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Contribution of Proton-Translocating Proteins to the Virulence of *Salmonella enterica* Serovars Typhimurium, Gallinarum, and Dublin in Chickens and Mice

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We investigated the attenuating effects of a range of respiratory chain mutations in three *Salmonella* serovars which might be used in the development of live vaccines. We tested mutations in *nuoG*, *cydA*, *cyoA*, *atpB*, and *atpH* in three serovars of *Salmonella enterica:* Typhimurium, Dublin, and Gallinarum. All three serovars were assessed for attenuation in their relevant virulence assays of typhoid-like infections. Serovar Typhimurium was assessed in 1-day-old chickens and the mouse. Serovar Gallinarum 9 was assessed in 3-week-old chickens, and serovar Dublin was assessed in 6-week-old mice. Our data show variation in attenuation for the *nuoG*, *cydA*, and *cyoA* mutations within the different serovar-host combinations. However, mutations in *atpB* and *atpH* were highly attenuating for all three serovars in the various virulence assays. Further investigation of the mutations in the *atp* operon showed that the bacteria were less invasive in vivo, showing reduced in vitro survival within phagocytic cells and reduced acid tolerance. We present data showing that this reduced acid tolerance is due to an inability to adapt to conditions rather than a general sensitivity to reduced pH. The data support the targeting of respiratory components for the production of live vaccines and suggest that mutations in the *atp* operon provide suitable candidates for broad-spectrum attenuation of a range of *Salmonella* serovars.

Many serotypes of *Salmonella enterica* subsp. *enterica* have been identified, mainly as a result of isolation from humans and animals, where infection often results in diarrhea with the pathogen remaining localized in the alimentary tract. However, some serotypes typically produce typhoid-like infections in a few host species. These include serovar Typhi and serovar Paratyphi, which cause typhoid and paratyphoid in humans, and serovar Gallinarum, which causes fowl typhoid, while similar diseases are caused by serovar Choleraesuis in pigs, serovar Dublin in cattle, and serovars Abortusovis and Abortusequi in sheep and horses, respectively (49, 65). A number of these serotypes, including serovars Choleraesuis, Dublin, Abortusovis, Typhimurium, and Enteritidis, also produce typical typhoid in mice (44, 49). Infection is normally by the oral route, and for the serotypes that produce typhoid-like disease, the salmonellae invade quickly from the alimentary tract and soon afterwards can be detected in the gut-associated lymphoid tissue and later in the liver and spleen. The infection process involves invasion of host cells, and the ability to multiply within and kill particular subsets of macrophages is considered to be important for virulence (39, 40, 51).

Much *Salmonella* research has focused on serovar Typhimurium infection in mice, which provides a convenient model, although chickens (5, 33) and calves (60) have provided alternative, more relevant hosts for serovars Typhimurium, Gallinarum, and Dublin. Work with all these different models has identified many *Salmonella* genes required for virulence and particularly for intracellular survival and multiplication, many of which are also regarded as "housekeeping genes." They include genes associated with environmental sensing and transcriptional regulation, such as *cya*/*crp*, *ompR*/*envZ*, *phoP*/*phoQ* (11, 19, 47), *rpoS* (21), and *rpoE* (31) and genes that influence DNA supercoiling, including *hns* (28) and *hupA* (57). Bacterial stress induced by the intracellular environment is indicated by the requirement for genes such as *htrA*, *clpB*, and *dksA* (12, 57, 63). Nutrient biosynthesis genes, including those for purines, pyrimidines, and aromatic amino acids, are also required for full virulence (22, 30). More recently, mutations in glutamine biosynthesis and transport have been found to be attenuating for serovar Typhimurium in mice (36). Other genes involved in the uptake of Fe^{2+}, Mg^{2+}, and Cu^{2+} have also been found to be associated with virulence in *Salmonella* and other bacteria (29, 61).

Bacteria can derive energy from carbon sources by respiration which involves electron transport and an electron acceptor, such as oxygen, or by fermentation. Respiration in *Escherichia coli* and *Salmonella enterica* has been reviewed by Gennis and Stewart (25). NADH is an important primary electron donor for electron transport. For *E. coli* and other bacteria, electrons are transferred from NADH to the NADH dehydrogenase I complex in the cytoplasmic membrane and then to quinones. Electrons are then transferred to a penultimate electron acceptor, such as the two cytochrome oxidases *o* and *d*, before being transferred to a terminal electron acceptor, such as oxygen. Electron transport is coupled to the transfer of

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protons out of the cell across the cytoplasmic membrane, generating a proton gradient (27). Bacteria can use substrates other than NADH, such as succinate, pyruvate, or lactate, as primary electron donors and a number of alternative electron acceptors, such as dimethyl sulfoxide, fumarate, and nitrate, in addition to oxygen. *E. coli*, *Salmonella*, and other bacteria possess two cytochrome oxidases, *d* and *o* (25). The cytochrome *d* oxidase is active under low oxygen tensions but is less energy efficient, transporting only one proton per electron compared to two transported by cytochrome *o* oxidase under higher oxygen tensions. The proton gradient generated by electron transport is used by the F_0F_1 proton-translocating ATPase for ATP synthesis, flagella rotation, and nutrient uptake (10, 45). During fermentative growth, the bacterial F_0F_1 proton-translocating ATPase hydrolyzes ATP to generate the proton gradient. The structure and function of the F_0F_1 ATPase of *E. coli* has been reviewed (27, 45).

Despite the extensive understanding of bacterial electron transport and proton translocation, little is known about the relative contribution of the relevant proton-translocating enzymes to the growth and survival of pathogens in the host in either the gut or the intracellular environment. We have sought to initiate such studies by determining the involvement of some of these protein complexes in the virulence of serovars Typhimurium and Gallinarum in chickens and of serovar Dublin in mice. We mutated genes crucially affecting enzyme activity, including *nuoG*, which codes for a key polypeptide of the NADH dehydrogenase I complex (2, 20), *cyoA* and *cydA*, which code for subunits of cytochrome *o* and cytochrome *d* oxidases, respectively (25), and *atpB* and *atpH*, which code for polypeptides of the F_0 and F_1 components, respectively, of the F_0F_1 proton-translocating ATPase (27).

MATERIALS AND METHODS

Bacterial strains and culture media. The bacterial strains and mutants used in this study are shown in Table 1. Serovar Typhimurium C5 is virulent for very young chickens and mice (32). Serovar Typhimurium F98 is highly virulent for newly hatched chickens and has been studied previously (5, 57). Serovar Gallinarum 9 produces a typical systemic fowl typhoid with high mortality (53). Serovar *S*. Dublin 2229 was isolated from a diseased calf, and its virulence for mice has been well characterized (4). For ease of enumeration in the alimentary tract, spontaneous nalidixic acid-resistant mutant derivatives of these strains were used which were produced by selection on Luria-Bertani (LB) agar containing sodium nalidixate (50 μg/ml). This has been found previously not to affect their virulence (54). An F-prototrophic *E. coli* K12 strain (proto) (6) was used as a noninvasive control strain.

Unless otherwise stated, broth cultures consisted of 10-ml volumes of LB broth (Difco) incubated for 24 h at 37°C in a Gallenkamp shaking incubator (150 rpm; Gallenkamp, Scientific Supplies, Nottingham, United Kingdom). On LB agar these contained between 1×10^9 and 3×10^9 CFU ml^{-1}.

Construction of mutants. Mutations in serovars Typhimurium, Gallinarum, and Dublin were produced in a similar way. The DNA homologies were such as to permit use of the same inactivated serovar Typhimurium sequences in serovars Gallinarum and Dublin. Construction of the *nuoG* and *atp* defined mutations has been described elsewhere (67). These mutations are polar and were constructed by replacement of several hundred nucleotides of the relevant open reading frame (ORF) with a Km^r GenBlock insertion cassette (Amersham-Pharmacia Biotech). These were intended to block the function of each protein complex rather than the individual gene, and polarity within each operon was, therefore, of lesser concern. The *nuoG* gene is the sixth gene in a single large, complex operon of 14 genes (*nuoA* to *nuoN*). Mutations in this gene, which encodes a structural-regulatory subunit, have been shown to abolish activity of the whole NADH dehydrogenase I complex (2). Both the F_0 and F_1 subunits of the proton pump would be inactivated by the polar mutation in *atpB*, whereas the *atpH* mutation prevents biosynthesis of the F_1 subunit only. However, since the F_0 subunit pore opens only upon interaction with the F_1 subunit, these mutants ought to behave similarly (27).

TABLE 1. Bacterial strains used in this study

Strain (mutant gene)	Reference or source
Serovar Typhimurium C5	32
Serovar Typhimurium C5 (*nuoG*)	67, this paper
Serovar Typhimurium C5 (*cydA*)	67, this paper
Serovar Typhimurium C5 (*cyoA*)	This paper
Serovar Typhimurium C5 (*atpB*)	67, this paper
Serovar Typhimurium C5 (*atpH*)	This paper
Serovar Typhimurium F98	5, 57
Serovar Typhimurium F98 (*nuoG*)	67
Serovar Typhimurium F98 (*cydA*)	67
Serovar Typhimurium F98 (*cyoA*)	This paper
Serovar Typhimurium F98 (*atpB*)	67
Serovar Typhimurium F98 (*atpH*)	67
Serovar Gallinarum 9	53
Serovar Gallinarum 9 (*nuoG*)	66
Serovar Gallinarum 9 (*cydA*)	This paper
Serovar Gallinarum 9 (*cyoA*)	This paper
Serovar Gallinarum 9 (*atpB*)	This paper
Serovar Gallinarum 9 (*atpH*)	This paper
Serovar Dublin 2229	4
Serovar Dublin 2229 (*nuoG*)	This paper
Serovar Dublin 2229 (*cydA*)	This paper
Serovar Dublin 2229 (*cyoA*)	This paper
Serovar Dublin 2229 (*atpB*)	This paper
E. coli K12 proto	6
E. coli K12 SM10λ*pir*	52

The *cyoA* mutation was constructed by PCR amplification from the serovar Typhimurium chromosome. Oligonucleotide primers were chosen that allowed a fragment to be amplified in two parts before being joined together by an additional overlap extension PCR using the same two parts as a template. This allowed the introduction of a *Kpn*I site in the middle of the fragment and a *Sal*I and *Xba*I site at each end. The construct was then incorporated into the suicide vector pDM4 (47), and the Km^r GenBlock insertion mutant was introduced into the *Kpn*I site. The construct thus consisted of a fragment from between bases 108 and 728 of the *cyoA* ORF with the Km^r GenBlock inserted after base 438.

An Spc^r-Str^r (spectinomycin and streptomycin resistance) insertion was made in the *cydA* gene in the same way, but this time the construct included bases 87 to 725 inclusive of the ORF. The cassette was in pHP45ΩSpc, obtained from H. Krisch, Département de Biologie Moleculaire, Université de Genèva, Switzerland. A single base-pair change generated a *Bam*HI site in the middle of the fragment that enabled an Spc-Str resistance cassette to be inserted after base 406 of the ORF, and *Xba*I sites were incorporated into each end of the fragment for cloning into pDM4.

These pDM4 derivatives were maintained in *E. coli* strain SM10λ*pir* (Table 1) (52) and were introduced into the recipient *Salmonella* strains by conjugation. Transconjugants were isolated on selective medium supplemented with either streptomycin or kanamycin (25 μg/ml), and their sensitivity to chloramphenicol was then tested to identify those that resulted from a double recombinational crossover event that had not incorporated any pDM4 DNA. The mutants were checked by PCR using primers from the 3′ end of the cassette and the 5′ end of the structural gene which generated a single DNA fragment in each of the mutants but not in the parent strain.

The phenotypes of these mutations were also checked. In all four operons, *nuoA to nuoN*, *cydA and cydB*, *cyoA to cyoE*, and *atpA to atpI*, no other genes are present which are associated with functions other than that of these genes. The *nuoG* mutation in serovar Typhimurium abolished NADH dehydrogenase activity (67). Under aerobic conditions the *cyd* and *atp* mutants grew more slowly than the parent strain on LB agar and minimal salts agar with glucose as the sole carbon source, producing much smaller colonies (18). Under anaerobic conditions, with glucose as the carbon source and no exogenous electron acceptor, very

little growth of the *atp* mutants occurred and they were nonmotile (27). Motility and an increase in growth yield were obtained by the addition of nitrate to these anaerobic cultures, suggesting that these mutants were able to generate a proton gradient in the presence of this oxidizing agent (27). The *atp* mutants also grew poorly with citrate as the sole carbon source (45).

All mutants were checked for nonagglutination with 5% acriflavine-HCl to confirm that their lipopolysaccharide remained intact.

Experimental animals. For virulence studies, unsexed, specified-pathogen-free Rhode Island Red chickens were used from the Institute for Animal Health flock. These are susceptible to systemic salmonellosis. They were housed in metal cages held at 30°C at 1 day of age, decreasing to 20°C at 3 weeks of age. They were reared on a vegetable-based protein diet (Special Diet Services, Manea, United Kingdom). Chickens were used either within 24 h of hatching (referred to as newly hatched) or as 3-week-old birds. Mice were BALB/c females (Charles River Laboratories, Manston, United Kingdom) weighing 20 g (approximately 6 weeks old).

Virulence assays. Virulence was assessed by oral inoculation of groups of animals with 0.1 ml (newly hatched chickens) or 0.3 ml (3-week-old chickens) of an undiluted culture or with 50 μl of a culture concentrated 10-fold by centrifugation (5, 66). Virulence in mice was assessed by oral inoculation with 50-μl volumes of a broth culture diluted to contain 10^4 CFU in this volume (11, 12). Morbidity and mortality were recorded over a 3-week period. Animals showing signs typical of salmonellosis were killed humanely. Signs in chickens included anorexia and disinclination to drink, standing with head and wings lowered, and caked feces around the vent. Mice became unsteady and had a "starry" coat. These signs are generally predictive of severe disease and death if the animals are left.

Intestinal invasiveness of serovar Typhimurium mutants in newly hatched chickens was assessed by counting viable bacteria in the ceca, spleen, and liver at intervals after oral inoculation (5). This was done because chickens have no lymph nodes.

Survival and multiplication in the organs were assessed by counting viable bacteria in organ samples at 1-h and longer intervals after intramuscular (gastrocnemius muscle) inoculation of 2-day-old chickens with 10^4 CFU (5).

Bacteria were counted on Brilliant Green agar (CM 263; Oxoid, Basingstoke, United Kingdom) containing sodium nalidixate (20 μg/ml) and novobiocin (1 μg/ml).

Invasion and persistence of *Salmonella* in primary chicken kidney cells. Chick primary kidney cells (CKC) were prepared from the kidneys of 1- to 2-week-old Rhode Island Red chicks as previously described (6). Bacterial cultures were diluted in LB to10^8/ml, and 100 μl was added to the CKC to give a multiplicity of infection of 10 bacteria per chicken kidney cell. Cells were incubated for 1 h at 37°C in an atmosphere including 5% CO_2. Extracellular bacteria were then killed by incubating cells for 1 h at 37°C in Dulbecco's modified Eagle medium (DMEM) containing 100 μg of gentamicin (Sigma)/ml. To determine bacterial invasion, infected CKCs were washed three times in Hanks' buffered saline solution (HBSS) and then lysed with 1 ml of 1% Triton X-100 (Sigma) for 30 min at 37°C. Viable counts of the intracellular bacteria in the lysate were made on LB agar. To determine intracellular persistence, cells were washed three times in HBSS and then incubated in RPMI 1640 containing 25 μg of gentamicin/ml to prevent growth of any bacteria released from lysed cells. Cells were incubated at 37°C in an atmosphere including 5% CO_2 for up to 24 h and then lysed, and counts were determined as described above.

Invasion and persistence of mutants of serovar Typhimurium in chick splenic macrophages. Splenic macrophages were isolated from 3-day-old Rhode Island Red chicks through a modification of the methods of Wigley et al. (64). Briefly, chicks were killed by neck dislocation, and the spleens were removed aseptically and placed in 2 ml of ice-cold DMEM. A cell homogenate was formed by passing the tissue through a 70-μM-pore-size cell strainer (Becton Dickinson). The cell homogenate was seeded at 5×10^6 cells ml in 100-μl volumes in a 96-well cell culture tray. Cells were incubated at 42°C under 5% CO_2 for 4 h to allow adherence of macrophages. Cells were then washed vigorously four times with DMEM to remove nonadherent cells. This resulted in a monolayer of 90 to 95% macrophages at approximately 5×10^4/ml. Invasion and persistence of the serovar Typhimurium parent strain and *cydA, cyoA, nuoG*, and *atpB* mutant strains in chick macrophages was determined at a multiplicity of infection of 10. Extracellular bacteria were killed by incubating cells for 1 h at 37°C in DMEM containing 100 μg of gentamicin (Sigma)/ml. To determine the extent of bacterial invasion, infected monolayers were washed three times in HBSS and then lysed with 1 ml of 1% Triton X-100 (Sigma) for 30 min at 37°C. Viable counts of the intracellular bacteria in the lysate were made on LB agar. To determine intracellular persistence, cells were washed three times in RPMI 1640 and then incubated in RPMI 1640 containing 25 μg of gentamicin/ml to prevent growth of any bacteria released from lysed cells. Cells were then incubated at 37°C in an atmosphere including 5% CO_2 for up to 24 h and then lysed, and counts were determined as described above. Since numbers of macrophages isolated from chicks are low, we determined persistence at 4 and 24 h postinvasion only.

Invasion and persistence of mutants of serovar Typhimurium in the mouse macrophage-like cell line, J774A.1. This was performed as described by Bowe and Heffron (9). J774A.1 cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, United Kingdom) and were grown as monolayers in 12-well cell culture trays using DMEM containing 10% fetal calf serum and 1 M glutamine (DMEM+). Bacterial cells for the assay were grown statically at 37°C overnight, harvested, resuspended in 20× volume normal mouse serum, and incubated at 37°C for 30 min. Antibiotic-free DMEM+ was then added to the bacterial cells before inoculating them onto the macrophages at a multiplicity of infection of 100. The bacteria were centrifuged onto the macrophage layer at $1{,}100 \times g$ for 5 min, and the plates were incubated for 1 h at 37°C in an atmosphere including 5% CO_2. Invasion and persistence were determined as described above.

Measurement of RpoS expression. Two 20-bp primers were used to amplify a fragment of 982 bp from position of 120 bp within the *rpoS* start sequence to 862 bp upstream of the start codon, within the *nlpD* gene and containing the known promoter site (37). This was first ligated into a TOPO vector, pR2.1 (Invitrogen, Paisley, United Kingdom) before being excised and ligated into the *lacZYA* reporter plasmid, pRW50 (42), and the construct was electroporated (Gene Pulser; Bio-Rad, London, United Kingdom) into serovar Typhimurium F98 and the *nuoG*, *cydA*, and *atpB* mutants. The cultures were purified and cultured in LB broth containing 10 μg of tetracycline/ml with shaking. Viable counts and optical density were measured regularly, and β-galactosidase activity was determined at 4 and 12 h postinoculation using standard procedures (46).

Measurement of acid tolerance response. A standard assay was used (7). Strains to be tested were grown overnight at 37°C with shaking in minimal E medium (minE), pH 7.6, supplemented with 0.04% glucose. The glucose concentration was increased to 0.2%, and the bacteria were grown for a further 3 h with shaking. Bacteria were diluted 1 in 10 in minE, pH 5.6, for adaptation to acidic conditions or pH 7.6 for unadapted control cells and incubated for 3 h at 37°C with shaking. Cultures were then diluted 1 in 100 in minE, pH 3.3, and returned to 37°C. At specified time intervals, samples were removed for determination of viable counts. Each mutant was tested in triplicate. Data are expressed as a percentage of viable cells compared with those prior to transfer to the pH 3.3 medium.

RESULTS

Mutations in the *nuo*, *cyd*, *cyo*, and *atp* operons attenuate some *Salmonella* serotypes. The virulence of serovar Typhimurium strain C5 and serovar Gallinarum strain 9, together with their mutants, was assessed in newly hatched or 3-week-old chickens, and that of C5 and serovar Dublin 2229 and its mutants was assessed in mice (Table 2).

Following oral inoculation with the virulent serovar Typhimurium strain C5, more than 90% of newly hatched chickens became severely ill from salmonellosis. Significantly fewer of the newly hatched chickens that were inoculated with the mutants showed signs of the disease. The *cyoA* mutant was the least attenuated, resulting in salmonellosis in 70% of the chicks, while the *cydA* mutant and both the *atpB* and *atpH* mutants were most attenuated, resulting in 9% or less of the chicks suffering salmonellosis. Very similar results were obtained with the same mutations in the avian virulent strain serovar Typhimurium F98 (data not shown).

Three-week-old chickens were inoculated with serovar Gallinarum. At this age such birds are resistant to systemic disease with serovar Typhimurium infection but are still susceptible to serovar Gallinarum infection (5, 53). In contrast to the parent strain, which produces typical signs of fowl typhoid in a high proportion of the chickens inoculated, the *nuoG*, *cydA*, *cyo*, and *atpB* mutants were all attenuated. The *nuoG* and *atpB* mutants were most attenuated, with the former pro-

TABLE 2. Virulence of mutants of serovar Typhimurium C5 and serovar Gallinarum 9 for chickens and of serovar Typhimurium C5 and serovar Dublin 2229 for mice

Mutant gene	No. of animals that succumbed to salmonellosis/no. inoculated (%) [χ^2 value; P value] using[a]:			
	Newly hatched chickens, serovar Typhimurium C5	3-wk-old chickens, serovar Gallinarum 9	Mice	
			Serovar Typhimurium C5	Serovar Dublin 2229
None (parent)	18/20 (90)	16/20 (75)	10/10 (100)	7/9 (78)
nuoG	6/20 (30) [$\chi^2 = 15$; $P < 0.01$]	0/20 (0) [$\chi^2 = 26.2$; $P < 0.01$]	8/10 (80) [$\chi^2 = 2.22$; $P = 0.15$]	5/10 (50) [$\chi^2 = 1.53$; $P = 0.4$]
cydA	2/20 (10) [$\chi^2 = 40$; $P < 0.01$]	6/20 (30) [$\chi^2 = 10.0$; $P < 0.01$]	6/10 (60) [$\chi^2 = 5.0$; $P = 0.03$]	7/10 (70) [$\chi^2 = 0.17$; $P = 0.6$]
cyoA	14/20 (70) [$\chi^2 = 2.5$; $P = 0.03$]	4/20 (20) [$\chi^2 = 14.4$; $P < 0.01$]	7/10 (70) [$\chi^2 = 3.53$; $P = 0.6$]	8/10 (80) [$\chi^2 = 0.01$; $P = 0.85$]
atpB	0/20 (< 10) [$\chi^2 = 32$; $P < 0.01$]	1/15 (7) [$\chi^2 = 18.5$, $P < 0.01$]	0/10 (0) [$\chi^2 = 20$; $P < 0.01$]	0/10 (0) [$\chi^2 = 12.45$; $P < 0.01$]
atpH	0/10 (< 10) [$\chi^2 = 32$; $P < 0.01$]	Not done [not done]	Not done [not done]	Not done [not done]

[a] Chickens were inoculated orally within 24 h of hatching with 0.1 ml of bacterial culture containing approximately 3×10^8 CFU (serovar Typhimurium) or with 0.3 ml of culture containing approximately 10^9 CFU (serovar Gallinarum). Morbidity and mortality were recorded over 3 weeks, and χ^2 comparisons were carried out. Six-week-old BALB/C mice were inoculated orally with 5×10^7 CFU in 50 μl of culture; morbidity and mortality were recorded over 2 weeks.

ducing no salmonellosis in any of the 20 chickens inoculated (<5%) and the latter producing salmonellosis in only 1 chicken out of 15 (7%).

The situation with mice was quite different. With the dose used, the parent strains serovar Typhimurium and serovar Dublin 2229 were highly virulent (78 to 100% of the mice succumbed). The *atpB* mutant was highly attenuated (<10% of mice succumbed), and the *nuoG* and *cydA* mutants of serovar Typhimurium C5 and the *nuoG* mutant of serovar Dublin 2229 showed some reduction in virulence, which was, nevertheless, of little significance. Other attenuations were much less marked.

Mutations in *atp* but not *nuoG*, *cydA*, or *cyoA* significantly affect the in vivo invasiveness of serovar Typhimurium F98. The effect of the mutations on different stages in the pathogenesis of systemic salmonellosis was studied using serovar Typhimurium F98 in newly hatched chickens. Intestinal invasiveness was tested by isolation from the liver and spleen, since chickens have no lymph nodes. The parent serovar Typhimurium strain and its *nuoG*, *cydA*, and *cyoA* mutants behaved similarly, and the results for the parent and the *atpB* mutant strains only are shown in Table 3. High bacterial densities of both strains were observed in the ceca during the course of the experiment. The *atpB* mutant was isolated from the liver and spleen considerably later than were the other strains. This was of significance because the 50% lethal dose of serovar Typhimurium for chickens by parenteral inoculation increases over the first 24 and 48 h by more than 1 and 2 orders of magnitude, respectively (5).

TABLE 3. Intestinal invasiveness of serovar Typhimurium C5 (Nal^r) and its *atpB* derivative

Time (h) after inoculation	Log_{10} viable count[a] after oral inoculation with:					
	Serovar Typhimurium C5 (Nal^r)			Serovar Typhimurium C5 (Nal^r *atpB*)		
	Liver	Spleen	Ceca	Liver	Spleen	Ceca
6	<2	2.77	9.64	<2	<2	8.94
12	3.83	3.54	10.0	<2	<2	9.78
24	2.85	3.36	9.51	<2	<2	9.77
48	3.88	4.22	9.32	2.91	3.13	9.84

[a] Median log_{10} count from three birds. Three-week-old chickens were inoculated orally with 10^9 CFU in 0.1 ml of culture.

In vitro invasiveness into cultured chicken kidney cells is reduced by mutation of *atpB*. The recovery of bacterial cells at times after in vitro infection of a primary culture of chicken kidney epithelial cells with serovar Typhimurium F98 and mutant derivatives is shown in Table 4. Cultured epithelial cells were studied as an in vitro model of invasiveness. In comparison with the noninvasive prototrophic *E. coli* K12, all strains were fairly invasive. At both early time points smaller numbers of the *atpB* and *cydA* mutants were recovered in comparison with the parent strain and the *nuoG* and *cyoA* mutants, but this was significant only at 1 h postinfection. By 24 h only the *atpB* mutant was recovered in reduced numbers, but this difference was not significant. In all cases a considerable part of the cell sheet (>70%) was still present at 24 h. Most damage had occurred with the parent strain and *cyoA* mutant, but this had not greatly affected the high recovery level for these two strains.

Mutations in *nuo*, *cyd*, or *atpB* compromise the persistence and multiplication of serovar Typhimurium F98 in the reticuloendothelial system of chickens. The persistence of serovar Typhimurium F98 and its mutants in the reticuloendothelial system of 2-day-old chickens was assessed by intramuscular inoculation followed by sampling from the liver and spleen at progressive time points thereafter. Intramuscular inoculation was chosen because of the great technical difficulties of intravenous inoculation in birds of this age. The results for the *nuoG* and *cydA* mutants were similar to each other, and for this reason the results for the parent strain and *cydA* and *atpB* mutants only are shown (Table 5). The numbers of the parent strain in the spleen increased rapidly and remained high during the course of the experiment, whereas the bacterial numbers in the liver began to decrease by 5 days postinfection (Table 5) but also persisted. In contrast, the *nuoG* and *cydA* mutants reached similarly high numbers in these organs by day 5, and thereafter their numbers declined more rapidly. The *atpB* mutant reached lower numbers in the spleen, also followed by a decline, until it was not isolated from either this organ or the liver after 10 days.

Mutations in *atpB* and *cydA* reduced the persistence and multiplication of serovar Typhimurium F98 in chicken macrophages. All mutants of serovar Typhimurium F98 invaded or were taken up by the chicken macrophages to a similar degree, with the exception of the *nuoG* mutant, which was recovered in

TABLE 4. In vitro invasiveness of and persistence in chicken kidney cells by serovar Typhimurium C5 and mutants

Time (h) after infection	Log_{10} viable bacterial number (geometric mean ± standard error) of strain recovered from chicken kidney cells[a]					
	Serovar Typhimurium (parent)	Serovar Typhimurium (*nuoG*)	Serovar Typhimurium (*cyoA*)	Serovar Typhimurium (*cydA*)	Serovar Typhimurium (*atpB*)	*E. coli* K12 proto
1	4.55 ± 0.03	4.54 ± 0.02	4.26 ± 0.11	3.19 ± 0.08*	3.61 ± 0.16*	1.91 ± 0.06
2	4.51 ± 0.27	4.90 ± 0.03	4.57 ± 0.01	4.57 ± 0.03	4.09 ± 0.06	2.32 ± 0.11
24	5.41 ± 0.14	5.61 ± 0.13	5.77 ± 0.11	5.88 ± 0.03	5.24 ± 0.03	2.15 ± 0.09

[a] Mean of three replicates carried out on two separate occasions, with standard error of mean. The asterisk indicates significant difference ($P < 0.05$) from the parental strain as determined by *t* test.

lower numbers (Fig. 1). All strains persisted at a similar level in macrophages after 4 h, with the exception of the *cydA* mutant, which showed a significant decrease in its persistence. At 24 h the parent strain and *nuoG* and *cyoA* mutants had multiplied in the macrophages, as indicated by an increase in the numbers of salmonellae recovered. In contrast, the *atpB* mutant was unable to multiply within the macrophages, its recovery rate being significantly lower than that of the other strains. Some multiplication of the *cydA* mutant was found, since the numbers recovered had increased from the 4-h time point. However, as with the *atpB* mutant, the numbers recovered from chick macrophages were significantly lower than that for the parent strain.

Mutations in *atpB* reduced the persistence of the mouse-virulent serovar Typhimurium C5 in cultured murine J774A.1 macrophage-like cells. The persistence of the parent C5 strain and its mutant derivatives in murine J774A.1 macrophage-like cells is shown in Fig. 2. This study was set up to compare the avian and murine infection models. All strains invaded to similar degrees. Statistically significant differences were observed from 4 h onwards, with the *atpB* mutant showing reduced survival and the *cydA* mutant showing increased persistence. These differences were greatly enhanced by 24 h. Considerable loss of the cell sheet occurred with the parent strain, and this occurred least with the *atpB* mutant, indicating that the reduction in bacterial counts was not related to the structural integrity of the cell sheet. Very similar results were observed with the *atpH* mutant when it was tested separately (results not shown).

TABLE 5. Viability of serovar Typhimurium F98 and its *cydA* and *atpB* derivatives in the livers and spleens of 2-day-old chickens

Time (days) after inoc.[a]	Log_{10} viable count[b] after intravenous inoculation of:					
	Serovar Typhimurium F98 (parent)		Serovar Typhimurium F98 (*cydA*)		Serovar Typhimurium F98 (*atpB*)	
	Liver	Spleen	Liver	Spleen	Liver	Spleen
0	<2	1.75	<2	1.63	<2	1.48
3	3.89	5.56	2.89	3.99	2.69	4.60
5	4.24	5.79	4.62	6.01	3.67	3.78
7	3.33	5.65	1.87	4.34	<2	2.45
10	2.66	4.34	<2	3.09	<2	1.98
13	2.34	3.77	<2	1.87	<2	<2
17	3.08	3.56	<2	1.22	<2	<2

[a] inoc., inoculation.
[b] Median log_{10} count from three birds. All birds were inoculated in gastrocnemius muscle with 10^4 CFU.

Mutation of *atpB* or *atpH* reduces acid resistance. We tested the sensitivity to pH 3.3 of mid-log-phase serovar Typhimurium F98 and its mutants with and without adaptation at pH 5.6 (Fig. 3), since disruption of the proton gradient could affect this characteristic, which might have considerable bearing on resistance to an acidic intracellular environment. All the strains were more resistant to pH 3.3 following an adaptive period at pH 5.6. The *atpH* mutant was the most acid-sensitive, with no viable bacteria detected after 20 min at pH 3.3 even after adaptation at pH 5.6. The *atpB* mutant was slightly more acid resistant than the *atpH* mutant, showing an average of approximately 1% survival after 60 min, but was still significantly more acid sensitive than the parent strain, which showed an average of approximately 6% survival at this same time point. The *cydA* mutant showed enhanced adaptation to pH 3.3 compared to the parent strain. After 120 min, this mutant showed an average of approximately 54% survival compared to 3% for the parent strain. At this time point the *nuoG* mutant also showed slightly slightly better survival than the parent strain.

Effect of mutations in *nuoG*, *cydA*, or *atpB* on *rpoS* expression. Energy generation in the intracellular environment is likely to affect key genes in intracellular virulence. The effect of mutations in *nuoG*, *cydA,* and *atpB* on *rpoS* expression was therefore studied. The levels of β-galactosidase expressed from the *lacZYA* genes of plasmid pRW50 containing the upstream promoter region of *rpoS* were measured at two time points within the aerobic bacterial growth cycle. The effects of mutations in *nuoG*, *cydA*, and *atpB* on this expression are shown in Fig. 4. Expression in the wild-type strain F98 showed an increase when bacterial growth had reached early stationary phase. The mutation in *cydA* had little effect on this, whereas mutations in *nuoG* and *atpB* increased expression considerably.

DISCUSSION

We have shown that in the animal typhoid-like infections produced by serovar Typhimurium in newly hatched chicks, serovar Gallinarum in more mature chickens, and serovar Dublin infection in mice, mutations in genes encoding proton-translocating enzymes are frequently attenuating. The mutations that we have investigated included those in genes encoding component peptides of the electron transport chain components, NADH dehydrogenase I (*nuoG*), and cytochrome *d* and *o* oxidases (*cydA* and *cyoA* respectively), which contribute to generating the proton gradient across the cyto-

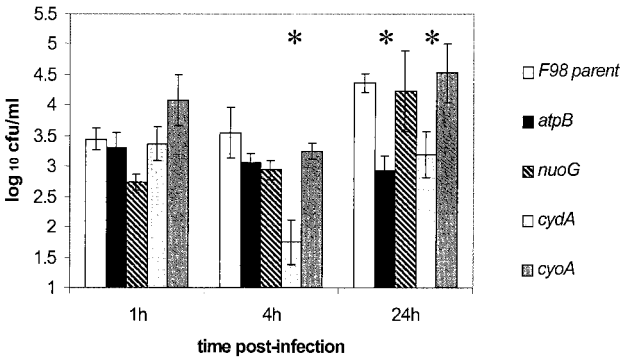

FIG. 1. Persistence of serovar Typhimurium F98 and mutant derivatives in chick splenic macrophages. Splenic macrophages isolated from 3-day-old chicks were challenged with approximately 10 bacteria per cell. After 1 h at 42°C to allow invasion, extracellular bacteria were killed by addition of gentamicin to the culture medium, and intracellular bacteria were counted on LB agar after lysis of macrophages with 1% Triton X-100. Numbers of salmonellae persisting were determined 4 and 24 h later; results are expressed as means of seven values, with significant differences ($P < 0.01$) indicated as asterisks and calculated by Student's *t* test.

plasmic membrane. The F_0F_1 proton-translocating ATPase (*atpB* and *atpH*) was also investigated. This enzyme uses the proton gradient for ATP synthesis and can also use ATP hydrolysis to generate the proton gradient.

The degree of attenuation varied in each of the infection models, with only mutations in *atp* being attenuated in them all, suggesting subtle differences between serovars. The virulence of serovar Typhimurium was assessed in chicks that were newly hatched only because chickens more than a few days old are resistant to systemic infection (5). Newly hatched chickens, which are immunologically immature and possess a relatively simple gut flora, are very much more susceptible to systemic disease with a number of serovars (54). All initial infections were made by oral inoculation, and the experimental evidence showed that attenuation was expressed primarily when the bacteria were multiplying in the cells of the reticuloendothelial system, since, with the exception of the *atpB* mutant, invasiveness was not reduced. The greater attenuation observed with some of the mutations (*nuoG* and *cydA*) in mice suggests that this may be largely a host species characteristic rather than a *Salmonella* serotype characteristic.

The mutation in *nuoG*, abolishing NADH dehydrogenase I, was not significantly attenuating for serovar Typhimurium or serovar Dublin in mice. The greatest attenuation was observed for serovar Gallinarum, and this is thought to be associated primarily with poor survival and multiplication in the reticuloendothelial system of the chicken (66). NADH dehydrogenase I is induced under microaerophilic and stationary-phase growth conditions under the differential regulatory control of ArcA, FNR, NarL (2, 8), and Fis (58). Mutations in *nuo* genes are known to affect a number of aspects of microbial physiology and biochemistry (16, 56), which undoubtedly has consequences for virulence.

The degree of attenuation induced by mutations in *cydA* or *cyoA* varied significantly depending on the *Salmonella* serotype and host species. The cytochrome *d* oxidase has a higher affinity for oxygen than cytochrome *o* and so is more important for electron transport at lower oxygen tensions, but it is less efficient at translocating protons (25). In addition, mutations in the *cyd* or *cyo* operon increase susceptibility in *E. coli* to oxidative stress (41), and *cyd* mutants are thought to be depleted in intracellular iron, indicated by the overproduction of siderophores (17). Mutations in *cyoA* or *cydA* were attenuating to various degrees in chickens but much less so in mice, suggesting that oxygen may be an electron acceptor of differing sig-

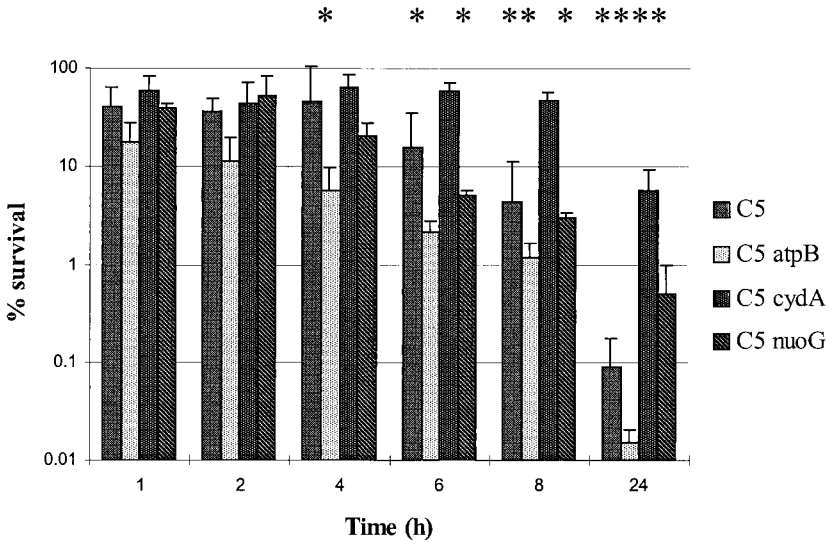

FIG. 2. Persistence of serovar Typhimurium C5 and mutant derivatives in the mouse macrophage-like cell line J774A.1. Monolayers of J774A.1 cultured macrophage-like cells were seeded with serovar Typhimurium cells, grown statically at 37°C overnight at a ratio of approximately 100 bacteria per macrophage. After 1 h at 37°C to allow invasion, extracellular bacteria were killed by the addition of gentamicin to the culture medium. Viable counts of surviving intracellular bacteria were then determined at the time intervals indicated. Results are expressed as means of six values from two experiments with each of three replicates. Asterisks show statistically significant differences ($P < 0.01$) measured by Student's *t* test.

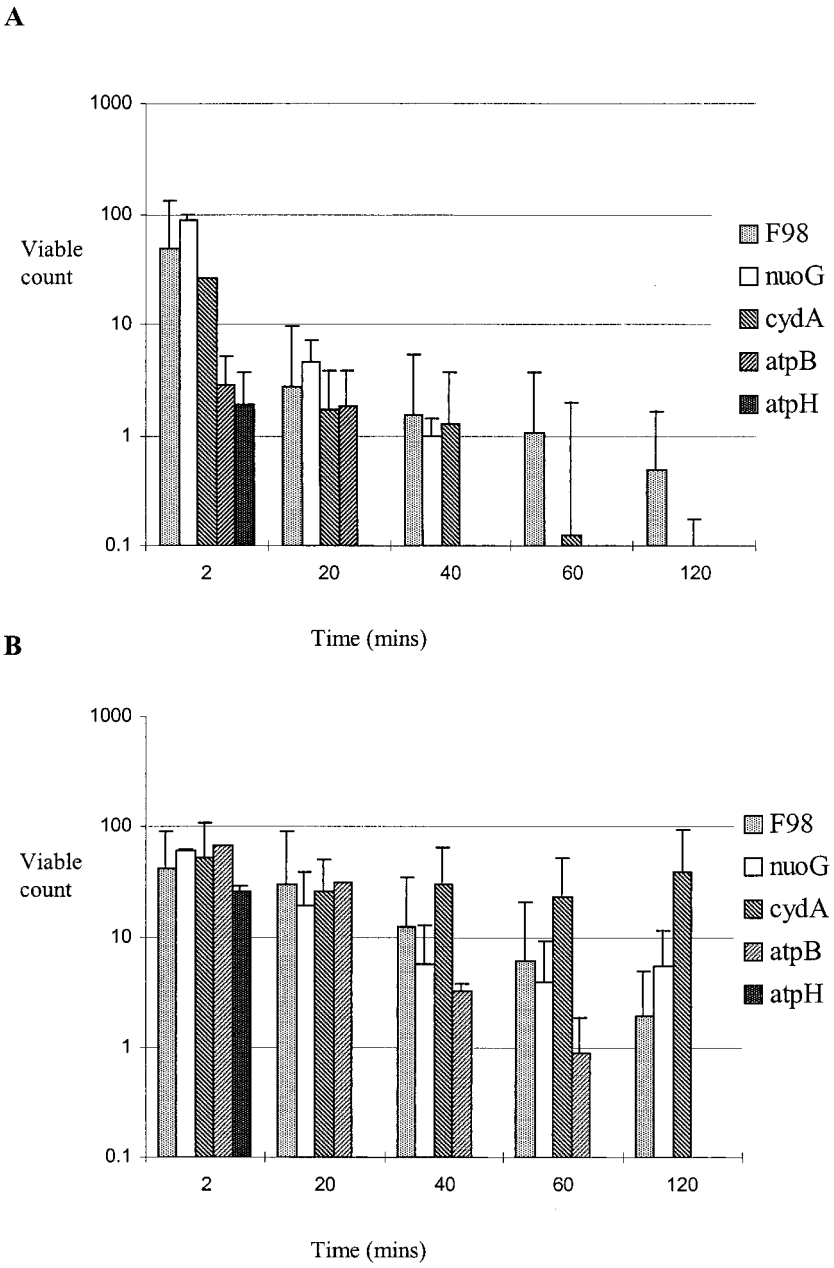

FIG. 3. Susceptibility of the serovar Typhimurium F98 strain and mutant derivatives to acidic conditions without adaptation (A) and following a period of adaptation at pH 5.6 (B). Bacteria were cultured overnight at 37°C in minE medium supplemented with glucose and then diluted into minE medium at pH 7.6 for unadapted controls (graph A) or at pH 5.6 for adaptation to low pH (graph B). After 3 h at 37°C to allow adaptation to occur, cultures were transferred to minE medium at pH 3.3. Viable counts were determined at the time intervals shown after this transition to low pH. The mean and standard error of five values were calculated.

nificance for *Salmonella* in chickens and mice during infection. The greater attenuation of the *cydA* mutant than the *cyoA* mutant for chickens also suggests a microaerophilic environment within the chicken macrophages, and the attenuation was supported by the poor survival in chicken macrophages. The lack of attenuation of these mutations for serovar Dublin in mice may be because, for this serovar, loss of one cytochrome oxidase may be complemented by the other, as has been suggested previously for *E. coli* (18, 59), or because oxygen is not a major terminal electron acceptor for serovar Dublin during infection of mice. An assessment of the virulence of a *cyoA cydA* double mutant may indicate which of these is true. Previous work with *Shigella dysenteriae* has shown that mutations affecting cytochrome *d* oxidase levels attenuate virulence, both for mice after intranasal inoculation and in the size of plaques produced in cell monolayers (62). The attenuating effects were considered to be mainly the result of reduced survival in the intracellular environment, although it was speculated that this

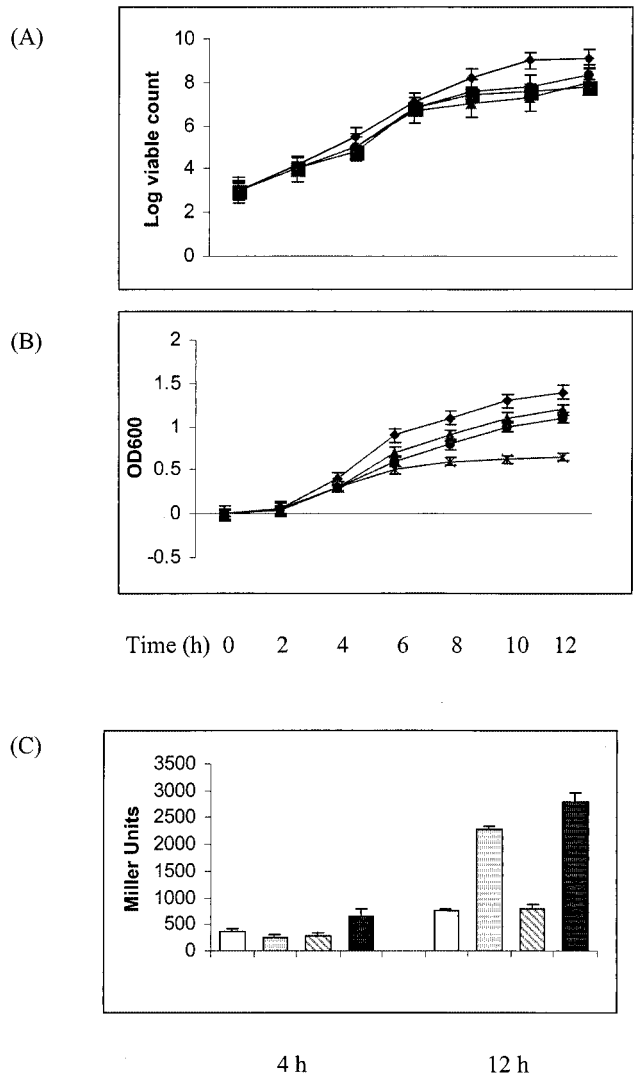

FIG. 4. β-galactosidase concentrations (Miller units) expressed for the *rpoS* promoter region cloned into the *lacZYA* expression plasmid pRW50 in different mutants of serovar Typhimurium. Cultures were incubated aerobically at 37°C in 10-ml aliquots of LB broth for the times shown, and Log_{10} viable counts (A)and optical densities (B) are indicated. ♦, serovar Typhimurium parent strain; ▲, *nuoG* mutant; ■, *cydA* mutant; ×, *atpB* mutant. Results in Miller units are shown in panel C. White bars, parent strain; grey bars, *nuoG* mutant; hatched bars, *cydA* mutant; black bars, *atpB* mutant. Values are expressed as means and standard errors.

might also affect survival in the lower alimentary tract, where oxygen would be present in low concentrations. With chickens, at least, this is unlikely, since neither *cydA* nor *cyoA* contributes to intestinal colonization by serovar Typhimurium (P. A. Barrow and M. A. Lovell, unpublished observations). In contrast to the other mutations, the *cydA* mutation in serovar Typhimurium conferred enhanced tolerance to low pH and enhanced survival in J774A.1 macrophage-like cells. The tolerance to low pH may be explained if such mutants accumulated a metabolite that had a buffering capacity, such as has been observed for *iso*-citrate dehydrogenase mutants that are more acid tolerant, probably due to increased intracellular levels of citrate and *iso*-citrate (23).

Only mutations in *atp* were equally attenuating in all the serovars. Although it is likely that the *atpH* mutant can synthesize the F_0 component of the ATPase, the pore in this membrane protein does not open until contact is made with the F_1 component (27). Therefore, the phenotypes of the *atpB* and *atpH* mutants would be expected to be similar, and this was generally the case, except that the *atpH* mutant was more susceptible to acidic pH. This may be due to the F_0 component allowing leakage of protons across the cytoplasmic membrane. In addition to poor survival and inability to multiply in the spleen, the *atpB* mutant showed reduced invasiveness in vivo. This was also reflected in reduced in vitro invasiveness, and in addition, previous findings (67) suggest that the reduced in vivo invasiveness may also reflect poorer intestinal colonization. Previous work (24) has shown that mutations in *atp* genes attenuate serovar Typhimurium for mice and result in reduced acid tolerance. These authors (24) showed that despite the reduced invasiveness from the gut after oral inoculation, there was no evidence for reduced tolerance of stomach acidity, since dosing the animals with sodium bicarbonate did not affect the result. In the present study, of the mutants tested, the *atp* mutants were the most sensitive to low pH, with or without adaptation. This suggests that the F_0F_1 ATPase has a vital role in countering low environmental pH (27, 34) and that such environments are encountered during the infection process. The *atpB* and *atpH* mutants of serovar Typhimurium were less able to persist in the cultured J774A.1 murine macrophage-like cell line, and decreases in pH of the *Salmonella*-containing vacuoles following the entry of serovar Typhimurium into macrophages have been demonstrated (1, 50, 55). The importance of the F_0F_1 ATP synthase in internal pH regulation is now well documented (24, 27, 34), and its significance in survival within the phagosome is likely to be considerable. However, the reduced acid tolerance and increased RpoS expression are, to some extent, incongruous and remain to be resolved. In all such in vitro virulence assays, viable counts from macrophage survival assays should be treated with caution because reduction in bacterial numbers can result not only from bacterial killing, as probably occurred with at least the *atp* mutant, but also from macrophage death either by necrotic lysis (15, 26) or by apoptosis (48). This did not appear to be a major problem during this study, since it was taken into account.

The proton-translocating enzymes have a central role in cellular metabolism, and consequently, mutations affecting these enzymes are pleiotropic. Probably the most important effect of such mutations is a change in their ability to use carbon sources efficiently to derive energy with oxygen as an electron acceptor. The effect of two of the three mutations on *rpoS* expression supports the case for the importance in ATP generation through the electron transport chain in the intracellular environment. RpoS is expressed in the intracellular environment (14) and also contributes to *Salmonella* virulence (17). Although its regulatory effects on the *spv* genes of the virulence plasmid are well known (13), it also modulates the acid tolerance response (38), which is likely to be of relevance in the phagolysosome and may explain the effects on RpoS expression and acid tolerance, for the *atp* mutants at least. The reduced activity of the Lon protease in *atp* mutants, probably

associated with lower intrabacterial concentrations of ATP (3), may also contribute to the attenuating phenotype. However, the *nuo*G and *cydA* mutants behaved differently, and the attenuating effects of mutations in these genes are likely to have been effected in other ways. The different behavior of the *cydA* mutant correlated with its increased survival intracellularly and at low pH.

An increasing number of genes have been identified as being required for virulence in animal typhoid models, including several required for the synthesis of different nutrients. However, few genes whose protein products are required in central metabolic pathways have been confirmed as required for virulence (35, 43). As we have shown here, mutations in proton-translocating enzymes, which are central to metabolism, can be attenuating in some *Salmonella*-host combinations. Klose and Mekalanos (36) have suggested that mutants defective in central metabolism should be effective live vaccines, since such mutations may not affect virulence gene expression. The *nuoG* mutant of serovar Gallinarum (66) is an example of this, and it is possible that other proton-translocating enzyme mutations may prove useful in the development of live attenuated vaccines against *Salmonella* and other pathogens.

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