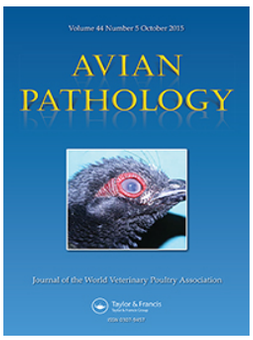

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ORIGINAL ARTICLE

The contribution of aerobic and anaerobic respiration to intestinal colonization and virulence for *Salmonella typhimurium* in the chicken

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The basic mechanism whereby *Salmonella* serovars colonize the chicken intestine remains poorly understood. Previous studies have indicated that proton-translocating proteins utilizing oxygen as terminal electron acceptor do not appear to be of major importance in the gut of the newly hatched chicken and consequently they would be even less significant during intestinal colonization of more mature chickens where the complex gut microflora would trap most of the oxygen in the lumen. Consequently, alternative electron acceptors may be more significant or, in their absence, substrate-level phosphorylation may also be important to *Salmonella* serovars in this environment. To investigate this we constructed mutants of *Salmonella enterica* serovar Typhimurium defective in various aspects of oxidative or substrate-level phosphorylation to assess their role in colonization of the chicken intestine, assessed through faecal shedding, and virulence. Mutations affecting use of oxygen or alternative electron acceptors did not eliminate faecal shedding. By contrast mutations in either *pta* (phosphotransacetylase) or *ackA* (acetate kinase) abolished shedding. The *pta* but not the *ackA* mutation also abolished systemic virulence for chickens. An additional *ldhA* (lactate dehydrogenase) mutant also showed poor colonizing ability. We hypothesise that substrate-level phosphorylation may be more important than respiration using oxygen or alternative electron acceptors for colonization of the chicken caeca.

Introduction

Salmonella enterica serovar Typhimurium and *S. Enteriditis* are the two *S. enterica* serovars most frequently associated with human food-poisoning, with 40,828 laboratory confirmed cases in the USA in 2009 and with more than 95,000 cases in the European Union in 2011 (CDC, 2011; Eurosurveillance editorial team, 2013).

Adult (56-week-old), healthy chickens generally show no clinical disease following oral infection with these serovars (Smith & Tucker, 1980) with asymptomatic caecal colonization and persistent faecal shedding of organisms, which results in carcass contamination at slaughter (Gast, 2008).

Although considerable progress has been made, the basic mechanism whereby *S. enterica* serovars colonize the intestine is still poorly understood. Screening randomly generated mutant libraries of *S. typhimurium* and more directed studies have provided some insights into the bacterial genes required for colonization of chickens which are several weeks old and possess a gut flora (Craven, 1994; Turner *et al*., 1998; Morgan *et al*., 2004; Chaudhuri *et al*., 2013). Type I and other fimbriae, including Stb, Csg and Sth (Morgan *et al*., 2004; Chaudhuri *et al*., 2013), are thought to be involved in bacterial attachment to the mucosal layer or even epithelial cells. Lipopolysaccharide is also thought to be involved but it is unclear how (Craven, 1994; Turner *et al*., 1998; Morgan *et al*., 2004), although a role has been suggested for preventing entrapment in mucin and/or for resistance to bile. Other factors, including some secreted proteins, contribute in different host species but again it is unclear how (Turner *et al*., 1998; Morgan *et al*., 2004). Additionally, global regulatory genes (Methner *et al*., 2004) and a number of metabolic functions including serine and citrate utilization, together with response to heat shock conditions, appear to contribute to the process in adult birds (Turner *et al*., 1998; Morgan *et al*., 2004). Although some of the genes identified indicate that a close association with the gut mucosa is important in *Salmonella* colonization, the metabolic behaviour of bacteria, and specifically how they generate energy is still not understood. Colonization of birds which possess a complex gut flora is likely to involve severe competition for nutrients and electron acceptors with numerically dominant obligate anaerobes. Carbon sources such as 1,2-propanediol, propionate and ethanolamine are catabolized with electron acceptors such as tetrathionate, at least in young birds (Harvey *et al.,* 2011) and in mice the initial inflammatory response in the intestine is thought to release

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ethanolamine and tetrathionate (Winter *et al.*, 2010; Thiennimitr *et al.*, 2011).

Salmonella and *E. coli* are able to derive their energy from a variety of carbon sources by respiration (Gennis & Stewart, 1996) or, if terminal electron acceptors are not available, by substrate-level phosphorylation through fermentation (Unden & Dünnwald, 2008). Respiration is modular since a number of electron donors can be combined with a variety of terminal electron acceptors depending on the ambient redox conditions and substrate availability. Oxygen is the preferred electron acceptor since glucose catabolism allows for 10 NADH molecules which allows for 38 ATP molecules. In the absence of oxygen, a hierarchy of electron acceptors are selected on the basis of their availability and the redox environment. The spectrum of energy generated varies from O_2/H_2 at +818 mV to fumarate/succinate at +30 mV (Gennis & Stewart, 1996).

Since proton-translocating proteins utilizing oxygen do not appear to be important in the gut of the newly hatched chicken (Zhang-Barber *et al.*, 1997; Dhawi *et al.*, 2011; Harvey *et al.*, 2011) they might be expected to be even less significant during intestinal colonization of chickens with the complex gut microflora trapping oxygen in the lumen. Multiplication of facultative anaerobes then takes place largely at the periphery of the lumen in contact with the mucosa where nutrients and oxygen will be present in higher concentrations (Poulsen *et al.*, 1995; Harvey *et al.*, 2011).

Alternative electron acceptors may be more significant or, in their absence, substrate-level phosphorylation may also be important. We have constructed a series of mutants of *S. typhimurium* defective in various aspects of oxidative or substrate-level phosphorylation to assess their role in colonization of the chicken intestine measured by faecal shedding and also for virulence for the chicken. Our model of colonization uses newly hatched chickens administered a gut flora preparation to reduce any variation in susceptibility that might occur from birds acquiring their own.

Materials and Methods

Bacteria and culture conditions. *S. enterica* serovar Typhimurium F98 is virulent for chickens and also colonizes the chicken alimentary tract (Turner *et al.*, 1998). For ease of enumeration in the alimentary tract, spontaneous nalidixic acid-resistant mutant derivatives of this strain were used which were produced by selection on nutrient agar (Oxoid, Basingtoke, UK) containing sodium nalidixate (50μg/ml). This has been found previously not to affect their virulence (Smith & Tucker, 1980).

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

Production of mutants. We produced a series of tagged deletion mutations in genes affecting the ability to utilize different terminal electron acceptors (Table 1) using standard allele replacement technology as described previously (Turner *et al.*, 2003). All mutations were transduced into a fresh parental background using bacteriophage P22 and selecting for antibiotic resistance. 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. Chloramphenicol sensitivity ensured that the mutants were the result of a double cross-over event that did not include pDM4 DNA.

Mutations in *nuoG, cyd*, *cyo* and *atp* have been characterised previously (Zhang-Barber *et al.*, 1997). Under aerobic conditions mutants grew more slowly than the parent. Mutations in *dmsA* and *frdA* were unable to grow anaerobically on formate as electron donor and DMSO or fumarate, respectively, as electron acceptor; *torC* and *narG/narZ/napA* mutants were unable to grow anaerobically with lactate as carbon source and either TMAO or nitrate, respectively, as electron acceptor. The *pta* and *ackA* mutants were unable to use acetate as a carbon source and were non-motile. Double mutants were produced in the same way and by transducing one mutation into the strain housing the second mutation checking genotype by PCR.

The Lambda-Red techinique (Datsenko & Wanner, 2000) was used to generate two additional mutants inactivating the *poxB* and *ldhA* genes. The kanamycin cassette (Km^r) replaced the open reading frame of *poxB* gene and a chloramphenicol cassette (Cm^r) replaced *ldhA* gene. Cm^r and Km^r colonies were selected on nutrient agar (Oxoid) containing chloramphenicol or kanamycin at concentration of 20 and 25 μg/ml, respectively. The correct site of insertion of each cassette was checked by PCR with selected colonies. After confirming the mutations, the *poxB* and *ldhA* mutations were transduced to a clean background using P22.

Experimental animals. Specified pathogen-free (SPF) Rhode Island Red chickens were used. 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, UK). Chickens were used within 24 h of hatching.

Intestinal colonization. Groups of 20 chickens, less than 24h old, were received as newly hatched.. Fresh faeces from an adult (40-week-old) SPF hen were incubated in 10 ml LB broth statically overnight at 37°C and 0.1 ml of this broth culture was administered to each bird within 24 h of hatching to provide a gut flora (Barrow *et al.*, 2003). Four days later 0.1 ml of an overnight LB broth culture of the *Salmonella* strain to be tested was administered to each chicken in the group.

The experimental design is described in Table 2. To assess intestinal colonizsation, three experiments were carried out. In experiment 1, chickens were divided in seven groups of 20 and then challenged with mutants with the inability to use oxygen as terminal electron acceptor and also with a *pta* mutant (groups 1 to 6); chickens of group 7 received the parent strain which was included as a control for comparison. Faecal excretion was assessed by taking cloacal swabs at weekly intervals for a period of 6 weeks.

In experiment 2, chickens were divided in 14 groups of 20 and then challenged with mutants with inabilities to use alternative electron acceptors and also with *pta* and *ackA* mutants (groups 1 to 13); one group of chickens received the parent strain and was included as control for comparison. Faecal excretion was assessed by taking cloacal swabs weekly for four weeks. The *ldhA* and *poxB* mutants together with the parent strain were assessed in a third experiment involving groups of 20 birds.

Cloacal swabs were plated in a standard manner (Smith & Tucker, 1980) on Brilliant Green agar (Oxoid) containing sodium nalidixate (20 μg/ml) and novobiocin (1 μg/ml). The number of birds at each time point expressed as a percentage which were excreting was calculated. Excretion rates were compared to that in the control group by Chi-square analysis.

Virulence assays. Virulence was assessed by oral inoculation of groups of birds with 0.1 ml (20 chickens within 24 h of hatching) of an undiluted culture of selected strains. Morbidity and mortality were recorded over a 3-week period. Birds 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 faeces around the vent. Mortality rates were analysed using the Chi-square test. Infection was confirmed by re-isolation of the inoculated strains from the liver.

Ethical compliance

All experimental work involving animals was carried out under UK Home Office project and personal licences and was approved by the local Animal Welfare and Ethical Review Body. All laboratory health and safety procedures were complied with during the course of the reported work.

Table 1. Salmonella Typhimurium *wild-type and mutant strains used in this study.*

Salmonella strain (mutated gene)	Function of mutated gene	Relevant resistance (all strains are Nal^r)	Source or reference
S. F98 (*S.*F98)	Wild-type strain	–	Turner *et al.* (1998)
*S.*F98 (*nuoG*)	NADH dehydrogenase I	Km^r	Zhang-Barber *et al.* (1997)
*S.*F98 (*cydA*)	cytochrome d oxidase	Km^r	Zhang-Barber *et al.* (1997)
*S.*F98 (*cyoA*)	cytochrome o oxidase	Km^r	Turner *et al.* (2003)
*S.*F98 (*atpB*)	F_0F_1 ATP synthase	Km^r	Zhang-Barber *et al.* (1997)
*S.*F98 (*atpH*)	F_0F_1 ATP synthase	Spc^r	Zhang-Barber *et al.* (1997)
*S.*F98 (*dmsA*)	DMSO reductase	Km^r	This study
*S.*F98 (*phsA*)	Thiosulphate reductase	Km^r	This study
*S.*F98 (*torC*)	TMAO reductase	Spc^r	This study
*S.*F98 (*narG*)	Nitrate reductase	Km^r	This study
S. F98 (*narZ*)	Nitrate reductase	Spc^r	This study
S. F98 (*narG*,*narZ*)	Nitrate reductase	Spc^r, Km^r	This study
S. F98 (*narG*,*napA*)	Nitrate reductase	Spc^r, Km^r	This study
S. F98 (*narZ*,*napA*)	Nitrate reductase	Spc^r, Km^r	This study
S. F98 (*napA*)	Nitrate reductase	Spc^r, Km^r	This study
S. F98 (*frdA*)	fumarate reductase	Km^r	This study
S. F98 (*pta*)	phosphotransacetylase	Km^r	This study
S. F98 (*ackA*)	acetate kinase	Km^r	This study
S. F98 (*ttrR*)	tetrathionate reductase	Km^r	This study
S. F98 (*ttrS*)	tetrathionate reductase	Km^r	This study
S. F98 (*poxB*)	pyruvate oxidase	Km^r	This study
S. F98 (*ldhA*)	lactate dehydrogenase	Cm^r	This study

Nal^r = sodium nalidixate resistant; Km^r = kanamycin resistant; Cm^r = chloramphenicol resistant; Spc^r = spectinomycin resistant.

Results

Faecal excretion. The patterns of faecal excretion over a period of 6 weeks, in comparison with the parental S. typhimurium F98, of strains with mutations in *nuoG*, *cyoA, cydA, atpB, atpH* or *pta* are shown in Figure 1. The patterns for the *nuoG* and *cydA* mutants were very similar to that of the parent strain. The *cyoA*, *atpB* and *atpH* mutants showed a reduced duration of excretion which was statistically significant ($P < .01$). The *pta* mutant was eliminated rapidly not being isolated after 2 weeks post-inoculation.

The faecal excretion of mutants with the inability to use alternative electron acceptors over a period of 4 weeks is shown in Figure 2. The *pta* and *ackA* mutants were not isolated at all during the experiment. Although some variation was observed between the other strains in the initial phase of the experiment, apart from *ackA* and *pta*, the only two mutants to show a marked reduction in excretion in comparison with the parent strain were the double mutant *narG,napA* ($P < .05$) and the *torC* mutant ($P < .01$), although these were still being excreted by a small number of birds at the end of the experiment (4 weeks). In birds challenged with the *torC* mutant, a reduced level of excretion was observed from weeks one to four of monitoring ($P < .01$). Apart from the *narG,napA, torC, pta* and *ackA* mutants none of the differences in excretion by the other mutants was significant ($P > .05$). The apparent inability of the *pta* and *ackA* mutants to colonize the chicken intestine was not a result of inability to culture these strains since both were able to grow on the selective Brilliant Green agar when inoculated from a broth culture which also grew well.

Two enzymes responsible for anaerobic metabolism of pyruvate to lactate (lactate dehydrogenase, *ldhA*) or for oxidation to acetate (pyruvate oxidase, *poxB*) were also assessed independently for their contribution to colonization ability (Figure 3). Both *ldhA* and *poxB* mutants were shed in the faeces much less than was the parent strain, the reduction being much more marked in the case of *ldhA* ($P < .01$) with no isolations beyond 2 weeks post-inoculation.

Virulence. The virulence of the mutants unable to utilize anaerobic electron acceptors for newly hatched chickens is shown in Table 3. Virulent *Salmonella* strains produced a systemic disease with high morbidity. Most mutants, including the *ackA* mutant, were fully or almost completely virulent in chickens. The *ttrS* and *ttrB* mutants produced reduced morbidity which was statistically significant with re-isolation of the inoculated strain from the livers of

Table 2. *Experimental design of intestinal colonization assay.*

Experiment	Duration	Number of groups of 20 chickens	Strains used to challenge birds
1	6 weeks	7	*nuoG*[a], *cyoA*[a], *cydA*[a], *atpB*[a], *atpH*[a], *ptu* and parent strain
2	4 weeks	14	*narG*[b], *narZ*[b], *narZ,narG*[b], *napA*[b], *narZ,napA*[b], *narG,napA*[b], *frdA*[b], *dmsA*[b], *torC*[b], *ttrB*[b], *ttrS*[b], *pta*, *ackA* and parent
3	4 weeks	3	*ldhA*, *poxB* and parent

[a]Strains with inability to utilize oxygen as terminal electron acceptor.
[b]Mutants with inability to utilize alternative electron acceptors.

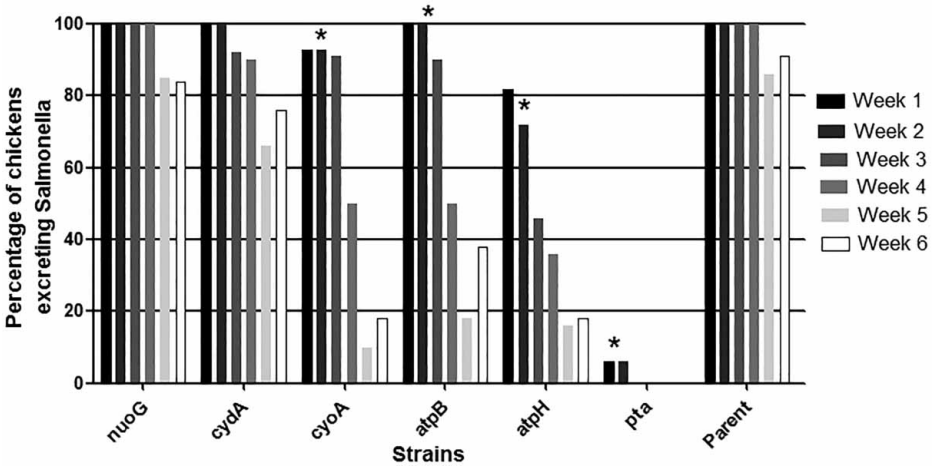

Figure 1. *Faecal excretion of mutants with inability to use oxygen as terminal electron acceptor or of* pta *mutant. Asterisks mean significant difference between the percentage of excretion of mutant and parent strain during the experiment (*P < .01).*

diseased chickens. The *pta* mutant produced no morbidity and the strain was not re-isolated from the livers when the experiment was terminated after 3 weeks.

Discussion

Our model, using newly hatched chickens administered with a gut flora preparation, has been used previously to ensure that all birds have obtained an identical flora rather than relying on natural acquisition which can introduce variation in susceptibility (Barrow *et al*., 2003).

Terminal electron acceptors for respiration used in the presence of oxygen or under anaerobic redox conditions did not appear to be essential for effective colonization, measured by faecal shedding, when mutated individually or, in the case of different nitrate reductases, when combined into double mutants.

Mutations affecting the function of NADH dehydrogenase I and cytochrome d-oxidase have been found to contribute little to the growth of *S. typhimurium* in the newly hatched chicken, where most *Salmonella* multiplication takes place at the lumen/mucosal border (Harvey *et al*., 2011) as in the mouse (Winter *et al*., 2013). Previous work also showed little effect of mutations in *cydA* or *atpH* on metabolism in the gut of young chickens (Zhang-Barber *et al*., 1997). In the present study, the *atpH* mutant was also less able to colonize the alimentary tract. In mutants which do not have a functional F_0F_1 ATP synthase energy generation is largely replaced by substrate-level phosphorylation. Under anaerobic conditions such mutants are non-motile because the proton motive force is not generated which itself drives the flagellar motor and is also responsible for uptake of a number of nutrients (Stewart *et al*., 2002).

None of the mutations affecting individual alternative electron acceptors reduced colonization to the extent of complete elimination of the mutants. The exception was perhaps the *torC* mutant where colonization was significantly reduced although some chickens were still excreting at the end of the experiment. The *torC* gene is the first in the *torCAD* operon which is associated with the generation of a proton motive force using a variety of N- and S-oxides including TMAO, pyridine N-oxide, DMSO and tetramethylene sulphoxide. In contrast, mutations in *dmsA* affect DMSO reductase but this enzyme complex can also use a wide spectrum of electron acceptors, including the substrates used by Tor. In *E. coli* DMSO reductase is required for specific respiration with DMSO or methionine sulphoxide as the substrate. Therefore, as in mice (Jones *et al*., 2011),

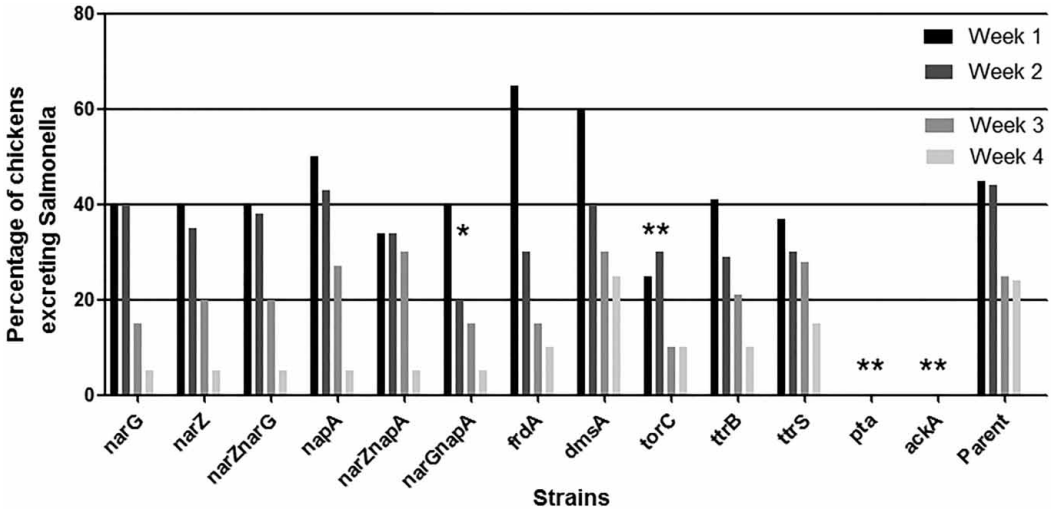

Figure 2. *Faecal excretion of mutants with inability to utilize alternative electron acceptors and of* pta *and* ackA *mutants. Asterisks mean significant difference between the percentage of excretion of mutant and parent strain in four weeks of experiment (*P < .05, **P < .01).*

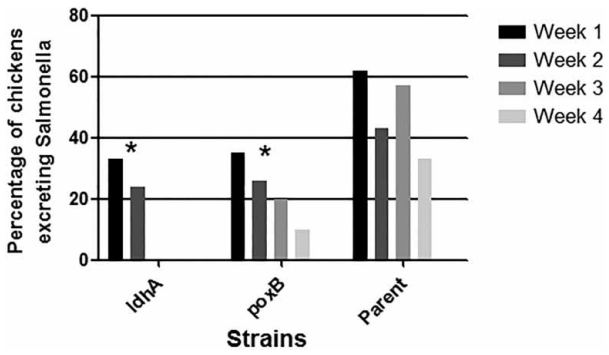

Figure 3. *Faecal excretion of ldhA and poxR mutants. Asterisks mean significant difference between the percentage of excretion of mutant and parent strain during the experiment (*P < .01).*

N- or S-oxide compounds in the chicken intestine appear to be available to *S. typhimurium* as electron acceptors.

Apart from the double mutant *narG,napA*, mutants with affected utilization of nitrate as electron acceptor had no considerable changes in their colonization ability. Nitrate reduction is energetically more favourable than other non-oxygen electron acceptors. *E. coli* and *Salmonella* have multiple nitrate reductases. Fumarate reductase, which is able to use a variety of electron donors and generates a proton motive force, did not appear to be important in faecal shedding.

The *ttrR* and *ttrS* genes encode regulatory proteins of the tetrathionate reductase operon (Price-Carter *et al*., 2001) responsible for the anaerobic degradation of 1,2-propanediol and ethanolamine using cobalamin as cofactor. Infection of mice with *S. typhimurium* results in inflammation releasing ethanolamine as a result of host epithelial cell degradation (Thiennimitr *et al*., 2011) but also generates tetrathionate as a result of reactive oxygen species on sulphur compounds in the gut (Winter *et al*., 2010). Combinations of *ttr*, *pdu* (1,2 propanediol degradation), *eut* (ethanolamine degradation), *cob* and *cbi* (cobalamin synthesis) and *btu* (cobalamin uptake) mutations were also found not to affect colonization in the chicken in a major way (Barrow, Berchieri & Lovell, unpublished results). This may also reflect the extensive metabolic flexibility in enteric bacteria and the ability to utilize melibiose and ascorbate as carbon sources *in vivo*

Table 3. *Virulence of mutants for newly hatched chickens.*

Mutant	Number of chickens dead or killed of 20 infected orally	χ^2	*P*
narG	20	…	…
narZ	19	…	…
napA narZ	20	…	…
narG napA	20	…	…
napA narZ	20	…	…
dmsA	20	…	…
torC	20	…	…
ttrS	12	7.6	<0.01
ttrB	8	14.4	<0.01
pta	0	36	<0.01
ackA	19	…	…
Parent	20	…	…

(Harvey *et al*., 2011). Microarray studies indicate a role for 1,2-propanediol and propionate in the gut of very young chickens (Harvey *et al*., 2011), at least. In older birds these are likely to be metabolised via propionyl-CoA with the generation of ATP via substrate-level phophorylation.

The almost complete elimination of colonization in the *ackA* and *pta* mutants suggested a role for glycolysis and fermentation in energy generation during colonization of the caeca although additional perturbations in central metabolism may clearly also have contributed . In the gut carbon sources such as N-acetylglucosamine, N-acetylgalactosamine, d-galactose, fucose, sialic acid, glucuronate, galacturonate, gluconate and ethanolamine are likely to be important, some generated by the degradation of host cell membranes (Harvey *et al*., 2011). With a fully active glycolytic pathway with glucose or related substrates, such as the above, much of the aerobic metabolic flux is directed at acetate production (Dittrich *et al*., 2005). The production of acetate through the *pta-ackA* pathway from pyruvate via acetyl-CoA generates ATP for bacterial growth. Mutations in this pathway increase diversion to lactate production although normally this is thought to be a minor part of the normal flux in wild-type cells. Even under aerobic conditions a significant part of the metabolic flux is directed through the *pta-ackA* pathway with the generation of acetate. This is also likely to be the case with amino acids, such as threonine, which are deaminated to pyruvate. Morgan *et al*. (2004) found evidence for a significant role for threonine and serine metabolism in the chicken gut. However, the role of this pathway under these circumstances is unclear, that is, whether this is a primarily route for ATP generation or rather for recycling acetyl-CoA and NAD.

Fermentation is thus likely to be an important source of energy and for other vital processes during intestinal colonization in the chicken. In addition to its contribution to the energy balance in the cell, the *pta-ackA* pathway is also important in recycling acetyl-CoA (Pascal *et al*., 1981) for NADH oxidation to maintain redox balance. Acetyl phosphate is also an internal global signalling molecule (McCleary & Stock, 1994), important for internal homeostasis and environmental signalling through its ability to take part in the phosphorylating reactions associated with two component signal transduction activity including the PhoP-PhoQ system and OmpR amongst others (Wanner, 1995). Acetyl phosphate may also be involved in the generation of polyphosphate, an important agent of internal ATP homeostasis (McMeechan *et al*., 2007). Mutations in the *pta-ackA* pathway also affect regulation of a number of stress response genes encoding DnaK, GroEL, GroES and ClpB, some of which are associated with intestinal colonization and virulence (Wolfe, 2005).

In the chicken, the caeca are the main organs colonized and they go through cycles of emptying and filling 2–4 times a day involving soluble material (chyle) from the ileum with caecal bacteria alternating between periods of exponential and stationary phase (Barrow, unpublished results). Under conditions where respiration is possible the log phase growth is associated with acetate excretion with its import and utilization when stationary phase is reached (Wolfe, 2005) coupled with increasing activity of pyruvate oxidase, the product of the *poxB* gene (Dittrich *et al*., 2005). Pyruvate acts as a node with a branch point between lactate dehydrogenase catalysing the formation of lactate and pyruvate formate lyase which generates acetyl-CoA and ethanol or acetate the latter via phosphotransacetylase and acetate

kinase. A mutation in *ldhA* should generate a greater flux through acetyl-CoA and increasing activity in Pta and AckA. The virtual elimination of colonization activity in the *ackA* and *pta* mutants suggests that these enzymes encode activity other than simply ATP generation and phosphorylation activity of acetyl phosphate.

The results of the virulence studies showed that none of the mutants which were compromised in their ability to use alternative electron acceptors were completely attenuated. We did find complete attenuation of the *pta* mutant for the chicken, as found previously by Kim *et al*. (2006) and a degreee of attenuation in the *ttrB* mutant. Phosphotransacetylase is not only involved in the interconversion of acetyl-CoA and acetyl phosphate but is involved in the utilization of propionate excreted during growth on 1,2-propanediol (Palacios *et al*., 2003) through the interconversion of propionyl-CoA and propionyl phosphate. Interestingly the *ackA* mutant was almost completely virulent. The *ackA* mutants are able to generate acetyl phosphate whereas *pta* mutants are not (Wolfe, 2005) again indicating a contribution by acetyl phosphate to virulence.

Our results suggest that for intestinal colonization of the chicken caeca substrate-level phosphorylation is likely to be more imprtant than respiration involving anaerobic electron acceptors. The contribution of *ackA* and *pta* mutants to virulence in chickens was more difficult to interpret given the higher redox conditions during systemic infection.

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