regions of the host.

Recent technical advances have made it possible to observe aspects of pathogen physiology during infection of a host. One such technique is differential fluorescence induction (DFI), which gives a view of a bacterium's genetic response to its environment. Central to DFI is the segregation of bacterial populations by fluorescence-activated cell sorting (FACS) (50). The fluorescence comes from the *Aquorea victorea*-derived green fluorescent protein fused to a promoter library constructed from the bacterial genome. Bacteria that carry an active promoter-*gfp* fusion are separated from clones that do not. Successive sorts from different environments yield bacteria that are fluorescent in vivo but not in vitro, and the differentially induced promoters can be identified by DNA sequence analysis.

DFI was initially used to identify *Salmonella enterica* serovar Typhimurium promoters activated by acid stress in Luria-Bertani (LB) broth culture (50). Subsequently, Valdivia and Falkow expanded the technique to identify serovar Typhimurium promoters induced during invasion of macrophages (51). Other groups have studied other bacteria in other broth and tissue culture models (4–6, 28, 37, 43, 45). Recently, Marra et al. described the first use of DFI to identify promoters

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induced during an animal infection (36). They sorted *Streptococcus pneumoniae* clones from infections of the middle ear, mouse lung, and peritoneum. The latter involved recovery of *S. pneumoniae* clones from intraperitoneal chamber implants.

In the present study we sought to sort *E. coli* directly from lavage fluid from a mouse with peritonitis. This is an in situ use of DFI that marks the first time this technique been applied to the study of an *Enterobacteriaceae* strain during host infection. Background fluorescence and sorting inefficiencies revealed that FACS of bacteria directly from inflammatory tissue carries technical limitations. Nonetheless, 11 sequences were identified which promote green fluorescent protein expression inside the mouse but not in LB broth culture. Disruption mutations of the associated genes were made, and the mutants were tested in mice in competition with the wild type. Mutation of one gene in particular, *degS*, caused virulence attenuation both within the mouse peritoneum and in the urinary tract. DegS is required for the activity of the alternative, extracytoplasmic sigma factor σ^E; thus, the findings here may be the first indication that σ^E is required for virulence in *E. coli*.

MATERIALS AND METHODS

Strains, growth conditions, and reagents. Uropathogenic *E. coli* strain CFT073, serotype O6:H1:K2, was isolated from the blood of a woman with acute pyelonephritis (35, 42). Except as noted, bacteria were grown at 37°C in LB broth with shaking or on LB agar. As appropriate, 50 μg of nalidixic acid, 50 μg of kanamycin, or 250 μg of carbenicillin/ml was added. These and other reagents were obtained from Sigma Chemical Co., St. Louis, Mo., and recombinant enzymes were from Promega Corp., Madison, Wis. (T4 ligase and *Taq* polymerase); Roche Diagnostics, Indianapolis, Ind. (*Pwo* polymerase); and New England Biolabs, Beverly, Mass. (restriction enzymes).

Construction of promoter library. Chromosomal DNA from CFT073 was partially digested with *Sau*3A, and fragments of from 0.5 to 4 kb were purified after excision from an agarose gel. The fragments were ligated into the *Bam*HI site of pFPV25 immediately before the promoterless *gfp*mut3 (50). Ligation products were electroporated into CFT073, and eight pools, each containing ca. 2,000 transformants, were assembled and stored at −80°C in LB medium-glycerol.

Mouse peritoneal infection. Individual bacterial strains or pooled transformants were grown overnight, washed, and diluted with phosphate-buffered saline (PBS) to an inoculum of ca. 5×10^7 CFU in 200 μl. This was injected into the peritoneal cavity of Swiss-Webster female mice (Harlan Teklad, Madison, Wis.). After 6 h, mice were sacrificed by CO_2 asphyxiation. Dissection was performed to reveal the intact ventral peritoneal wall, and lavage with 2 ml of PBS was carried out. Recovered lavage fluid was cultured on LB agar or directly and immediately analyzed by flow cytometry or sorted by FACS. Spleens from mice were collected by dissection and excision and then were homogenized in 1 ml of PBS. Dilutions of the homogenate were cultured.

DFI. Aliquots of each pool of CFT073/pFPV25 genomic library transformants were used to start a culture in LB broth. After overnight growth, the bacteria were washed and diluted in PBS then inoculated transperitoneally into mice. The bacteria were recovered by lavage 6 h later and immediately sorted, with nonfluorescent and constitutively fluorescent CFT073/pFPV25 clones used as controls (5). The most fluorescent (uppermost 1%) were collected and grown on plates. The following day, the colonies on LB agar were scraped to pool them and then sorted by FACS. The least fluorescent (lowest 5%) were collected. After overnight growth, the clones were again inoculated into mice, recovered by peritoneal lavage after 6 h, and sorted for high fluorescence, collecting and culturing the uppermost 1%. Individual colonies were subcultured, and then each was individually tested in mice and LB broth to confirm high and low fluorescence, respectively, by flow cytometry.

Sequence analysis. Plasmid inserts were sequenced (ACGT, Inc., Northbrook, Ill.), and the sequences were aligned to the genome of CFT073 at http://www.genome.wisc.edu/. BLAST searches for sequence homology were carried out at http://www.ncbi.nlm.nih.gov.

Generation of mutants. Deletion mutants of in vivo-induced loci were generated by the linear recombination (λ Red) method of Datsenko and Wanner (17). More than 90% of the putative coding sequence of each locus was deleted,

leaving behind the 5′ and 3′ ends. A kanamycin resistance cassette was inserted in place of the deleted sequence, and kanamycin was used for selection. The insertion cassette was derived from the nonpolar (in frame, with added ribosome binding site) plasmid template pKD4 (17). The deletion and insertion mutations were confirmed by PCR.

A disruption mutation of *degS* was constructed by homologous recombination. A 500-base internal *degS* fragment was generated by PCR with genomic DNA from strain CFT073 as a template. The fragment extends from bases 248 to 748 (NCBI accession no. AE016767, bases 175543 to 176043) of the predicted 1,063-base *degS* open reading frame (ORF). *Kpn*I and *Sac*I restriction sites were incorporated into the primers and, after digestion with these enzymes, the 500-bp fragment was ligated into the multiple cloning site of pEP185.2, a conjugative, π-dependent suicide vector (5). This construct, pWAM2851, was placed into the *E. coli* donor strain S17λ*pir*, and mating introduced pWAM2851 into CFT073. Antibiotic selection (nalidixic acid and chloramphenicol) isolated WAM2853, produced by crossover of pWAM2851 into the *degS* chromosomal locus. WAM2854 is WAM2853 transformed with a plasmid carrying *degS*. This plasmid was constructed by insertion of full-length *degS* between *Bam*HI and *Pst*I sites on pACYC177. For this cloning, the *degS* insert was generated by PCR from wild-type CFT073 chromosomal DNA (AE016767, bases 175208 to 176372) by using primers incorporating *Bam*HI and *Pst*I restriction sites.

In vitro phenotype screening. Colony size and morphology were compared on LB agar and MacConkey agar. Guinea pig and human erythrocyte agglutination was used to assess type 1 and P fimbriation, respectively (39). Mannose resistance was checked by the addition of 25 mM mannose to red blood cells agglutinated by bacteria. Hemolysin activity was determined by comparison of colony lysis zones on sheep erythrocyte agar and by liquid hemolysis assay (7). Culture stabs into 0.3% LB agar were used to compare motility. Growth curves were determined by serial optical density at 600 nm readings from LB broth cultures at 37, 43, and at 37°C in LB broth containing 3% (vol/vol) ethanol. Matching readings of the optical density at 600 nm were confirmed by quantitative cultures. Tolerance to oxidative stress was determined by monitoring growth at 37°C in LB broth containing 750 μM H_2O_2. Tolerance to envelope stress was also examined by monitoring of growth in the presence of 1.5 U of polymyxin B/ml. Serum resistance was assayed by quantitative recovery of bacteria incubated in Veronal-buffered saline plus 50% nonimmune human serum for 1 h at 37°C (32). Lipopolysaccharide (LPS) profiles were obtained by whole-cell lysis, followed by proteinase K digestion and then separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining (27).

Competitive murine urinary tract infection. CBA/J female mice (Harlan Teklad, Madison, Wis.), 6 to 8 weeks old, were anesthetized and inoculated transurethrally by using a catheter and syringe. The inoculum was prepared from LB broth cultures, grown without shaking at 37°C over 6 days, with passaging into fresh broth on the second and fourth days. After centrifugation and resuspension in PBS, type 1 fimbriation was assessed by guinea pig red blood cell agglutination and, if equal, the two strains to be compared (wild-type CFT073 and mutant) were mixed 1:1 to create the inoculum for a total of ca. 5×10^8 bacteria suspended in 50 μl of PBS. Two days after inoculation, mice were sacrificed by CO_2 asphyxiation, and kidneys and bladder were retrieved by dissection. These tissues were homogenized with PBS inside sterile plastic field specimen bags (Whirl-Pak; Nasco, Fort Atkinson, Wis.) by rolling compression by using a round glass bottle. Homogenates were diluted and cultured on LB agar, and the mutant and wild type were distinguished by antibiotic sensitivity (patching or replica plating onto kanamycin- or chloramphenicol-treated LB agar).

Statistical analysis. Analysis of relative recovery of strains from competitive mouse infections was done by Mann-Whitney U testing by using the Instat statistical program (GraphPad Software).

RESULTS

DFI analysis. We sought to identify promoters induced by exposure to conditions inside the mouse peritoneum but repressed by growth in rich medium. A DFI enrichment strategy was used, as described above. From a promoter trap library of ca. 16,000 clones, 11 promoters were found after screening and sequencing steps, to be unique (Table 1). The ratio of in vivo to in vitro fluorescence ranged from 2.9 to 24. All 11 clones matched sequence in CFT073, but three matched putative promoter regions for ORFs of uncertain function. The eight remaining were linked to genes encoding a diverse range of

TABLE 1. *E. coli* CFT073 clones with differentially induced GFP expression

Clone	GenBank accession no.[a]	Insert match (base range)	Induction[b]	Putative induced locus	Function
301	AE016767	174120–176043	2.9	*degS*	σ^E regulation
504	AE016757	183205–183893	5.3	*sucA*	Succinate dehydrogenase
508	AE016757	73190–74849	4.6	*ybdL*	Unknown
514	AE016767	26654–27564	7.9	*yqjH*	Unknown
606	AE016761	115753–114223	4.4	*ydiJ*	Unknown
723	AE016762	115531–118130	9.6	*irp2*	Yersiniabactin synthesis
957	AE016767	156345–157876	6.4	*gltB*	Glutamate synthesis
1031	AE016766	143717–140819	19	*iucA*	Aerobactin synthesis
1053	AE016766	124661–123556	18	*iha*	Adherence (?)
1217	AE016757	63076–66096	23	*entC*	Enterobactin synthesis
1218	AE016770	210782–211820	24	*metA*	Methionine synthesis

[a] NCBI GenBank accession number (sections of CFT073 genome).
[b] Ratio of fluorescence (geometric mean) in peritoneal versus LB broth preparations. The average (mean) of three determinations is given.

proteins involved in siderophore synthesis, metabolism, regulation, and adherence.

Iron acquisition. Of the 11 promoters, 3 appear to be linked to iron acquisition genes. The siderophore enterobactin is represented on clone 1217, which contains on its insert the promoter of *entC*, encoding the first enzyme in enterobactin synthesis (49). Another clone, 1031, contains the start of the aerobactin synthesis operon, *iucABCD*, while a third clone, 723, contains the promoter for *irp2*, the first gene of the operon for yersiniabactin synthesis (19, 24).

Two other clones show sequences that may be associated with iron acquisition. One, 514, carries the first 42 bases of *yqjH*, an ORF of unknown function but whose predicted protein product has 28% identity to ViuB, a cytoplasmic vibriobactin utilization protein in *Vibrio cholerae* (11). The plasmid insert in another, clone 1053, contains the promoter for *iha*, a gene not present in the K-12 *E. coli* strain MG1655 but described in *E. coli* O157:H7 as the IrgA homolog adhesin ("*iha*") (46). The *iha* locus in strain CFT073 has also been annotated as ORF R4, the "exogenous ferric siderophore receptor" on the basis of protein homology (26).

Metabolic enzymes. Three clones carry genes with metabolic functions. One of these, clone 504, has the promoter for the *sucAB* operon. The product of *sucAB* forms, with that of *lpd*, the 2-ketoglutarate dehydrogenase complex, an enzyme in the tricarboxylic acid cycle (15). Another, clone 1218, carries the promoter for *metA*, which encodes the enzyme for the first committed step in the methionine synthesis (25). The promoter and initial 137 bases of *gltB* appear on the insert of a third, clone 957. The *gltBDF* operon encodes the large subunit of glutamate synthase.

Unknowns. Clone 514 features the promoter for *yqjH*, an ORF of unknown function but, as noted above, with protein homology to a vibriobactin utilization protein. Two others, 508 and 606, carry promoters for *ybdL* and *ydiJ*, ORFs with no known function or homology.

Regulation. The product of *degS*, whose promoter appears in clone 301, plays a regulatory role. This gene encodes a cytoplasmic membrane serine protease whose only known target is another inner membrane protein, RseA (3). RseA binds and represses σ^E, a sigma factor associated with the bacterial response to envelope stress (1). When RseA is degraded by DegS, σ^E is free to activate its regulon.

Mutant survival in mice. Deletion mutants were made of the loci associated with the identified promoter regions, and deletions were replaced by an antibiotic resistance marker. If the promoters appeared to be linked to an operon, the first gene of that operon was mutated. In the case of clone 723, two mutants were made: CFT073 *irp2* and CFT073 *fyuA*. The former gene encodes a yersiniabactin synthesis enzyme, whereas the latter encodes an outer membrane yersiniabactin uptake receptor. Both are transcribed from the same promoter in a long operon in the *Yersinia enterocolitica* high-pathogenicity island (HPI) (8). In the case of clone 301, the *degS* locus could not be deleted by the λ Red method, but a single crossover disruption mutation could be made (see above).

The mutants were inoculated into the mouse peritoneal cavity in competition with the wild type. Bacteria were recovered by culture of the lavage fluid and spleen after 6 h, and the relative numbers of mutant and wild type were compared. None of the mutants showed a statistically significant competitive disadvantage except CFT073 *degS* (WAM2853, Table 2). CFT073 *degS* recovery from the spleen and lavage fluid could be restored to that of the wild type by plasmid complementation of *degS* (Fig. 1A).

The CFT073 *degS* mutant was also tested in a mouse model of ascending urinary tract infection. Here, relative numbers of mutant and wild type organisms were compared in bladder and

TABLE 2. Survival of mutants compared to wild-type after mixed peritoneal infection of mice[a]

CFT073 mutation	Mutant vs wild-type spleens			Mutant vs wild-type lavage		
	Ratio	n	P	Ratio	n	P
degS	0.05	10	0.04	0.01	10	0.004
sucA	0.6	6	0.70	0.4	6	0.40
yqjH	1.1	6	0.99	1.0	6	0.93
ybdH	1.0	6	0.59	0.9	6	0.94
ydiJ	1.2	6	0.48	1.2	6	0.70
irp2	1.3	6	0.31	1.4	6	0.18
fyuA	1.2	5	0.55	1.5	5	0.55
gltB	0.7	6	0.70	0.8	6	0.40
iha	1.3	6	0.81	1.1	6	0.81
metA	1.0	6	0.94	1.5	6	0.70

[a] Ratio, recovered mutant/number of recovered wild-type CFU; *n*, number of mice; *P*, comparison as determined by Mann-Whitney U test.
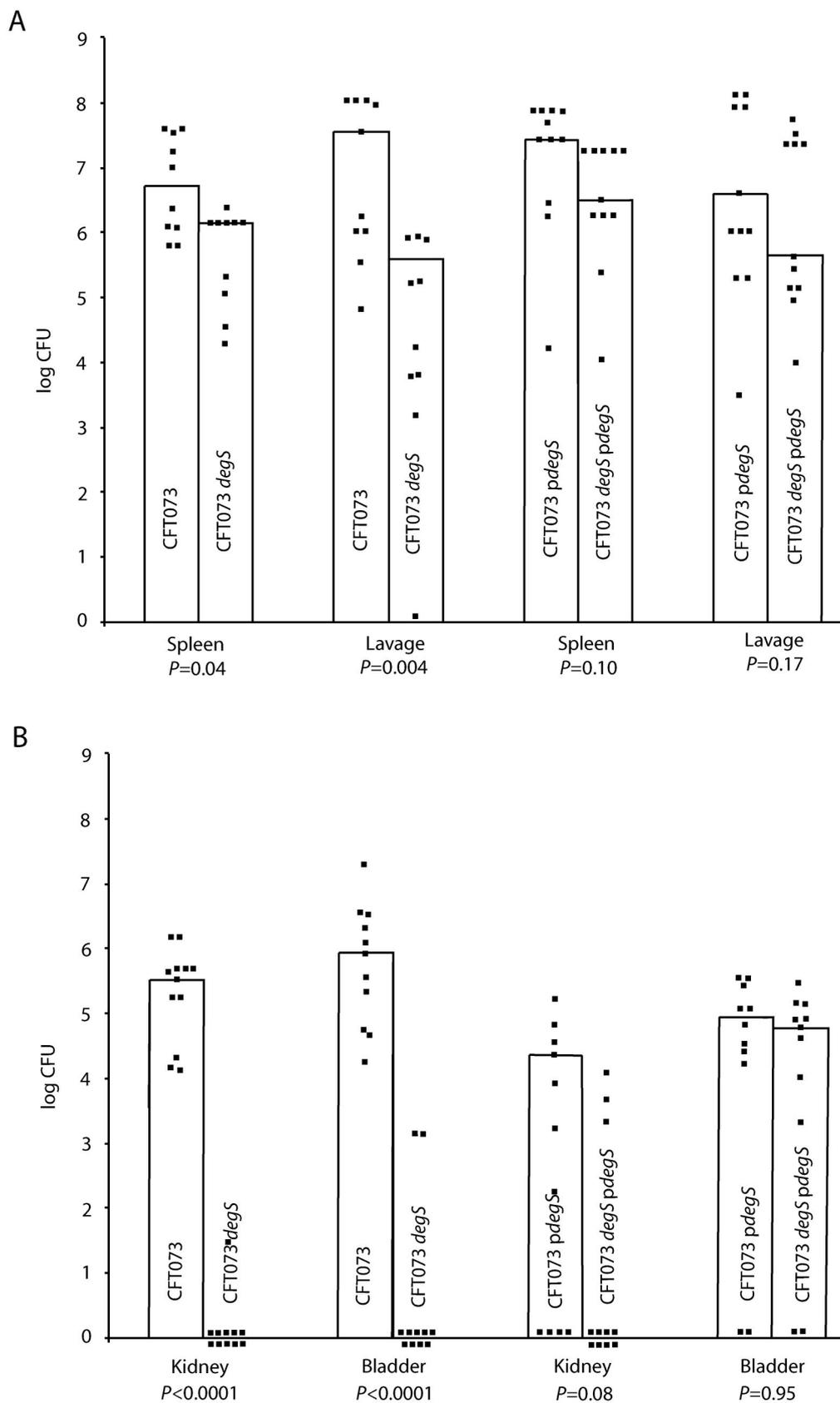
FIG. 1. Competitive infections in mice. Squares denote the CFU counts in individual mice. The bar height is the median number of CFU per gram (spleen, bladder, and kidney) or per milliliter (lavage fluid culture). (A) Wild-type versus CFT073 *degS* recovery from spleen and lavage fluid after a 1:1 mixed inoculation of the peritoneum. Recovery of wild type and mutant with plasmid-borne *degS* is also shown. (B) Wild-type versus CFTO73 (*degS*) recovery from bladder and kidney after mixed inoculation of bladder. Complemented strain recovery is also shown. *P* values were determined by using the Mann-Whitney U test.
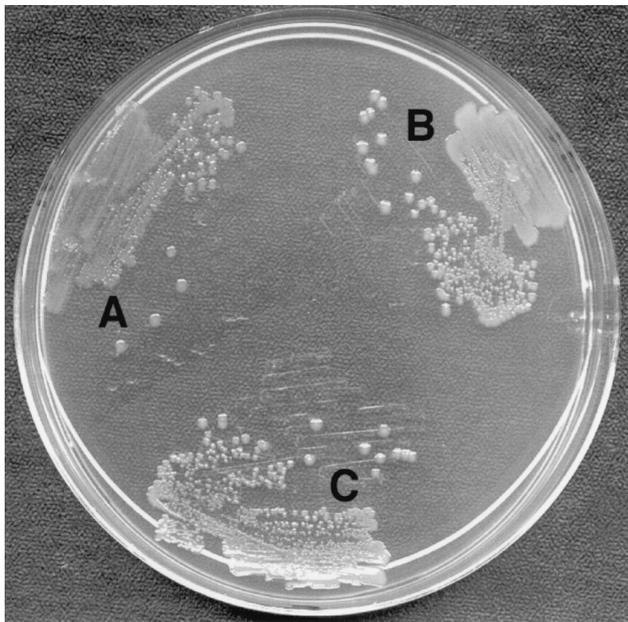
FIG. 2. Colony appearance on LB agar. (A) CFT073 *degS*; (B) CFT073 wild type; (C) CFT073 *degS*/p*degS*.

kidney cultures made 2 days after transurethral inoculation. Again, the mutant showed diminished in vivo survival compared to wild type in both culture sites. Plasmid complementation (WAM2854) brought up CFT073 *degS* numbers in bladder cultures to match those of wild type. In the kidney, too, complementation with *degS* statistically improved mutant survival, but in many mice the complemented CFT073 *degS* strain could not be recovered from the kidneys (Fig. 1B).

CFT073 *degS* in vitro phenotypes. The *degS* mutant has a colony size and morphology similar to that of the wild type on LB agar and MacConkey medium. However, CFT073 *degS* colonies are more translucent than wild-type colonies (Fig. 2). Plasmid complementation restores colony opacity, so that the complemented *degS* mutant resembles the wild type (Fig. 2). The mutant and wild type are motile. Both agglutinate red blood cells and are equally hemolytic. No difference between the *degS* mutant and the wild type was detectable when LPS was compared on a silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel (data not shown). No difference was seen in serum sensitivity after exposure to 50% nonimmune human serum. The *degS* mutant grows as well in LB broth as does the wild type in the presence of polymyxin B (1.5 U/ml) or hydrogen peroxide (750 μM) (data not shown).

The growth kinetics of CFT073 *degS* do not, however, match those of the wild type in conditions of heat or ethanol stress. At 37°C in LB broth the growth curves are similar, but at 43°C the *degS* mutant shows a longer lag phase, a prolonged exponential phase, and a stationary-phase plateau lower than those of the wild type (Fig. 3B). Plasmid complementation of the *degS* mutant restores its growth curve to match that of the wild type. The addition of ethanol to LB broth (3% [vol/vol]) moderately impairs wild type but markedly impedes the growth of the *degS* mutant at 37°C (Fig. 3B). Again, plasmid complementation of

the CFT073 *degS* mutant restores its growth kinetics to match those of wild type in 3% ethanol.

DISCUSSION

The present study sought to examine the response at the genetic level of a pathogenic bacterium to its host environment. DFI was used, with sorting of clones directly from mouse peritoneal lavage fluid. Of eight pools of clones, 11 promoters in the extraintestinal *E. coli* strain CFT073 were found to be active in mouse peritonitis but relatively quiescent in LB medium.

This undoubtedly is not a complete list of in vivo-induced promoters. A major hindrance to the DFI technique in our system is the considerable amount of debris present in the mixture of bacteria and inflammatory peritoneal lavage fluid put through the cell sorter. Autofluorescent particles within the bacterial size range tested the discriminatory abilities of the FACS instrument. Of 10,000 "events" sorted as highly fluorescent, ca. 1,000 showed up in culture as viable bacteria. Even with successive high- and low-fluorescence sorting, most of the isolated clones did not demonstrate differential induction when tested individually in mice and broth. Thus, the combination of small bacterial size and background fluorescence pushes the limits of FACS capabilities and leads to errors and decreased sensitivity in sorting.

The same constraints limit the feasibility of using animal tissue to do the obverse of what was done here, that is, use of FACS to identify promoters that are active in LB culture but downregulated in mouse peritoneum. This is because the enrichment series to isolate such clones would require sorting nonfluorescent bacteria from the peritoneal fluid. The efficiency of FACS at this end of the fluorescence spectrum is particularly low due to the masking effect of background autofluorescence.

Technical issues such as these are probably the reason that almost all previous studies with DFI have been done with in vitro model systems. Nonetheless, despite its limitations, DFI in the present study provides a glimpse of the genetic response of a bacterium directly interacting with attack by the immune response and other environmental stresses inside the mouse.

Notable among the 11 in vivo-induced promoters is the number associated with iron acquisition genes. These include not only the three siderophores but also *iha* and *yqjH*, based on the homology as noted above. Thus, almost half of the 11 loci identified potentially relate to iron, a testament to the importance of this element, both as a nutrient and potentially as a signal to the pathogen of entry into the host.

As in other in vivo induction analyses, we found a variety of genes with metabolic functions. Why such "housekeeping" genes should be induced in vivo but not in vitro is in many cases not clear, but in some a particular stimulus can be postulated. For example, glutamate synthetase, the product of *gltB*, may be upregulated in response to nitrogen limitation in the mouse peritoneum (22). Homoserine transsuccinylase (the *metA* product) may be upregulated to maintain sufficient levels of this enzyme. In fact, this gene product, which catalyzes the first step of methionine synthesis, has been described as the most temperature-sensitive biosynthetic enzyme and, since it also is inactivated by stressors such as ethanol and cadmium, it has
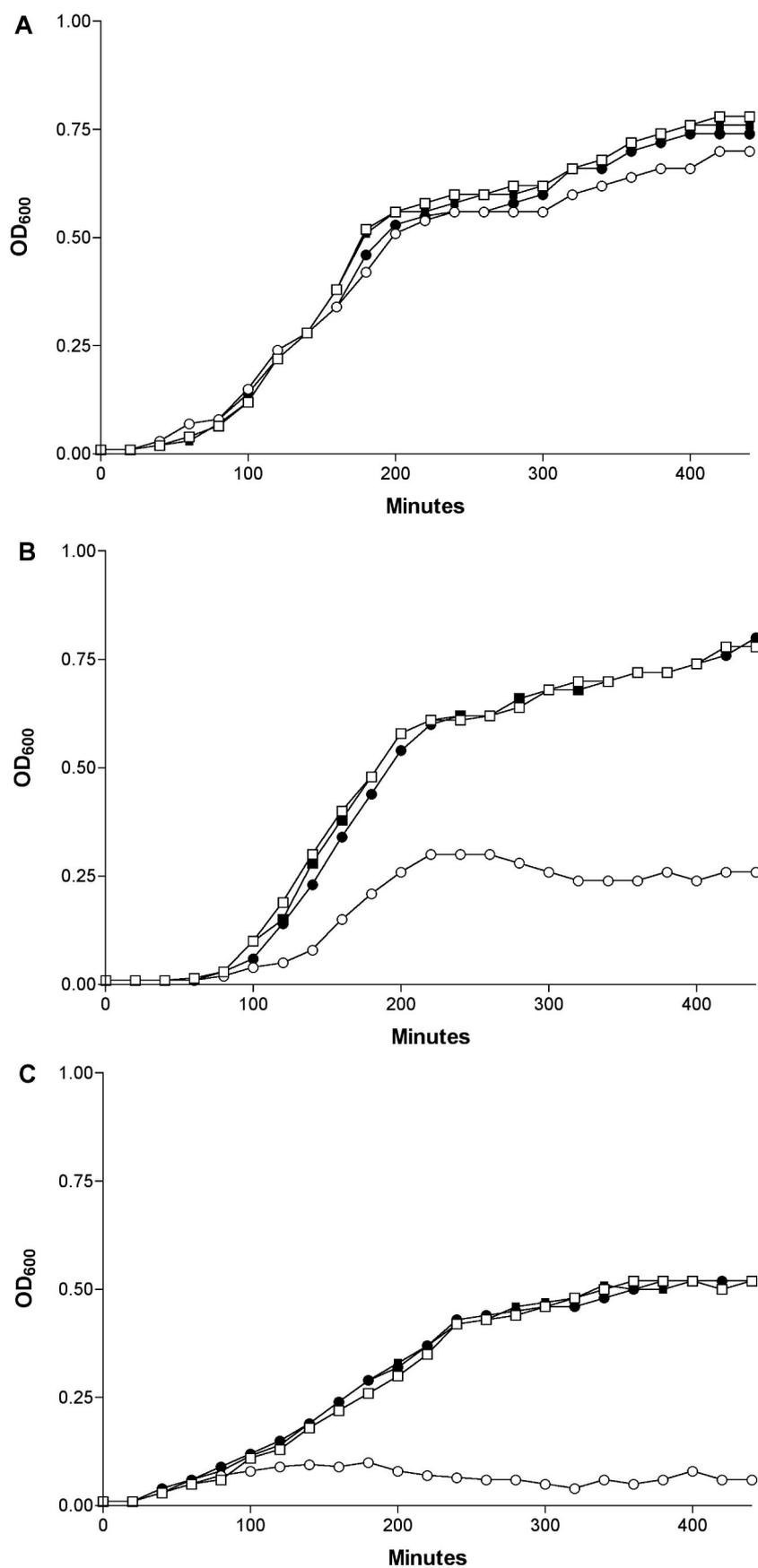
FIG. 3. Growth curves in LB broth (average of three determinations) at 37°C (A), 43°C (B), or 37°C (C) in LB broth plus 3% ethanol. Symbols: □, wild-type CFT073; ■, CFT073/pWAM2851; ○, CFT073 *degS*; ●, CFT073 *degS*/pWAM2851.

been suggested that MetA denaturation itself serves as an environmental sensor (9). The present study marks the third time *suc* has been identified by in vivo screening (12, 13). Why this tricarboxylic acid cycle component is upregulated inside a host is unclear. Bryk et al. showed that in *Mycobacterium tuberculosis* parts of the succinyltransferase complex can shunt reducing equivalents to sustain the specialized antioxidant defenses of the *Mycobacterium* (10). Whether a connection such as this between intermediary metabolism and pathogenesis exists in the *Enterobacteriaceae* has yet to be demonstrated.

Mutants were made of 9 of the 11 loci linked to the identified promoters. Two of the in vivo-induced genes, *entC* and *iucB*, have been studied in vivo previously (48) and were excluded from further analysis. Mutants at the nine other loci were tested in competition with the wild type in the mouse peritonitis model. In these infections, the only mutant showing attenuation was CFT073 *degS*.

The apparently undiminished virulence of the majority of the mutants may be a demonstration that redundant pathways exist for the functions carried out by their deficient gene products. The in vivo redundancy of iron uptake systems in strain CFT073 has been shown previously (48). In some cases, however, the lack of attenuation is surprising compared to studies in other bacterial strains. For example, the HPI present in CFT073 matches closely the island in *Yersinia pestis* and *Yersinia pseudotuberculosis* (41). This HPI contains the synthesis genes and uptake system for yersiniabactin, as well as a transcription regulator, YbtA. In *Y. pestis*, loss of the HPI has been shown to be associated with a marked increase in the 50% lethal dose when injected subcutaneously in mice (8). Similarly, an extraintestinal *E. coli* strain producing yersiniabactin was significantly more lethal than an isogenic, synthesis-defective mutant when injected subcutaneously into mice (44). However, in CFT073 deletion of the first gene of the synthesis operon, *irp2*, or deletion of the yersiniabactin uptake receptor gene, *fyuA*, had no effect on mutant survival compared to wild type in the first 6 h of mouse peritoneal infection (Table 2). This suggests either that CFT073 is different from other extraintestinal *E. coli* strains or that animal virulence assays have different sensitivities.

The only mutant significantly outcompeted by the wild type in peritoneal infection is CFT073 *degS*. The *degS* mutant was further tested in competition with the wild type in the mouse urinary tract. Here, too, it is attenuated, as measured by its recovery compared to that of wild type in bladder and kidney cultures. Complementation of the mutant with full-length *degS* on a plasmid significantly improves its in vivo survival.

DegS plays a major role in the central event of σ^E regulation, the degradation of RseA. Liberation of σ^E from RseA inner membrane repression frees it to activate its regulon, which includes *rpoE*, the gene for σ^E. In *E. coli* K-12 strains, *rpoE* is essential, and *degS*, too, is essential (3, 18). The failure of linear recombination to delete the *degS* locus in CFT073 may reflect its essential nature in this pathogenic *E. coli* strain as well. However, functional disruption was accomplished by recombination into *degS* of a plasmid introduced by conjugation. This suggests that *degS* may not be essential in CFT073 or that this gene disruption method is more permissive, allowing the emergence of mutants with secondary, suppressor mutations.

The CFT073 *degS* mutant studied here, therefore, may well contain a second, uncharacterized mutation. The nature of this mutation is that it permits the mutant to grow in rich medium at 37°C. The mutant also is tolerant of oxidative stress, unlike σ^E mutants in other bacterial species (47). The mutant does not, however, tolerate heat or ethanol stress as well as the wild type, nor does it appear to tolerate the in vivo stress of peritoneal or urinary tract infection. All of the these deficits can be restored by *degS* complementation, so the secondary mutation, if there is one, only partially compensates for the loss of DegS function.

The regulon of σ^E in a K-12 *E. coli* strain in one study was found to contain 25 promoters (16). Almost half of these were linked to genes of unknown function. The remainder were linked to transcription factors (*rpoE*, *rpoH*, and *rpoD*), periplasmic folding enzymes, and LPS biogenesis enzymes. Some of these genes (e.g., *rpoH* and *rpoD*) are transcribed from multiple promoters. Two members of the regulon, *yqjA* and *yaeL*, have been found to be essential. The function of *yqjA* remains unknown, but the product of *yaeL* was reported last year to be, like DegS, an RseA degradase (2, 31). Thus, degradation of the σ^E repressor, RseA, appears to be a two-step process (2). Interestingly, *yaeL* was recently found among genes in a cDNA-based screen of avian *E. coli* genes upregulated during infection of chickens (21).

The degradation of RseA by DegS (and YaeL) is necessary for the release of σ^E and activation of the σ^E regulon. The genes needed for virulence would therefore appear to be one or more members of that regulon. Of the genes in *E. coli* K-12 described thus far as σ^E dependent, none are known virulence genes per se (16). However, nine remain unknown, and the σ^E regulon could be larger or different in CFT073, so further characterization is needed of the function and extent of genes regulated by σ^E in this strain.

σ^E has previously been implicated in virulence, but not in *E. coli*. In *S. enterica* serovar Typhimurium and nontypeable *Haemophilus influenzae*, σ^E has been found to be necessary for survival inside macrophages (14, 29). In *V. cholerae* σ^E is needed for survival inside the intestine (33). In *Pseudomonas aeruginosa* AlgU (σ^E) is needed to counter hyperosmolar, oxidative, and heat stresses (38, 54). AlgU also activates the expression of genes needed for alginate production, a secreted polysaccharide required for bacterial persistence in human lungs (38). Alginate therefore serves as an example of a well-established virulence factor linked to σ^E.

Envelope stress, like iron depletion, may serve as a signal to the pathogen that it has entered its host or changed locales within the host. Transduction of this signal could trigger specialized pathways involved in virulence. Hung et al. found that CpxR activates transcription of the *pap* gene cluster, the synthesis and assembly genes of the P pilus. These investigators also report that it may increase the transcription of other virulence factors, since they see putative CpxR binding sites upstream of genes for hemolysin, cytotoxic necrotizing factor, and type 1 pili (30). This regulator, which with CpxA transduces signals of envelope stress, is thus linked to genes on pathogenicity islands. Whether the transmission of similar information by DegS and σ^E activates virulence mechanisms in *E. coli* will be a subject for further study in this laboratory.

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Editor: A. D. O'Brien