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Identification of a Mutation in the *pst-phoU* Operon That Reduces Pathogenicity of an *Escherichia coli* Strain Causing Septicemia in Pigs

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We used transposon (Tn*phoA*) mutagenesis to study the role of virulence factors of pathogenic *Escherichia coli* strains associated with septicemia in calves and piglets. We have produced an avirulent and serum-sensitive mutant of wild-type pathogenic strain 5131 O115:K″V165″:F165 and have localized and identified the Tn*phoA* insertion in the *pstC* gene of the *pst-phoU* operon. This operon encodes the PstSCAB transporter and PhoU protein that negatively regulate the phosphate (Pho) regulon. This mutation is pleiotropic and could have an effect on pathogenicity and on the production of the surface polysaccharides of strain 5131. The mutant demonstrated restored repressibility of alkaline phosphatase and regained the capacity to resist serum and to survive systemically for at least 5 days in experimentally inoculated pigs when complemented with plasmid pAN92, bearing the *pst-phoU* operon.

Escherichia coli has been associated with intestinal and extraintestinal diseases in humans and animals. Extraintestinal *E. coli* is a frequent cause of septicemia, urinary tract infection, and meningitis (18). The virulence mechanisms of such strains appear to be complex and are only partially understood. Several virulence factors, including lipopolysaccharide (LPS), capsule, fimbriae, cytotoxins, and aerobactin, have been associated with the ability of *E. coli* to cause extraintestinal infections.

Transposon mutagenesis has been used to identify virulence factors in a variety of pathogens. A collection of Tn*phoA* mutants was generated in the wild-type pathogenic strain 5131 of *E. coli* O115:K″V165″:F165 (7). This strain, which was isolated from a diarrheic piglet at the Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec, Canada (3), has several attributes of extraintestinal *E. coli*. It is serum resistant, aerobactin positive, and ColV positive and has DNA sequences similar to both the *pap* and *sfa* operon sequences, which encode fimbriae $F165_1$ (Prs-like) (8, 12) and $F165_2$ (F1C-like) (9), respectively. In experimental inoculation studies, this strain induced septicemia in piglets. The collection of 5131 Tn*phoA* mutants was screened in order to evaluate the role of virulence determinants in the pathogenesis of *E. coli* septicemia.

One of these mutants, mutant 2, was serum sensitive, alkaline phosphatase positive, and avirulent in 1-day-old chickens (7). In pigs, mutant 2 did not produce any clinical signs within 8 days after inoculation and was rapidly cleared from the bloodstream, whereas strain 5131 persisted in the blood, colonized extraintestinal organs, and induced septicemia (16). Mutant 2 demonstrated a lower total carbohydrate content in the supernatant of its bacterial cells heated at 60°C than did strain 5131. This supernatant extract contains capsular antigen, LPS, and proteins (17). Mutant 2 had a smaller amount of surface layer as measured by electron microscopy (16). However, this mutant produced smooth LPS chains similar to those of the parent strain 5131 (16). In vitro, this mutant attached to and was ingested by porcine polymorphonuclear leukocytes to the same extent as the parental strain 5131, and both strains equally resisted killing by these cells (unpublished data). In this report, we identify the Tn*phoA* insertion which reduces the pathogenicity of strain 5131 to the *pstC* gene of the *pst-phoU* operon.

Cloning, mapping, and sequencing of the mutated gene. The Tn*phoA* element is present as a single insertion in the chromosome of mutant 2 as determined by Southern blotting analysis (7). Thus, cloning of the flanking sequences at the 5′ end of the gene fusion could be accomplished in one step by using the kanamycin resistance gene in the Tn*phoA* element as a selectable marker. Cloning of the mutated gene was performed by cleaving 3 μg of total cellular DNA from the mutant 2 strain with *Eco*RV and ligating the DNA to 1 μg of plasmid pBR322 (1) that was digested with the same enzyme and prepared as described elsewhere (13). The ligation mixture was transformed into strain HB101 (2). Transformants carrying the desired recombinant plasmid were selected on Luria-Bertani agar plates (5 g of yeast extract, 10 g of tryptone, 10 g of NaCl per liter; Difco Laboratories, Detroit, Mich.) supplemented with ampicillin (40 μg/ml) and kanamycin (40 μg/ml). By this procedure, we obtained a plasmid, designated pFD2X, which carried a 6-kb insert and was used for further analysis and characterization.

The Tn*phoA* insertion was physically mapped by screening the Kohara bank of the *E. coli* chromosome, which was contained in phage lambda clones (11), with a probe derived from plasmid pFD2X. Phage lysates were transfered to nitrocellulose filters (Xymotech Biosystems Inc., Mont-Royal, Québec, Canada) by the plaque lift procedure for DNA (13). For the probe, 1.0 μg of a 1.6-kb *Eco*RV-*Cla*I fragment from plasmid pFD2X was isolated and labeled by using the DIG DNA labeling and detection kit (Boehringer Mannheim Canada Ltd., Laval, Québec, Canada) according to the manufacturer's instructions. The probe was found to hybridize to phage 561 (not shown). Thus, the junction between IS*50*L from Tn*phoA* and the chromosomal DNA from the mutant 2 was located at approximately 83 min on the Kohara (11) restriction map of the *E. coli* genome.

To precisely identify the mutated gene, the sequences of the

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TABLE 1. PhoA activities and carbohydrate contents of *E. coli* O115 strain 5131 and its mutants[a]

Strain	PhoA activity (U/min)		Carbohydrate content (μg/ml)	
	Medium		Medium	
	Low P_i[b]	High P_i[c]	Low P_i[b]	High P_i[c]
5131	119.0 ± 7.0	5.0 ± 1.0	84.0 ± 3.0	84.7 ± 6.4
2	129.3 ± 3.1	142.0 ± 6.0[d]	78.0 ± 1.0	54.3 ± 3.3[d]
2(pAN92)[e]	111.0 ± 4.5	5.7 ± 1.2	79.3 ± 1.5	70.2 ± 6.0

[a] Results are the means ± standard deviations for at least three distinct tests.
[b] Trace amount of P_i (4).
[c] 10 mM P_i (5).
[d] Significantly different ($P < 0.05$) from the parent strain 5131 as determined by the *t* test.
[e] Mutant 2 transformed with plasmid pAN92.

double-stranded plasmid DNA at the junction between IS*50*L and the site of the Tn*phoA* insertion in the plasmid pFD2X was determined by the dideoxy-chain reaction termination method described by Sanger et al. (20), as modified for use with a T7 sequencing kit (Pharmacia LKB Biotechnology Inc., Baie d'Urfé, Québec, Canada) according to the instructions of the manufacturer. An oligonucleotide (5′AATATCGCCCTGA GC3′) corresponding to nucleotides 72 to 86 of Tn*phoA*, synthesized on a Gene Assembler (Pharmacia LKB Biotechnology, Inc.), was used as the primer. Nucleotide sequence analysis with the GenBank data bank demonstrated that the Tn*phoA* insertion was located within the *pstC* gene of the *pst-phoU* operon that maps at about 83.5 min on the *E. coli* chromosome. These results agree with the physical mapping data of the Kohara bank. The site of insertion is at 300 bp from the beginning of the *pstC* gene. The sequence analysis demonstrated that Tn*phoA* was inserted in the wrong orientation, opposite to the direction of transcription, indicating that no fusion protein would be produced. Thus, the alkaline phosphatase activity detected in mutant 2 would result from the constitutive induction of the endogenous *phoA* gene of a deregulated phosphate (Pho) regulon.

One of the P_i transport systems of *E. coli* is the Pst system encoded by the *pst-phoU* operon. This operon of five genes codes for the PstSCAB transporter and a protein called PhoU, whose function is still unclear (24). The presence of an intact *Pst-phoU* operon is essential for phosphate transport by means of the Pst system. The Pst system is typical of a class of periplasmic permeases with two cytoplasmic membrane proteins (PstA and PstC), one periplasmic binding protein (PstS), and one cytoplasmic protein (PstB) (28). The Pst system has two independent functions in *E. coli*: (i) the transport of P_i and (ii) the negative regulation of the Pho regulon (19). More than 30 genes belonging to the Pho regulon are regulated by separate transcriptional units. These genes are controlled by a two-component regulatory system consisting of a positive regulator, PhoB, and a sensor, PhoR (23). Mutation in the *pst-phoU* operon, as in mutant 2, leads to the induction of the Pho regulon, whereby products of the Pho regulon, such as alkaline phosphatase, are synthesized constitutively at a high level (26, 27). The products of the Pho regulon are usually expressed only under conditions of phosphate starvation.

The mutant 2 strain was transformed with plasmid pAN92 (10), which carries an entire *pst-phoU* operon. This transformed mutant was called mutant 2(pAN92). Bacterial colonies of mutant 2(pAN92) on culture in a medium containing the alkaline phosphatase substrate XP (5-bromo-4-chloro-3-indolyl phosphate) (Sigma Chemical Company, St. Louis, Mo.), were white. Low alkaline phosphatase activity was detected (Table 1), and no PhoA band was seen by Western blotting (immunoblotting) (not shown). Thus, acquisition of pAN92 restores *phoA* repression.

In *E. coli*, the Pho regulon products are induced when bacteria are grown in a low-P_i medium and repressed in a high-P_i medium (27). The PhoA activities of the wild-type strain and mutant 2(pAN92) were very low in a high-P_i medium and increased in a low-P_i medium (Table 1), thus being dependent on the phosphate concentration of the medium. As a control for the influence of copy effect, wild-type strain 5131 was also transformed with plasmid pAN92. The transformed strain 5131(pAN92) had the same level of PhoA activity as the wild-type strain grown under the same conditions (data not shown). The PhoA activity of mutant 2 was always high, thus being independent of the growth medium. The total carbohydrate content of heated cell extracts, which were produced as described previously (16, 17), of mutant 2 grown on high-P_i medium was significantly less than that of parental extract and also less than that of mutant 2(pAN92) (Table 1). The total carbohydrate contents of bacteria grown on low-P_i medium were not significantly different for the wild-type, transformed, or mutated strain (Table 1). This suggests that the carbohydrate content could be independent of the induction of the Pho regulon. Examination for survival in 90% fresh rabbit serum, as described previously (16, 25), demonstrated that mutant 2(pAN92) was resistant to the bactericidal effect of serum at a level similar to that of the parental strain 5131, in contrast to the serum-sensitive mutant 2 (Fig. 1). The wild-type strain 5131, which was grown in low-P_i medium, was also resistant to the bactericidal effect of serum (data not shown). This also suggests that the survival in serum of strain 5131 was independent of the induction of the Pho regulon.

The pathogenicity of mutants in pigs was tested essentially as described previously (16). Briefly, colostrum-deprived newborn pigs were inoculated intragastrically with 1 ml of a culture containing approximately 10^{10} CFU ml^{-1} of *E. coli* and were kept under germ-free conditions. As found previously, wild-type strain 5131 colonized the small and large intestines, was found in the blood, colonized various extraintestinal organs, and resulted in the development of clinical signs of septicemia and death within 48 h (Table 2) (16). Mutant 2 colonized the intestines but was found in small numbers in extraintestinal organs at 1 day after inoculation and was nonpathogenic even at 5 days after inoculation. On the other hand, mutant 2(pAN92) colonized the intestines and was found in the blood

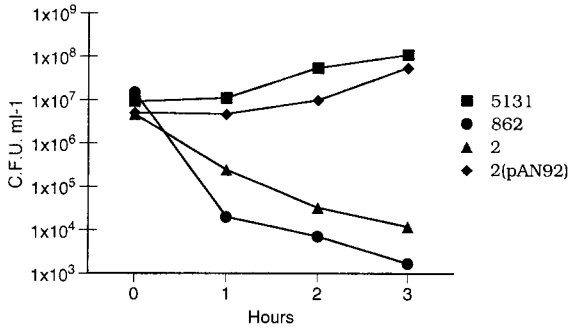

FIG. 1. Serum resistance. Survival and growth of *E. coli* O115 strain 5131 and its mutants in 90% fresh rabbit serum. Wild-type strain 862 (O115:K^-) was used as a negative control. The numbers of viable cells were counted by plating the cells on Luria-Bertani medium at different times after inoculation.

TABLE 2. Bacterial colonization of organs from pigs inoculated with *E. coli* O115 strain 5131 and its mutants

Strain	Time (days postinoculation)	No. of pigs examined	Presence of clinical signs[a]	Colony count in[b,c]:			
				Intestine	Lung	Liver	Blood
5131	1	1	Yes	++++	++	++++	+++
	2	1	Yes	++++	+	++++	++
2	1	1	No	+++	±	±	±
	2	1	No	+++	−	−	−
	5	2	No	+++	−	−	−
2(pAN92)	1	1	No	++++	++	++	++
	2	1	No	++++	+	+	++
	5	2	No	+++	+	+	+

[a] Clinical signs of septicemia included anorexia, lameness, reluctance to move, or lack of motor coordination.
[b] ±, <5 colonies in the first quadrant; +, ≥5 colonies in the first quadrant; ++, ≥5 colonies in the second quadrant; +++, ≥5 colonies in the third quadrant; ++++, ≥5 colonies in the fourth quadrant.
[c] With mutant 2(pAN92), colonies were selected on Luria-Bertani plates containing 20 μg of chloramphenicol ml^{-1}.

and extraintestinal organs of inoculated pigs up to 5 days after inoculation, although it did not result in septicemia (Table 2). Thus, the complemented mutant 2(pAN92) can survive and multiply in the bloodstream in a manner similar to that of the wild-type strain but is not pathogenic for pigs.

Thus, we have demonstrated that a Tn*phoA* insertion, which renders a septicemia-inducing *E. coli* strain serum sensitive and nonpathogenic, is within the *pstC* gene of the *pst-phoU* operon, localized at 83.5 min on the *E. coli* map. This operon codes for the Pst system, one of the two systems responsible for phosphate transport in *E. coli*. In the pathogenic strain 5131, this mutation in *pst-phoU* is pleiotropic, since it not only affects P_i transport but also affects resistance to the bactericidal effect of serum and the production of surface polysaccharides. Most importantly, this mutation renders the mutant strain nonpathogenic in pigs. This mutation induces the Pho operon, which becomes expressed constitutively in this mutated strain. The induction of the Pho regulon by phosphate starvation does not seem to be involved in serum sensitivity and carbohydrate expression in the wild-type strain 5131, as shown by the results of growth of strains in a low-P_i medium. This mutation, which disrupted phosphate transport via the Pst system, may have a direct effect on the virulence by an unknown function of the phosphate transporter, or it may affect virulence indirectly by regulation of another gene(s) unrelated to the Pho regulon. Thus, we suggest that the virulence of strain 5131 is modulated by the phosphate transport system encoded by the *pst-phoU* operon and is probably not regulated by or under the regulation of a gene belonging to the Pho regulon. We do not know if the observed effects are caused directly by the mutated *pstC* gene or by a polar effect on downstream genes such as *phoU*. The latter acts as a repressor of the Pho regulon (15, 24) and may be involved in intracellular metabolism (22). The role of PhoU in phosphate transport is not well defined (15, 22). A similar mutation in the *pst-phoU* operon of an enteropathogenic porcine *E. coli* strain, which decreased the attaching-effacing ability of this strain, has been isolated in our laboratory (6). In contrast, a *pst-phoU* mutation was found to enhance the ability of a laboratory strain containing a large invasion plasmid derived from an enteroinvasive *E. coli* clinical isolate to invade cultured HEp-2 cells (21). However, this effect on invasion was less pronounced when the mutation was in a clinical isolate rather than in a laboratory strain.

Virulence factors can be modulated by various environmental stimuli (14). This can result in the coordinate expression of sets of genes, including virulence genes, whose products bear little or no apparent functional relationship. Thus, it is possible that the phosphate transport system coded by *pst-phoU* operon is involved either directly or indirectly in the pathogenicity of the septicemia-inducing strain 5131. Studies of mutations in a regulatory network, such as phosphate regulation, may give insight into new approaches to the development of nonpathogenic *E. coli* strains.

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REFERENCES

1. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**95–113.
2. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–472.
3. **Fairbrother, J. M., A. Broes, M. Jacques, and S. Larivière.** 1989. Pathogenicity of *Escherichia coli* O115:K″V165″ strains isolated from pigs with diarrhea. Am. J. Vet. Res. **50:**1029–1036.
4. **Gerdes, R. G., and H. Rosenberg.** 1974. The regulationship between the phosphate-binding protein and a regulator gene product from *Escherichia coli*. Biochem. Biophys. Acta **351:**77–86.
5. **Gibson, F., G. B. Cox, J. A. Downie, and J. Radik.** 1977. A mutation affecting a second component of the F_0 portion of the magnesium ion-stimulated adenosine triphosphatase of *Escherichia coli* K12. Biochem. J. **164:**193–198.
6. **Harel, J., J. M. Fairbrother, and H. An.** 1995. Personal communication.
7. **Harel, J., C. Forget, M. Ngeleka, M. Jacques, and J. M. Fairbrother.** 1992. Isolation and characterization of adhesin-defective Tn*phoA* mutants of septicaemic porcine *Escherichia coli* of serotype O115:K^-:F165. J. Gen. Microbiol. **138:**2337–2345.
8. **Harel, J., C. Forget, J. Saint-Amand, F. Daigle, D. Dubreuil, M. Jacques, and J. Fairbrother.** 1992. Molecular cloning of a determinant coding for fimbrial antigen F1651, a Prs-like fimbrial antigen from porcine septicaemic *Escherichia coli*. J. Gen. Microbiol. **138:**1495–1502.
9. **Harel, J., M. Jacques, J. M. Fairbrother, M. Bossé, and C. Forget.** 1995. Cloning of determinants encoding F1652 fimbriae from porcine septicaemic *Escherichia coli* confirms their identity as F1C fimbriae. Microbiology **141:** 221–228.
10. **Jans, D. A., A. L. Fimmel, L. Langman, L. B. James, J. A. Downie, A. E. Senior, G. R. Ash, F. Gibson, and G. B. Cox.** 1983. Mutations in the *uncE* gene affecting assembly of the c-subunit of the adenosine triphosphatase of *Escherichia coli*. Biochem. J. **211:**717–726.
11. **Kohara, Y., K. Akiyama, and K. Isono.** 1987. The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. Cell **50:**495–508.
12. **Maiti, S. N., L. DesGroseillers, J. M. Fairbrother, and J. Harel.** 1994. Analysis of genes coding for the major and minor fimbrial subunits of the Prs-like fimbriae F1651 of porcine septicemic *Escherichia coli* strain 4787. Microb. Pathol. **16:**15–25.
13. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a

laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Mekalanos, J. J.** 1992. Environmental signals controlling expression of virulence determinants in bacteria. J. Bacteriol. **174:**1–7.
15. **Muda, M., N. N. Rao, and A. Torriani.** 1992. Role of PhoU in phosphate transport and alkaline phosphatase regulation. J. Bacteriol. **174:**8057–8064.
16. **Ngeleka, M., J. Harel, M. Jacques, and J. M. Fairbrother.** 1992. Characterization of a polysaccharide capsular antigen of septicemic *Escherichia coli* O115:K″V165″:F165 and evaluation of its role in pathogenicity. Infect. Immun. **60:**5048–5056.
17. **Ørskov, F., and I. Ørskov.** 1984. Serotyping of *Escherichia coli*. Methods Microbiol. **14:**43–112.
18. **Ørskov, I., and F. Ørskov.** 1985. *Escherichia coli* in extraintestinal infections. J. Hyg. **95:**551–575.
19. **Rao, N. N., and A. Torriani.** 1990. Molecular aspects of phosphate transport in *Escherichia coli*. Mol. Microbiol. **4:**1083–1090.
20. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
21. **Sinai, A. P., and P. M. Bavoil.** 1993. Hyper-invasive mutants define a novel Pho-regulated invasion pathway in *Escherichia coli*. Mol. Microbiol. **10:**1125–1137.
22. **Steed, P. M., and B. L. Wanner.** 1993. Use of the *rep* technique for allele replacement to construct mutants with deletions of the *pstSCAB-phoU* operon: evidence of a new role for the PhoU protein in the phosphate regulon. Infect. Immun. **175:**6797–6809.
23. **Stock, J., A. Ninfa, and A. Stock.** 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. **53:**450–490.
24. **Surin, B. P., H. Rosenberg, and G. B. Cox.** 1985. Phosphate-specific transport system of *Escherichia coli*: nucleotide sequence and gene-polypeptide relationships. J. Bacteriol. **161:**189–198.
25. **Taylor, P. W., and H.-P. Kroll.** 1983. Killing of an encapsulated strain of *Escherichia coli* by human serum. Infect. Immun. **39:**122–131.
26. **Torriani, A.** 1990. From cell membrane to nucleotides: the phosphate regulon in *Escherichia coli*. BioEssays **12:**371–376.
27. **Wanner, B. L.** 1987. Phosphate regulation of gene expression in *Escherichia coli*, p. 1326–1333. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
28. **Webb, D. C., H. Rosenberg, and G. B. Cox.** 1992. Mutational analysis of *Escherichia coli* phosphate-specific transport system, a member of traffic ATPase (or ABC) family of membrane transporters. J. Biol. Chem. **267:**24661–24668.

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