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An Unusual *pagC*::Tn*phoA* Mutation Leads to an Invasion- and Virulence-Defective Phenotype in Salmonellae

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Two phenotypes believed to contribute to the pathogenesis of *Salmonella* infections are macrophage survival and invasion of epithelial cells. It was recently observed that the *Salmonella* macrophage survival factor PagC has significant amino acid similarity to the *Yersinia* invasion factor Ail. This observation raised the possibilities that macrophage survival is in part determined by the pathway of entry and that PagC confers an entry mechanism that does not trigger the microbicidal activities of the macrophage. Thus, we sought to investigate the role of PagC in invasion by examining (i) the invasion phenotype of *pagC* mutants and (ii) the invasion phenotype of *Escherichia coli* carrying *pagC*. A previously identified invasion-defective Tn*phoA* insertion mutant of *Salmonella enteritidis* was found to have Tn*phoA* inserted into the signal sequence-encoding region of *pagC*; the *pagC* allele from this mutant, SM5T, was designated *pagC64*. In contrast, *Salmonella typhimurium* carrying the *pagC1* allele (a Tn*phoA* insertion mutation, downstream of the region encoding the signal sequence) was not defective for invasion. Further analysis of these two *pagC* alleles suggested that the invasion-defective phenotype associated with *pagC64* is not due to the loss of PagC function but rather is due to the synthesis of a hybrid PagC-alkaline phosphatase protein that is aberrantly localized, most likely to the inner membrane, and thus may prevent proper localization or function of a factor(s) required for efficient invasion. The observation that *pagC* did not confer an invasive phenotype to *E. coli* further suggests that PagC is not an invasion factor. A cloned *pagC* gene complemented the macrophage survival defect of *S. typhimurium pagC1* mutants, but the cloned *ail* gene did not. Together these results suggest that the structural similarity between PagC and Ail may not extend to a similarity in function. Interestingly, *S. enteritidis* carrying the *pagC64* allele that results in both an invasion defect and a macrophage survival defect was less virulent for mice infected intragastrically or intraperitoneally than was *S. enteritidis* carrying the *pagC1* allele that results only in a macrophage survival defect.

There are an estimated 400,000 to 4,000,000 cases of salmonellosis in the United States per year with an economic impact of >50 million dollars (7). Unlike many infectious diseases, the incidence of *Salmonella* gastroenteritis in developed countries has been rising steadily over the past 30 years and now accounts for 10 to 15% of all instances of acute gastroenteritis in the United States (7, 38). The pathogenesis of salmonellosis is not well understood, but several aspects have received considerable attention recently. These include the ability of salmonellae to survive in macrophages (5, 11, 12, 18, 31, 32) and to invade eukaryotic cells in tissue culture (9, 14, 15, 17, 22, 23, 41). Both of these properties have been correlated with virulence; strains with mutations that affect either macrophage survival or invasion are less virulent in murine models of infection (12, 15, 17, 31, 32).

One locus identified in *Salmonella typhimurium* that affects survival within macrophages is *pagC* (31). Insertion of Tn*phoA* into *pagC* results in the synthesis of a fusion protein (PagC-alkaline phosphatase [AP]) whose expression is positively regulated by PhoP (31). This mutant is significantly less virulent for mice than the wild-type strain. The *pagC* gene was sequenced and was found to encode an 18-kDa outer membrane protein with significant amino acid identity to Ail, a *Yersinia enterocolitica* invasion protein (33, 36). This observation suggested that PagC may itself play a role in entry into various eukaryotic cells. Thus, the macrophage survival defect of *pagC* mutants may result from the loss of an entry mechanism that either localizes bacteria in a protected compartment or does not activate the microbicidal activities of the macrophage.

PagC has not previously been implicated as a mediator of invasion of epithelial cells. Invasion of tissue culture cells by nontyphoid salmonellae appears to be a multifactorial process requiring several genes (17, 41); the best-characterized locus is one at 57 to 60 min on the *S. typhimurium* chromosome that contains the *invA*, *invB*, *invC*, and *invD* genes (17). We recently isolated Tn*phoA* insertion mutants defective for invasion of tissue culture cells; these mutants were separated into six classes based on their phenotype in each of three different cell lines (HEp-2, CHO, and MDCK) (41). Some mutants were essentially noninvasive for all cell types, whereas others exhibited cell line-specific defects.

Given the similarity of PagC to the *Y. enterocolitica* invasion factor, Ail, we investigated the ability of *pagC* to confer an invasive phenotype to *Escherichia coli* and the possibility that one or more of our invasion-defective mutants have an insertion of Tn*phoA* in *pagC*. PagC did not confer an invasive phenotype to *E. coli*; however, the class II invasion-defective mutants (SM3, SM4, and SM5) did contain insertions in *pagC*. Further analysis indicated that the invasion defect of these mutants was not due to the loss of PagC function.

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TABLE 1. Strains

Species and strain	Parent	Relevant genotype or phenotype	Reference(s)
S. enteritidis			
CDC5str	CDC5	Streptomycin resistance	41
SVM42	CDC5str	*phoP102*::Tn*10*d-cam	This study
SM5T	CDC5str	*pagC64*	41, this study
SVM43	SM5T	*pagC64 phoP102*::Tn*10*d-cam	This study
SVM48	CDC5str	*pagC1*	This study
SVM47	CDC5str	*pagC1*	This study
SVM46	SVM48	*pagC1 phoP102*::Tn*10*d-cam	This study
SVM45	SVM47	*pagC1 phoP102*::Tn*10*d-cam	This study
S. typhimurium ATCC 14028			
CS119	ATCC 14028	*pagC1*	31
CS015	ATCC 14028	*phoP102*::Tn*10*d-cam	31
CS120	ATCC 14028	*pagC64*	This study
SVM39	ATCC 14028	*pagC64*	This study
SVM44	SVM39	*pagC64 phoP102*::Tn*10*d-cam	This study

MATERIALS AND METHODS

Bacterial strains. Bacterial strains were maintained at −70°C in Luria broth (LB) medium containing 25% (vol/vol) glycerol or on LB agar plates (29). *Salmonella* strains are listed in Table 1. *E. coli* HB101 (1) and *E. coli* DH5α (19) were maintained on LB agar or LB agar containing the appropriate antibiotics for plasmid maintenance. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 15 μg/ml; and kanamycin, 50 μg/ml. *Salmonella* phage P22 HT*int* was propagated and used in transductions as previously described (8). AP was assayed as previously described from 12- to 18-h cultures of bacteria grown in LB (28). The cosmids pLAFR3 and pWP061 were mobilized from *E. coli* to *S. typhimurium* by triparental mating with *E. coli* MM294 containing pRK2013 (16).

Nucleic acid purification and probe preparation. Chromosomal DNA was isolated by the method of Mekalanos (27) as modified by Stone et al. (41). Plasmid DNA was isolated by the alkaline lysis method (25) or with Qiagen columns (Qiagen, Chatsworth, Calif.). The PagC probe was prepared as follows. Plasmid pCDRI, carrying the *pagC1* locus from *S. typhimurium* (30), was digested with *Hpa*I, and the fragments were separated by electrophoresis through a 0.7% agarose gel. The 1.6-kb fragment was purified from the agarose gel slice with Geneclean (Bio 101, La Jolla, Calif.). The purified fragment was then labelled with [^{32}P]dCTP by the random primer method (10). This probe contains ~200 bp of the 3′ end of Tn*phoA*, all but the first 100 bp of *pagC*, and ~900 bp downstream of *pagC* (36). A probe containing only *phoP* sequences was prepared as follows. Plasmid p5AE, carrying the *phoPQ* genes from *S. typhimurium* (30), was digested with *Eco*RV, and the fragments were separated by electrophoresis through a 0.7% agarose gel. The 514-bp fragment containing the majority of *phoP* was purified and labelled with [^{32}P]dCTP as described above (10). DNA restriction enzymes, T4 DNA ligase, and Klenow fragment were purchased from New England Biolabs (Beverly, Mass.) and used according to the manufacturer's instructions.

Southern hybridization analysis. Chromosomal DNA was digested to completion with the indicated restriction endonuclease, and the fragments were separated by electrophoresis through a 0.7% agarose gel. The separated DNA fragments were transferred to nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.) by the method of Southern (40). Hybridizations were performed at medium stringency, and the filters were washed as described elsewhere (35).

DNA sequencing. Chromosomal sequences upstream of the Tn*phoA* in *Salmonella enteritidis* SM5T were cloned as follows. Chromosomal DNA from SM5T was digested with *Sal*I (there is a unique *Sal*I site in Tn*phoA* downstream of the Kan^r gene) and ligated to *Sal*I-digested pUC19 (Amp^r). The ligation reaction was then used to transform *E. coli* DH5α. Transformants carrying the desired recombinant plasmid were selected on LB plates supplemented with ampicillin and kanamycin. All such transformants carried an ~18-kb *Sal*I fragment; one such plasmid, designated pVM119, was used for further analysis. An oligonucleotide from Tn*phoA* was used as a primer to sequence chromosomal DNA 5′ of the fusion junction carried on pVM119. The dideoxy method of Sanger et al. (39), as modified for use with Sequenase (U.S. Biochemicals), was used for double-stranded plasmid DNA sequencing.

Tissue culture cells, tissue culture invasion assay, and macrophage survival assay. Human laryngeal epithelial (HEp-2), Chinese hamster ovary (CHO), and Madin-Darby canine kidney (MDCK) cells were maintained and prepared for the invasion assay as previously described (13). Quantitative tissue culture invasion assays, using gentamicin for selection of intracellular bacteria, were performed as described elsewhere (41).

Intracellular survival of bacteria by using either bone marrow-derived BALB/c macrophages or the J774.1 cultured macrophage line was measured by the method of Lissner et al. (24) as modified by Buchmeier and Heffron (5). The macrophage survival index was the mean bacterial count at 24 h divided by that obtained 1 h after gentamicin exposure.

Western blot (immunoblot) analysis. Membrane and periplasmic fractions from 1.5 ml of cultures grown to saturation in LB with aeration were obtained as described previously (26). Samples were stored at −20°C until use. Enzyme assays were performed on fractions from CDC5str carrying pRS454 (*lacZ* Amp^r) to test the purity of the various fractions; β-galactosidase was used as a marker for cytoplasm, β-lactamase was used as a marker for periplasm, and NADH oxidase was used as a marker for membrane. The membrane fraction contained only 1.5% of the β-galactosidase activity and no β-lactamase activity. The periplasm had 2.3% of the β-galactosidase activity and 2.9% of the NADH oxidase activity. Thus, on the basis of these enzyme values, there was minimal cross contamination between the fractions.

For Western analysis the samples were mixed with sodium dodecyl sulfate (SDS)-sample buffer containing dithiothreitol and boiled for 5 min, and the proteins were separated by electrophoresis through a 12.5% polyacrylamide gel (21). The proteins were then transferred to nitrocellulose and incubated with antibody to AP as described elsewhere (6). After incubation with the primary antibody, immunoblots were incubated with a second antibody conjugated to AP. Antibody binding was visualized by incubating the filter with 5-bromo-4-chloro-3-indolylphosphate and *p*-nitroblue tetrazolium chloride (4).

LD_{50} determination. Bacteria grown overnight with aeration at 37°C in LB were washed, suspended, and diluted in phosphate-buffered saline (PBS). Six- to seven-week-old female BALB/c mice were obtained from Charles River

TABLE 2. Invasion by *E. coli* carrying derivatives of *pagC*

Plasmid	% Invasion of[a]:	
	HEp-2 cells	CHO cells
pLAFR3	0.019 ± 0.001	0.094 ± 0.025
pWPO61	0.024 ± 0.002	0.072 ± 0.008
pUC19	0.019 ± 0.004	0.149 ± 0.008
pWPL4	0.016 ± 0.002	0.128 ± 0.015
pWPL3	0.014 ± 0.002	0.232 ± 0.009
pWPL10	0.015 ± 0.001	0.171 ± 0.008
pWPL8	0.016 ± 0.004	0.279 ± 0.073

[a] The invasion phenotype of *E. coli* HB101 carrying the indicated plasmids was determined for HEp-2, CHO, and MDCK cells as described previously (41). For MDCK cells, all values were <0.001. The results are expressed as follows: % invasion = 100 × (no. of intracellular bacteria/no. of bacteria added).

Breeding Laboratories. For 50% lethal dose (LD_{50}) determinations, 0.2 ml of the bacterial suspensions were injected intraperitoneally (i.p.) or the mice were infected through the oral route by gavage with 0.25 ml. In each LD_{50} experiment, five dilutions were tested with five mice per group; each strain was tested at least twice by each route of infection. The mice were scored for viability twice daily for 2 weeks, and the LD_{50} was calculated by the method of Reed and Meunch (37). In each experiment five mice were mock infected with PBS; all of these mice survived.

Plasmid constructions. Cloning of the wild-type *S. typhimurium pagC* gene into the cosmid vector pLAFR3 was described previously, and this plasmid was designated pWP061 (36). Plasmids pWPL3 and pWPL4 were constructed by ligation of the 5.3-kb *Eco*RI restriction fragment from pWP061 containing the *pagC* gene into pUC19. The orientation of *pagC* in pWPL3 is such that *pagC* cannot be expressed from the pUC19 *lac* promoter, while in pWPL4 *pagC* can be expressed from this heterologous promoter. Plasmid pWPL8 was generated by deletion of the 800-bp *Bgl*II-*Sma*I fragment downstream of the *pagC* gene in pWPL3. This was accomplished by digestion of pWPL3 with the restriction endonuclease *Bgl*II, end filling of the 5′ overhang with Klenow polymerase, digestion with *Sma*I endonuclease, and ligation with T4 ligase. Plasmid pWPL10 was generated by deletion of a 2.2-kb *Pst*I fragment that is 1.5 kb upstream of the start codon of *pagC* in pWPL4. *Salmonella* and *E. coli* strains containing these plasmids all produced an 18-kDa protein which was immunoreactive in Western blot analysis with antisera raised against a synthetic peptide corresponding to the deduced amino acid sequence of PagC (data not shown).

RESULTS

Phenotype of *pagC* clones in *E. coli*. The *ail* gene from *Y. enterocolitica* confers an attachment and invasive phenotype to *E. coli* HB101 (34). Given the similarity of PagC to Ail (36), the invasive phenotype of *E. coli* expressing *pagC* was examined (Table 2). No significant increase in invasion was observed with *E. coli* HB101 carrying the *pagC* clones as compared with *E. coli* HB101 carrying the cloning vector (pLAFR3 or pUC19) alone. In comparable assays, *E. coli* carrying the *ail* clone, pVM102, gave invasion levels of 0.37 and 12.7% for HEp-2 and CHO cells, respectively (34). Although *pagC* in *trans* could complement the macrophage survival defect of the *S. typhimurium pagC1* mutant CS119 (see below), the *ail* clone, pVM102, could not complement this defect of the *S. typhimurium pagC* mutant CS119 (data not shown). Thus, it appears that although there is considerable amino acid conservation between Ail and PagC, they are not functionally equivalent. It may be that unlike Ail, PagC requires an additional factor(s) or a cell membrane environment that is present in *Salmonella* strains but not in *E. coli*. Therefore, we investigated the effect of *pagC* on invasion by salmonellae.

Class II invasion-defective mutants of *S. enteritidis* have insertions of Tn*phoA* in *pagC*. We previously identified 13 mutants with an invasion-defective phenotype after screening a bank of Tn*phoA* insertion mutants of *S. enteritidis* CDC5str (41). These mutants were grouped into six classes (I through VI) based on their invasion phenotypes and chromosomal map locations. The class II mutations (SM3, SM4, and SM5) hybridized to the fragments corresponding to min 25 on the *S. typhimurium* chromosome (41), which is close to where *pagC* maps (30). We were therefore interested in determining whether any of the *S. enteritidis* invasion-defective mutants had Tn*phoA* insertions in *pagC*. Southern hybridization analysis of chromosomal DNA from each of the 13 invasion mutants was performed using a *pagC* probe (Fig. 1). The 3 class II mutants had a single Tn*phoA* insertion into the *Eco*RV fragment that contains *pagC*, while the other 10 mutants appeared to have a wild-type *pagC* locus. The other 10 invasion-defective mutants had Tn*phoA* insertions distant from the *pagC* locus; thus, the faint hybridization signals observed for some of the mutants were due to the short Tn*phoA* sequence that was part of the PagC probe (see Materials and Methods). Class II mutant SM3 has two Tn*phoA* insertions (41), which is why two hybridization signals were observed for this mutant and not the other class II mutants, SM4 and SM5.

The class II mutants (SM3, SM4, and SM5) were previously shown to be defective for invasion of CHO and MDCK

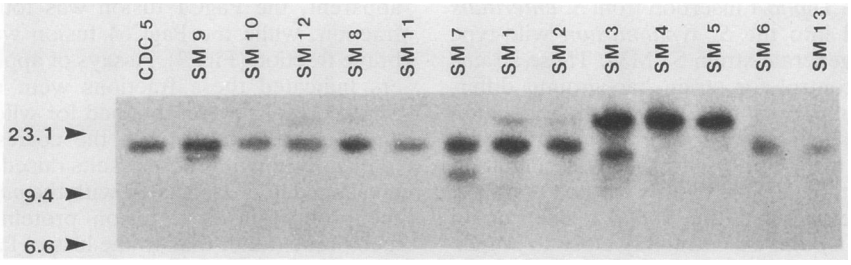

FIG. 1. Southern analysis of wild-type *S. enteritidis* and invasion-defective mutants by using a PagC probe. Chromosomal DNA was purified from the indicated strains and digested with *Eco*RV. Preparation of the PagC probe and hybridizations were performed as described in Materials and Methods. Strain CDC5 is the wild-type *S. enteritidis* strain. Strains SM1 through SM13 are previously described invasion-defective mutants of CDC5 (41). The positions and sizes (in kilobases) of λ *Hin*dIII fragments are indicated on the left.

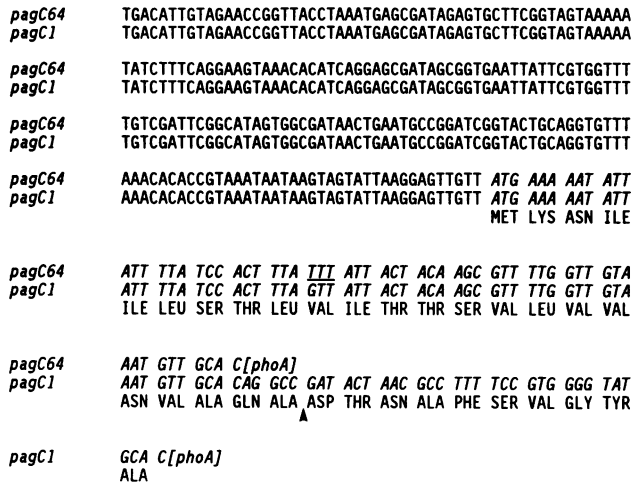

FIG. 2. Nucleotide sequence 5′ of the Tn*phoA* insertion in *S. enteritidis* SM5T. The chromosome-Tn*phoA* junction was cloned and sequenced as described in Materials and Methods. The sequence from *S. enteritidis pagC64* is shown aligned with the sequence from *S. typhimurium pagC1* (36). The site of the Tn*phoA* insertion is indicated in brackets. Underlined is the position of the *S. enteritidis pagC* codon that differs from the *S. typhimurium pagC* sequence. The structural gene sequence is in italics with the derived amino acid sequence of the *S. typhimurium* PagC indicated below. The position of the signal sequence cleavage site is indicated by the arrowhead.

cells but invaded HEp-2 cells at nearly wild-type levels (41). In addition, all three of these mutants were missing an 18-kDa membrane protein, similar in size to the product of *pagC* (41). As these three mutants were previously shown to be indistinguishable by a variety of assays, SM5 was chosen as a representative of this class of mutants for further analysis. To ensure that the invasion-defective phenotype of SM5 was due to the Tn*phoA* insertion, the Tn*phoA* insertion mutation from SM5 was transduced into the wild-type *S. enteritidis* strain CDC5str by using phage P22. All transductants had the same invasion defect as the parent (SM5) (data not shown), indicating that the invasion defect was indeed due to the Tn*phoA* insertion. One such transductant was named SM5T and used in subsequent analyses.

S. typhimurium CS119 carries the Tn*phoA* insertion that identified *pagC* (31). This mutation (*pagC1*) was moved into the wild-type *S. enteritidis* strain (CDC5str) by transductional cross using a phage P22 lysate grown on CS119. Transductants carrying the *pagC1* allele were identified by selecting for the Kan^r of Tn*phoA*. Two such transductants, designated SVM47 and SVM48, were used in subsequent analyses. Similarly, the Tn*phoA* insertion from *S. enteritidis* SM5T was transduced into the *S. typhimurium* wild-type strain ATCC 14028 to generate strain SVM39. These strains were constructed to control for strain background differences in subsequent experiments. Southern hybridization analysis of these strains using the *pagC* probe indicated that the Tn*phoA* insertion from SM5T and the *pagC* mutation from CS119 were indistinguishable by this method (data not shown). Thus, it appeared that the Tn*phoA* insertion in SM5T was either within *pagC* or extremely close to *pagC*.

To confirm that class II invasion mutants of *S. enteritidis* (e.g., SM5T) have Tn*phoA* inserted in *pagC*, the chromosome-Tn*phoA* junction was cloned and the nucleotide sequence was determined (Fig. 2). The sequence obtained was identical to the published sequence of *pagC* from *S. typhimurium* (36) with the exception of a single nucleotide change in the 10th codon, resulting in a change of valine (*S. typhimurium*) to phenylalanine (*S. enteritidis*). The Tn*phoA* insertion in *S. enteritidis* SM5T would result in a protein fusion of AP to the penultimate amino acid of the proposed signal sequence of PagC. The Tn*phoA* insertion originally isolated in *S. typhimurium* CS119 is 36 nucleotides downstream of the insertion in SM5T. The *pagC*::Tn*phoA* insertion mutations from *S. enteritidis* SM5T and *S. typhimurium* CS119 will be referred to as *pagC64* and *pagC1*, respectively.

Invasion and macrophage survival phenotype of *pagC* mutants. The *S. typhimurium pagC1* mutant was shown previously to have a decreased ability to survive within bone marrow-derived BALB/c macrophages (31) or the macrophagelike cell line J774.1 as compared with that of the wild-type strain (30). Therefore, survival in J774.1 cells of the wild-type *S. enteritidis* strain CDC5str and invasion-defective mutant SM5T was determined (Table 3). The wild-type strain was clearly able to survive within these cells and actually increased ninefold over a 24-h period. In contrast, over the same 24-h period the mutant was killed. These results are comparable to what has been observed for the wild type and the *pagC1* mutant of *S. typhimurium* (30, 31).

Tissue culture invasion assays (Table 3) indicated that the *pagC64* mutants (*S. enteritidis* SM5T and *S. typhimurium* SVM39) were decreased about 12-fold in CHO cells and 22-fold in MDCK cells relative to the wild-type strains (*S. enteritidis* CDC5str and *S. typhimurium* ATCC 14028). In contrast, the *pagC1* mutants (*S. enteritidis* SVM48 and *S. typhimurium* CS119) invaded tissue culture cells at levels similar to those of the wild types, although a two- to threefold decrease was occasionally observed. None of these mutants have a known defect other than the loss of PagC (36, 41); thus, the different invasion phenotypes of these two types of *pagC* mutants were somewhat surprising.

Cellular localization of PagC-AP fusions. The most striking difference between the two mutations is that the insertion in *pagC64* is within the signal sequence-encoding region of *pagC*, whereas the insertion in *pagC1* is in the coding sequence for the mature protein. Therefore, a possible explanation for the difference in the phenotypes of the two mutations is that the two fusion proteins are located in different cellular compartments and that cellular localization influences the fusion protein's effect on the cell. This was investigated by examination of periplasmic and membrane fractions of the wild-type *S. enteritidis*, *S. enteritidis pagC64* mutant (SM5T), and *S. enteritidis pagC1* mutant (SVM48) (Fig. 3). The PagC-AP fusion proteins were detected by Western analysis with antibody to AP. As was readily apparent, the PagC1 fusion was found in the periplasmic fraction, while the PagC64 fusion was found in the membrane fraction (Fig. 3). Assays of appropriate enzyme markers indicated these fractions were more than 90% pure. Similar results were obtained for wild-type *S. typhimurium* and *S. typhimurium* with the corresponding *pagC* alleles (data not shown). These results raised the possibility that the invasion defect associated with the *pagC64* allele was due to the production of a fusion protein that remains in the membrane rather than to the loss of PagC. If this is the case, some predictions can be tested. First, reduced synthesis of the fusion protein should alleviate the invasion defect. Second, a wild-type clone of *pagC* should not complement the invasion defect of the *pagC64* mutation, since loss of

TABLE 3. Invasion by wild-type *Salmonella* strains and *pagC* and *phoP* mutants

Strain	Relevant phenotype	% Invasion (fold decrease) in[a]:		MSI[b]
		CHO cells	MDCK cells	
S. enteritidis				
CDC5str	PagC$^+$ PhoP$^+$	7.36 ± 0	8.49 ± 0.4	9.13 ± 4.3
SVM42	PagC$^+$ PhoP$^-$	6.25 ± 0.22 (0.8)	2.23 ± 0.12 (3.8)	ND
SM5T	PagC64 PhoP$^+$	0.66 ± 0.01 (11.2)	0.36 ± 0.01 (23.6)	0.02 ± 0.02
SVM43	PagC64 PhoP$^-$	9.14 ± 1.19 (0.8)	2.96 ± 0.12 (2.9)	ND
SVM48	PagC1 PhoP$^+$	9.77 ± 0.99 (0.8)	6.79 ± 0.33 (1.3)	0.08 ± 0.03
SVM46	PagC1 PhoP$^-$	5.93 ± 0.29 (1.2)	4.79 ± 0.29 (1.8)	ND
S. typhimurium				
ATCC 14028	PagC$^+$ PhoP$^+$	7.12 ± 0.58	10.63 ± 0.56	ND
CS015	PagC$^+$ PhoP$^-$	3.57 ± 0.12 (2.0)	5.64 ± 0.37 (1.9)	ND
SVM39	PagC64 PhoP$^+$	0.37 ± 0.03 (12.7)	0.49 ± 0.07 (21.7)	ND
SVM44	PagC64 PhoP$^-$	3.20 ± 0.31 (2.2)	3.53 ± 0.75 (3.0)	ND
CS119	PagC1 PhoP$^+$	6.98 ± 0.05 (1.0)	6.73 ± 0.38 (1.6)	ND

[a] The invasion phenotypes of the indicated strains were assayed by using CHO and MDCK tissue culture cell lines as described elsewhere (41). The results are expressed as follows: % invasion = 100 × (no. of intracellular bacteria/no. of bacteria added). The decrease in invasion is as compared with the wild type parental strain.

[b] MSI, macrophage survival index (± the standard deviation) at 24 h postinfection by using the J774.1 macrophage cell line; determined as described previously (5, 24). ND, not determined.

PagC function is not responsible for this defect. Furthermore, a wild-type clone of *pagC* should not complement the macrophage survival defect of the *pagC64* mutation, as synthesis of the PagC64 fusion protein would block proper localization of wild-type PagC in the outer membrane.

Effect of mutations in *phoP* on the phenotype of *pagC*::TnphoA mutants. PhoP is a positive regulator of *pagC* expression (31); hence, a mutation in *phoP* should result in reduced synthesis of the PagC-AP fusion proteins. To test whether decreased PagC64 production could eliminate the invasion defect, a *phoP102*::Tn*10*d-cam mutation from *S. typhimurium* CS015 was introduced into *S. enteritidis* CDC5str, SM5T, and SVM48 and into *S. typhimurium* SVM39 by phage P22 transduction. The presence of the *phoP102*::Tn*10*d-cam mutation was confirmed by Southern hybridization analysis with a *phoP* probe (data not shown). The resulting strains (*S. enteritidis* SVM43 and SVM46, and *S. typhimurium* SVM44) showed decreased expression of PagC-AP fusion proteins as determined by Western blot analysis with antibody to AP (Fig. 3 and data not shown) and by analysis of AP activity (Table 4). Protein cross-reactive with α-AP was not detected in strains carrying the *phoP102*::Tn*10*d-cam mutation alone (SVM42). Introduction of the *phoP* mutation into *S. enteritidis* SM5T (*pagC64*) and SVM48 (*pagC1*) resulted in an ~200-fold reduction in AP activity. Introduction of this mutation into *S. typhimurium* SVM39 (*pagC64*) resulted in a 56-fold reduction in AP activity. Together these data confirm that PhoP is required for expression of the fusion protein.

The *phoP102*::Tn*10*d-cam mutation in the wild-type strain backgrounds (*S. enteritidis* CDC5str and *S. typhimurium* ATCC 14028) resulted in a slight but reproducible decrease in invasion (Table 3). Similarly, this *phoP* mutation in the *S. enteritidis pagC1* mutant (SVM46) resulted in a slight de-

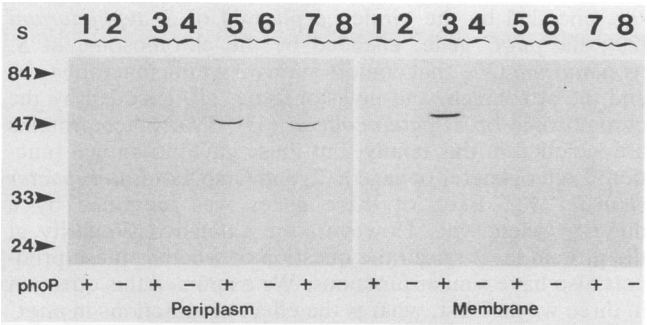

FIG. 3. Western analysis of periplasmic and membrane fractions from *pagC* mutants. Periplasmic and membrane fractions were prepared from the indicated strains. The proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose as described in Materials and Methods. The filters were then incubated with antibody to AP. + and −, presence or absence of a wild-type *phoP* locus. The indicated lanes contain protein isolated from the following strains (the *pagC* allele is indicated in parenthesis): 1, CDC5str (wild-type *pagC*); 2, SVM42 (wild-type *pagC*); 3, SM5T (*pagC64*); 4, SVM43 (*pagC64*); 5, SVM47 (*pagC1*); 6, SVM45 (*pagC1*); 7, SVM48 (*pagC1*); 8, SVM46 (*pagC1*). The positions and sizes (in kilodaltons) of protein standards (S) are indicated on the left.

TABLE 4. AP activity of PagC-AP fusions

Strain	Relevant phenotype	U of AP (fold decrease)[a]
S. enteritidis		
CDC5str	PagC$^+$ PhoP$^+$	0.53 ± 0.05
SVM42	PagC$^+$ PhoP$^-$	0.55 ± 0.01
SM5T	PagC64 PhoP$^+$	635.68 ± 4.4
SVM43	PagC64 PhoP$^-$	2.90 ± 0.09 (219)
SVM48	PagC1 PhoP$^+$	720.96 ± 4.26
SVM46	PagC1 PhoP$^-$	4.00 ± 0.12 (180)
S. typhimurium		
ATCC 14028	PagC$^+$ PhoP$^+$	0.40 ± 0.01
CS015	PagC$^+$ PhoP$^-$	0.99 ± 0.0
SVM39	PagC64 PhoP$^+$	695.69 ± 17.82
SVM44	PagC64 PhoP$^-$	12.33 ± 0.08 (56)
CS119	PagC1 PhoP$^+$	941.07 ± 12.60

[a] The amount of AP activity (± the range) for the indicated strains was determined as described elsewhere (28) from cultures grown overnight at 37°C in LB broth with aeration. The decrease in AP activity is as compared with a strain carrying the wild-type *phoP* locus.

TABLE 5. Effect on invasion and macrophage survival of the cloned *pagC* gene in *S. enteritidis*

Strain	Plasmid	% Invasion for CHO cells[a]	MSI[b]
CDC5str	pLAFR3	5.81 ± 0.35	1.12 ± 0.12
CDC5str	pWP061	14.45 ± 0.49	1.05 ± 0.44
SM5T	pLAFR3	0.44 ± 0.06	0.07 ± 0.02
SM5T	pWP061	0.29 ± 0.03	0.02 ± 0.01

[a] The invasion phenotypes of the indicated strains were assayed by using CHO cells as described previously (41). The results are expressed as follows: % invasion = 100 × (no. of intracellular bacteria/no. of bacteria added).

[b] MSI, macrophage survival index (± the standard deviation) at 24 h postinfection by using bone marrow-derived BALB/c macrophages; determined as described elsewhere (5, 24).

TABLE 6. Effect of *pagC* mutations on the LD_{50} of *S. enteritidis* in mice

Strain	LD_{50}[a] (no. of bacteria)	
	Oral	i.p.
S. enteritidis		
CDC5str	2.5×10^6	<10
SM5T (*pagC64*)	1.1×10^{10}	3.6×10^5
SVM47 (*pagC1*)	5.4×10^8	4.6×10^3
S. typhimurium		
ATCC 14028	ND	<20
CS120 (*pagC64*)	ND	6×10^4
CS119 (*pagC1*)	ND	7×10^3

[a] Determined after either intragastric (oral) or i.p. inoculation as described in Materials and Methods. ND, not determined.

crease in invasion. However, the *phoP* mutation in the *pagC64* background resulted in an increase in invasion of both CHO and MDCK cells to levels similar to that of the wild-type strains carrying the *phoP* mutation alone (compare SVM43 with SVM42 and SVM44 with CS015) (Table 3). This result suggests that synthesis of the PagC64 fusion protein rather than the loss of PagC function was responsible for the invasion defect of strains carrying the *pagC64* mutation. Consistent with this is the observation that most invasion-positive revertants of SM5T no longer synthesized the fusion protein, although they retained the Tn*phoA* insertion in *pagC* (data not shown).

Complementation of *pagC* alleles with wild-type *pagC*. The second prediction is that a wild-type clone of *pagC* should not complement the invasion defect or the macrophage survival defect of the *pagC64* allele. This prediction was tested by mating a wild-type *S. typhimurium pagC* clone, pWP061 (36), into CDC5str and SM5T (*pagC64*) and measuring the invasion and macrophage survival phenotypes of the resulting strains. Western analysis using an antibody to PagC indicated that PagC was expressed from pWP061 in these strains (data not shown). Neither the cloning vector pLAFR3 nor the plasmid carrying *pagC*, pWP061, restored the invasive phenotype to SM5T (Table 5). CDC5str (pWP061) was consistently two- to threefold more invasive than the control strain CDC5str(pLAFR3). This may have been due to the increased aggregation observed with cultures of CDC5str(pLAFR3) as opposed to any of the other strains listed in Table 5. As in the invasion assay, neither the vector pLAFR3 nor the *pagC*-containing plasmid pWP061 complemented the macrophage survival defect of strain SM5T (*pagC64*). In contrast, a *pagC* clone did complement the macrophage survival defect of a *pagC1* mutation; CS119(pWP061) was 41-fold more resistant to bone marrow-derived macrophages than CS119.

Virulence phenotype of *pagC64* and *pagC1* mutants. The *pagC1* mutant of *S. typhimurium* (CS119) was previously shown to be less virulent than the wild type by the i.p. route of infection in BALB/c mice (31). The in vitro invasion phenotype is hypothesized to be a reflection of the ability of salmonellae to penetrate the intestinal wall (a step that is bypassed during i.p. infections) or to invade cells during the subsequent systemic infection. Therefore, we sought to examine the effect of these *pagC* mutations on virulence by both the i.p. and oral routes of infection (Table 6). Both *pagC* mutants had higher LD_{50}s (were less virulent) than the wild type when administered orally, but the *pagC64* mutation of SM5T resulted in a 50-fold higher LD_{50} than the *pagC1* mutation of SVM47. Similar results were obtained by using the i.p. route of infection; both mutants were significantly less virulent than the wild-type strain. However, the *pagC64* mutant again was even less virulent than the *pagC1* mutant, with an LD_{50} 10^4 times higher than that of wild type and more than 70-fold higher than the LD_{50} of the *pagC1* mutant SVM47. Intraperitoneal infection of BALB/c mice with wild-type *S. typhimurium* or strains carrying either *pagC1* or *pagC64* gave results similar to those observed with *S. enteritidis* (Table 6). These results indicated that *pagC* is a virulence factor for *S. enteritidis* as well as for *S. typhimurium* and suggested that the invasion defect of the *pagC64* mutation or other unidentified pleiotropic effects also contribute to the virulence phenotype. This effect on virulence was not restricted to mice infected by the oral route, suggesting that the properties affected by these mutations may act throughout an infection or act subsequent to penetration of the intestinal epithelium.

DISCUSSION

Recently a number of virulence genes whose products have a significant level of amino acid identity (ranging from 31.6 to 53.3% identical residues, depending on the pair) have been described. These include the serum resistance gene, *rck*, encoded by the virulence plasmid of *S. typhimurium* (20); the *pagC* gene, encoded by the chromosome of *S. typhimurium* (36), that confers survival within macrophages; and the attachment and invasion gene, *ail*, encoded by the chromosome of *Y. enterocolitica* (33). Two other proteins are included in this family, but these have no known function: Lom of bacteriophage λ (2) and OmpX of *Enterobacter cloacae* (42). Each of these genes was identified by a different phenotype. However, the extensive similarity at the protein level raised the question of whether these products also have similar functions. We examined this question in three ways. First, what is the effect of mutations in *pagC* on tissue culture invasion by salmonellae? Second, can *pagC* confer an invasive phenotype for tissue culture cells to *E. coli*? Third, can *ail* complement a *Salmonella pagC* mutant in a macrophage survival assay? The results presented above indicate that PagC and Ail do not confer similar properties to their host bacteria. Although the cloned *ail* gene was expressed in *Salmonella* cells, it did not restore survival of *Salmonella pagC* mutants in macrophages, and unlike *ail* (34), a cloned *pagC* gene did not confer an invasive phenotype to *E. coli*. The effect of mutations in *pagC* on the invasion phenotype of salmonellae initially gave conflicting results. One mutant, SM5T, which carries a Tn*phoA* inser-

tion in the *pagC* gene of *S. enteritidis* exhibited an invasion defect for CHO and MDCK cells. In contrast, the *S. typhimurium pagC* mutant CS119 invaded these tissue culture cell lines to the same degree as its wild-type parent.

The different invasion phenotypes of these two Tn*phoA* insertion mutations of *pagC* were not due to differences in strain background, since each mutation retained its phenotype regardless of whether it was in *S. typhimurium* or *S. enteritidis*. Sequence analysis of these mutations indicated that the invasion-defective mutant, SM5T (*pagC64*), has a Tn*phoA* insertion in the penultimate codon of the PagC signal sequence, whereas the invasion-proficient mutant, CS119 (*pagC1*), has an insertion of Tn*phoA* 11 codons downstream (36). The fusion proteins produced as a result of these two mutations were found in different cellular fractions, with the PagC1 fusion in the periplasm and the PagC64 fusion in the membrane. This suggests that the signal sequence of PagC64 is not cleaved and that consequently the fusion protein remains associated with the membrane. When the PagC64 fusion protein is not synthesized because of a mutation in the regulatory gene *phoP*, tissue culture invasion is restored even though the bacteria still lack PagC. This suggested that the invasion defect of SM5T was not a result of the loss of PagC function, which is consistent with the observation that a plasmid expressing *pagC*, pWP061, did not complement either the macrophage survival-defective or the invasion-defective phenotype of SM5T (*pagC64*). Conversely, a plasmid carrying the wild-type *pagC* locus did complement the macrophage survival defect of CS119 (*pagC1*). A model accounting for these results is as follows. The fusion protein produced by the *pagC64* mutation is abnormally localized to the inner membrane, where it blocks proper localization of proteins necessary for efficient invasion. Thus, the invasion defect is probably an indirect effect due to the production of an unusual fusion protein, while the macrophage survival defect is due to the loss of PagC.

Taken together these results suggest that the amino acid similarity between Ail and PagC (and probably the other family members as well) may not reflect a conservation of function. The amount of amino acid identity of the proposed membrane-spanning domains of any two family members is very high (50.7% for Ail and PagC), whereas the amino acid identity of proposed cell surface domains is quite low (9.8% for Ail and PagC). Thus, it may be that the cell surface domains of Ail and PagC have diverged to perform different functions, while the remainder of the protein sequences are conserved to maintain proper localization and orientation within the outer membrane (3). While this is an appealing explanation, this model needs to be examined by further functional studies of the family members and by studies on the actual topology of these proteins in the membrane.

Virulence studies using BALB/c mice indicated that regardless of the route of infection, mutants carrying the *pagC64* allele are even less virulent than mutants carrying the *pagC1* allele. This result suggests that the invasion defect observed in tissue culture cells for strains carrying the *pagC64* mutation may be contributing to a lethal outcome. While it is possible that in addition to decreased invasion, the *pagC64* mutation has other pleiotropic defects, strains carrying this mutation have the same growth rate, motility, and lipopolysaccharide profile as the wild type when grown in vitro (41). In addition, the protein profiles of cytoplasmic, periplasmic, and membrane fractions of the mutant and wild type differ only in the loss of PagC in the mutant (41). These results provide further support for the hypothesis that the ability to invade cells plays a role in the virulence of salmonellae.

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