Inorganic polyphosphate is essential for long-term survival and virulence factors in *Shigella* and *Salmonella* spp.

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The importance of inorganic polyphosphate (poly P) and poly P kinase (PPK), the enzyme principally responsible for its synthesis, has been established previously for stationary-phase survival of *Escherichia coli* and virulence in *Pseudomonas aeruginosa*. The gene (*ppk*) that encodes PPK is highly conserved among many bacterial pathogens, including *Shigella* and *Salmonella* spp. In view of the phylogenetic similarity of the enteropathogens and the frequency with which virulence factors are expressed in stationary phase, the *ppk* gene of pathogenic *Shigella flexneri*, *Salmonella enterica* serovar Dublin, and *Salmonella enterica* serovar typhimurium have been cloned and deleted. In some of these mutants lacking *ppk*, the phenotypes included features indicative of decreased virulence such as: (*i*) growth defects, (*ii*) defective responses to stress and starvation, (*iii*) loss of viability, (*iv*) polymyxin sensitivity, (*v*) intolerance to acid and heat, and (*vi*) diminished invasiveness in epithelial cells. Thus PPK may prove, as it has with *P. aeruginosa*, to be an attractive target for antibiotics, with low toxicity because PPK is not found in higher eukaryotes.

Inorganic polyphosphate (poly P) is a chain of tens or many hundreds of phosphate residues linked by "high-energy" phosphoanhydride bonds. Poly P is ubiquitous, having been found in every cell examined (1), and performs varied functions depending on the cell and circumstances (2). *Escherichia coli* mutants lacking polyphosphate kinase (PPK), the enzyme responsible for the synthesis of poly P from ATP, are deficient in responses to stresses and stringencies and fail to survive in stationary phase (3, 4). The gene *ppk* that encodes PPK is highly conserved in Gram-negative bacteria (5), including some 20 pathogens. Mutation of *ppk* in six enteropathogens rendered them impaired in motility on a semisolid agar surface (6), indicative of a loss in ability to invade and establish systemic infections in host cells. A *ppk* mutant of *Pseudomonas aeruginosa* was also defective in quorum sensing and the dependent virulence factors, elastase and rhamnolipid; the mutant was also deficient in biofilm formation and was not lethal in a burned-mouse pathogenesis model (7). *Vibrio cholerae ppk* mutants also show defects in growth, motility, and surface attachment, features linked to virulence (8).

Poly P is involved in the expression in *E. coli* of RpoS (9), the sigma factor responsible for activation of more than 50 genes required for survival during starvation, UV radiation, oxidative damage, and osmotic stress (10, 11). In addition to a decrease in long-term survival in the stationary phase, increased sensitivities to oxidative, osmotic, and heat stresses, and defects in adaptive growth in minimal media are among the phenotypic features exhibited by the *ppk* mutant (3); these can all be linked to a decreased expression of the *rpoS* gene (9). On the basis of all these factors, it would seem that poly P is likely needed for the virulence of *Shigella* and *Salmonella* spp.

Shigella flexneri, a facultative intracellular pathogen, is the etiological agent of bacillary dysentery. The capacity of this bacterium to enter human epithelial cells depends on secreted proteins encoded by a regulon of virulence genes. Expression of the genes is controlled by multiple environmental signals (12). The ability of *S. flexneri* in stationary phase to survive for several hours at pH 2.5 likely accounts for the low infective dose in shigellosis. The acid resistance depends on expression of *rpoS*; a deletion mutant is highly acid sensitive (13). Interdependence with *rpoS* expression in *E. coli* (9) can be added to the similarity of *S. flexneri* in the expressions of several invasion operons (e.g., *viz*, *ipa*, *mxi*, and *spa*) that are maximal in stationary-phase cultures (13, 14). Evidence that infective *S. flexneri* is in a stationary, nondividing state and that expression of stationary-phase-specific genes is essential for survival and virulence provides support for a role for poly P in its pathogenesis.

Salmonellae are Gram-negative facultative anaerobes and, when acquired by the ingestion of contaminated food or water, can cause a range of diseases depending on the serovar and host (15–17). *Salmonella enterica* serovar typhimurium (*S. typhimurium*) causes inflammatory diarrhea in calves and also elicits a systemic disease in mice. It shows a broad host range, infecting cattle, pigs, sheep, horses, poultry, and rodents (18); similar diseases caused by *Salmonella enterica* serovar Dublin (*S. dublin*) are found principally in cattle.

In salmonellae, as in *S. flexneri*, evidence exists for the regulation of virulence by RpoS. *S. typhimurium* harbors a large (80–100 kDa) plasmid (19), the absence of which results in attenuation or loss of virulence. The *spvABCD* (salmonella plasmid virulence) operon found on this plasmid greatly enhances the ability of the bacteria to proliferate in extraintestinal tissues and thus is required for the induction of systemic disease in mice (20).

To investigate the role of poly P in the virulence of *Shigella* and *Salmonella* spp., null mutants of *ppk* were prepared and their phenotypes, with particular relation to virulence factors, were examined.

Materials and Methods

Reagents. ATP, creatine kinase, DNase I, and RNase IIIa were from Roche Molecular Biochemicals. Creatine phosphate, 3-(*N*-morpholino)propanesulfonic acid (Mops), kanamycin, ampicillin, tetracycline, amino acids, and BSA were from Sigma. [γ-^{32}P]ATP, Hybond-N$^+$ nylon membranes, and carrier-free $^{32}P_i$ were from Amersham Pharmacia. Polyethyleneimine-cellulose F TLC plates were from Merck. Restriction enzymes were from New England Biolabs. The DIG (digoxigenin) DNA labeling kit was from Boehringer Mannheim, and [^{32}P]poly P was as described (21).

Strains, Plasmids, and Phages. Strains, plasmids, and phages are listed in Table 1. Plasmids were introduced into *E. coli* by

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Abbreviations: poly P, inorganic polyphosphate; PPK, polyphosphate kinase; PPX, exopolyphosphatase; *S. typhimurium*, *Salmonella enterica* serovar typhimurium; *S. dublin*, *S. enterica* serovar Dublin; WT, wild type; cfu, colony-forming unit.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF085682).

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Table 1. Strains, phage, and plasmids

	Relevant characteristics	Source or ref.
Strains		
S. typhimurium		
S. typhimurium	SL7519 [F⁻] *mutL111*:::Tn*10*	B. A. D. Stocker
CF 7519	SL7519 Δ*ppk* Δ*ppx*::*kan*	This study
S. typhimurium WRAY	WT	B. A. D. Stocker
S. typhimurium FIRN	Nal^r, WT	This study
SF11687	Nal^r, *S. typhimurium* FIRN, *ppk*::*kan*	This study
S. dublin		
S. dublin	SVA47 WT	B. A. D. Stocker
S. dublin N2	SVA47 Δ*ppk* Δ*ppx*::*kan*	This study
S. flexneri		
S. flexneri	WT 2a	A. T. Maurelli
S. flexneri M1	Δ*ppk* Δ*ppx*::*kan*	This study
E. coli		
DH5α	F′ *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF–lacZYA*) *U169* (ϕ80*dlacZ*Δ*M15*)	Lab collection
S17-1 (λ pir)	Tp^r *recA thi pro hsdR*$^-$*M*$^+$ *RP4:2-Tc:MuKm* Tn*7*, λ pir	Lab collection
CF5802	K-12 (MG1655) Δ*ppk* Δ*ppx*::*kan*	Lab collection
KL16	Hfr	
CF16	KL16 Δ*ppk* Δ*ppx*::*kan*	This study
Phage and Plasmids		
λ DASHII	λ DASHII vector digested with *Bam*HI	Stratagene
pBluescript II KS (+)	Ap^r, ColE1, high-copy-number cloning vector	Stratagene
pUC4K		Pharmacia
pKNG101	Sm^r, oriR6K, mobRK2, *sacB*, suicide vector	22
pKS7	pBluescript II SK (+) derivative harboring 5.7-kb fragment containing *ppk* and *ppx* genes of *S. typhimurium* FIRN	This study
pKS10	pBluescript II SK (+) derivative harboring 2,072-bp *ppk* PCR fragment	This study
pKS10-1	pKS10, *Apa*I, *Sal*I, and *Eco*RI sites in pKS10 were removed	This study
pKS10-6	pKS10-1 derivative containing *ppk* coding region interrupted with Km cassette (*ppk*::*km*)	This study
pKSS1	pKNG101 containing *ppk*::*km* cassette	This study

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transformation and into *S. typhimurium* strains by electrotransformation with a Bio-Rad Gene Pulser.

DNA Manipulations and Analysis. DNA manipulations and analysis were as described by Sambrook *et al.* (23).

Preparation of Part of *ppk* Gene of *S. typhimurium* WRAY. Partial sequence of *S. typhimurium ppk* was obtained by a BLAST search of the TIGR (The Institute for Genomic Research) genome sequence database by using the *E. coli ppk* sequence. This sequence was amplified by PCR with two synthetic primers (*Sal*I forward primer, 5′-CCGTGAATAAAACGGAGTATAGGTAG-3′; *Sal*I reverse primer, 5′-AAAATGTCATCCAGGCAG-3′); genomic DNA of *S. typhimurium* was the template.

Genomic Library Construction and Screening of *S. typhimurium* WRAY. Wild-type (WT) DNA was partially digested with *Sau*3AI. The fragments were ligated into *Bam*HI-digested λ DASHII and packaged by using Gigapack II Gold (Stratagene). The library was screened by using the labeled PCR fragment (partial *ppk* gene, 659 bp) to obtain clones that carry the entire *ppk* gene. From the positive clones, a 5.7-kb *Xba*I–*Kpn*I fragment was identified that contained the whole *ppk* gene. This fragment was cloned into pBluescript II KS (+) (Stratagene) that had been digested with *Xba*I and *Kpn*I, yielding the plasmid pKS7. The 5.7-kb fragment in pKS7 was sequenced.

Construction of *ppk*-Deletion Mutant of *S. typhimurium*. A 2.072-kb PCR fragment containing the *ppk* coding sequence was generated with pKS7 as the template and the primers 5′-CGTGAATAAAAACGGAGTAT-3′ and 5′-ATGAAAGCTGTTTGAGCCG-3′. This fragment was ligated into pBluescript II KS (+) that had been digested with *Sma*I to construct the plasmid pKS10. It was further digested with *Apa*I and *Eco*RI to remove the *Sal*I site in the vector. The resulting 5.1-kb fragment was treated with T_4 DNA polymerase to create blunt ends, and the ends were then self-ligated. The plasmid pKS10-1 thus prepared was digested with *Sal*I to remove a 800-bp fragment from the midportion of the *ppk* gene to obtain a 4.3-kb fragment. It was ligated to a kanamycin-resistance gene cassette contained within a *Sal*I restriction fragment of pUC4K. The resulting plasmid, pKS10-6, was digested with *Kpn*I and *Xba*I; the fragment was blunt-ended by using T_4 DNA polymerase and cloned into pKNG101, yielding pKSS1. The plasmid pKSS1 was introduced into the WT *S. typhimurium* FIRN from *E. coli* S17-1 λ pir by conjugal transfer. Cointegrant conjugants containing no plasmid and representing a single homologous recombination were isolated by plating on LB agar plates supplemented with nalidixic acid (50 μg/ml) and carbenicillin (60 μg/ml), and the genotype was confirmed by PCR. The strains were subjected to sucrose selection; clones with a double-homologous recombination event (streptomycin-sensitive and kanamycin-resistant) were identified and further tested by PCR and assay of PPK.

Construction of *S. enterica* Serovar Dublin Δ*ppk* Δ*ppx*::*kan* Mutants. $P1_{vir}$ lysate of *E. coli* strain CF5802 was used to transduce the Δ*ppk* Δ*ppx*::*kan* mutation into *E. coli* Hfr strain KL16. Conjugation was performed between KL16 Δ*ppk* Δ*ppx*::*kan* (donor) and *S. typhimurium* strain SL7519 [F⁻] *mutL111*::Tn*10* (recipient). A P22 lysate prepared from the resultant SL7519 Δ*ppk* Δ*ppx*::*kan* strain was then used to transduce the mutation into *S. dublin* SVA47. Three independent *S. dublin* Δ*ppk* Δ*ppx*::*kan* mutants were isolated and verified by PCR.

Construction of *S. flexneri* Δ*ppk* Δ*ppx*::*kan* Mutants. $P1_{vir}$ lysate of *E. coli* strain CF5802 was used to transduce the Δ*ppk* Δ*ppx*::*kan* mutation into WT *S. flexneri* strain 2a.

Table 2. PPK, PPX, and poly P in cell lysates

Strain	PPK, units/mg protein		PPX, units/mg protein		Poly P, nmol/mg protein
	Membrane	Soluble	Membrane	Soluble	
S. flexneri					
WT	3,520	<50	2,950	<50	21
Mutant	90	<10	<10	<10	<0.1
S. typhimurium FIRN					
WT	5,889	488	7,050	200	25
Mutant (SF11687)	<60	<10	213	200	<0.1
S. dublin					
WT	5,530	<50	4,750	<100	15
Mutant	<100	<10	120	<10	<0.2
*E. coli**					
WT	1,800	120	1,610	<40	25
Mutant	<50	<10	<30	<10	<0.1

Values are the averages of three independent experiments.
*Data calculated from Crooke *et al.* (4).

Biochemical Assays. PPK and exopolyphosphatase (PPX) activities were assayed as described (24, 25). Poly P levels were determined by the nonradioactive method (26).

Survival. *Survival of stationary-phase cells.* See legend to Fig. 3.

Heat-shock survival. See legend to Fig. 4.

Acid tolerance. Stationary-phase cells grown overnight (≈20 h) in LB were washed in saline, resuspended in acidified LB (pH 3.0 for *S. flexneri* and 3.3 for *Salmonella* spp.), and incubated aerobically at 37°C; cultures were diluted and plated on LB to measure viability.

Polymyxin B resistance. See legend to Fig. 5 for details.

Surface attachment. As described (8), 96-well assay plates of polyvinyl chloride (Falcon 3911 Microtester III flexible) were from Becton Dickinson.

Gentamycin-protection assays. See legend of Fig. 6.

Nucleotide Sequence Accession Number. The GenBank accession number for the sequence of *S. typhimurium* WRAY reported in this study is AF085682.

Results

PPK, PPX, and poly P in WT and Mutant Pathogens. Levels of PPK, PPX, and poly P in the enteric pathogens *S. flexneri*, *S. typhimurium*, and *S. dublin* were similar to those observed for *E. coli* (4) (Table 2). PPK of the pathogens was localized, as in *E. coli*, in the membrane fraction but at 2 to 3 times the level. PPX downstream in the *ppk* operon resembled *E. coli* PPK in levels and membrane localization. The null *ppk* mutants of all these enteric strains exhibited barely detectable levels of PPK, PPX, and poly P (Table 2).

Growth in Rich Media. Of the several enterics, only the *S. flexneri ppk* mutants show a defect in growth and short-term loss of survival (Fig. 1). By contrast, the *Salmonella* spp. *ppk* mutants showed relatively little loss of viability even after 2 days of incubation, but as with *E. coli* (3), suffered profound losses in survival after many days of incubation (see below).

In LB medium, *S. flexneri* grew to an OD_{540} of 4 and a cell density of 4×10^9 colony-forming units (cfu)/ml. Unlike WT, the *ppk* mutant, after a normal rate of growth for 3 h, declined in the next 2 h to an OD_{540} of 0.3 and a cell density of 8×10^6 cfu/ml. Thereafter, the mutant cultures recovered to near WT levels by about 16 h. The increase in mutant cell density after the abrupt drop coincided with appearance of a small-colony phenotype; these variants represented about 60% of the number of typical large WT colonies present in overnight (≈16-h) cultures.

The *ppk* mutant of *S. flexneri* also differed from WT; when the cells were grown aerobically in nutrient broth (Difco) the growth rate was significantly slower up to 6 h, and a lower cell density was reached after 16 h of growth. Remarkably, mutants grown in either LB or nutrient broth aggregated and settled out within a few minutes, unlike WT, which remained uniformly dispersed in the growth medium (data not shown).

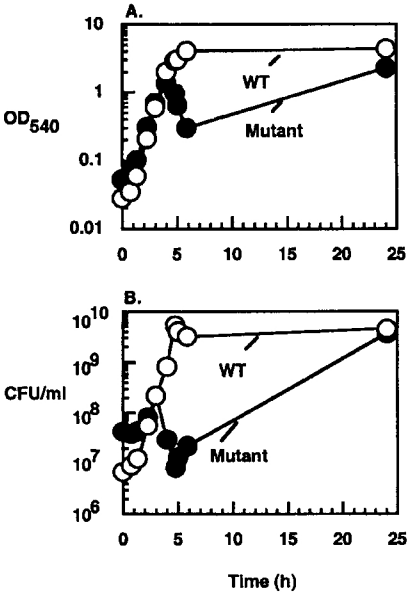

Fig. 1. Growth studies. WT and mutant *S. flexneri* were grown aerobically at 37°C in LB medium. Samples were analyzed for growth (OD_{540}) and viability (cfu/ml).

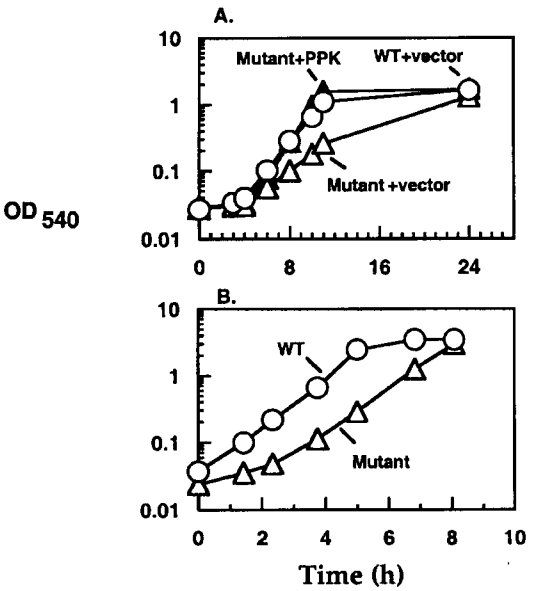

Fig. 2. Growth after downshift. (*A*) *S. typhimurium* WT complemented with vector, and mutant with either vector or vector containing *ppk* gene, were grown aerobically at 37°C in LB for about 20 h. The cultures were then diluted (1:100) into prewarmed (37°C) Mops-buffered minimal medium with 2 mM P_i (26) and incubated aerobically at 37°C; growth was monitored by OD_{540}. (*B*) The *S. dublin* WT and mutant diluted in Mops medium in a similar way were compared.

Table 3. Survival of *S. flexneri* and *Salmonella* spp. in acidic medium

Strain	cfu/ml		Survival, %
	Before	After	
S. flexneri			
WT	1.98×10^9	2.38×10^5	0.012
Mutant	1.85×10^9	0.04×10^5	0.0002
S. typhimurium			
WT	2.7×10^9	0.26×10^9	9.6
Mutant	3.4×10^9	0.12×10^9	3.5
S. dublin			
WT	1.4×10^9	0.83×10^9	59.0
Mutant	2.65×10^9	0.27×10^9	10.0

Stationary-phase cells (20 h in LB medium) of *S. flexneri* were incubated aerobically at 37°C for 1 h in LB medium adjusted to pH 3.0 with HCl. After incubation, cultures were diluted and plated to measure viability. In the case of *Salmonella* spp., the stationary-phase cells were incubated for 1.5 h in LB medium adjusted to pH 3.3. Values are the averages of three independent experiments.

Growth in Minimal Media. PPK mutants of *Salmonella* spp. could be adapted to grow in Mops-buffered minimal medium (27), but the *ppk* mutant of *S. flexneri* grew poorly. Supplementation of the medium with amino acids restored the growth of the mutant to WT levels (data not shown).

Growth After Nutrient Downshift. *S. typhimurium* WT grown in LB medium and diluted (1:100) into Mops-buffered minimal medium suffered a lag of about 4 h before the start of exponential growth with a generation time of 76 min. After a similar lag, the *ppk* mutant grew more slowly, with a generation time of about 120 min (Fig. 2*A*). WT growth was restored when the mutant was complemented with a plasmid containing *ppk*. *S. dublin* WT subjected to a similar nutrient downshift grew with no lag and a doubling time of 60 min; the *ppk* mutant grew slowly to about 3 h before the onset of WT exponential growth (Fig. 2*B*).

Effect of pH on Growth. Many bacteria endure transient encounters with very low or high pH, well outside the growth range (13, 14). The Enterobacteriaceae must cope with low-pH stress during their passage through the stomach to the intestine (29, 30). Growth of WT and the *ppk* mutant were compared in LB medium supplemented with 0.4% glucose and adjusted to pH values ranging from 3.0 to 8.0. *S. flexneri* WT cells grew well across a broad pH range of 5 to 8 and even at pH 4.3 after a prolonged lag period; the *ppk* mutant also grew well from pH 5 to 8 but failed to grow at all at pH 4.3; neither WT nor mutant cells could grow at pH 3.3 (data not shown).

The WT and mutant of *S. typhimurium* did not differ in growth at pH values between 5.0 and 8; the density of the WT was about 2-fold higher after 7 h of growth between pH 5.0 and 7.0. Both WT and mutant did not grow at pH 3.0; growth of the WT was better than the mutant at pH 4.0; WT reached a density of 0.8 after 7 h compared with 0.3 for the mutant. The growth rate of both WT and mutant *S. dublin*, like *S. typhimurium*, did not differ at pH values between 5.0 and 8.0; but the density of the WT was about 3-fold higher after 7 h at pH 5.0–7.0. Both WT and mutant did not grow at pH 3.0. Growth of the WT and the mutant at pH 4.0 commenced after a 4-h lag, but no significant difference occurred in the growth curves (data not shown).

Virulence Factors. *Acid tolerance.* Stationary-phase cells grown in rich medium were exposed to pH 3.0 (for 1 h at 37°C). Survival of WT *S. flexneri* was reduced by 10^4-fold, but was still 50-fold better than that of the mutant (Table 3). Unlike *S. flexneri*, less difference occurred in acid tolerance in *Salmonella* spp. between WT and

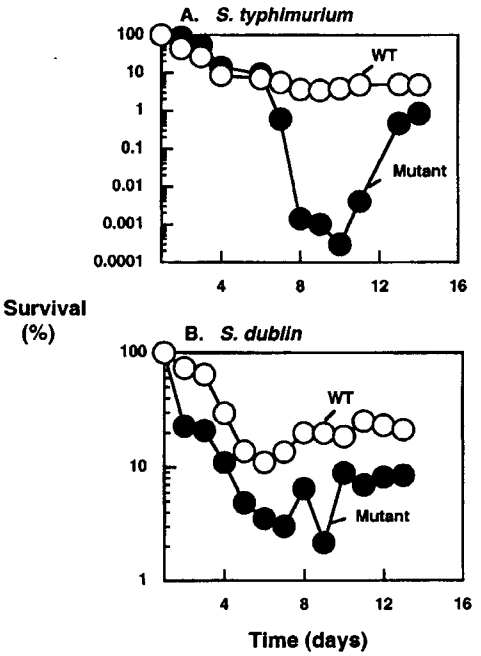

Fig. 3. Long-term survival. WT and mutants of *S. typhimurium* (*A*) and *S. dublin* (*B*) were tested. Long-term survival in LB was assayed as described (3).

mutants. The *S. typhimurium* mutant showed a 3-fold lesser acid tolerance than WT; the *S. dublin* mutant showed a 6-fold lower acid tolerance (Table 3).

Long-term survival. In LB medium, survival of the *S. typhimurium* mutant declined to about 0.001% after 8 days of incubation and still further after 10 days, whereas the WT remained at about 6% of the initial value (Fig. 3). At 10 days, the colony size of the mutant was like that of the WT, but in the next 4 days the culture grew back to about 1% of the initial number with two-thirds of the colonies being variants of small size. When stationary-phase cultures of the WT and mutant were present at a 1:1 ratio, the survival of the mutant increased to about 20% of the initial value until 8 days; thereafter, viability of both WT and mutant in the mixed culture dropped drastically to about 0.001% (data not shown).

The *S. dublin* mutant showed only a modest loss of viability to about 3% after 7 days of incubation, whereas the WT remained at about 20% of the initial value. When stationary-phase cultures of the WT and mutant were present at a 1:1 ratio, the viability of the mutant decreased rapidly to about 0.2% of the initial value after 3 days of incubation compared with 20% for the WT. Thereafter, the mutant population decreased another 10-fold after 9 days, whereas the WT remained near the same level (data not shown).

Heat resistance. On entry into stationary phase, the *E. coli* WT develops a tolerance to heat at 55–57°C (3). Stationary-phase WT *S. flexneri* exposed to 55°C for 3 min retained 70% of their viability compared with less than 0.5% for the mutant (Fig. 4*A*). *S. typhimurium* WT survival after 2 min was 30% compared with less than 4% for the mutant (Fig. 4*B*). The heat resistance of *S. dublin* was relatively greater than that of *S. typhimurium*; little loss of viability occurred after 4 min and, even after 10 min, 9% of the WT survived compared with 2% for the mutant (Fig. 4*C*).

Polymyxin B resistance. Survival of mutant *S. typhimurium* in the stationary phase in polymyxin B at 10 μg/ml was 65% compared with 100% for WT; at 20 μg/ml only 15% of the mutant survived compared with 65% for WT (N.N.R. and A.K., unpublished data). In a repetition of this experiment with a wider range of polymyxin B levels, the resistance of the mutant was again significantly less

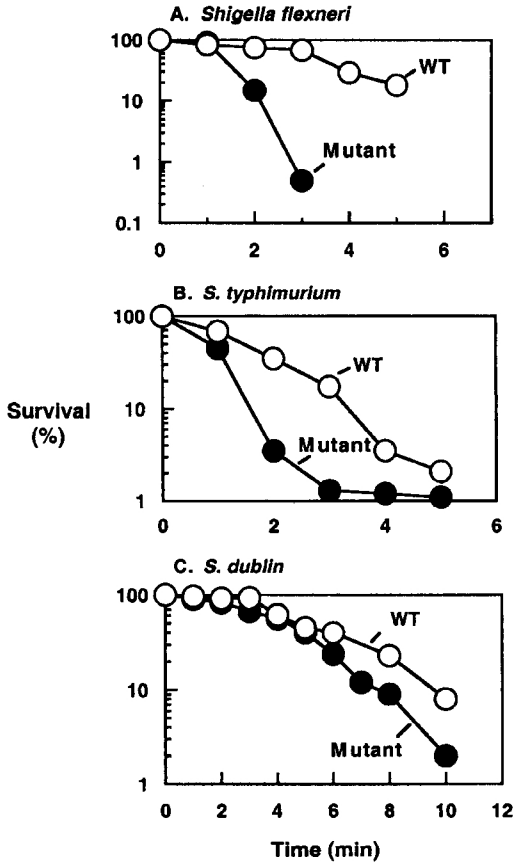

Fig. 4. Heat-shock survival. WT and mutants of *Salmonella* spp. and *S. flexneri* were grown overnight (≈16 h) in LB. The stationary-phase cells were washed and diluted in 0.9% NaCl to a cell density of about 5×10^3 per ml. Samples (2 ml) in glass tubes were prewarmed to 55°C and 0.1-ml samples were plated directly on LB for viable cell numbers.

than that of the WT and could be restored to WT levels by complementation with *ppk* (Fig. 5). Similar results were obtained with *S. dublin* (data not shown).

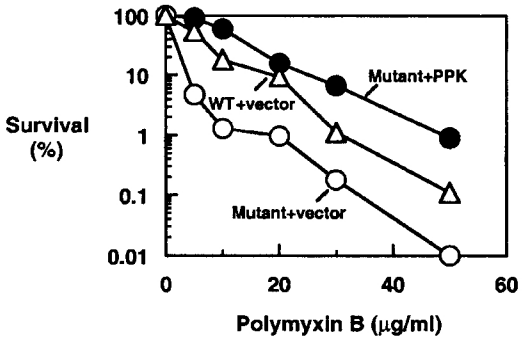

Fig. 5. Polymyxin B resistance. In *ppk* mutant of *S. typhimurium*, resistance is restored after complementation with PPK. For induction of PPK, cells were grown to an OD_{540} of 0.8 in LB to which 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added and incubated further aerobically at 37°C for 3 h. Cells were washed, diluted in sterile 0.85% saline to about 5×10^3 cells per ml, and exposed for 1 h at 37°C to various concentrations of polymyxin B. Survivors were measured by plating on LB.

Motility and surface attachment. The mutants of *Salmonella* spp. were impaired in their swimming motility as observed with *E. coli* and other bacteria (6). WT *S. flexneri* is nonmotile. With regard to adherence to an abiotic surface, predictive of a capacity to form biofilms, the *Salmonella* spp. mutants showed a 20–35% decrease relative to WT in their adherence to polystyrene (data not shown).

Invasion and growth in epithelial cells and survival in macrophages. The *S. typhimurium* mutant was only half as invasive in HEp-2 epithelial cells measured at 2 h and was still at half the WT level after 24 h (Fig. 6*A*), as measured by a gentamycin-protection assay. Survival in the macrophage RAW 264.7 was unaffected for the WT, but the mutant declined progressively when assayed after 4 and 24 h (Fig. 6*B*).

Discussion

The purpose of this study was to confirm and extend the hypothesis that poly P and PPK, the enzyme that makes it, are needed for virulence in the important enteric pathogens *S. flexneri*, *S. typhimurium*, and *S. dublin*. The hypothesis was based on several facts: (*i*) poly P participates in activation of stationary-

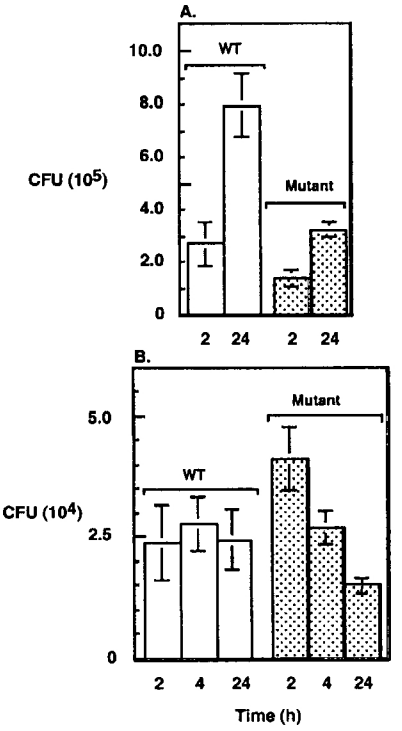

Fig. 6. Invasion and growth in epithelial cells and survival in macrophages. The gentamycin-protection assays were as described (28). (*B*) WT and mutant of *S. typhimurium* were grown overnight in LB to stationary phase. The cells were diluted in PBS and opsonized for 30 min in PBS containing 20% normal mouse serum. They were added to 2.5×10^5 macrophage RAW 264.7 cells (ATCC TIB-71) seeded in 24-well tissue culture plates at a multiplicity of infection of 10:1 and incubated at 37°C for 30 min. Infected monolayers were then treated with gentamycin (100 μg/ml) for 90 min and were lysed or further incubated for 4 and 24 h in the presence of 10 μg/ml gentamycin. Infected cells were then washed twice with PBS and lysed with 1% Triton X-100 in PBS and plated on LB for measurement of cfu. (*A*) HEp-2 (ATCC CCL-23) epithelial cells were maintained in RPMI medium 1640 supplemented with 10% FCS. Gentamycin protection assays were as described above except that WT and mutant *S. typhimurium* were grown under an invasive condition [overnight in LB static culture, diluted 1:50 in LB-high-salt (0.3 M NaCl) medium followed by static incubation at 37°C for 4.5 h]. Bacteria were inoculated into 8×10^4 HEp-2 cells seeded in 24-well tissue-culture plates at a multiplicity of infection of 10:1 for 30 min.

phase responses and starvation in *E. coli* and so is essential for its survival (2, 3); (*ii*) virulence factors of many pathogens are expressed in stationary phase (1, 4, 31); (*iii*) the *ppk* gene is highly conserved among these and many other pathogens (2, 5); and (*iv*) virulence in mice of *P. aeruginosa* (7) and virulence factors of *Neisseria meningitidis* (32), *V. cholerae* (8), and *Helicobacter pylori* (C.D.F., C.-M. Tzeng, and A.K., unpublished results) all depend on the intact *ppk* gene. An additional incentive in this study was to widen the range of comparisons and relationships in the enzymology, metabolism, genetics, and physiology of poly P.

Organization of the *ppk* operon and the levels and membrane location of PPK were similar in the three enteric pathogens and resembled those of *E. coli* (Table 2). Although knockout of *ppk* reduced PPK and poly P to levels below detection, it should be noted that another PPK activity (PPK2) has been discovered in *ppk* mutants of *P. aeruginosa* that was not detected by the standard assays for PPK; this pathway is likely responsible for significant accumulation of poly P in the *ppk* mutants of this organism (H. Zhang and A.K., unpublished results).

Of the three enteric pathogens, only *S. flexneri* showed a strong defect in the extent of growth in both rich and minimal media (Fig. 1), which was accompanied by a profound loss in viability in a few hours. By contrast, the *Salmonella* spp. showed only a modest diminution of growth rate after a nutrient downshift (Fig. 2) and lost viability only after many days (Fig. 3). With the loss of viability, the large WT colony type was succeeded by the emergence of a small-colony variant, much as had been observed with *E. coli* (3). When mutant and WT *Salmonella* spp. were cultured together in a 1:1 ratio, both the WT and mutant *S. typhimurium* lost viability, whereas under similar conditions the *S. dublin* WT was spared. The basis for these results remains to be studied.

The phenotypes of the enteric pathogens include defects in several factors (Table 4), some of which have been related to virulence. These virulence factors include diminished capacity to withstand low pH or elevated temperature or polymyxin B, impairment in motility, attachment to an abiotic surface, and invasiveness in eukaryotic cells in culture. On the basis of these *in vitro* criteria of virulence, tests of the mutants in an animal host are now clearly indicated.

Cloning of the *ppk* gene of the three enteric pathogens makes their overexpression feasible, as well as making their PPKs available for structural and functional studies. These should complement the comparative studies of the PPKs of *E. coli*, *P. aeruginosa*, *V. cholerae*, and *H. pylori*, which have revealed striking differences in their kinetics and specificities (5).

Table 4. Defects in PPK mutants

Assay	Defect relative to WT			
	S. flexneri	*S. typhimurium*	*S. dublin*	*E. coli*
Loss of PPK	+++	+−+	+++	+++
Loss of PPX	+++	+−+	+++	+++
Lack of poly P	+++	+−+	+++	+++
Growth in LB	+++	−	+	+
Loss of survival				
Short-term	+++	−	+	+
Long-term	NA	+−+	+++	+++
Growth at low pH	+++	+−	+−	ND
Resistance to				
Acid	+−+	−	+−	ND
Heat	+−+	+−	+	+−+
Polymyxin B resistance	ND	+−	+−	ND
Surface attachment	ND	−	+−	+−
Epithelial cell invasion	ND	+−	ND	ND
Survival in macrophage	ND	+−	ND	ND
Motility	NA	+−+	+++	+++

Defect has been graded from high (+++) to low (+), based on data presented in *Results*. NA, not applicable; *S. flexneri* loses viability in the short term, and the WT is nonmotile. ND, not determined.

PPK is attractive as a target for antibiotics, because the absence of any similar enzyme in higher eukaryotic species makes toxicity less likely. Large-scale screening for inhibitors of *E. coli* and *P. aeruginosa* PPKs have produced candidates, unique among known kinases, and active at low concentration (S. Lee, ICOS Corp., Bothell, WA, personal communication). Such compounds may prove useful not only as drugs but also as reagents for studies in which prompt inhibition of PPK can be achieved and for which conditional mutants are not yet available.

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