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Review

Towards an understanding of *Salmonella enterica* serovar Typhimurium persistence in swine

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Abstract

Salmonella enterica is an important food borne pathogen that is frequently carried by swine. Carrier animals pose a food safety risk because they can transmit *S. enterica* to finished food products in the processing plant or by contamination of the environment. Environmental contamination has become increasingly important as non-animal foods (plant-based) have been implicated as sources of *S. enterica*. The prevalence of *S. enterica* in swine is high and yet carrier animals remain healthy. *S. enterica* has developed a highly sophisticated set of virulence factors that allow it to adapt to host environments and to cause disease. It is assumed that *S. enterica* also has developed unique ways to maintain itself in animals and yet not cause disease. Here we describe our research to understand persistence. Specifically, data are presented that demonstrates that detection of most carrier animals requires specific stresses that cause *S. enterica* to be shed from pigs. As well, we describe a phenotypic phase variation process that appears to be linked to the carrier state and a complex set of factors that control phenotypic phase variation. Finally, we describe how the composition of the gut bacterial microbiome may contribute to persistence and at the least how *S. enterica* might alter the composition of the gut bacterial microbiome.

Keywords: *Salmonella*, carrier, phase variation, transportation stress, virulence, microbiome, *Lawsonia*, swine.

Introduction

This paper is based in part on a keynote presentation given (by REI) to the Bacterial Pathogenesis Section at the 2014 Conference of Research Workers in Animal Diseases.

Salmonella enterica is a gram negative, non-lactose fermenting, facultative anaerobic rod in the family Enterobacteriaceae. It is a frequent cause of diarrhea and can be found in most species of animals. *S. enterica* is a facultative intracellular bacterium. It has an intracellular lifestyle in animals and this allows *S. enterica* to evade many of the host's innate and adaptive immune clearance mechanisms (Broz *et al.*, 2012). *S. enterica* can be found colonizing the ileum, cecum, and colon of pigs. Studies on the pathogenesis of diarrheal disease caused by *S. enterica* have shown that this microbe invades M-cells in the ileum of the small intestines (Carter and Collins, 1974; Hohmann *et al.*, 1978; Jones *et al.*, 1994; Penheiter *et al.*, 1997) as well as epithelial cells (Takeuchi, 1967). *S. enterica* also is frequently found in mesenteric lymph nodes and spleens. In severe cases of salmonellosis, *S. enterica* can enter the blood stream and spread systemically causing sepsis. There are more than 2500 known serovars of *S. enterica*. Host adapted serovars, like the swine

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pathogen *S. enterica* serovar Choleraesuis, are most likely to cause systemic infections and cause enteric fever. To invade epithelial cells and maintain its intracellular lifestyle, *S. enterica* has a well-coordinated set of virulence genes many of which are involved in the synthesis of type three secretion systems (TTSS) (Troisfontaines and Cornelis, 2005; Coburn *et al.*, 2007). TTSSs are known for their abilities to inject effector proteins into target cells. For example, one TTSS encoded in the *Salmonella* pathogenicity island 1 (SPI1) (Lostroh and Lee, 2001) mediates the initial invasion of *S. enterica* in to epithelial cells and is essential for disease.

Other host-adapted serovars of *S. enterica*, including Typhi (human) and Dublin (cattle), cause severe disseminated disease. The non-host adapted serovars of *S. enterica* Typhimurium and Enteritidis are the two most common serovars causing food borne illnesses (CDC, 2013). The Centers for Disease Control has estimated that *S. enterica* is responsible for over 1 million cases of food-borne illness per year in the USA resulting in over 19,000 hospitalizations and 378 deaths (Scallan *et al.*, 2011). *S. enterica* has been ranked as the leading cause of food borne disease as measured by the combined cost of illness and Quality Adjusted Life-Year (Batz *et al.*, 2011). It has been estimated that the economic losses due to salmonellosis in humans in the USA exceeds $3.5 billion per year (Batz *et al.*, 2011). Pork is frequently associated with food borne illnesses caused by *S. enterica* in humans (Bryan, 1988; Bean and Griffin, 1990; EFSA, 2012; Brendan *et al.*, 2013).

It is well known that the prevalence of *S. enterica* in most livestock species is high including pigs, cattle, and chickens and in most cases these animals are healthy carriers. Estimates from the National Animal Health Monitoring System (NAHMS) (Anonymous, 2009) put the farm prevalence of *S. enterica* in pigs at 50% in the USA. Other studies have shown that the prevalence of *S. enterica* is even higher (Bahnson *et al.*, 2006a). In naturally infected animals, most never experience overt disease. It is well known that naïve pigs can be infected with *S. enterica* early in life and consequently become long-term carriers of this microbe (Williams and Newell, 1970). Wood *et al.* (1989) demonstrated that pigs orally challenged with *S. enterica* serovar Typhimurium (*S.* Typhimurium) shortly after weaning became long-term carriers of the challenge strain but when pigs were 28 weeks of age, only low numbers of the challenge strain could be found in their feces. Wood *et al.* (1989) also looked for internal reservoirs of the challenge *S.* Typhimurium strain and found that it was most consistently found in the tonsils. However, detection of the challenge strain was sporadic and currently the location of the animal's natural reservoir remains unknown. Most experimentally challenged pigs do experience diarrhea, though the disease usually is mild and self-limiting. However, most become persistently infected (carriers) yet remain clinically healthy. While *S. enterica* is generally thought to be a pathogen, the emerging picture suggests that *S. enterica* may be a component of the normal intestinal microbiota. Based on the broad distribution of *S. enterica* among animals and the apparent lack of salmonellosis in these animals we suggest that *S. enterica* can be both a pathogen and a commensal microbe.

The goal of this paper is to review our data describing *S. enterica* persistence in swine and to then summarize a unique phenotypic phase variation process that may explain how *S. enterica* can be a pathogen and a commensal. In addition, we will describe data demonstrating an interaction between *S. enterica* and another pig pathogen, *Lawsonia intracellularis,* how *L. intracellularis* may contribute to the development of a carrier state, and describe the alteration to the composition of the intestinal microbiota when pigs carry *S. enterica*.

Additional evidence that pigs can be persistent carriers of *S.* Typhimurium

S. Typhimurium is the most common serovar found in pigs that causes food borne salmonellosis (CDC, 2013). We wanted to confirm that this serovar could persistently infect pigs. We orally challenged pigs and followed the number of pigs shedding the challenge strain and the amount of cells actually being shed. In addition, we wanted to know whether stressing pigs could increase shedding of the challenge strain. To do this we challenged pigs with a nalidixic acid-resistant mutant of *S.* Typhimurium strain 798 after weaning (4-weeks of age) and collected fecal samples at various intervals to both detect, which pigs were shedding the challenge strain and in what quantities (Isaacson *et al.*, 1999b). A two-step enrichment protocol was used for detection (Bahnson *et al.*, 2006a, b) and a three tube most probable number analysis test was used to quantify the challenge strain in feces. The final stage of the culturing process was by plating the enrichments on XLT4 agar containing nalidixic acid. One-week post challenge *S.* Typhimurium strain 798 was detected in the feces of approximately 75% of the pigs (group size was 16 pigs) (Fig. 1). However, over time the number of pigs actively shedding the challenge strain decreased. By 4 months post challenge only 4% of pigs were shown to be shedding *S.* Typhimurium strain 798. Likewise, the quantity of *S.* Typhimurium strain 798 in feces decreased over time. One-week post challenge we detected approximately 10^9 *S.* Typhimurium cells per gram of feces. But at 4 months post challenge the quantity of with *S.* Typhimurium strain 798 in feces decreased to a mean concentration of less than 1 colony forming unit per gram of feces (Fig. 2). These data demonstrated that there was a slow but continual clearance of *S.* Typhimurium from the pig feces. However, based on the work of Williams and Newell (Williams and Newell, 1970) we wondered whether the challenge organism was really cleared from these animals or if it was only cleared from their feces? Therefore, we stressed the pigs by subjecting them to feed withdrawal (0, 6, 12 or 24 h prior to transport) followed by a 2-h ride in a truck (group size was 15 pigs per group). After the truck ride, the pigs were necropsied and contents of their ceca were cultured to detect the infecting strain of *S.* Typhimurium (Fig. 3). The pigs that did not receive the feed withdrawal process showed a slight increase in the prevalence of *S.* Typhimurium strain 798. But pigs that were not fed for 24 h exhibited a large increase in shedding: almost 80% of the pigs were now positive for *S.* Typhimurium strain 798. The

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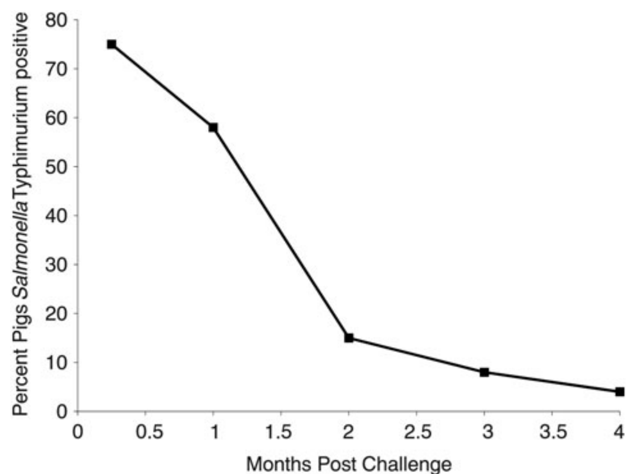

Fig. 1. The number of pigs actively shedding the challenge strain was determined at the indicated times using feces collected from pigs orally challenged with a nalidixic acid resistant *S.* Typhimurium strain 798 using a double enrichment (tetrathionate broth and Rappaport-Vassiliadis R10 Broth) followed by plating on XLT-4 agar containing nalidixic acid.

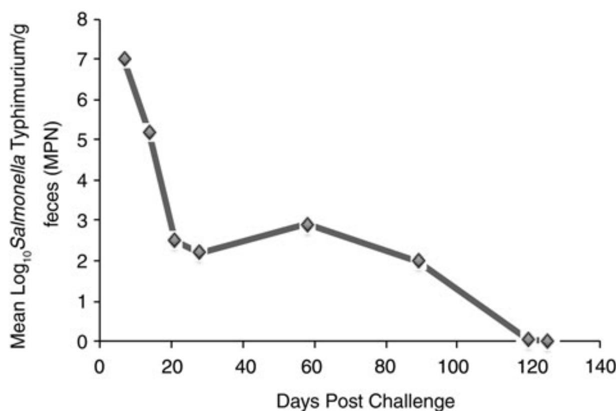

Fig. 2. Quantitative measurement of a nalidixic acid-resistant *S.* Typhimurium strain 798 in pig's feces at the indicated times post oral challenge. Quantitative measurement utilized a most probable number (MPN) protocol.

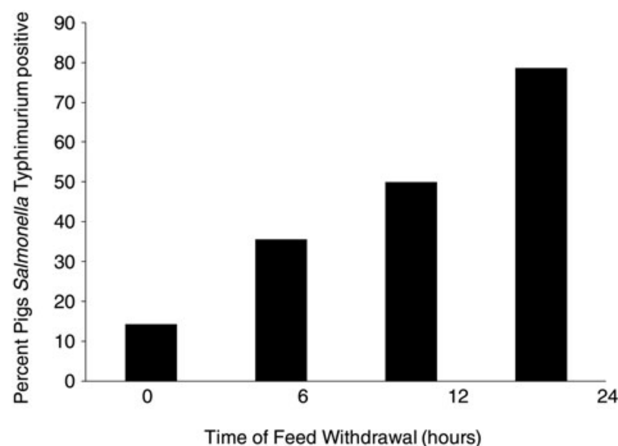

Fig. 3. Percentage of pigs actively shedding the nalidixic acid-resistant *S.* Typhimurium strain 798 approximately 4 months post challenged. Prior to sample collection pigs were subjected to feed withdrawal (0, 6, 12 or 24 h) and then loaded onto a truck with separate partitions for each feed withdrawal group and transported for 2 h prior to collection of cecal contents. Challenge strain positive pigs were determined after using a double enrichment (tetrathionate broth and Rappaport-Vassiliadis R10 Broth) followed by plating on XLT-4 agar containing nalidixic acid.

interpretation of that data was that the majority of pigs in the study had remained colonized with *S.* Typhimurium strain 798 even though they were not actively shedding this microbe in their feces 4 months post challenge. The stress associated with feed withdrawal and transporting them was sufficient to cause them to return to a shedding state. The exact compartment(s) where the challenge strain was hiding remains unknown. Based on the work of Wood *et al.*, a likely reservoir was the tonsils (Wood *et al.*, 1989). The reason that cecal contents were collected at necropsy rather than feces was because pigs that had been in the 24 feed withdrawal group did not have feces that could be collected from their rectums. Whether cecal contents and feces represent an essentially identical sample is not known and thus could have biased the outcome. We know that feces and colonic tissues are not the same in terms of physical properties (nutrient availability, water, pH, etc.). There are three hypotheses that might explain the increased shedding after feed withdrawal and transport. The first is that the challenge strain of *S.* Typhimurium strain 798 was present in all pigs and feed withdrawal coupled with the stress of transportation caused a reactivation of the challenge strain and increased shedding. The second hypothesis is that we would have detected the challenge organisms in cecal contents prior to feed withdrawal and transport had we assayed them. Thus, the difference was due to sample differences. The third hypothesis is that relatively few pigs actually shed the *S.* Typhimurium challenge strain at the time of feed withdrawal, but that during transport stress those few pigs that were shedding the challenge strain provided the inoculum for the other pigs that began to shed *S.* Typhimurium after transport. This conclusion is consistent with the work of Hurd *et al.* (2002) where they noted that commercial pigs held in lairage prior to slaughter began to shed serotypes of *S. enterica* not seen in those animals during on farm sampling. Their assumption was that the pigs held in lairage pens picked up additional strains of *S. enterica* in this new environment and rapidly became shedders of these new strains. Supporting evidence that a brief exposure to *S. enterica* in the environment could rapidly result in shedding is based on the work of Fedorka-Cray *et al.* (1995). They demonstrated that *S.* Typhimurium could be detected in the intestines of pigs within 3 h post challenge by the intranasal route in pigs that had their esophagus surgically ligated. Presumably the *S.* Typhimurium gained entry in to the blood stream via the respiratory tract and then homed to the gut lumen. Thus, shedding of *S.* Typhimurium strain 798 within the 2 h transport time frame is plausible. However, an important difference between

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our experiment and the one using esophagotomized pigs is that the inoculum used in the esophagotomized pigs was (10^9), which is quite high and it is unlikely that a similar concentration of *S.* Typhimurium would be found in the truck during transport. In addition, we found that the increase in the number of pigs positive for the challenge strain of *S.* Typhimurium strain 798 correlated with the length of feed withdrawal suggesting that the degree of stress related to duration of feed withdrawal is an important contributor in the conversion of apparent *S.* Typhimurium negative pigs to *S.* Typhimurium positive pigs. It is likely that the resumption of shedding of *S. enterica* by carriers is due to many factors including stress induced as well as exposure to *S. enterica* contamination in different environments.

S. Typhimurium can grow in two phenotypic phases

In our studies to understand how *S.* Typhimurium was able to enter a carrier state within pigs, we found that one strain, *S.* Typhimurium strain 798, was able to grow in one of two phenotypes (Isaacson and Kinsel, 1992). *S.* Typhimurium strain 798 was initially isolated from a pig with diarrhea. But subsequent work with this strain showed that it could cause persistent non-clinical infections of pigs (Wood *et al.*, 1989). Initially, the observation that *S.* Typhimurium strain 798 grew with two phenotypes was based on the ability of *S.* Typhimurium strain 798 to grow in two different and distinct colony morphologies when grown on blood agar plates: small, non-mucoid, cohesive colonies compared with larger, mucoid, non-cohesive colonies. Cells from each colony type were tested for their abilities to attach to enterocytes isolated from baby pigs. The larger colonies were shown to be highly adhesive while the smaller colonies did not attach to enterocytes (Isaacson and Kinsel, 1992). When colonies of either phenotype were picked and re-plated, a small number of colonies would arise that appeared to be of the other phenotype. The larger the numbers of total colonies on the plate the more likely that colonies of the other phenotype would be present. When these cells were tested for adhesion to enterocytes, they either became adhesive (if they originated from the non-adhesive phenotype) or become non-adhesive (if they originated from the adhesive phenotype). Repeating this process always resulted in the same result indicating that the two phenotypes could at low frequency transition to the other phenotype.

Further characterization of cells in the two phenotypes revealed many other differences, which are summarized in Table 1. Based on their adhesive phenotype to porcine enterocytes we designated the phenotypes as non-adhesive phenotype or adhesive phenotype. Data demonstrated that type 1 fimbria was responsible for adhesion to enterocytes and type 1 fimbriae were only produced by cells in the adhesive phenotype (Isaacson and Kinsel, 1992; Althouse *et al.*, 2003). Further investigations of cells in the adhesive phenotype showed that they also were highly invasive and once inside a mammalian cell were able to survive (Isaacson and Kinsel, 1992). This is a clear link to virulence and the intracellular lifestyle of *S. enterica*. Cells in the non-adhesive phenotype were much less invasive and once internalized were rapidly killed. In studying the differences between cells of the two phenotypes, we noted that all traits associated with a phenotype were coordinately regulated. That is all were either expressed or not expressed based on their phenotype and when a phenotypic variant was selected by plating on agar, all of the traits were either expressed or not expressed consistent with the phenomenon called phase variation (reviewed by (Henderson *et al.*, 1999)).

Table 1. Some characteristics of the two phenotypes of *S.* Typhimurium strain 798

Adhesive phase	Non-adhesive phase
Adhesive to porcine enterocytes	Non-adhesive to porcine enterocytes
Type 1 fimbriae	No Type 1 fimbriae
10–15 unique envelope proteins	1–2 unique envelope proteins
Long O-antigen	Short O-antigen
Enhanced invasion	Reduced invasion
Intracellular survival in phagocytes	Killed within phagocytes
Resistant to serum complement	Sensitive to serum complement
White on Evans blue-uranine agar (EBU)	Blue on Evans blue uranine agar (EBU)
Delayed growth in broth containing no NaCl	Normal growth in broth containing NaCl

Because we knew that some of the co-expressed genes associated with the adhesive phenotype would encode membrane-associated proteins (e.g. type 1 fimbriae), we used the transposon Tn*phoA* to create a library of mutants that were in genes that encode membrane proteins. Tn*phoA* encodes alkaline phosphatase (PhoA). If Tn*phoA* inserts into a gene causing an in-frame gene fusion and if the protein encoded by that gene is exported from the cell by virtue of having a leader peptide, an active extra-cellular alkaline phosphatase will be produced. If cells are then plated on an agar medium containing 5-bromo-4-chloro-3-indolyl phosphate (XP), a substrate for alkaline phosphatase, colonies will be blue. Using the Tn*phoA* library, we showed that the short O-antigen trait was related to differential expression of the gene *rfaL*, which is an O-antigen ligase (Kwan and Isaacson, 1998). Expression of the PhoA::RfaL fusion protein was shown to be phase variable. In addition, cells expressing the PhoA::RfaL fusion protein retained all features of the adhesive phenotype except the long O-antigen. Conversely, cells that did not express the PhoA::RfaL fusion protein retained the traits associated with the non-adhesive phenotype.

Because of the diverse number of differences between the two phenotypes, we decided to determine the complete repertoire of coordinately controlled genes in the two phenotypic phases. To do this, RNAseq was used to compare global gene expression in cells of both phenotypes (Patterson *et al.*, 2012). Eighty-three genes were shown to be up regulated in cells in the adhesive phenotype, while 31 genes were shown to be down regulated. Many of the up-regulated genes encode

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known virulence factors. In particular, all genes in the *Salmonella* pathogenicity island SPI1 were shown to be up regulated. The genes in this pathogenicity island are known to encode a TTSS that is involved in invasion of epithelial cells including enterocytes and M-cells (Wallis and Galyov, 2000; Lostroh and Lee, 2001). In addition to the SPI1 genes, the genes that encode the metabolic pathway for propanediol utilization were upregulated. The propanediol pathway has been shown to be important for survival in macrophages (Klumpp and Fuchs, 2007). Several genes encoded in SPI2 (sopA, sopB, sopD, and sopE) also were up regulated. These genes are part of a second TTSS, one that is important in intracellular survival (Hensel, 2000).

Measuring the rate of phenotypic phase variation

When the two phenotypes of *S.* Typhimurium strain 798 were discovered, it was noted that cells could switch between the phenotypes. A rough estimate of the rate of transition was between 10^{-2} and 10^{-4} per cell per generation (Isaacson and Kinsel, 1992). This rate was considered to be too frequent to be caused by a mutation and was more similar to the process of phase variation that is known to control expression of *E. coli* type 1 pili (Brinton, 1959; Eisenstein, 1981). During our genetic analysis of *S.* Typhimurium strain 798 we noticed that when cells of either phenotype were plated on Evans blue-uranine agar (EBU) colonies from cells in the non-adhesive phenotype were blue while colonies from cells in the adhesive phase were white. Furthermore, colonies of either phenotype yielded small numbers of colonies of the opposite color on EBU plates similar to the colony variations we previously saw on blood agar plates. Based on analysis of several traits, we confirmed that these colonies represented phase variants. Using EBU agar as a differential medium to more effectively identify colonies of the two phenotypes, we were able to more accurately estimate the frequency of phase variation from the non-adhesive to the adhesive phenotype (Patterson *et al.*, 2012). The rate was estimated to be 1.7×10^{-4} per cell per generation. However, while we were able to find the occasional colony that varied from the adhesive to the non-adhesive phenotype, the rate was too low on EBU agar plates to accurately measure the rate of phase variation. The limit of detection using EBU plates was approximately 10^{-5} per cell per generation and the actual rate was presumed to be lower.

In our studies to characterize the adhesive and non-adhesive phenotypes, we found that cells of the non-adhesive phenotype, when inoculated into Luria broth containing no NaCl had a very long lag phase (2.5–7.5 h). However, cells in the adhesive phenotype exhibited normal growth characteristics with a short lag phase. We reasoned that a colony containing cells from the adhesive phase would contain a small fraction of cells that had varied to the non-adhesive phase even though the number was too low to accurately estimate the number. If such a colony was inoculated into LB broth containing no NaCl and the antibiotic ampicillin so that any actively growing adhesive phase cells would be killed by ampicillin while any non-adhesive phase cells in the extended lag phase would remain viable. Using this ampicillin enrichment procedure, and incubating the culture for 2 h we were able to increase the sensitivity of the assay to measure phase variation and in doing so accurately measured the rate of phase variation from adhesive to non-adhesive phases at 1.5×10^{-6}. Thus, in broth conditions (LB with no NaCl), *S.* Typhimurium strain 798 had a rate of phase variation from non-adhesive to adhesive phase that was about 100-times more frequent than the other direction. Because we propose that phenotypic phase variation is important for *S.* Typhimurium in animals it is possible that the rates could be quite different in pigs compared with broth cultures.

What controls phase variation in *S.* Typhimurium strain 798?

In an attempt to understand what controls phase variation, a library of transposon (Tn*10d*-Cam) mutants was created in cells of the adhesive phenotype and then transduced into non-adhesive phase cells using the bacteriophage P22 (Elliott and Roth, 1988). The goal was to identify mutants that had altered rates of phase variation compared with wild type cells. The transductants were plated on LB agar plates. Individual colonies were picked and plated on EBU plates. Colonies that appeared to phase vary to light-colored (adhesive phase) more frequently were identified. Insertion mutants were identified that had increased rates of phase variation. Several colonies exhibited a 10-fold increase in the rate of phase variation from the non-adhesive phenotype to adhesive phenotype. To confirm that the insertion mutation was the cause of the increased rate of phase variation and not a spurious result, the Tn*10d-Cam* insertion was transduced again into non-mutant, non-adhesive phase cells. All transductants demonstrated the 10-fold increase in the rate of phase variation from non-adhesive to adhesive phenotype. Sequence data from these insertion mutations indicated the transposon insertion sites were just upstream of the gene *lrhA*.

To confirm the association between phase variation and LrhA, a second genetic screen was used to identify mutations with alterations in phase variation. The screen used a novel two-color identification scheme. A *fimA*::Tn*phoA* mutant that had been previously created was one part of the screen (Althouse *et al.*, 2003). FimA is the major subunit of type 1 fimbriae that we have shown to be involved in adhesion to pig enterocytes and is expressed as part of the adhesive phenotype (Althouse *et al.*, 2003). The *fimA*::Tn*phoA* fusion was transduced into adhesive phase *S.* Typhimurium strain 798 cells using bacteriophage P22. Cells growing on agar medium containing XP yielded blue colonies if the PhoA::FimA fusion was expressed. A second mutation was introduced into either *sig*E or *pef*B using adhesive phase cells using the transposon Tn5*lac*ZY. These two genes were selected because they encode known virulence factors and were expressed at high levels in cells of the adhesive phase but were not encoded near the type 1 fimbrial gene cluster. Rather they are encoded in SPI1. Cells expressing either *sig*E or *pef*B grew as red colonies when plated on agar containing

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6-chloro-3-indolyl-β-D-galactoside (Red-Gal) because of the expression of β-galactosidase. Double mutants containing *Pho*A::*fim*A and either *sig*E::*lac*ZY or *pef*B::*lac*ZY were plated on agar containing XP and Red-Gal. If both gene fusions were expressed the colonies were purple. If neither of the two gene fusions were expressed, the colonies were white. The double mutants were subsequently mutagenized with the transposon Tn*10d-Cam* to simultaneously knockout expression of both *fim*A and *Sig*E or *pef*B. A Tn*10d-Cam* transposon library was screened for white colonies. White colonies represented either knockouts of the phase variation process or were simply phase variants. Using EBU plates, we were able to identify colonies that contained phase variants because they would have the low percentage mix of blue and white colonies. Phase variants were eliminated. We then cloned and sequenced what appeared to be mutations that knocked out two genes simultaneously (Tn*10d-Cam* mutants) and were not phase variants and all were found to be in the gene *lrh*A. This is the same gene mutagenized in the non-adhesive phase cells that resulted in an increase in phase variation.

Using DNA sequencing, the sites of the transposon insertions again were shown to be located just upstream of the *lrhA* gene. Since the transposon insertions were located upstream of the start site (320 bp in two insertion sites and 1010 bp in two other insertion sites) we wondered if the mutation increased or decreased expression of *lrhA*. Previous studies in *Salmonella* and *Escherichia coli* identified insertions in similar sites upstream of *lrhA* (Cunning, and Elliott, 1999). These mutations actually increased the expression of *lrhA* because they likely knocked out repression of *lrhA*. Defined *S*. Typhimurium mutants that were known to either increase or knock out expression of *lrhA* were obtained (Cunning and Elliott, 1999). The defined mutations were moved into non-adhesive and adhesive phase cells and the rates of phase variation were calculated for each of the mutants. Strain 7637-8, which over expresses *lrhA*, was found to phase vary from non-adhesive phase cells to adhesive phase cells on EBU plates at a rate of 9.29×10^{-4} per cell per generation. This is almost 10-fold higher than the rate compared with non-adhesive phase cells (1.70×10^{-4} per cell per generation, $P = 0.0003$) and was similar to the rate of phase variation of the hyper variable mutants obtained in the first screen. The rate of phase variation for the null mutation in *lrhA* (7638-8) was 1.37×10^{-4} per cell per generation. This was not statistically different from wild type non-adhesive phase cells ($P = 0.233$). Mutants that over expressed *lrhA* were assayed to determine if they showed altered rates of phase variation. No changes were detected. Hence we concluded that over production of LrhA led to an increased rate of phase variation from non-adhesive to adhesive phenotype but did not alter phase variation from the adhesive phenotype to the non-adhesive phenotype.

LrhA has been shown to result in the degradation of the alternate RNA polymerase sigma factor, RpoS, in *E. coli* and possibly in *Salmonella* (Cunning and Elliott, 1999). Therefore, we looked at the effect of an *rpoS* null mutation (Brown and Elliott, 1996) on the rate of phase variation. Strain 6168-8 (non-adhesive/*rpoS*::Tn*10d*-Cam) did increase the rate of phase variation from blue to light-colored colonies (non-adhesive phase to the adhesive phase) by approximately 10-fold. Strain 6168-9 (adhesive/*rpoS*::Tn*10d*-Cam) did not exhibit a change in the rate of phase variation from adhesive to non-adhesive phase cells. Oddly, and in contradiction to this result, based on our RNAseq data *rpo*S is expressed at an approximately 10-fold higher rate in non-adhesive phase cells compared with adhesive phase cells. However, if RpoS is not involved in phase variation from adhesive to non-adhesive phase cells the level of expression of *rpo*S in adhesive phase cells is irrelevant. Thus, as the data shows, LrhA reduces RpoS in non-adhesive phase cells and does so through a regulatory effect.

Is *S.* Typhimurium strain 798 unique in its ability to phase vary?

Since strain 798 is a clinical isolate capable of persistently infecting pigs but is not highly virulent in the BalbC mouse model of disease (oral $LD_{50} \sim 10^9$, Isaacson *et al.*, 1999a; Althouse *et al.*, 2003), we wanted to know whether other *S*. Typhimurium isolates undergo phenotypic phase variation and in particular if more highly virulent strains of *S*. Typhimurium were capable of phase variation between the adhesive and non-adhesive phenotypes. Two high virulence strains, SL1344 (Hoiseth and Stocker, 1981; Garcia-Quintanilla and Casadesus, 2011) and 14028 (ATCC® 14028™) were plated on EBU agar to see if blue colonies were present. These strains are normally light-colored on EBU. We used the ampicillin enrichment procedure to increase the likelihood of finding blue colonies (Patterson *et al.*, 2012). After enrichment blue colonies were isolated from SL1344 and 14028. The rates of phase variation from the adhesive phenotype to the non-adhesive phenotype were calculated and both were statistically the same for both strains compared with *S*. Typhimurium strain 798 (Table 2). We used blue colonies from both non-adhesive phase *S*. Typhimurium strain 14028 and SL1344 to measure the rates of phase variation back to the adhesive phase. Both strains reverted to the adhesive phase almost 100-fold more frequently compared to *S*. Typhimurium strain 798 (Table 2). Thus, phenotypic phase variation discovered in *S*. Typhimurium strain 798 also occurred in two additional and highly virulent strains. It is unknown if other serotypes also undergo a similar phenotypic phase variation process.

How phenotypic phase variation may contribute to the development of the *S. enterica* carrier pig

Based on the recognition that *S*. Typhimurium cells in the adhesive phenotype also produce an array of important virulence genes while cells in the non-adhesive phenotype do not, it is logical to believe that the adhesive phenotype cells are virulent while non-adhesive phenotype cells are not. Since *S*. Typhimurium cells appear to be able to transition from one phenotype to the other, we would assume that highly virulent strains would have a larger proportion of cells in the adhesive

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Table 2. Rate of phenotype phase variation for three strains of *S.* Typhimurium

Strain	Adhesive to non-adhesive	Non-adhesive to adhesive
798	1.5×10^{-6}	1.7×10^{-4}
SL1344	3.0×10^{-6}	3.2×10^{-2}
14028	4.8×10^{-6}	1.2×10^{-2}

or virulent phenotype. When the two high virulence strains of *S.* Typhimurium (SL1344 and 14028) were analyzed for rates of phenotypic phase variation, the rates were consistent with these strains being most likely to be found in the adhesive or virulent phenotype (Table 2). That is, the rate of phase variation was four orders of magnitude higher going from the non-adhesive phenotype to the adhesive phenotype. The LD_{50} values for these two strains in the BalbC mouse model are between 10^4 and 10^5 (Hoiseth and Stocker, 1981). On the other hand, based on the measured rates of phenotypic phase variation, *S.* Typhimurium strain 798 is less likely to be in the adhesive or virulent phase compared with *S.* Typhimurium strains SL1344 or 14028. *S.* Typhimurium strain 798 also is much less virulent having a mouse $LD_{50} > 10^9$. We would expect that if all three *S.* Typhimurium strains were used to challenge pigs, that the two high virulence strains would cause typical salmonellosis, perhaps severe disease, while we already know that *S.* Typhimurium strain 798 causes a milder disease. Because of the differences in virulence, *S.* Typhimurium strain 798 might be more likely to cause asymptomatic, persistent infections. Our experience with this strain is that it indeed does cause mild diarrhea that lasts no longer than 24 h and then causes persistent but asymptomatic infections of pigs (Isaacson *et al.*, 1999b).

A model of how phase variation contributes to disease is shown in Fig. 4. Cells in the adhesive phenotype produce an array of virulence factors and would be expected to attach to M-cells and villus absorptive enterocytes in the intestines and then invade into these intestinal cells. *S.* Typhimurium in the M-cells would escape into the sub epithelial layer and enter dendritic cells, macrophages, and/or neutrophils but because of the expression of virulence factors encoded in SPI2 (and other virulence genes), would survive and grow intracellularly. These phagocytes would then migrate to local mesenteric lymph nodes where the *S.* Typhimurium would eventually be controlled. *S.* Typhimurium within the enterocytes also would proliferate and ultimately spread to other enterocytes, stimulate an inflammatory response and thus be exposed to inflammatory neutrophils in the intestinal tissues and the gut lumen, which has been shown to enhance growth of *S.* Typhimurium (Stecher *et al.*, 2007; Winter *et al.*, 2010).

On the other hand, *S.* Typhimurium cells in the non-adhesive phenotype would not attach to enterocytes because, among other things, they do not produce type 1 fimbriae (Althouse *et al.*, 2003). These cells would still enter M-cells because M-cells are constantly sampling the environment in the lumen and because these cells also produce other adhesins, but would ultimately be killed by resident dendritic cells or macrophages found under the M-cells. *S.* Typhimurium cells remaining in the lumen of the intestines would be cleared by peristalsis and eliminated in feces.

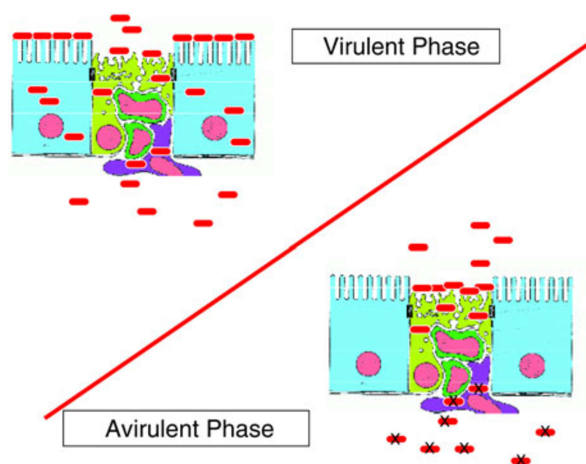

Fig. 4. Model of the interactions of *S. enterica* (red rod shaped figures) with enterocytes in the intestinal tract (light blue), their brush borders, and M-cells (light green). The two cartoons represent the expected interactions that occur when the *S. enterica* are in the two phenotypic phases.

Based on this information, we hypothesize that the development and maintenance of a persistent carrier state is related to the proportion of *S.* Typhimurium cells in a population in the two phenotypes based on rates of phenotypic phase variation. If the rate of phenotypic phase variation from the non-adhesive and adhesive phenotypes was modulated such that there was a sufficient number of adhesive phase cells to maintain a mucosal and/or intracellular population but was too low to cause disease, a carrier state would develop. After a pig is exposed to *S.* Typhimurium, cells in the non-adhesive phase would be rapidly cleared from the lumen of the intestines because they did not attach to intestinal cells, or be killed by phagocytic cells. The remaining *S.* Typhimurium cells would be those in the adhesive phenotype. Because phenotypic phase variation is a dynamic process, the remaining cells in the adhesive phenotypic phase would continually spin off cells in the non-adhesive phenotype. The actual number of cells that varied to the non-adhesive phenotype is related to the actual rate of phase variation in vivo. However, since non-adhesive phase cells would be rapidly cleared from the lumen or killed intracellularly after the initial exposure period, the only non-adhesive phase cells present would be those derived from the remaining adhesive phase cells and these too would be rapidly cleared or killed. Thus, depending on the actual *in vivo* rates of phenotypic phase variation it would be possible to maintain a small but consistent population of *S.* Typhimurium in pigs. This population would not be large enough to cause disease and thus pigs would remain asymptomatic but remain infected. It should be noted that while the hypothesis stated is related to pig growth, we have not measured rates of phase variation *in vivo* to confirm the hypothesis.

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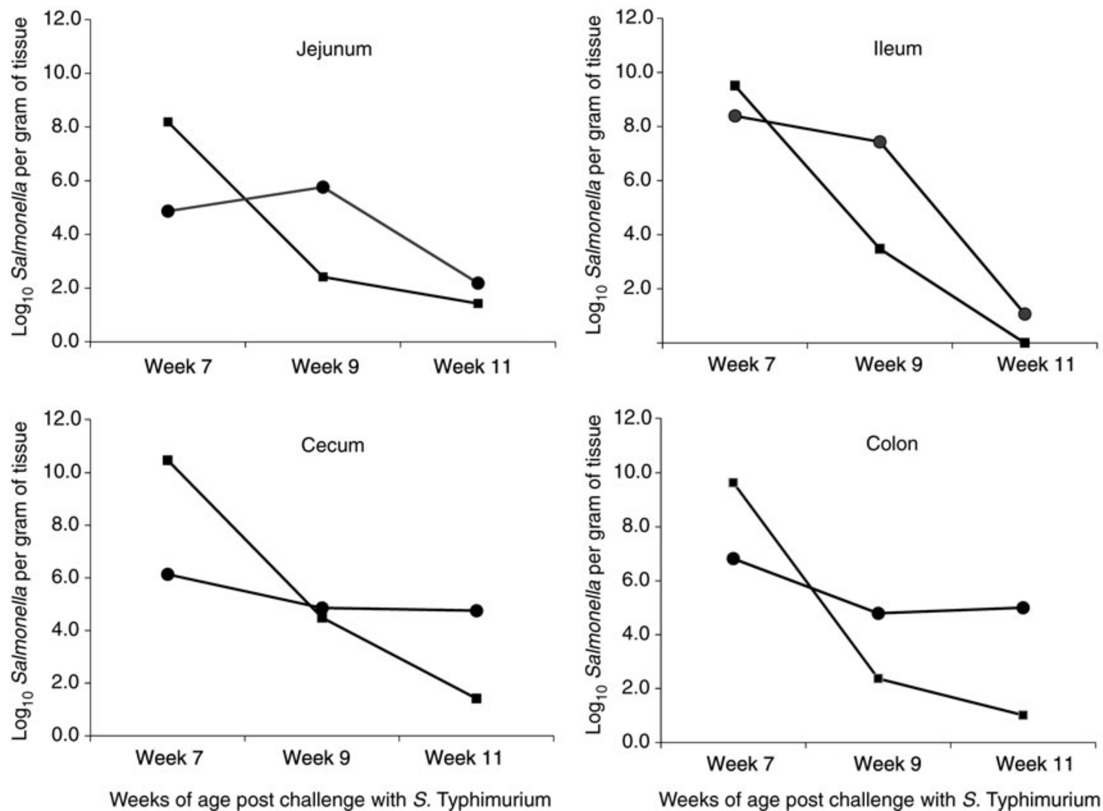

Fig. 5. Shedding at the indicated times of the nalidixic acid resistant *S.* Typhimurium strain 798 in pigs challenged only with this strain (■) or also challenged with *L. intracellularis* (●). Intestinal tissue samples were collected from the jejunum, ileum, cecum, and colon of pigs, the luminal surfaces scraped and the number of *S.* Typhimurium determined using most probable number (MPN) protocol employing a double enrichment (tetrathionate broth and Rappaport-Vassiliadis R10 Broth) followed by plating on XLT-4 agar containing nalidixic acid.

S. enterica shedding is enhanced by co-infection with *Lawsonia intracellularis*

Other factors that may contribute to the levels of *S. enterica* shedding prevalence, shedding levels, and persistence include interactions of *S. enterica* with other members of the intestinal microflora. For example, recent work by Beloeil *et al.* (2004) proposed that co-infection of pigs with pathogens such as *Lawsonia intracellularis* or porcine respiratory and reproductive syndrome virus might predispose them to shed *S. enterica*. In their study, 105 French pork production farms were sampled and a statistically significant association (Odds Ratio 3.2, 90% Confidence Interval 1.4 to 7.2) between infections with *L. intracellularis* and carriage of *S. enterica* was found. That work is consistent with the hypothesis that *L. intracellularis* interacts with *S. enterica* and/or other members of the gut microbiome and that these interactions lead to increased colonization and shedding of *S. enterica. L. intracellularis* is the cause of porcine proliferative enteritis.

To experimentally determine if *L. intracellularis* had any effects on the levels of *S. enterica* in pigs, we performed a challenge study. Groups of 5 week old pigs were challenged with *L. intracellularis, S.* Typhimurium strain 798, or both. A fourth group of pigs served as non-challenged controls. At various times, pigs were euthanized and the quantity of *S.* Typhimurium in the jejunum, ileum, cecum, and colon of pigs was determined using a most probable number analysis. The results are shown in Fig. 5. When the pigs were 7 weeks of age (1 week post challenge with *S.* Typhimurium and 2 weeks post challenge with *L. intracellularis*) the *S.* Typhimurium challenge strain was found in all pigs that had been challenged regardless of tissue site. The challenge strain was not found in any of the pigs that had not been challenged with *S.* Typhimurium strain 798. In jejunal and ileal tissues from pigs challenged with the nalidixic acid-resistant *S.* Typhimurium strain 798, the challenge strain was found at the highest levels in 7-week old pigs. Over the next 4 weeks the tissue levels dropped to ≤2 $\log_{10}$ regardless of whether the pigs also had been challenged with *L. intracellularis*. However, the culture results from the cecal and colonic tissues showed that tissues from pigs co-challenged with *L. intracellularis* did not show decreases in the overall concentration of the *S.* Typhimurium challenge strain and were statistically significantly higher at 11 weeks of age compared with pigs challenged only with *S.* Typhimurium ($P < 0.05$). The cecal levels of *S.* Typhimurium in the animals co-challenged with *S.* Typhimurium and *L. intracellularis* were 4 $\log_{10}$ higher ($P < 0.05$) compared with those only challenged with only *S.* Typhimurium while the colonic levels were 3 $\log_{10}$ higher ($P < 0.05$). In pigs challenged only with *S.* Typhimurium, a consistent decrease in the level of *S.* Typhimurium in the cecum or

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colon was observed and by 11 weeks of age all pig tissues contained ≤2 $\log_{10}$ *S* Typhimurium strain 798 per gram of tissue. This level of colonization is what we typically see after pigs are challenged with this strain of *S.* Typhimurium (Isaacson *et al.*, 1999b).

We also looked at the levels of *L. intracellularis* in the same tissues. Co-infection with *S.* Typhimurium strain 798 had no effect on the concentration of *L intracellularis* in any of the intestinal tissues.

The concept of pathogen super shedders has long been postulated and for *Escherichia coli* O157:H7, super shedders have been identified (Matthews *et al.*, 2006). However, for *S. enterica* the identification of super shedders has not occurred. Huang *et al.* (2011) showed that within a population of pigs orally challenged with *S.* Typhimurium, high and low shedders could be identified. However, this is unlikely to be the identification of super shedders because high shedding status is only for a short period of time (approximately 2 weeks). The observations that *L. intracellularis* can promote prolonged shedding of *S.* Typhimurium and at higher levels might represent a situation where a super shedder is created.

Does *S. enterica* change the composition of the microflora in the colon of pigs?

Pigs experimentally challenged orally with *S.* Typhimurium strain 798 have been shown to have altered compositions of their gut microbiota (the bacterial microbiome) (Borewicz *et al.*, 2015). Commercial pigs that are naturally infected with *S. enterica* and that are shedding this organism also exhibit altered compositions of the fecal microbiomes (Borewicz *et al.*, 2015). The challenged and naturally infected pigs at 10 weeks of age showed similar shifts in the microbiota composition, with significant changes in the relative abundance of *Barnesiella, Pseudobutyrivibrio, Prevotella, Lactobacillus, Anaerobacter, Roseburia, Fastidiospila, Campylobacter,* and *Succinivibrio.* Because there were similar shifts in microbiome composition between experimentally challenged pigs and those naturally exposed to 'presumably' low doses of *S. enterica*, we hypothesize that the shifts were due to the presence of *S. enterica* and not an effect of a microbial composition shift making the pigs more susceptible to *S. enterica*. This further suggests that while it has been assumed that one function for normal microflora is to exclude pathogens, in the case of *S. enterica* this may not hold true. One of the microbial shifts observed in older experimentally challenged pigs is the increased levels of *Akkermansia*. While this observation was observed in a sample of colon from one pig at 22 weeks of age (Borewicz *et al.*, 2015), it is known that *Akkermansia* does contribute to infection with *S. enterica* presumably by degrading mucin in the gut and increasing gut inflammation (Ganesh *et al.*, 2013).

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