RESEARCH ARTICLE

Identification of novel attenuated *Salmonella* Enteritidis mutants

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Abstract

Salmonella Enteritidis is a major food-borne pathogen that causes nontyphoidal diarrhoea in humans. Infection of adult egg-laying hens usually results in symptomless carriage but in young chicks it may cause paratyphoid disease. It is not known whether *S.* Enteritidis requires genes additional to known virulence genes for systemic infection of young chickens. A transposon insertion library was created using *S.* Enteritidis 10/02, which yielded 1246 mutants. Of 384 mutants screened in chickens for attenuation (30.8% of insertion library), 12 (3.1%) had a 50% lethal dose at least 100 times that of the parental strain. Sequencing revealed insertions in genes involved in the biosynthesis of lipopolysaccharide, cell membrane, ATP biosynthesis, transcriptional regulation of virulence and the *yhbC* gene, which has an unknown function. Evaluation of *in vitro* virulence characteristics of a Δ*yhbC* mutant revealed that its ability to invade HeLa cells and survive within a chicken macrophage cell line (HD11) was significantly reduced. It was also less resistant to reactive oxygen and nitrogen intermediates and had a retarded growth rate. Chickens challenged with the Δ*yhbC* mutant cleared the organism from the liver and spleen 1 week faster than the parental strain and were able to develop specific serum IgG antibodies against the Δ*yhbC* mutant.

Introduction

There are over 2400 different serovars of *Salmonella enterica* (Brenner *et al.*, 2000) and almost all are believed to be able to cause some illness in humans. One of the most important *Salmonella* serovars is *Salmonella* Enteritidis, which is responsible for a substantial number of *Salmonella* infections in humans each year (Patrick *et al.*, 2004). *Salmonella* Enteritidis is a major food-borne pathogen that causes mainly nontyphoidal diarrhoea in humans. Infection occurs mainly through the consumption of contaminated poultry products, particularly raw or undercooked eggs (Kimura *et al.*, 2004). The presence of *S.* Enteritidis in or on the eggs occurs through faecal contamination of eggshells or invasion of partially formed eggs via transovarian transmission (Keller *et al.*, 1995). This occurs because *S.* Enteritidis is able to colonize the tissues of the ovary and oviduct without causing clinical disease in egg-laying hens, which then allows it to invade the eggs as they are being formed (Keller *et al.*, 1995). In newly hatched or young chicks, however, infection with *S.* Enteritidis can lead to paratyphoid disease, resulting in illness and high mortality (Dhillon *et al.*, 1999).

Because the majority of *S.* Enteritidis-related cases of salmonellosis in humans may be linked to chicken eggs, one approach to reduce this incidence is to eliminate *S.* Enteritidis from the food chain or, more specifically, from egg-laying hens. For this strategy to be effective, a good vaccination programme is required and the vaccine would have to be able to elicit strong mucosal immunity in order to prevent *S.* Enteritidis from colonizing. Several live oral *Salmonella* vaccines have been created through the deletion of particular gene(s) that are involved in virulence as well as various other functions. When the mutation gives rise to an attenuation effect of sufficient magnitude, these mutants may be possible live vaccine candidates, provided they also elicit a strong immune response and are genetically stable (Curtiss *et al.*, 1993).

The primary aim of our study was to identify any *S.* Enteritidis genes involved in virulence in young chickens, which have not been described previously and have potential

for possible live vaccine development. Because *S.* Enteritidis is able to infect a wide range of hosts, including chickens, rodents and humans, they would be expected to possess many different genes to deal with the different environments of the different hosts. Genes that are necessary for infection of a particular host may not be as important for infection in a different type of host. It is known that certain virulence determinants do not contribute equally to different disease outcomes in different hosts (Santos *et al.*, 2001). Most chicken studies use older chickens that are less susceptible to paratyphoid disease. Therefore, little is known about the systemic infection of young birds and whether other genes additional to known virulence genes are required for virulence. Although the genes in SPI-2 have been established as being important for replication in mice, we do not really know whether there are other additional genes important for survival within chickens. Our study describes the creation and identification of mutants attenuated for virulence in young chickens and the assessment of the *in vitro* virulence characteristics of one of these mutants, Δ*yhbC*.

Materials and methods

Chickens

Newly hatched *Salmonella*-free commercial layers were purchased from a hatchery in Singapore. One-day-old chickens were housed in clean cardboard boxes with unrestricted access to commercial antibiotic-free starter feed and water. Boxes were kept in different rooms for different treatments. For long experiments, chickens were transferred to wire mesh cages at 2 weeks of age with unrestricted access to food and water.

Bacterial strains and culture

Salmonella enterica serovar Enteritidis 10/02 was used in this study and was obtained from the Agri-Food and Veterinary Authority of Singapore. It was originally isolated from the ovary of a 2-year-old layer. We used the API 20E biochemical test kit (Biomerieux) as well as PCR, with genus- and serotype-specific primers, to confirm that the isolate was indeed *S.* Enteritidis. The primers used were OMPCF and OMPCR (genus-specific) and ENTF and ENTR (serotype-specific) as described previously (Alvarez *et al.*, 2004). All *Salmonella* and *Escherichia coli* strains were cultured at 37 °C in Luria–Bertani (LB) broth or on LB agar for 18–20 h. Kanamycin (50 μg mL^{-1}) was added to the media for the culturing of *Salmonella* mutants. Xylose lysine deoxycholate (XLD) agar, with or without kanamycin (50 μg mL^{-1}), was used for the isolation of *S.* Enteritidis mutants and wild-type *S.* Enteritidis 10/02, respectively, from the organs of chickens.

Construction of insertion library and Δ*yhbC* mutant

A random insertion library was constructed from *S.* Enteritidis 10/02 using the EZ-Tn5^{TM} < KAN-2 > Transposome^{TM} Kit (Epicentre Biotechnologies) following the protocol provided by the manufacturer (Hoffman *et al.*, 2000). Briefly, electroporation was used to transform *S.* Enteritidis 10/02 cells with 1 μL of Transposome, a stable complex of the transposase enzyme and EZ-Tn5 < KAN-2 > transposon. Kanamycin-resistant colonies were isolated, creating a library of 1246 insertion mutants. One of the mutants, with an insertion in the *yhbC* gene, was identified from the mutant library as being attenuated for virulence in chickens. Subsequently, it was selected for further study because no description of its association with virulence in chickens had been published previously and because of the potential for further characterization of the gene product, which has an unknown function. A new *yhbC* deletion mutant was recreated by deleting the whole *yhbC* gene, except for the first and the last 10 nucleotides, using the Lambda Red recombinase system (Datsenko & Wanner, 2000). We followed the protocol exactly, except that 10–30 mM of L-arabinose was used to induce transcription of the lambda red genes from pKD46 instead of 1 mM. Briefly, a set of primers, yhbCF (ACAGCCAAAATTTC TTTGTTCATCGCGGGCTTTTCACCTCATCCAGACTGTT AAAAGTGGGTGTAGGCTGGAGCTGCTTC) and yhbCR (TTTATGTCTTGGGGGTGGGCTTGTCCACATTAGAGCA AAAATTAACAGAGATGATTACAGTTCCGGGGATCCGT CGACCT), was created in order to generate the PCR product that was required for deleting the *yhbC* gene through homologous recombination. Each primer was 80 nucleotides in length and contained 60 nucleotides flanking the start or the end of the *yhbC* gene and 20 nucleotides corresponding to the P1 or P4 sequence on the pKD13 vector, which carried the kanamycin resistance gene that would replace the target gene to be deleted on the chromosome. Once PCR products were created, they were introduced into *S.* Enteritidis 10/02 carrying the plasmid pKD46, which encodes the Lambda Red recombinase, using electroporation by standard methods (Sambrook & Russell, 2001). All nucleotide sequences were based on the nucleotide sequence of *S. enterica* Enteritidis PT4 NCTC 13349, available from the Sanger Institute, UK (http://www.sanger.ac.uk/Projects/Salmonella/). Another set of primers, yhbCF-2 (CATCGATCTCCTGCTCATAT) and yhbCR-2 (ATGTCCTTCGTAGCTGGATT), were used for PCR and DNA sequencing to confirm the deletion of the correct gene.

Identification of attenuated mutants *in vivo*

In this study, we used a definition of attenuation as having a 50% lethal dose (LD_{50}) that was at least 100 times that of the

parental strain. The LD_{50} of the parental strain was determined to be *c.* 2×10^7 bacteria. About 30% of the *S.* Enteritidis mutant library, consisting of 384 mutants, was screened directly in 3-day-old chickens for attenuation. Five 3-day-old chicks per mutant were challenged subcutaneously using a dose of *c.* 2×10^9 bacteria, suspended in 100 μL of phosphate-buffered saline (PBS) and delivered to the neck with a 1 mL syringe and a 26 g needle. This dose was about 100 times the LD_{50} of *S.* Enteritidis 10/02, with the method was described previously (Reed & Muench, 1938). All deaths were recorded over a 5-day period. We found that most deaths occurred within 3 days after being challenged with *S.* Enteritidis 10/02 and so a 5-day observation period was sufficient to identify any attenuated mutants. However, for the determination of the LD_{50} of the Δ*yhbC* mutant, the observation period was extended to 10 days. Positive (*S.* Enteritidis 10/02) and negative (PBS) controls were included in each experiment. All animal protocols adhered to the guidelines provided by the National Advisory Committee for Laboratory Animals in Research (NACLAR) in Singapore, and protocols were approved by the IACUC. The genes that were disrupted by the transposon, within attenuated mutants, were identified by direct sequencing of genomic DNA using the BigDye® Terminator v3.1 Cycle Sequencing Kit (PE Biosystems) and primers that were homologous to the ends of the transposon. The sequencing primers were Kan-2 FP-1 (ACCTAC AACAAAGCTCTCATCAACC) and Kan-2 RP-1 (GCAATGT AACATCAGAGATTTTGAG), which were provided with the Transposome kit. Approximately 4 μg of purified genomic DNA, which was extracted from overnight bacterial broth cultures using the Wizard® Genomic DNA Purification Kit (Promega), and 10 pmol of primer were added to 20 mL reactions containing 8 mL of the terminator ready reaction mix. The cycle sequencing program consisted of one cycle at 95 °C for 4 min, followed by 60 cycles of 30 s at 95 °C, 20 s at 55 °C and 4 min at 60 °C. Sequence similarity searches were performed using BLAST at the NCBI website (www.ncbi.nlm.nih.gov/blast).

Persistence in chickens and serum response from a live challenge with the Δ*yhbC* mutant

Two groups of 30, 3-day-old chickens were challenged subcutaneously with an overnight culture of the Δ*yhbC* mutant. One group was injected with a dose of 5.2×10^7 bacteria per chick, in a volume of 100 μL, using a 1 mL syringe and a 26 g needle. The other group received injections of 5.2×10^8 bacteria per chick. The positive control consisted of a group of 60 chickens challenged with a sublethal dose of 6.1×10^6 bacteria per chick. The negative control was made up of 30 chickens that received injections of 100 μL of PBS. After 1 week and then at weekly intervals thereafter until all the birds were sampled, blood (500–1500 μL) was either collected from the wing vein of birds older than 3 weeks or from the jugular vein of younger birds (5 birds group^{-1}) before euthanasia. Cloacal swabs were also taken and plated out onto XLD agar to determine the duration of shedding of the Δ*yhbC* mutant in the faeces. The liver and spleen were collected from euthanized birds, weighed and then homogenized in plastic sealable bags with PBS and a roller bottle, which was used to crush the organs. The homogenate was then serially diluted in PBS and plated out onto XLD agar and incubated at 37 °C for 24–48 h. The blood that was collected was allowed to clot at 37 °C for 2 h or at 4 °C overnight and then centrifuged at 13 000 ***g*** for 5–10 min. The serum was collected and then used in an enzyme-linked immunosorbent assay (ELISA) to determine the serum (IgG) response to infection with the Δ*yhbC* mutant. The antigen used in the ELISA was purified recombinant g,m flagellin antigen, which is characteristic for serovar Enteritidis (Yap *et al.*, 2001). Wells of 96-well microtitre trays (NUNC) were coated with 150 ng of purified antigen in 0.1 M sodium bicarbonate buffer (pH 9.6) in 100 μL volumes. Trays were incubated at 37 °C for 2 h or 4 °C overnight. Wells were subsequently blocked with 5% skim milk in 0.05% PBS-Tween 20 (PBS-T) for 1 at 37 °C. Later, 100 μL of test serum (1 : 50 dilution) in PBS-T, containing 1% skim milk, was added to each well, and the trays were incubated for 1 h at 37 °C. After five washes with PBS-T, 100 μL of horseradish peroxidase (HRP)-conjugated rabbit anti-chicken IgG antibody (1 : 2000 dilution) was added to each well and incubated at 37 °C for 1 h. Wells were washed five times again, and 100 μL of the substrate *o*-phenylenediamine dihydrochloride was added. The reaction was allowed to proceed at room temperature for 5–10 min and then stopped with 2 M H_2SO_4. The OD was recorded at 490 nm in an automatic ELISA plate reader (Sunrise™ microplate reader, Tecan, Switzerland).

Growth rate of Δ*yhbC* mutant

Overnight cultures of bacteria that had been grown in LB at 37 °C were diluted to *c.* 10^6 bacteria mL^{-1} with LB. One hundred microlitres of this suspension was used to inoculate 20 mL of fresh LB, in a 50-mL polypropylene centrifuge tube and incubated at 37 °C. Samples (100 μL each) were taken every hour for 10 h and diluted out in PBS and plated out onto LB agar. Agar plates were incubated at 37 °C for 24 h.

Resistance to reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI), sodium deoxycholate and normal chicken serum

We attempted to identify any differences between the response of the Δ*yhbC* mutant and the parental strain to a wide range of *in vitro* tests. Tests for resistance to ROI and

RNI were performed as described previously (Lu *et al.*, 1999). To determine the resistance to ROI, a lawn culture of bacteria was exposed to 30 μL of a 3% hydrogen peroxide solution, placed onto a sterile filter paper disc on an LB agar plate. Plates were incubated at 37 °C overnight and the zone of inhibition was measured. To determine the resistance to RNI, samples were taken at 0, 1, 3, 4.5 and 6 h after *c.* 10^9 bacteria were exposed to 20 mM sodium nitrite in 2 mL of LB (pH 5), diluted and then plated out onto LB agar. To examine any differences in resistance to sodium deoxycholate and chicken serum, *c.* 10^9 CFU of Δ*yhbC* mutant in 500 mL of PBS was mixed with 500 mL of 1% sodium deoxycholate or 20% normal chicken serum, which had been either heat inactivated (56 °C for 2 h) or left untreated. Viability was determined by agar plate counts.

Survival of the Δ*yhbC* mutant in chicken macrophages, at the stationary growth phase and under acidic conditions

Any differences in the ability of the Δ*yhbC* mutant, compared with the parental strain, to survive under acidic conditions and at the stationary growth phase were determined by the assays described previously (Lu *et al.*, 1999). Overnight cultures of bacteria were diluted with LB (pH 4) to *c.* 10^6 bacteria mL^{-1} in 5 mL and incubated at 37 °C without shaking. Samples were taken at 0, 1, 2, 3 and 4 h, diluted and plated out onto LB agar. For the assay determining viability under prolonged stationary phase, bacteria were grown in 100 mL of LB at 37 °C for 6 days with shaking. Samples were taken every 24 h and the number of viable cells was determined by plate counts. Assays measuring the ability of the Δ*yhbC* mutant to survive in the continuous chicken macrophage cell line HD11 (Beug *et al.*, 1979) were conducted as described previously (Kramer *et al.*, 2003) without minor modifications. Briefly, *c.* 10^5 macrophages were infected with 10^6 CFU of bacteria and incubated at 37 °C for 30 min in 5% CO_2 after which media containing gentamicin (200 μg mL^{-1}) were added for 30 min to kill any extracellular bacteria. After two washes, cells were incubated in media containing 20 μg mL^{-1} of gentamicin. At various time points (1, 4, 7, 24 and 48 h), cells were lysed with 0.1% Triton X-100 and the number of viable intracellular bacteria was determined by a plate count. *Escherichia coli* DH5α was used as a negative control.

Invasion of HeLa cells

The ability of the Δ*yhbC* mutant to invade cultured cells was assessed by invasion assays, which were performed exactly as described previously (Lu *et al.*, 1999), except that after the addition of bacteria, culture plates were not centrifuged. The percentage of invasion was derived by taking the number of bacteria added divided by the number of bacteria recovered multiplied by 100.

Complementation of *yhbC*

For gene complementation, *yhbC* was amplified from *S.* Enteritidis 10/02 using primers yhbC-comp-F5 (CCGGGGGATCCAGGAGGATATTCATATGTCACC TCAT CCAGACTGTTAAAAGTGG), which contained a BamHI site, and yhbC-comp-R5 (CCGGGGTCGACTTAACAGA GATGATTACAGC), which contained a SalI site. The PCR product was cloned into pBR322 and transformed directly into the Δ*yhbC* mutant. Plasmid pB*yhbC* was isolated from the ampicillin-resistant transformants and the presence of the *yhbC* insert was verified by PCR. A chicken challenge experiment using 3-day-old chicks was performed to assess whether expression of *yhbC* from a plasmid could restore virulence. Doses of *c.* 5×10^6, 10^7, 10^8 and 10^9 bacteria per bird were given as described for identification of attenuated mutants *in vivo*. *Salmonella* Enteridis 10/02, the parental strain, was injected into birds that were used as a positive control and PBS was used as the negative control. The resulting deaths were recorded over a 7-day period.

Results

Identification of attenuated mutants *in vivo*

Out of the 384 mutants that were screened in chickens (30.8% of insertion library), 12 (3.1%) were found to have an LD_{50} at least 100 times that of the parental strain (Table 1). Six of these 12 mutants had insertions in genes that were involved in the biosynthesis of the lipopolysaccharide core or the O side chain. The remaining mutants all had insertions in well-characterized genes or genes with an assigned function, except for one, the *yhbC* gene. Currently, the function of *yhbC* is still unknown. The other mutants with insertions in genes with a known function included one mutant that had an insertion in the *ompR* gene, which is part of the OmpR-EnvZ two-component regulatory system and is a known virulence gene. There were three mutants with insertions in genes encoding either components of the inner membrane or involved in the assembly of outer membrane proteins. They included the *tolA*, *yfiO* and *asmA* genes. Finally, sequencing data revealed that in one mutant, the transposon had inserted into the *aceE* gene, which codes for the E1 enzyme of the multi-enzyme pyruvate dehydrogenase complex.

Growth rate of the Δ*yhbC* mutant

The growth rate of the Δ*yhbC* mutant, compared with the parental strain, was measured over a 10-h period. Although 10 h was not enough time for the cultures to reach the

Table 1. *Salmonella* Enteritidis mutants attenuated for virulence in chickens

No.	Insertion library ID	Gene	Function of gene product	Challenge dose (CFU bird^{-1})	No. of survivors
1	SEM1A5	*ompR*	Transcriptional regulator	3.1×10^9	4/5 (80%)
				3.43×10^9	18/18 (100%)
2	SEM1C3	*waaJ*	Lipopolysaccharide core biosynthesis	2.66×10^9	5/5 (100%)
3	SEM2B12	*rfbE*	Lipopolysaccharide O-chain biosynthesis	3.24×10^9	5/5 (100%)
4	SEM2F1	*waaG*	Lipopolysaccharide core biosynthesis	8.0×10^9	5/5 (100%)
5	SEM2F8	*yfiO*	Assembly of outer membrane proteins	3.5×10^9	5/5 (100%)
				1.06×10^{10}	9/10 (90%)
6	SEM2H6	*yhbC*	Unknown	1.39×10^9	9/10 (90%)
				4.6×10^9	6/10 (60%)
7	SEM3A7	*asmA*	Assembly of outer membrane proteins	1.91×10^9	10/10 (100%)
8	SEM3C7	*rfbD*	Lipopolysaccharide O-chain biosynthesis	3.0×10^9	4/5 (80%)
9	SEM3F2	*aceE*	ATP biosynthesis	2.15×10^9	5/5 (100%)
10	SEM4B5	*waaO*	Lipopolysaccharide core biosynthesis	1.09×10^9	5/5 (100%)
11	SEM4B6	*tolA*	Membrane spanning protein	1.15×10^9	5/5 (100%)
12	SEM4C4	*rfbF*	Lipopolysaccharide O-chain biosynthesis	1.66×10^9	5/5 (100%)

Three-day old chickens were challenged subcutaneously and mortality was recorded over a period of 5–10 days. The challenge dose was c. 100 times, or higher, the LD_{50} of the parental strain.

stationary phase, it was sufficient to gain an insight into the growth characteristics of this mutant. The number of Δ*yhbC* mutant cells was 2.18×10^4 CFU at the start of the assay, which was about threefold lower than the initial number of the parental strain, at 6.3×10^4 CFU. However, after 10 h of growth the Δ*yhbC* mutant was nearly two logs lower than the parental strain and appeared to have a slightly longer lag phase, taking almost 2 h longer to enter the exponential phase (Fig. 1). On solid media such as LB agar, the Δ*yhbC* mutant requires around one and a half times longer incubation period to reach the same colony size as the parent strain (data not shown). The Δ*yhbC* mutant clearly has a retarded growth rate compared with its parent.

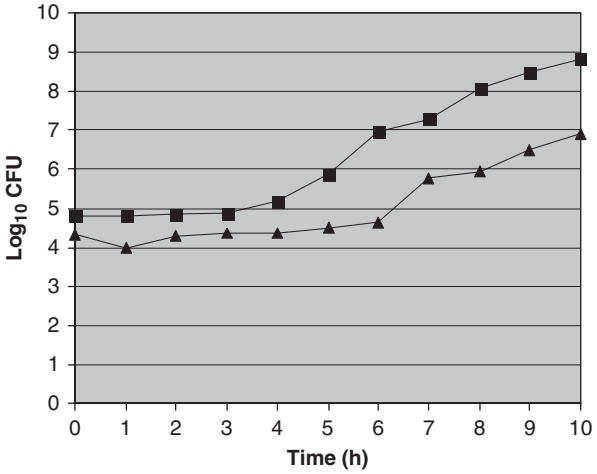

Fig. 1. Growth curve of the Δ*yhbC* mutant (▲) and *Salmonella* Enteritidis 10/02 (■) over a 10-h period. Data are from a representative assay typical of numerous assays and clearly display the slower growth rate of the Δ*yhbC* mutant compared with the parental strain. Although the initial number of bacteria for Δ*yhbC* mutant (2.18×10^4 CFU) was about three times lower than the parental strain (6.3×10^4 CFU), by 10 h it was nearly two logs lower than the parental strain. It also seems to have a slightly longer lag phase, taking about 2 h longer to enter the exponential phase. At 10 h, the cultures had not reached the stationary phase.

Virulence characteristics of the Δ*yhbC* mutant

No significant difference was found between the Δ*yhbC* mutant and the parental strain in their ability to grow after a prolonged stationary phase or their ability to resist low pH conditions, sodium deoxycholate and chicken serum. However, the assays in which there was a significant difference included invasion, resistance to ROIs and RNIs and survival within macrophages (Table 2). The Δ*yhbC* mutant was significantly less able to penetrate HeLa cells, with intracellular bacterial numbers reaching only 1% of that of the parental strain. It was also significantly less resistant to ROI and RNI. For ROI, average zones of inhibition were considered to be significant if it was greater than 3.76 cm because this value was equal to the average zone size of the WT strain (3.67 cm), plus three SDs (0.03 cm). The average zone size for the Δ*yhbC* mutant was 3.98 cm, which was significant. For resistance to RNIs, the population of both the Δ*yhbC* mutant and the parental strain declined steadily over time upon exposure to sodium nitrite, and the number of surviving bacteria was recorded over for a 6-h period. After 4.5 h, the population of the Δ*yhbC* mutant had been reduced to zero whereas the population of the parental strain remained at *c.* 10^4 CFU and was only reduced to zero at the next sampling point at 6 h. For the macrophage assay, the chicken macrophage cell line HD11 was able to reduce the intracellular numbers of both the Δ*yhbC* mutant and the

Table 2. *In vitro* virulence assays where there was a difference in response between Δ*yhbC* mutant and the parental strain, *Salmonella* Enteritidis 10/02

		Resistance to ROI	Resistance to RNI				Survival in macrophages					
			Log_{10} CFU at				Log_{10} CFU at					
In vitro assay	% Invasion of HeLa cells	Zone of inhibition (cm)	0 h	1 h	3 h	4.5 h	6 h	0 h	1 h	4 h	7 h	24 h	48 h
S. Enteritidis 10/02	1.44%	3.67 ± 0.03	10.1 ± 0.74	8.50 ± 0.84	7.17 ± 0.62	4.01 ± 0.44	0	6.55 ± 0.07	3.60 ± 0.06	3.57 ± 0.05	3.40 ± 0.05	2.79 ± 0.07	2.94 ± 0.03
Δ*yhbC* mutant	0.02%	3.98 ± 0.03	9.53 ± 0.42	8.79 ± 0.45	7.10 ± 0.41	0	0	6.70 ± 0.04	3.45 ± 0.07	3.30 ± 0.04	3.05 ± 0.06	0	0

parental strain. Bacterial numbers declined steadily from the first sampling time point at 1 h postinfection. However, by 24 h, intracellular organisms of the Δ*yhbC* mutant had been completely eliminated as no bacteria could be recovered by a plate count. In contrast, the parental strain was still recoverable, numbering in the hundreds of CFU $well^{-1}$ after 24 h, and by 48 h this number had not declined but had increased slightly, indicating that it could resist complete killing by HD11 cells. For the negative control, *E. coli* cells were almost completely killed off after 7 h.

Serum response to challenge with the Δ*yhbC* mutant

The serum IgG response of chickens challenged with the Δ*yhbC* mutant was measured using ELISA, and the target antigen used in the assay was recombinant flagellin containing the g,m epitope specific for *S.* Enteritidis. A subcutaneous challenge with 5.2×10^7 and 5.2×10^8 organisms of Δ*yhbC* mutant induced a specific IgG response (Fig. 2). Both challenge doses induced a bell-shaped curve typical of primary exposure to an antigen. The response from challenge with the higher of the two doses induced a slightly better IgG response. However, this was still much lower than the IgG levels seen in chickens that were challenged with a sublethal dose of only 6.1×10^6 organisms of the parental

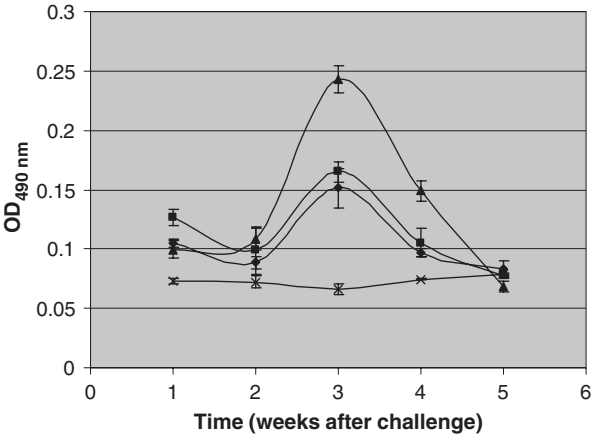

Fig. 2. Serum IgG response of chickens challenged with the Δ*yhbC* mutant. Sera were diluted 1 : 50 and tested by ELISA using purified recombinant flagellin of *Salmonella* Enteritidis 10/02 as the target antigen. Each data point represents the average value from three separate assays and represents the mean of five different serum samples collected from chickens at weekly intervals. Bars represent the SD. Chickens were challenged subcutaneously with 5.2×10^7 organisms of the Δ*yhbC* mutant (•) or 5.2×10^8 organisms of the Δ*yhbC* mutant (■). Serum IgG responses of Δ*yhbC* mutant-challenged birds were compared with serum from birds injected with PBS (×) and serum from birds challenged with 6.1×10^6 organisms of WT *S.* Enteritidis 10/02 (▲). $OD_{495\,nm}$ values above 0.85 were considered to be positive (mean of negative control ± 3 SD).

strain, which was about one to two logs lower than the doses used for the Δ*yhbC* mutant.

Persistence of the Δ*yhbC* mutant in chickens

Only one death was recorded in the group of birds challenged with 5.2×10^7 CFU of the Δ*yhbC* mutant (3.3%) and two deaths in the group challenged with 5.2×10^8 CFU (6.6%). In contrast, 31 deaths out of 70 (44.3%) birds challenged with 6.1×10^6 CFU of the parental strain were recorded. In order to determine how long it would take for complete clearance of the Δ*yhbC* mutant from the liver and spleen of chickens, samples were taken at weekly intervals until bacteria could no longer be recovered. The livers of chickens that had been challenged subcutaneously with 5.2×10^7 CFU of the Δ*yhbC* mutant had completely cleared by the second week postchallenge. The livers from chickens challenged at the higher dose of 5.2×10^8 CFU took 3 weeks to clear and in contrast, birds challenged with the WT strain took 5 weeks (Fig. 3a). Clearance from the spleen took longer but at the lower dose, the Δ*yhbC* mutant could not be recovered from four out of five birds sampled and was completely cleared from all birds sampled at 4 weeks postchallenge. At the higher dose, it took as long as the WT strain, around 5 weeks (Fig. 3b).

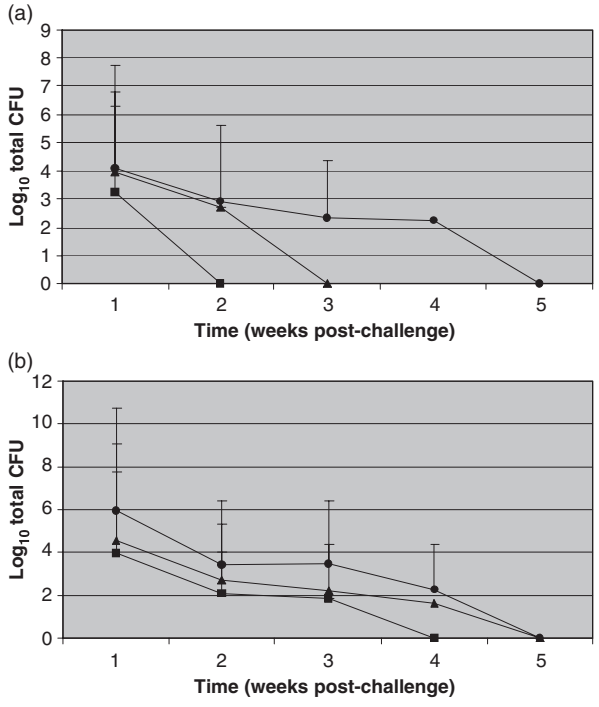

Fig. 3. Persistence of the Δ*yhbC* mutant in chickens challenged subcutaneously at 3 days old. (a) Bacteria recovered from the liver (b) Bacteria recovered from the spleen. (•) *Salmonella* Enteritidis 10/02; (■) 5.2×10^7 CFU Δ*yhbC* mutant; (▲) 5.2×10^8 CFU Δ*yhbC* mutant. Each data point represents the mean of five different samples. Bars represent the SD.

Complementation of *yhbC*

The Δ*yhbC* mutant, when complemented with pB*yhbC*, was able to kill chickens at a level that was almost identical to the parental strain. No chickens survived when given a dose of *c.* 5×10^8 or 5×10^9 CFU of either the WT strain or the Δ*yhbC* mutant complemented with *yhbC*. At a lower dose of 5×10^7 CFU, after 7 days postchallenge, two chickens and one chicken survived, respectively, of the WT strain and the complemented strain. At the lowest dose of 5×10^6 CFU, seven chickens survived in both groups.

Discussion

In our study, chickens were challenged with *S.* Enteritidis mutants subcutaneously, which bypassed the initial phase of entry into the host. Therefore, we did not expect to find mutants that were attenuated for invasion but those that had lost the ability to survive or replicate inside the host, which are characteristics important for systemic infection. Identification of the lipopolysaccharide and *ompR* mutants validated our *in vivo* screening procedure in the chicken because *ompR* and the lipopolysaccharide genes are well-known virulence genes of *Salmonella*. Six of the 12 attenuated mutants identified from our screening process were lipopolysaccharide mutants. Lipopolysaccharide is a major virulence determinant that has been associated with the ability of *Salmonella* to colonize the gastrointestinal tract of chickens and is responsible for serum resistance (Grossman *et al.*, 1987; Carroll *et al.*, 2004). There is a correlation between the length of the O side chains and virulence. Virulent isolates of *S.* Enteritidis possess longer O chains than avirulent isolates (Rahman *et al.*, 1997). Because the lipopolysaccharide mutants identified in this study had mutations in lipopolysaccharide genes encoding either core components or O-antigen, they would be expected to have either short or absent O chains, making them more susceptible to killing.

The *ompR* gene plays an important role as a regulator of transcription and its absence has a dramatic effect on virulence. An *ompR* mutant of *Salmonella* Typhimurium was shown to be attenuated in mice, with an LD_{50} that was 10^5 times that of the parental strain when delivered intravenously (Dorman *et al.*, 1989). The OmpR protein, apart from regulating expression of porin proteins like OmpC and OmpF in response to osmolarity, regulates the expression of another two-component regulatory system, SsrA-SsrB. This sensor-kinase system regulates the expression of the type III secretion system in *Salmonella* pathogenicity island 2 (SPI-2), which encodes genes that are required for intracellular replication (Lee *et al.*, 2000; Garmendia *et al.*, 2003). Lee *et al.* established that loss of virulence in *ompR* mutants

could be partly attributed to the loss of the transcriptional regulator of the SsrA-SsrB system- the OmpR protein. Expression of the genes in SPI-2 is essential for virulence in mice and although we did not carry out any further work with the *ompR* mutant, it is likely that there is a correlation between the expression of OmpR and virulence in chickens in a manner similar to that seen in mice.

The *tolA* mutant is a perisplasmic-leaky mutant and tends to lose its perisplasmic content due to the loss of integrity of the membrane (Amouroux *et al.*, 1991) and is more susceptible to surfactants like deoxycholate, found in bile. Moreover, *tolA* mutants are impaired in an O-antigen assembly (Vines *et al.*, 2005). The fragility of this mutant would compromise its ability to establish an infection in the host. Similarly, *yfiO* and *asmA* mutants are unable to synthesize certain outer membrane proteins, reducing their ability to establish infection. The *aceE* mutant, which has an insertion in the E1 enzyme (carboxylase component) of the pyruvate dehydrogenase complex, is the subject of another study currently in progress and will not be discussed here. Owing to the large number of lipopolysaccharide and membrane-damaged mutants identified in our study, it appears that systemic virulence in chickens depends heavily on the integrity of surface structures like lipopolysaccharide and the outer membrane once it has breached the initial host barrier.

To further our study, we decided to focus mainly on the *yhbC* gene because of its unknown function and the lack of literature associated with this gene. We propose that the attenuating effect of the Δ*yhbC* mutant in chickens is due primarily to its slow growth rate. We believe that the *yhbC* gene is not a *bona fide* virulence gene but is associated with virulence due to its role in some basic process within the bacterial cell that affects its growth rate. The growth of Δ*yhbC* mutant is much slower than the parental strain. After 10 h of growth in an environment containing freely accessible nutrients and oxygen, there was almost 100 times less number of Δ*yhbC* mutant cells compared with the parental strain. Naturally, within the harsh environment of the host and without free access to nutrients, the Δ*yhbC* mutant is expected to be even more slow-growing, thus giving the host a better chance to clear the organism. Because the *yhbC* gene is found within the *metY-nusA-infB* operon, which contains genes that are all involved in either transcription or translation, it is reasonable to speculate that the *yhbC* gene is also associated with one of these processes, which could ultimately affect its growth rate. It has been suggested that *yhbC* could have an Sm RNA-binding domain and is part of the LSm (like Sm) protein family (Anantharaman & Aravind, 2004). The LSm proteins form multimeric complexes that bind RNA (Khusial *et al.*, 2005). The organization of the *metY-nusA-infB* operon is similar to that of *E. coli* and begins with the *metY* gene encoding initiator tRNA, the *yhbC* gene encoding a cytoplasmic protein composed of 140 amino acids, the *nusA* gene encoding a transcription termination factor, the *infB* gene encoding the essential IF2 factor required for initiation of translation, the *rbfA* gene encoding a ribosome-binding factor and the *truB* gene encoding tRNA pseudouridine 5S synthase (Bylund *et al.*, 2001). In constructing the Δ*yhbC* mutant, care was taken to avoid creating polar mutations that would affect genes downstream in order to ensure that any effects of the mutation were solely from the loss of the *yhbC* gene.

The Δ*yhbC* mutant was significantly more susceptible to killing by ROI and RNI than the parental strain. In agreement with these data, the Δ*yhbC* mutant was unable to resist killing by chicken macrophages *in vitro* unlike the parental strain. Moreover, chickens challenged with the Δ*yhbC* mutant were able to eliminate the mutant faster than the parental strain, presumably as a result of clearance by macrophages from the liver and spleen, from where *Salmonella* is thought to reside during systemic infection. We propose that the increased susceptibility to ROI, RNI and killing by macrophages could be explained through its slower ability to synthesize the proteins and enzymes necessary for resistance rather than *yhbC* having some direct role in resistance. Similarly, its reduced ability to invade HeLa cells may also be attributable to its inability to synthesize the necessary proteins fast enough when required for invasion. When the Δ*yhbC* mutant was complemented with a copy of *yhbC* expressed from a plasmid, it regained its ability to kill 3-day-old chicks at a level comparable to the parental strain. This indicated that attenuation of virulence could be attributed solely to the deletion of *yhbC*.

Protection against *Salmonella* is mediated by both mucosal and serum antibodies. Our data demonstrated that the Δ*yhbC* mutant was able to induce specific serum IgG in chickens challenged with this mutant. However, it is not known whether the levels of serum antibodies would be sufficient for protection against a subsequent challenge by the WT strain. This could be tested in future studies on this novel mutant, now part of the ever-growing list of potential live vaccine candidates of *Salmonella*.

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Authors' contribution

J.C. and E.P. contributed equally to this study.

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