Glycogen production by different *Salmonella enterica* serotypes: contribution of functional *glgC* to virulence, intestinal colonization and environmental survival

Alisdair McMeechan,¹ Margaret A. Lovell,² Tristan A. Cogan,¹ Kerrie L. Marston,² Tom J. Humphrey¹ and Paul A. Barrow^{2†}

Correspondence

Paul A. Barrow

Paul.Barrow@bbsrc.ac.uk

¹School of Clinical Veterinary Science, University of Bristol, Langford House, Langford, Bristol BS40 5DU, UK

²Institute for Animal Health (IAH), Compton Laboratory, Compton, Newbury, Berkshire RG20 7NN, UK

In enteric bacteria, the contribution of endogenous energy sources to survival both inside and outside the host is poorly understood. The contribution of glycogen production to the virulence, colonization and environmental survival of different *Salmonella enterica* serotypes was assessed. Of 19 serotypes (339 strains) tested for glycogen production, 17 (256 strains) were positive. The avian-specific serovars *S. Gallinarum* (62 strains) and *S. Pullorum* (21 strains) did not produce glycogen. The sequence of *glgC* in three *S. Gallinarum* strains tested revealed an identical deletion of 11 consecutive bases, which was not present in *S. Pullorum*, and a CCC insertion after position 597. Transduction of *S. Gallinarum* and *S. Pullorum* to a glycogen-positive phenotype did not change the ability to colonize the intestine or affect virulence in the chicken. Mortality rates in chickens following oral infection with a *S. Typhimurium* glycogen mutant (*glgC*::km) were not significantly reduced, although colonization of the intestine was reduced over the first 4 weeks of the trial. Growth and yield of the *glgC*::km mutant were comparable to the parent. The *glgC* mutant survived less well in faeces and in water at 4 °C when the strain was grown in LB broth containing 0.5 % glucose, and in saline it died off more rapidly after 7 days. The data suggest that glycogen has a complex but comparatively minor role in virulence and colonization, but a more significant role in survival.

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INTRODUCTION

From the point of view of infection biology, *Salmonella enterica* can be divided into two broad groups (Parker & Collier, 1990). One group consists of a relatively small number of serotypes that typically produce typhoid-like systemic disease in a narrow range of host species. These include *S. enterica* serovars Typhi in man, Gallinarum in birds and Choleraesuis in pigs. In the absence of clinical disease, they colonize the gut poorly (Barrow *et al.*, 1988; Parker & Collier, 1990), and therefore rarely enter the human food chain. The remaining more than 2000 serovars rarely produce systemic disease, but are able to colonize the alimentary tract of food animals asymptotically.

For both types of *Salmonella*, the alimentary tract is a major niche. There is great flexibility and redundancy in the

metabolic capacity in both *Salmonella* and *Escherichia coli* (Neidhardt, 1996); however, the relative role of different metabolic pathways is not well understood. In a recent study, colonization of the murine intestine was only affected by mutations in sugar pathways. Colonization was unaffected by mutation in genes involved in phospholipid and amino acid catabolism, gluconeogenesis, the tricarboxylic acid cycle or the pentose phosphate pathway (Chang *et al.*, 2004).

Glycogen is a major energy storage compound in many bacteria, including *S. Typhimurium* (Steiner & Preiss, 1977), in addition to its importance in eukaryotes. It is a polysaccharide containing glucose units in a branched structure, comprising predominantly α -1,4 linkages and a smaller number of α -1,6 branching glucosyl linkages. It is produced predominantly under limiting growth conditions in which carbon sources such as glucose are present (Preiss, 1996).

The glycogen locus, both in *E. coli* and in *S. Typhi* and *S. Typhimurium*, consists of two adjacent operons transcribed

[†]Present address: School of Veterinary Science and Medicine, University of Nottingham, Sutton Bonington, Leicestershire LE12 5RD, UK.

Abbreviations: IAH, Institute for Animal Health; WT, wild-type.

unidirectionally, and encoding *glgB* (branching enzyme) and *glgX* (glycosyl hydrolase) in one operon, and *glgC* (ADP-glucose pyrophosphorylase), *glgA* (glycogen synthase) and *glgP* (glycogen phosphorylase) in the other (Preiss & Romeo, 1989; Preiss, 1996). The function of the products of these genes has been studied extensively in *E. coli*. The activities of GlgC, GlgA and GlgB increase in the presence of rich medium containing glucose, and expression also increases as the bacteria enter stationary phase (Preiss, 1984). Synthesis rates are also inversely proportional to growth rate when nutrients such as nitrogen are limited (Preiss, 1984). Under conditions of nutrient deficiency, glycogen accumulates, the amount depending on substrate availability and allosteric regulation by ADP-glucose pyrophosphorylase (Preiss, 1984). Expression of *glgC* and *glgA* is regulated by intracellular bacterial signals involved in denoting energy status. These include cAMP/CRP, CsrA and ppGpp (Romeo & Preiss, 1989; Romeo *et al.*, 1993). In *E. coli*, CsrA binding to *glgCAP* transcripts inhibits glycogen metabolism by promoting *glgCAP* mRNA decay (Baker *et al.*, 2002). Transcription of the glycogen operon is mediated through the major active form of RNA polymerase ($E\sigma^{70}$), and is subject to regulation by RpoS (Hengge-Aronis & Fischer, 1992). In addition, *rpoS* mutants of *E. coli* have been reported to accumulate less glycogen (Makinoshima *et al.*, 2003). The regulation of glycogen biosynthesis and catabolism is therefore adjusted to accommodate changes in the availability of easily utilizable energy sources.

Given the widespread existence of glycogen in enteric genera, including *Citrobacter*, *Enterobacter*, *Klebsiella*, *Serratia* and *Shigella* (Preiss, 1984), it seems reasonable that, under conditions of nutritional and other stresses associated with infection and environmental survival, the accumulation of energy-storage compounds such as glycogen would be important. We therefore examined the contribution of glycogen production to virulence, intestinal colonization and survival. We observed that *S. Gallinarum* and *S. Pullorum* do not accumulate glycogen. The significance of this to the biology of these serovars was therefore assessed. Since GlgC catalyses the first committed and rate-limiting step in glycogen biosynthesis in bacteria (Jin *et al.*, 2005), we constructed a defined mutant in *glgC* in *S. Typhimurium*.

METHODS

Bacterial strains and plasmids. Strains and plasmids used during this study are shown in Table 1. *S. Typhimurium* F98 produces systemic disease in young poultry and is able to colonize their alimentary tract (Barrow *et al.*, 1987b, 1988). *S. Gallinarum* 9 produces typical fowl typhoid in poultry (Smith, 1955; Barrow *et al.*, 1987a). *S. Gallinarum* 287/91, 21-92 and *S. Pullorum* strain 3 were isolated from diseased poultry. For intestinal colonization studies, mutants were used which were resistant to nalidixic acid. The *Nal^R* resistance determinant has previously been shown not to adversely affect growth, virulence or colonization (Barrow *et al.*, 1987c; Zhang-Barber *et al.*, 1997).

Table 1. Bacterial strains and plasmids

Amp^R, ampicillin resistance; Tet^R, tetracycline resistance; Cm^R, chloramphenicol resistance; Kan^R, kanamycin resistance; Glc+, glycogen positive.

Strain or plasmid	Description	Source or reference
Strains		
<i>S. Gallinarum</i>		
9	Fowl typhoid	Barrow <i>et al.</i> (1987a)
SG9Glc+	<i>S. Gallinarum</i> 9 Glc+ transductant	This study
287/91	Isolated from diseased poultry	This study
21-92	Isolated from diseased poultry	This study
<i>S. Pullorum</i>		
3	Pullorum disease in avian species	Barrow <i>et al.</i> (1987a)
SP3Glc+	<i>S. Pullorum</i> 3 Glc+ transductant	This study
<i>S. Typhimurium</i>		
F98	Wild-type mouse and chicken virulent	Barrow <i>et al.</i> (1987b)
<i>glgC</i>	F98 <i>glgC::kan</i>	This study
<i>S. Choleraesuis</i> B195	Non-colonizing control strain	Barrow <i>et al.</i> (1988)
<i>S. Enteritidis</i> P125109	Sequenced phage type 4 strain	IAH, Compton, UK
<i>E. coli</i> S17.1	Cloning strain, λ <i>pir</i>	Simon <i>et al.</i> (1983)
<i>E. coli</i> TOPO10	Cloning strain	Invitrogen
Plasmids		
pDM4	Suicide vector, Cm ^R	Milton <i>et al.</i> (1996)
pGEM-T Easy	T-cloning vector, Amp ^R	Promega
pCR2.1	Cloning vector, Amp ^R , Kan ^R	Invitrogen
pRW50:: <i>rpoS-lacZ</i>	Reporter plasmid, Tet ^R	Lodge <i>et al.</i> (1992)

Growth media and conditions used. The media used in this study were LB or M9 minimal medium (Sambrook & Russell, 1989) containing 0.5% (w/v) glucose. Unless otherwise stated, cultures were grown overnight at 37 °C with shaking at 150 r.p.m. The following growth factors were added for *S. Gallinarum* and *S. Pullorum*: 2 µg thiamine ml⁻¹, 100 µg nicotinic acid ml⁻¹, and glutamic acid, arginine, proline, cysteine, leucine and histidine all at 20 µg ml⁻¹. Phenylalanine, tryptophan, tyrosine, *para*-aminobenzoic acid and sodium dihydroxybenzoate, all at 100 µg ml⁻¹, were added for the *S. Typhi aroCaroD* strain. Where mentioned, casamino acids (Merck) were added at 0.5% (w/v).

Mutant construction. The defined mutation in *glgC* was produced as previously described (Zhang-Barber *et al.*, 1997; Turner *et al.*, 1998). Fragments of the *glgC* gene were amplified from *S. Typhimurium* F98 DNA by PCR using the primer pairs Gly01-*EcoRI* (5'-TGAAT-TCGTGAGTTTATAGAGAAGACGA-3') and Gly02-*KpnI* (5'-CAATT-CATACAGGTACCCATCACGCCGAACGCCGT-3'), and Gly03-*KpnI* (5'-ATGGGTACCTGTATGAATTGCTGG-3') and Gly04-*XbaI* (5'-CTCTAGACAGCATTTCACGCGTGACCA-3') (restriction sites are underlined). Selection for Km^RCm^S colonies indicated loss of the suicide vector and PCR confirmed the desired deletion.

Glycogen-positive (Glc+) transductants of *S. Gallinarum* and *S. Pullorum* were created as follows. A 485 bp fragment of the *glpD* gene, adjacent to the glycogen operon, was amplified from *S. Enteritidis* P125109 and cloned into pDM4. This construct was electroporated into *E. coli* S17.1 lambda *pir*, from which it was conjugated into *S. Enteritidis* P125109; a chloramphenicol-resistant mutant was then selected. This was confirmed by PCR to have resulted from the insertion of the construct into the *glpD* gene. P22 transduction was then used to transfer chloramphenicol resistance to *S. Gallinarum* 9 and *S. Pullorum* 3. A high proportion of the purified recipient colonies were found to produce glycogen. These colonies were checked for motility in soft agar to confirm that they were serovars of *Gallinarum* and *Pullorum* (non-motile), and not *Enteritidis* (motile).

Glycogen assay. The production of glycogen was detected using a standard procedure involving iodine staining after growth on glucose-rich agar (Govons *et al.*, 1969). Bacteria were grown on an agar medium comprising 0.1% K₂SO₄, 1.3% K₂HPO₄, 0.47% KH₂PO₄, 0.01% MgSO₄·7H₂O, 0.6% yeast extract and 1.5% Bacto agar (Difco). Filtered glucose was added at 1% (w/v) after sterilization.

DNA sequence analysis. From the published *S. Typhimurium glgC* sequence (GenBank X59281), primers were designed at intervals along the gene and used to amplify *glgC* from *S. Gallinarum* strains 9, 287/91 and 21-92, and *S. Pullorum* strain 3. The fragments were then cloned into pGEM-T Easy (Promega), and plasmid-specific primers were used as sequencing primers. DNA sequencing was carried out using a PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Inc.) and the Applied Biosystems 373 DNA sequencer. Nucleotide sequences were analysed by comparison with the published sequence for *S. Typhi* and *S. Typhimurium* (www.sanger.ac.uk/Projects/Salmonella/ and www.salmonella.org/).

Measurement of bacterial growth. For each strain to be tested, triplicate cultures were diluted to between 10³ and 10⁴ c.f.u. ml⁻¹ in M9 medium, and 300 µl was inoculated per well of a honeycomb microplate. Growth was measured using a Bioscreen C automatic turbidimetric analyser (Thermo Electron Corp., Basingstoke, UK). A higher inoculum (10⁶–10⁷ c.f.u. ml⁻¹) was used for *S. Gallinarum* and *S. Pullorum*, since preliminary studies demonstrated poorer growth in M9, compared with that of *S. Typhimurium*.

Intestinal colonization. Specific-pathogen-free chickens that were free of *Salmonella* and free of antibody against *Salmonella enterica* were used. Studies of intestinal colonization were carried out in two

ways. In the first method, the spread of infection was observed by infecting three out of 20 four-day-old Light Sussex chickens with 0.3 ml containing 10⁹ c.f.u. of the strain to be tested. At weekly intervals, cloacal swabs were taken (Smith & Tucker, 1975) and plated onto Brilliant Green agar (BGA) plates containing sodium nalidixate (20 µg ml⁻¹) and novobiocin (1 µg ml⁻¹). Bacteria that might be non-culturable by direct plating were enriched by incubating the swabs in selenite broth overnight at 37 °C, before plating on BGA, as before. In the second method, all 20 chickens were infected directly with the same inoculum level as that of the first method.

Virulence assays. For virulence assays, Rhode Island Red chickens from the IAH flock were used. These have moderate susceptibility to systemic salmonellosis and were reared as described previously (Barrow *et al.*, 1988). For infection with *S. Typhimurium*, day-old chicks were used, and for *S. Gallinarum*, three-week-olds were used. Groups of 20 chickens were inoculated orally into the crop with 3 × 10⁸ c.f.u. of the strain to be tested in 100 µl. Animals were observed for signs of salmonellosis, and when it became severe, were killed humanely. The livers of the birds were cultured for the inoculated strain on XLD and BGA (Oxoid). Groups of 10 BALB/c mice were inoculated orally directly into the stomach with 10⁷ c.f.u. of the strain to be tested in 50 µl. Mice were observed frequently, and those showing signs of salmonellosis were killed humanely and their livers cultured as above. Statistical analysis was carried out using the chi-square test.

Survival in faeces, water and saline. Survival in faeces was investigated as follows. Material was collected from the bottom of the cages of chickens that had been infected with either *S. Typhimurium* F98 or its *glgC* derivative 3 weeks previously. Fresh samples of faeces, which had been produced within the previous hour, were collected. They were pooled in a glass bottle, and each pool of samples was homogenized crudely with a pestle and mortar. Samples were incubated at room temperature and bacterial counts made at intervals. For survival in water, deionized water was collected into sterile tissue culture bottles, and 50 ml aliquots filtered into 500 ml sterile flasks for use. Bacterial cultures were washed twice in PBS and resuspended in water to a starting count of approximately 10⁶ c.f.u. ml⁻¹. They were incubated statically at 4 and 37 °C. Samples were withdrawn at intervals and the numbers of viable bacteria estimated. For survival in saline, strains were diluted to approximately 1 × 10⁴ c.f.u. ml⁻¹ in 0.85% NaCl and incubated without shaking at 37 °C. Bacterial cells were enumerated by plating appropriate dilutions onto Colombia base agar containing 5% defibrinated horse blood (Oxoid). Statistical analysis was carried out using a two-tailed *t* test.

Measurement of *rpoS* expression. The expression of *rpoS* in wild-type (WT) and *glgC* mutant was measured using reporter plasmid pRW50 carrying promoterless *lacZY* genes inserted behind the *rpoS* promoter (Lodge *et al.*, 1992). Overnight cultures harbouring the pRW50::*rpoS-lacZ* reporter plasmid were inoculated into 10 ml LB supplemented with 5 µg tetracycline ml⁻¹, and incubated with shaking for 2 h. This culture was diluted 1/100 into 100 ml fresh pre-warmed LB supplemented with tetracycline and 0.1 M glucose. WT and *glgC* mutant strains were analysed for *rpoS* expression at different times during growth by a β-galactosidase assay (Miller, 1972).

RESULTS

Glycogen production by different *Salmonella* serotypes

In total, 256/339 *Salmonella* strains produced glycogen (Table 2). None of 62 strains of *S. Gallinarum* or 21 strains of *S. Pullorum* produced glycogen (Table 2). In contrast,

Table 2. Glycogen production by different *Salmonella* serotypes and related genera

Serotype or related genus	Glycogen status	No. tested	Host range
Serotype			
Typhimurium	+	10	Broad host range
Enteritidis	+	10	Broad host range
Montevideo	+	2	Broad host range
Anatum	+	2	Broad host range
Munster	+	2	Broad host range
Virchow	+	2	Broad host range
Oranienburg	+	2	Broad host range
Senftenberg	+	2	Broad host range
Hadar	+	2	Broad host range
Heidelberg	+	2	Broad host range
Pullorum	–	21	Avian adapted
Gallinarum	–	62	Avian adapted
Dublin	+	208	Bovine adapted
Bovis-morbificans	+	1	Bovine adapted
Choleraesuis	+	5	Porcine adapted
Typhi	+	1	Human adapted
Abortusovis	+	2	Ovine adapted
Abortusequi	+	1	Equine adapted
Subsp. <i>arizonae</i>	+	2	Broad host range
Related genus			
<i>E. coli</i>	+	2	Broad host range
<i>Klebsiella</i>	+	3	Broad host range
<i>Enterobacter</i>	+	3	Broad host range
<i>Citrobacter</i>	+	3	Broad host range
<i>Shigella</i>	+	4	Human/primate

10/10 strains of *S. Enteritidis* and 208/208 strains of *S. Dublin* were positive for glycogen production. These are closely related serotypes, belonging to group D₁ (Li *et al.*, 1993). The phenotype of the *S. Typhimurium glgC* mutant and Glc+ transductants was confirmed after staining with potassium iodide.

glgC sequence analysis

The *glgC* gene sequence of *S. Gallinarum* 9 was 11 bp shorter than that of *S. Typhimurium* and had a CCC (proline) insertion after position 597 (Fig. 1a). Bases 459 to 471 inclusive (13 bp) were replaced by TT, such that the remaining

Fig. 1. Nucleotide sequence of section (424–623) of *glgC* of *S. Typhimurium*, indicating differences with respect to the corresponding sequences of (a) *S. Gallinarum* strains 9, 287/91 and 21-92, and (b) *S. Pullorum* strain 3. The *S. Typhimurium* sequence is displayed with nucleotide differences shown above, including the CCC insertion after position 597 and the deletion in the *S. Gallinarum* sequence between 459–469.

transcribed sequence would have been out of frame, leading to the generation of a number of stop codons. It is likely that the ADP-glucose pyrophosphorylase activity would be non-functional, since the substrate and allosteric effector binding site is thought to be between positions 567 and 594 (Leung & Preiss, 1987b). The complete *glgC* gene was also sequenced in *S. Gallinarum* strains 287/91 and 21-92. The sequences were identical, with the same CCC insertion and 11 bp deletion. In addition, recently published incomplete genome sequence information (www.sanger.ac.uk/Projects/Salmonella) indicates shortening of *glgB* by 52 bp in *S. Gallinarum* 287/91. The *glgC* sequence of a single *S. Pullorum* strain 3 was found to more closely resemble that of *S. Typhimurium* (Fig. 1b). The published incomplete genome sequence of *S. Pullorum* (www.salmonella.org/genomics/spu.all) also indicates considerable reductions in the size of *glgB* and *glgP*, although this remains to be confirmed.

Influence of *glgC* on growth

Yields and exponential-phase growth rates were higher when casamino acids were present (Fig. 2a). The growth rate of *S. Typhimurium glgC* was only marginally less than that of the parent and did not reach the same final OD in stationary phase. There was little effect of a Glc⁺ phenotype on the growth of *S. Gallinarum*; however, Glc⁺ *S. Pullorum* achieved a higher OD in log and stationary phase (Fig. 2b). This may be attributed to the increased cell density that occurs with accumulation of intracellular polysaccharide.

Intestinal colonization

The patterns of faecal excretion of *S. Typhimurium* F98 and its *glgC* mutant by chickens are shown in Fig. 3. The excretion rates are compared with those of *S. Choleraesuis* B195, a strain that has been shown not to colonize the avian

Fig. 2. (a) Growth of *S. Typhimurium* F98 WT (circles) and *glgC* mutant (squares) in M9 with (closed symbols) and without (open symbols) casamino acids at 37 °C; $n=9$. (b) Growth of *S. Gallinarum* 9 WT (●) and *S. Pullorum* 3 WT (▲) and their Glc⁺ derivatives (■ and ◆, respectively) in M9 with 0.5% casamino acids at 37 °C. Error bars show SEM; $n=3$.

Fig. 3. Intestinal colonization of chickens by *S. Typhimurium* F98 NaI^R and other strains expressed as percentage of chickens in each group excreting the inoculated strain at different times after infection. (a) All chickens inoculated directly; (b) chickens infected by placing them in contact with experimentally infected chickens. Excretion of parent strain (●); *glgC* mutant (■) and *S. Choleraesuis* B195 (▲) non-colonizing control strain.

alimentary tract or be excreted extensively in the faeces (Barrow *et al.*, 1988). The excretion rate of the *glgC* mutant was marginally less than that of the parent, indicating that it colonized less well, but the differences were not significant. Caecal carriage for the parent and *glgC* strain was 62% and 58%, respectively, at the end of the trial. When naïve chickens were mixed with those infected experimentally (Fig. 3b), the *glgC* mutant colonized less rapidly over the first 4 weeks, but at the end of the trial, excretion rates were similar.

Virulence of *glgC* mutants

S. Typhimurium F98 was highly virulent for chickens, with a mortality rate of 18/20 (90%) in chickens. A reduction of virulence was observed for the *glgC* mutant, producing mortality in 15/20 (75%) chickens. This was of marginal statistical significance ($P=0.02$). When *S. Typhimurium* F98 and its *glgC* derivative were cultured in LB broth without glucose, both strains were equally virulent for newly hatched chicks (data not shown). The virulence of the *S. Typhimurium glgC* mutant in mice was not statistically different from that of the parent (data not shown).

Both *S. Gallinarum* 9 and its Glc⁺ transductant produced severe fowl typhoid in all 20 three-week-old chickens inoculated, birds becoming ill at the same rate (data not shown).

Survival in faeces, water and saline

In faeces, the *S. Typhimurium glgC* mutant survived significantly less well than the parent strain (Fig. 4a). In water at 4 °C, the parent survived better than the *glgC* strain only after prior culture in LB broth containing 0.5% glucose. When cultured in LB without glucose, both the *glgC* mutant and the parent survived equally well during the period of observation (Fig. 4b). At 37 °C, the rate of decline in viability was more rapid, and this was more marked when grown in the presence of glucose (data not shown). When tested for survival in 0.85% NaCl, different patterns were observed with *S. Typhimurium*, *S. Gallinarum* and *S. Pullorum* (Fig. 4c, d, e). Up to 7 days, the *S. Typhimurium glgC* mutant survived as well as the parent, but the numbers fell away more rapidly after this time (Fig. 4c), and by day 12, the *glgC* mutant survived significantly less well ($P<0.05$). The Glc⁺ derivatives of *S. Gallinarum* and *S. Pullorum* survived as well as the respective parent strains (Fig. 4d, e).

rpoS expression in WT and *glgC* mutant

Since glycogen synthesis is under the control of *rpoS* (Hengge-Aronis & Fischer, 1992), we wanted to see if mutation in *glgC* affected *rpoS* expression in our *S. Typhimurium* F98 WT and *glgC* strains. Levels of *rpoS* expression in the parent strain rose nearly ninefold as the organisms approached stationary phase, as expected. In a *glgC* background, *rpoS* promoter activity was reduced by slightly less than 50%, compared to the parent (Fig. 5).

DISCUSSION

We have found that, in common with other members of the *Enterobacteriaceae*, most *Salmonella* serotypes produce glycogen. In contrast, none of the strains of the avian pathogens *S. Gallinarum* and *S. Pullorum* that were tested produced glycogen under the test conditions. To our knowledge, this is the first report of this phenotype in serovars *Gallinarum* and *Pullorum*. The loss of glycogen synthesis ability by these serovars is another example of pseudogene accumulation by host-restricted serovars, and is indicative of functions no longer required due to niche specialization. In addition, host adaptation is often concomitant with extensive gene deletions (Boyd *et al.*, 2003) and complex nutritional requirements, as observed in other host-adapted serovars, such as *S. Typhi*.

It was initially tempting to attribute the absence of ADP-glucose pyrophosphorylase activity to the major sequence change that was found in the *glgC* gene of the three strains of *S. Gallinarum*, which had been isolated from different parts of the world, particularly since the product of the *glgC* gene is responsible for considerable regulation of

Fig. 4. Survival of *S. Typhimurium* F98 WT (●) and *glgC* mutant (■) in faeces at 20 °C (a) and in water at 4 °C (b). In (b), the same strains are shown (circles and squares, respectively) cultured with (closed symbols) or without (open symbols) glucose. Survival at 37 °C in saline of *S. Typhimurium* F98 (c), *S. Gallinarum* 9 (d) and *S. Pullorum* 3 (e) WT and the respective *glgC* mutant (c) or Glc⁺ transductant (d, e). Mean values from three experiments are shown. ●, WT; ■, *glgC* mutant or Glc⁺ transductant. Error bars show SEM.

glycogen synthesis through allosteric competition by fructose 1,6-bisphosphate (Leung & Preiss, 1987a). However, the absence of this change in *S. Pullorum* suggested that this was not the sole reason. The ability to transduce these serotypes to a glycogen-positive phenotype suggested that additional mutations to a glycogen-negative phenotype had occurred elsewhere in an ancestor of both these serotypes and presumably within ~40 kb of the adjacent *glpD* gene. Analysis of the published incomplete sequence showed this to be the case, with different mutations in both *glgP* and *glgB* in both serotypes. The absence of the additional accumulation of mutations in the other genes in these operons suggests that the changes have taken place relatively recently, with several additional mutations occurring in the *glgC* gene of *S. Gallinarum*.

Glycogen is known to be a major energy reserve in the *Enterobacteriaceae*, the significance of which has been unclear until relatively recently. Glycogen reserves have been reported to be important for biofilm formation and virulence of *S. Enteritidis* (Bonafonte *et al.*, 2000). The production of excess glycogen can be induced by mutation in a number of regulatory genes, such as *glgX*, *glgR*, *glgQ* and *csrA*, the last, at least, of which is known to be pleiotropic (Preiss, 1996; Baker *et al.*, 2002; Dauvillee *et al.*, 2005) and to regulate the expression of some virulence genes (Altier *et al.*, 2000). Glycogen is known to be of importance to sporulating bacteria such as *Clostridium* and *Bacillus* (Preiss, 1984), but its known contribution to survival in *E. coli* has so far been limited to a role in prolonged survival under starvation conditions (Strange, 1968). The literature on the

Fig. 5. Expression of β -galactosidase from the *rpoS* promoter in plasmid pRW50, during growth in LB supplemented with 0.5% glucose, in *S. Typhimurium* F98 WT parent (● and black bars) and *glgC* mutant (□ and white bars). Error bars show SEM; $n=3$.

role of glycogen outside the host is apparently conflicting and species specific. The presence of large amounts of glycogen is thought to compromise survival ability in *Sarcina lutea* (Burleigh & Dawes, 1967). In contrast, accumulation of glycogen acts to promote survival of *Streptococcus mitis* under starvation conditions (Van Houte & Jansen, 1970). We have found that glycogen production contributes to the survival of *S. Typhimurium* in faeces and water. However, transductants of serovars Gallinarum and Pullorum expressing glycogen-synthesis ability did not display a fitness advantage. In saline, functional *glgC* appeared to contribute more to long-term survival. Henrissat *et al.* (2002) found that a higher proportion of obligately parasitic bacteria lack enzymes for glycogen elaboration and speculated that this is related to lifestyle. On this basis, the avian serovars Gallinarum and Pullorum are more highly adapted to their host than are the other pathogenic serovars. However, the results of our experimental work suggest that the explanation may be more complex.

A running hypothesis was that glycogen reserves increase virulence if the organism has had the opportunity to accumulate them. This occurs with some other storage compounds, such as ferritin (Ftn), so that growth in iron-rich media contributes to survival ability in iron-depleted media, but only when the organisms are pre-grown in iron-rich media (Abdul-Tehrani *et al.*, 1999). However, in our study, there was no indication that WT strains subjected to prior culture in the presence of glucose were more virulent than those pre-cultured in the absence of glucose, although this improved the recovery of organisms from samples. Glycogen production did not contribute to the virulence of serovars Typhimurium or Gallinarum in either an avian or murine model of salmonellosis.

The absence of a major contribution of glycogen to intestinal colonization by *S. Typhimurium* suggests that if it is

indeed produced in the gut, it is also utilized *in situ*. A recent school of thought suggests that most bacterial growth in the large intestine (caeca/colon) occurs at the interface of the mucosa and the plug/core of chyme (Poulson *et al.*, 1995). Here, bacteria may be able to utilize components of mucin that would contribute to glycogen synthesis. This may enhance survival rates in the core of the caecal contents, where microbial growth is likely to be virtually zero. It may be expected, therefore, that survival of the parent in faeces would be greater than that of a *glgC* mutant, which is indeed the case.

The relatively minor effect of mutations in *glgC* on *rpoS* expression in our experiments was interesting and unexpected, since during starvation, and when glycogen reserves are depleted, RpoS levels might be expected to increase. This shows that the interaction between energy-rich reserves and the small-molecular-mass intracellular metabolites that regulate RpoS expression is complex (Preiss, 1996; Romeo, 1998). Glycogen may provide a steady and non-varying supply of carbon for metabolism and biosynthesis during periods when growth rates may fluctuate considerably and where the replenishment of glycogen and other carbon sources may be intermittent.

In conclusion, under the conditions examined, glycogen has a complex but comparatively minor role in virulence and colonization, but has a more significant role in survival. The fact that *S. Typhimurium glgC* mutants die off more rapidly in saline, and survive less well in both faeces and water, suggests that glycogen has a role in full survival and, hence, in prolonging the infectivity of broad-host-range *Salmonella* outside the host.

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