Title: Novel two-step hierarchical screening of mutant pools reveals mutants under selection in chicks

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Abstract

Contaminated chicken/egg products are major sources of human salmonellosis yet the strategies used by *Salmonella* to colonize chickens are poorly understood. We applied a novel two-step hierarchical procedure to identify new genes important for colonization and persistence of *S.* Typhimurium in chickens. A library of 182 *S.* Typhimurium mutants each containing a targeted deletion of a group of contiguous genes (for a total of 2069 genes deleted) was used to identify regions under selection at 1, 3, and 9 days post- infection in chicks. Mutants in 11 regions were under selection at all assayed times ('colonization' mutants) and mutants in 15 regions that were under selection only at day 9 ('persistence' mutants). Second, we assembled a pool of 92 mutants, each deleted for a single gene, representing nearly all genes in nine regions under selection. 12 single gene deletion mutants were under selection in this assay, and we confirmed 6 of 9 of these candidate mutants via competitive infections and complementation analysis in chicks. *STM0580, STM1295, STM1297, STM3612, STM3615,* and *STM3734* are needed for *Salmonella* to colonize and persist in chicks and were not previously associated with this ability. One of these key genes, *STM1297* (*selD*), is required for anaerobic growth and supports the ability to utilize formate in these conditions, suggesting that metabolism of formate is important during infection. We report a hierarchical screening strategy to interrogate large portions of the genome during infection of animals, using mutant pools of low complexity. Using this strategy we have identified 6 genes not previously known to be needed during infection in chicks, one of which (*STM1297)* suggests an important role for formate metabolism during infection.

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51 **Introduction**

52 Non-typhoidal *Salmonella* spp. are the most common cause of bacterial food
53 borne disease with over 1 million cases and nearly 500 deaths in the United States
54 annually [1]. Contaminated poultry and eggs are a major source of human *Salmonella*
55 infection [2, 3] and are implicated as the cause of more than 50% of *Salmonella*
56 outbreaks in the United States [4]. Serotypes Typhimurium and Enteritidis can colonize
57 young chicks, and can persist sub-clinically for the life of the animal [5-7]. These 'carrier'
58 birds are a reservoir for this organism, shedding the organism in feces contaminating
59 the environment and the human food supply. These *Salmonella* colonized chickens are
60 hard to identify and remove from food production because they frequently do not have
61 clinical disease [8-10].

62 Despite the importance of chickens in contamination of the food supply, there is a
63 relative paucity of information on the strategies employed by *Salmonella* to colonize and
64 persist within this host. Flagellar motility and lipopolysaccharide biosynthesis are
65 necessary for *Salmonella* to colonize the intestine of both chickens and mammals [11-
66 16]. The type three secretion system-1 (TTSS-1) encoded on *Salmonella* pathogenicity
67 island-1 (SPI-1) plays a major role and the TTSS-2 a minor role in intestinal colonization
68 of mammalian hosts [17-19]. However, the available data suggest that the TTSS-1 and
69 TTSS-2 are both dispensable for intestinal colonization of chicks that have not been
70 seeded with adult intestinal microflora, prior to 6 days post infection [20, 21]. Mutants
71 deficient in pyrimidine and amino acid biosynthesis, central metabolism, and transport of

a variety of nutrients are predicted to be under negative selection in mutant screens but the role of these metabolic pathways in colonization has not been confirmed [22-24]. These studies highlight the similarities and differences in the strategies used by *Salmonella* during colonization of different hosts. Further studies in the chicken are needed to elucidate and confirm the repertoire of genes used by *Salmonella* to colonize and survive in the intestinal tract of poultry. This knowledge will be useful to develop new strategies to reduce *Salmonella* colonization of poultry.

Several genetic screens have been performed to identify *Salmonella* genes necessary for colonization in poultry [22-24]. Earlier screens performed in chicks employed 'signature-tagged mutagenesis' (STM) [22-24], a method to screen a pool random transposon (Tn) insertion mutants during infection [25]. Tens of thousands of Tn mutants are required to ensure near complete coverage of the genome for genetic screening. Yet, random loss from such complex pools due to biological 'bottlenecks' [26, 27] can create a high false positive discovery rate. An alternative to this approach is to generate pools containing fewer independent Tn insertions, but if the total number of Tn mutants to be screened remains constant, a greater number of animals is needed. Compounding this issue is the fact that each pool should be screened in multiple animals to ensure that the mutants identified as under selection are not specific to an individual. To have sufficient confidence in the identification of true positive mutants under selection in such screens, the use of random Tn insertions and the necessity for biological replicates drive up the number of animals needed for such studies, often to prohibitive levels.

Seeking a method for screening a larger fraction of the genes in the *Salmonella* genome, we constructed 182 mutants with targeted deletions in regions of multiple contiguous genes (multi-gene deletion mutants or MGD) in *Salmonella* Typhimurium ATCC14028 using lambda red recombination [28-30]. Collectively, these MGDs are deleted for ~2069 genes, and they can be pooled to identify those mutants under selection during infection. This method allows us to assay approximately half of the non-essential genome with a single, low complexity pool of less than 200 mutants.

We report a two-step screening approach that we have used to identify genes linked to mutants under selection in chickens. Our strategy employs a low-complexity pool of MGD mutants to identify regions of the genome needed for colonization of a host. Then, a second screen using a pool that contains deletions of individual genes (single gene deletion or SGD mutants) mapping to MGD regions under selection, identifies genes needed during infection. Using this strategy in four-day old SPF White Leghorn chicks, we ultimately confirmed six genes needed to colonize chick intestine. We linked the phenotypes of all six of these mutants directly to the corresponding candidate genes by complementation analysis.

We further explored the molecular role of selenophosphate synthetase (*selD)*, one of the genes we confirm as needed in chicks, during *Salmonella* growth. Using selenide and ATP, SelD synthesizes selenophosphate, a precursor needed for the formation of selenocysteine, a modified amino acid that can be incorporated into protein and tRNA. In *Salmonella* and *E. coli,* selenocysteine is incorporated into formate dehydrogenases (FDH), forming the catalytically active redox center of this enzyme [31]. Produced from pyruvate during anaerobic growth, formate is metabolized by FDHs

to carbon dioxide while donating electrons to an electron acceptor (Figure 6B). The FDH that is active, is reliant on the electron acceptor present, among other variables [32, 33]. We confirm that in anaerobic conditions, mutants lacking *selD* grow poorly, due to inability to utilize formate.

Our study illustrates a novel screening approach that allows for identification of mutants under selection in animal hosts using mutant pools of low complexity. We report 6 novel genes needed for colonization or persistence in the chick, an under-studied host of *Salmonella* that is a major source of contamination of the human food supply. Finally, we show, that one of genes identified in our study, *STM1297* (*selD*), is required for survival of *Salmonella* in anaerobic conditions and highlight the importance of formate metabolism for successful colonization of chicks.

Materials and Methods

Ethics Statement

The Texas A&M University Institutional Animal Care and Use Committee approved all animal experiments.

Bacterial strains and growth conditions

All strains used in this study are derivatives of *Salmonella enterica* serovar Typhimurium ATCC14028 (Manassas, VA) (Supplementary Table 1). Strains were routinely cultured in Luria-Bertani (LB) broth or M9 minimal medium at 37°C or 41°C where indicated and on LB plates supplemented with the appropriate antibiotics. Bacterial cultures used to infect chicks were grown in LB broth supplemented with the

appropriate antibiotics to stationary phase at 41°C with aeration. Antibiotics and other supplements were used at the following concentrations: 20 mg/L 5-bromo-4-chloro-3-indolyl phosphate (XP), 50 mg/L kanamycin (Kan), 20mg/L chloramphenicol (Cm), 100 mg/L nalidixic acid (Nal), 100 mg/L streptomycin (Strep), and 100mg/L carbenicillin (Carb).

Generation of mutant pools

Multi-gene deletion (MGD) and single gene deletion (SGD) mutants were generated using a modification of the lambda red recombinase method [28] as described previously [29, 30]. MGD mutations were moved into the wild type background by P22 transduction [34], to remove potential background mutations, and stored in 1 ml aliquots with 30% glycerol at -80°C.

Two separate pools were generated: the MGD mutant pool (MGD1029) and an SGD mutant pool. In order to generate the 'input' pool of mutants for screening in live animals, individual mutants were grown in 1ml of LB broth supplemented with kanamycin at 37°C with agitation to stationary phase. A single pool of mutants was generated by combining 200 µl of each mutant, and the resulting pool was divided in aliquots stored at -80°C until needed for animal infection. The composition of the MGD mutant pool is listed in Supplementary Table 3.

The SGD pool representing nearly all mutants from each of 9 regions under selection was generated by growing 1 ml of each SGD mutant in deep well 96 well dishes, and combining 100 µl of each mutant to generate the larger pool. The composition of SGD mutant pool is listed in Supplementary Table 4.

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164 Plasmid construction

165 All primers used in this study for the construction of complementing plasmids are
166 listed in Supplementary Table 2. Complementing plasmids containing intact *STM0580,*
167 and *STM3734* were generated as follows. Each gene was amplified using primers,
168 *STM0580*_HindIII_F and *STM0580*_KpnI_R for *STM0580*, and *STM3734*_EcoRI_F and
169 *STM3734*_HindIII_R for *STM3734*. PCR products, containing the full length open
170 reading frame (ORF) with approximately 200 base pairs upstream and 50-100 base
171 pairs downstream sequence were generated by PCR using *Pfu Turbo* DNA polymerase
172 (Agilent Technologies) in 50 μl volume at an annealing temperature of 58°C for 30
173 cycles. PCR products were digested with HindIII and KpnI for *STM0580*, EcoRI and
174 HindIII for *STM3734* as per manufacturer instructions (New England Biolabs, Ipswich,
175 MA). Digested PCR products were ligated into pWSK29 previously cut with the same
176 enzymes. Each ligation was transformed into *E. coli* XL1-Blue, and positive clones were
177 selected on LB plates containing carbenicillin. Correct inserts were confirmed by
178 restriction digest and sequencing.

179 Complementation of mutants *in cis* containing deletions in *STM1295, STM1297,*
180 *STM3612* and *STM3615* was performed by inserting intact copies of each of these
181 genes into the chromosome disrupting the *phoN* gene. The advantage of placing
182 complementing genes in this site is that such mutations have no effect on colonization
183 or virulence in murine or chick models [35] (and data not shown), and we have a simple
184 colorimetric assay for identifying disruptions in the *phoN* gene [35]. Inactivation of *phoN*,
185 encoding an alkaline phosphatase, abolishes the ability to cleave 5-bromo-4-chloro-3-

indolyl phosphate (XP) resulting in the formation of white colonies on LB plates supplemented with XP, while *phoN*+ strains appear blue. PCR products were amplified as described above and digested with BamHI and XhoI for *STM3612* and BamHI and KpnI for *STM1295, STM1297*, and *STM3615*. A plasmid derivative of pCLF3 [29] was generated bearing the multi-cloning site from pWSK29 plasmid [36] inserted into the SwaI site of pCLF3 upstream of the antibiotic resistance cassette, and the resulting plasmid was named pMR3.2. Digested PCR products were ligated into multi-cloning site on pMR3.2, previously cut with the same enzymes, and ligation products were transformed into *E. coli* S17λpir. Correct transformants were confirmed by restriction digest, and the resulting plasmids were named HA1440 (pMR3.2::*STM1297*), HA1442 (pMR3.2::*STM3615*), HA1450 (pMR3.2::*STM3612*) and HA1451 (pMR3.2::*STM1295*). Using these plasmids as a template, PCR products containing the intact genes for complementation and the flanking chloramphenicol resistance marker were generated using primers containing 20 bases of homology to the plasmid template and 45 bases of homology to either end of the *phoN* gene (Supplementary Table 2). PCR was carried out using ExTaq polymerase (Takara) in 60 µl total reaction volume at an annealing temperature of 55°C for 30 cycles. Purified and dialyzed PCR products were transformed into each mutant background expressing lambda red recombinase as previously described [29] and transformants were confirmed by PCR using primers in *phoN* flanking the site of insertion.

Chick hatching and screening of pools

Specific pathogen free (SPF) eggs were obtained from Charles River SPAFAS (North Franklin, CT). Eggs were incubated in an egg incubator (GQF Manufacturing Co.) at 38°C with 58-65% of humidity for 21 days. Eggs were periodically rotated for the first 18 days and then moved to the hatching tray for the last 3 days pre-hatch [21]. Chicks were housed in a poultry brooder (Alternative Design Manufacturing, Siloam Springs, AR) at 32°C to 35°C with *ad libitum* access to tap water and irradiated lab chick diet (Harlan Teklad, Madison, WI).

Screening of the pool of targeted MGD mutants in four-day old chicks was performed as follows. The inoculum for the 'input pool' was prepared by dilution of frozen aliquot of the pool (1 ml volume) into 50 ml of LB broth supplemented with kanamycin, and this culture was grown at 41°C overnight. This 'input pool' was spiked with strains marked with different antibiotic resistance markers in neutral locations in the genome to monitor biological bottlenecks during infection (See 'Fluctuation assay' in Materials and Methods).

Thirty specific pathogen free (SPF) White Leghorn chicks were orally infected with 1×10^9 CFU of the 'input pool' in 100 µl at 4 days post-hatch. Animals were monitored twice daily for signs of infection. On days 1, 3, and 9 post-infection, groups of 10 chicks were humanely euthanized. The cecal contents, cecal tissue, cecal tonsil, spleen, liver, and bursa were excised and homogenized in phosphate buffered saline (1 ml PBS for cecal tonsil, 3 ml for all other organs), serially diluted, and plated on three different media, LB plates containing either kanamycin, chloramphenicol or streptomycin to determine colony forming units (CFU) of the input MGD pool and each of the spiking strains. The remainder of each homogenate was grown overnight in LB broth

supplemented with kanamycin, and the bacteria were harvested by centrifugation to generate the 'output pool'. The 'input' and 'output' pools were simultaneously processed for analysis on Nimblegen tiling arrays as previously described [29].

Growth of the SGD pool inoculum, screening of the SGD pool, and analysis of SGD output pools was performed in a similar to the same procedures for the MGD pool described in the previous paragraph. We infected groups of 10 chicks and euthanized at days 1,3 and 9 post-infection. Mutant representation after infection was determined from organs as described above. Data were analyzed as for the MGD pool.

Fluctuation Test

In order to monitor biological bottlenecks during animal infection, two strains of ATCC14028 Nal^R that carry different antibiotic resistance markers in neutral location, in the *phoN* gene, were generated using the lambda red recombination method (HA530, Δ*phoN*::Cm^R, and HA697, Δ*phoN*::$Strep^R$) [21, 28]. Our screening 'input' pool was spiked with these two strains at different ratios, 1:200 using the choramphenicol resistant strain HA530, and 1:1000 using the streptomycin resistant isolate HA697. The ratio of each spiking strain to the total 'input' pool was compared to the ratio at which these spiking strains were recovered from the 'output' at each time point, and from each organ collected, to monitor for biological bottlenecks.

Nimblegen array use for comparison of the input and output pools

A Nimblegen tiling array consisting of approximately 387,000 50-mer oligonucleotides tiled along the entire *Salmonella enterica* serotype Typhimurium

14028s genome (GEO platform GPL14855 at http://www.ncbi.nlm.nih.gov/geo/) was used for detection of mutants in input and output pools. Preparation of labeled RNA and hybridization conditions were as previously described [29]. Briefly, total DNA of input or output mutant pools was sonicated, poly-A tailed, and PCR amplified with a primer targeting the shared portion of each mutant and a primer including oligo dT at the 3' end. The PCR product was subjected to reverse transcription from a T7 RNA polymerase promoter located inside each mutant and a mixture of NTPs that included a fluorescently labeled UTP. The RNA was purified using the RNeasy Mini Kit (Qiagen), and approximately 4 micrograms of labeled RNA was hybridized to the array at 42°C for 16 hours, as per manufacturer's recommendations. The arrays were washed according to the manufacturer's protocol, and scanned using a GenePix 4000B laser scanner (Molecular Devices, Sunnyvale, California) at 5 μm resolution. Data was uploaded into WebArrayDB [37, 38] and data analyzed for peak height in the DNA directly downstream of each mutant location. The relative signal of each mutant in the pool harvested from the chicks was compared to the relative signal in a corresponding array of the same pool prior to selection. A custom-built R script was used to automatically estimate the ratio of the signal strength at the expected nucleic acid positions for each mutant before and after selection. Representative automatic results were visually verified in graphs obtained during analysis in WebarrayDB.

Array data analysis and identification of candidate mutants

Array data from four organs (cecal tissue, cecal contents, bursa of Fabricius, and spleen) in nine chicks were analyzed to identify candidate regions under selection. The

signal strength ratios were tested for normality using an Anderson-Darling normality test (http://www.xuru.org/st/DS.asp). When the data were normally distributed, a mean score for each mutant was calculated using the mean score of multiple samples from different animals. Using this mean score and standard deviation, an 85% confidence interval (CI) was calculated. Mutants under selection in our screens were defined as those mutants with a score outside the 85% confidence interval of the mean scores.

Testing growth *in vitro*

Growth of candidate MGD mutants *in vitro* was measured in LB or M9 broth at different temperatures. Overnight culture of each candidate MGD mutant was sub-cultured at a dilution of 1:100 in 5 ml of fresh LB broth supplemented with kanamycin, and subcultures were grown for 24 hours at 41°C or at 37°C with agitation. For testing growth in M9 minimal broth, overnight cultures were washed twice with sterile M9 minimal broth and sub-cultured in fresh sterile M9 minimal broth supplemented with kanamycin, and grown for 24 hours at 41°C. The number of colony forming units (CFU) was evaluated at the beginning of subculture (T_0) and at the end (T_{24}). Each experiment was performed on three separate occasions.

Competitive infections and complementation analysis in chicks

Verification of mutant phenotypes predicted by our SGD screen and complementation experiments were performed using individual competitive infections in groups of 10-15 chicks. Four-day old SPF White Leghorn chicks were orally infected with 1×10^9 CFU of 1:1 mixture of wild type HA431 (ATCC14028 *ΔphoN*::Kan^R) [21] and mutant, or wild type HA877 (HA431 + pWSK29) and complemented mutant of interest.

The inoculum was serially diluted and plated on LB plates containing kanamycin and XP to determine the total CFU and the exact input ratio of wild type vs. mutant. Groups of five chicks were euthanized on days 1, 3, and 9 post infection for verification of mutant phenotypes and on each time point, ceca, cecal tonsil, bursa of Fabricius, spleen and liver were collected and homogenized in PBS (1 ml PBS for cecal tonsil, 3 ml for all other organs). For complementation of mutants, groups of five chicks were euthanized on days 3 and 9 post infection and sample collection and processing performed as for verification of mutant phenotype. The CFU of mutant and wild type organisms were determined by serial dilution and titer. Competitive index was determined by comparing the ratio of mutant to WT in chick tissue with that ratio in the inoculum. Data were analyzed using a two-tailed Student's *t*-test. Statistical significance was set at $P < 0.05$.

Anaerobic growth of *ΔseID* mutant

To assess bacterial growth without oxygen, strains were grown overnight in an anaerobic chamber. Bacteria were collected by centrifugation, transferred into the anaerobic chamber with internal atmosphere of 5% H_2, 5% CO_2, and 90% N_2 (Bactron I, ShelLab) and resuspended in LB broth pre-equilibrated for at least 16 hours in the anaerobic chamber. The resulting bacterial cultures were used to inoculate LB-broth, or LB-broth supplemented with 40 mM sodium formate or 40 mM sodium fumarate at 1:100 dilution. Aliquots were collected hourly, serially diluted, and plated on LB agar for CFU enumeration. All experiments were performed on at least three separate occasions. Bacterial generation number was calculated using the following equation: [log10(CFU final) - log10(CFU start)]/log10(2).

Plate assay for nitrate reductase-linked formate dehydrogenase (FDH_N) and hydrogen-linked formate dehydrogenase (FDH_H) activities

The ability of strains of interest mutant to produce active FDH_N was tested on MacConkey nitrate medium plates [39]. Plates were equilibrated in the anaerobic chamber with internal atmosphere of 5% H_2, 5% CO_2, and 90% N_2 (Bactron I, ShelLab) for at least 16 hours. Wild type, *ΔselD* (HA1557), complemented *ΔselD* (HA1472) and *ΔfdhE* (HA1558) strains were grown aerobically overnight, cells were collected by centrifugation, transferred to the anaerobic chamber and streaked on MacConkey nitrate plates. Plates were incubated at 37°C overnight in anaerobic chamber. Colonies lacking active FDH_N are red due to accumulation of formate [40]. In parallel experiments, strains were streaked on MacConkey nitrate plates and incubated overnight aerobically at 37°C. All plates were imaged after 20 hours of incubation.

To test the ability of strains of interest to produce active hydrogen-linked formate dehydrogenase (FDH_H), the wild type, *ΔfdhD* (HA1559) and strains listed in the preceding paragraph were grown anaerobically in LB-broth for 4 hours at 37°C, and streaked on LB plates. Plates and LB-broth used for this experiment were equilibrated in the anaerobic chamber for at least 16 hours prior to use to remove remaining dissolved oxygen. Inoculated plates were incubated at 37°C for 20 hours in anaerobic chamber. Benzyl viologen (BV) dye overlay agar was prepared as described by Mandrand-Berthelot et al. [41]. Within few minutes after removal of plates from the anaerobic chamber they were overlaid with 5 ml of melted BV agar. Colonies with active FDH_H

reduced the BV dye and developed a deep purple color. Colonies lacking active FDH_H remained white. Plates were imaged immediately after solidification of BV overlay.

Results

Colonization of four-day old chicks

In order to screen pools of mutants to identify those under selection in chicks, we needed a robust model for subclinical *Salmonella* colonization. Age is one of the important factors that influence the level of colonization and disease in chicks infected with *Salmonella* [42, 43]. Newly hatched chicks younger than 3 days post-hatch are highly sensitive to low dose infection with *Salmonella* Typhimurium and they develop systemic disease with high mortality [42, 43]. When infected after 3 days of age, chicks are heavily colonized with *Salmonella* but do not develop clinical disease. To screen for mutants unable to colonize and persist in the intestinal tract of chicks, we developed a model for subclinical colonization of *Salmonellae* using four-day old chicks. In our experiments, chicks were heavily colonized with *Salmonella* Typhimurium ATCC14028 in the intestinal tract from day 1 to day 9 post-infection (13 days of age) (Figure 1A), the latest time point before the rapidly growing White Leghorn broiler chicks become too large to house in our facility. Cecal contents in these chicks are most highly colonized with *Salmonella* as previously reported [44] containing ~$1x10^8$ CFU throughout the duration of infection. Systemic organs of four-day old chicks were lightly colonized after oral infection (Figure 1), yet these sites are more heavily colonized in chicks infected at four days post hatch than chicks infected at seven days post hatch [21].

Monitoring of biological bottlenecks of mutant pool during chick infection

Biological 'bottlenecks' cause random loss of mutants from the pool during animal infection, and such bottlenecks can be so severe as to make screening of highly diverse pools of mutants technically challenging. This 'bottleneck' problem is particularly problematic after oral infection and during transit of bacterial pathogens from the intestinal tract to systemic sites [26, 27, 45]. This problem has been mitigated in previous work by using very small pools of mutants for screening [23], but this approach severely limits the total number of mutants that can be screened when using random transposon mutants allowing only partial interrogation of the genome.

Evaluation of bottlenecks by measuring the fluctuation in representation of mutants in a pool used to infect an animal, in each infected animal and from each niche to be evaluated, increases the probability of successful identification of mutants under selection. We evaluated the fluctuation in the representation of strains used to infect in each animal, in each organ, and at each time point throughout our screen. We spiked our pool of mutants with two strains that have antibiotic resistance markers inserted in the *phoN* gene (HA530 ATCC14028s *ΔphoN::Cm*^{R} and HA697 ATCC14028s *ΔphoN::Strep*^{R}), and evaluated the representation of these strains in our 'input' pool and in the 'output' pools from each niche. HA530 was added to the pool at a 1:200 ratio, approximately the proportion of each individual mutant in our pool of 182 mutants, while HA697 was added to the pool at a five-fold lower concentration; 1:1000.

Each animal showed a unique pattern of fluctuation of the pools during infection, but surprisingly the representation of HA530 in pools recovered from the intestine generally did not fluctuate greater than 10-fold until day 9 post-infection with only a few exceptions (Figure 1B). Consistent with previous observations, the representation of

HA530 in the 'output' pools from systemic sites fluctuated more severely than from intestinal sites (Figure 1B, Spleen and Liver). Samples from each niche where HA530 fluctuated 5-fold or less (Figure 1B, horizontal dotted line) were used to determine the representation of each mutant in the pool using a Nimblegen tiling array.

Step One: Screening of pool of targeted multi-gene deletion mutants in chicks

An overview of our two-step screening strategy is shown (Figure 2). The pool of 182 MGD mutants in *Salmonella* Typhimurium ATCC 14028 was used to inoculate four-day old chicks by gavage. Chicks were humanely euthanized at days 1, 3, and 9 post-infection and output pools were collected from several organs. A total of seventeen samples, derived from four different organs (cecal contents, cecal tissue, bursa of Fabricius, and spleen) from nine different chicks, were analyzed by microarray for each time point. By looking at samples under selection in multiple niches in the host, we hypothesized that we could identify mutants that were most consistently under selection.

We identified a total of 26 regions under selection (outside the 85% confidence interval), with a combined 397 genes deleted (Table 1). These mutants fell into two categories. The first category represented mutants under selection at all times examined: days 1, 3, and 9 post-infection. We termed these mutants candidate 'colonization' MGD as they appeared to be unable to establish an infection from the earliest time points we measured. The second category of mutants colonized similar to the wild type organism early in infection, but were selected against only at the latest

time point we assayed: day 9 post-infection. We termed these mutants candidate 'persistence' MGD mutants.

We identified 11 'colonization' MGD mutants, of which 8 regions contain genes previously reported to be candidates for colonization of chicks [22, 23, 46, 47]. Furthermore, 8 of the 15 'persistence' MGD mutants are deleted for genomic regions containing at least one gene predicted to be necessary for colonization of chicks [22, 23, 46, 47]. Thus, our screening strategy identifies genomic regions already predicted to be necessary for colonization of the chicken host and identifies novel regions needed for both 'colonization' and 'persistence' within this host.

Growth of MGD 'colonization' and 'persistence' mutants *in vitro*

We tested the growth of 26 MGD mutants identified in our screen in chicks during growth in both LB and M9 minimal broth at 41°C, the normal body temperature of the chickens. All 26 MGD mutants grew similarly to wild type (HA431) in LB broth, but this was not the case in M9 minimal broth (Supplementary Figure S1). Of the candidate 'colonization' mutants assayed, three of the eleven (*ΔSTM0002-0011, ΔSTM3603-3651,* and *ΔSTM4416-4467)* had growth defects in M9 minimal media at 41°C (Supplementary Figure S1, panel A). Similarly, three of the fifteen candidate 'persistence' mutants (*ΔSTM1165-1156, ΔSTM2434-2450, ΔSTM2667-2672*) had noticeable growth defects in M9 minimal media at 41°C (Supplementary Figure S1, panel B). Growth characteristics similar to those noted at 41˚C were observed in both LB broth and M9 minimal media during growth at 37˚C (data not shown) excluding an effect of temperature on our observed growth phenotypes. We excluded from further analysis the six MGD mutants

that grow poorly in M9 media *in vitro*, although these may still encode genes of relevance to colonization or persistence in chicks.

Step two: Screening of single gene deletion mutants from MGD regions under selection

Of the deleted regions identified as under selection in our primary screen that did not have growth defects *in vitro*, we selected nine regions including three 'colonization' MGD mutants and six 'persistence' MGD mutants to pinpoint the individual genes responsible for the phenotypes we noted in chicks (Table 1, Bold). We generated a pool of targeted single gene deletion (SGD) mutants in nearly all genes from each of these nine MGD regions under selection, using a library of SGD mutants that we constructed previously [29, 30]. The SGD pool that we assembled contained deletion mutants in 92 of the 103 genes located in these nine regions (Supplementary Table 3), and this pool was screened in chicks using the same methodology described previously.

In this second round of screening, we identified twelve SGD mutants under selection in chicks (Figure 3). These genes map to seven of the original MGD regions that we identified as under selection (Table 2). Six mutants (Δ*STM0580,* Δ*STM1297,* Δ*STM3472,* Δ*STM3615,* Δ*STM3734,* and Δ*STM4290*) were under selection only on day 9 post-infection. We did not identify any SGD mutants with phenotypes in chicks that mapped to two MGD regions, *STM0102-0092* and *STM3626-3650*.

Confirmation of candidate phenotypes and complementation analysis

We retested nine of the twelve SGD mutants under selection in individual competition infections with wild type, HA431 (ATCC14028 *ΔphoN*::Kan^R) in chicks. Six SGD mutants (Δ*STM0580,* Δ*STM1295,* Δ*STM1297,* Δ*STM3612,* Δ*STM3615,* and Δ*STM3734)* had significantly reduced colonization in ceca (Figure 4). Colonization by the Δ*STM0580,* Δ*STM1295,* Δ*STM1297,* and Δ*STM3615* mutants was significantly reduced in ceca beginning from day 1 post-infection. The Δ*STM3612* and Δ*STM3734* mutants had significantly reduced colonization in ceca beginning from day 3 post-infection (Figure 4). We also observed reduced colonization by four mutants (Δ*STM0580,* Δ*STM1295,* Δ*STM1297,* and Δ*STM3734)* in cecal tonsil, bursa of Fabricius, spleen and liver (data not shown). We were unable to confirm the phenotype predicted by the screen for three mutants (Δ*STM3472*, Δ*STM3616*, and Δ*STM3942* Supplementary Figure S2*).*

We performed complementation of six confirmed SGD mutants (Δ*STM0580,* Δ*STM1295,* Δ*STM1297,* Δ*STM3612,* Δ*STM3615,* and Δ*STM3734)* using competition assays in chicks for each deletion mutant containing an intact copy of the corresponding gene, against derivatives of the wild type (either HA877 (HA431 + pWSK29) or HA431 (ATCC14028 *ΔphoN*::Kan^R)). For each deletion mutant that we attempted to complement, we successfully restored colonization to levels similar to wild type, definitively linking each of these genes to the phenotypes we observed during infection (Figure 5).

Formate metabolism is important for colonization and persistence of *Salmonella* in the chick intestine.

We selected one of our mutants *STM1297* (*selD*), confirmed to be under selection in chicks, for more detailed study. *selD* encodes selenophosphate synthetase [39] [48], an enzyme required for the generation of selenocystine and thus for the incorporation of selenium into proteins and tRNA (Figure 6A) [48]. In enterobacteria formate dehydrogenase (FDH) is an important enzyme needed for metabolism during anaerobic growth that requires selenocysteine for activity (at position 194 in FDH_N, position 140 in FDH_H, and position 196 in FDH_O). Thus, we were interested in whether *selD* mutants could grow in anaerobic conditions [49]. Mutants lacking *selD* had a growth defect compared to wild type when strains were grown anaerobically (Figure 6B). The growth of mutants lacking SelD could not be rescued by the addition of exogenous formate (Figure 6C), suggesting that *selD* mutants cannot utilize this metabolite because *selD* mutants cannot produce active FDH. In contrast, when media were supplemented with fumarate, an alternate energy source, the growth of the *ΔselD* mutant was restored to mirror the growth of the wild type (Figure 6D). These data suggest that the mutant lacking *selD* fails to grow in anaerobic conditions because it cannot metabolize formate specifically, rather than having a more generalized metabolic defect.

To test whether the absence of *selD* affects FDH activity, we used two different plate assays to assess activity of FDH_N and FDH_H in *ΔselD* mutants. When the wild type, a *ΔselD mutant*, a *ΔselD* mutant complemented *in cis*, and a *ΔfdhE* mutant strain were streaked on MacConkey nitrate plates and grown aerobically there was no difference in colony appearance between strains (Figure 6E) [39]. In contrast, when grown anaerobically the *ΔselD* mutant appeared red on MacConkey nitrate plates

506 similar to an FHD-deficient *ΔfdhE* strain (Figure 6F). This color change indicates
507 accumulation of formate in the colony and it was suppressed by providing an intact copy
508 of *selD in cis* (Figure 6F) [40]. Thus, in the *selD* mutant, FDH_N is not active in conditions
509 where this enzyme should normally be induced and active.
510 FDH_H is known to be able to reduce benzyl viologen (BV) [40]. To assess the
511 activity of this enzyme in *ΔselD* mutants we used a BV dye overlay assay [41].
512 Anaerobically grown colonies with active FDH_H develop deep purple color when overlaid
513 with benzyl viologen-containing top agar while colonies lacking formate dehydrogenase
514 activity remain colorless. In our BV assay, anaerobically grown wild type colonies
515 changed color as expected (Figure 6G). In contrast, colonies of the *ΔselD* mutant strain
516 remained colorless similar to a formate dehydrogenase-deficient strain lacking *ΔfdhD.*
517 Formate dehydrogenase activity was restored in the *ΔselD* mutant complemented *in cis*
518 (Figure 6H). These combined results suggest that deletion of *selD* abolishes the activity
519 of both FDH_N and FDH_H. Finally, these data support the hypothesis that colonization
520 and persistence defect of *ΔselD* mutant (Figure 4 and 5D) may be due to a growth
521 defect in anaerobic conditions, and more specifically due to an inability to utilize
522 formate.
523
524 **Discussion**
525 We report a novel screening methodology to identify genes necessary for a
526 pathogen to colonize an animal host. Our strategy uses two separate pools of low
527 complexity of targeted deletion mutants in a hierarchical process. First, we screened a
528 pool of targeted deletion mutants in multiple adjacent genes (MGD mutants) in oral

529 infection of 4-day old chicks. Next, we constructed a second pool of targeted deletion
530 mutants in single genes (SGD mutants) that corresponded to genes deleted in MGD
531 mutants identified as under selection from the MGD mutant screen. This pool was
532 screened in oral infection of chicks to identify SGD mutants under selection. Using this
533 screening strategy, we were able to effectively interrogate almost half of the *Salmonella*
534 genome during infection overcoming a major limitation to currently employed high-
535 density transposon mutant screens.

536 Our two-step screening strategy allowed us to use a relatively small number of
537 animals for screening while still maintaining high genome coverage and using multiple
538 biological replicates. We used only 60 animals to effectively screen 2069 genes with at
539 least three biological replicates per time point. A previous study utilized Tn mutants to
540 identify genes under selection in chickens [23]. This study provided only two biological
541 replicates and required 180 chickens [23]. Our studies provide similar genome
542 coverage, yet utilize far fewer animals.

543 In the first step of our screen, the use of an MGD mutant library allowed us to
544 cover about 50% of the non-essential *Salmonella* Typhimurium genome in a pool with
545 10-fold fewer clones than genes deleted. Use of this low complexity pool is
546 advantageous for screening in niches where the pool encounters biological bottlenecks,
547 including after oral infection in live animals [26, 27, 29]. We noted that our input pool
548 was sufficiently small that it did not experience significant bottlenecks in intestinal
549 niches. However, as expected the representation of our neutrally marked strains
550 fluctuated in systemic sites as transit out of the intestine represents a well established
551 bottleneck (Figure 1B). Use of the second screening step allowed us to rapidly narrow

down to a handful of individual candidate genes in a single experiment (Figure 2). This two-step strategy should prove useful to interrogate the genome of other pathogens where animal models are limited in supply or too costly to preclude adequate number of biological replicates for large numbers of mutant pools.

We developed a four-day old chick model for screening of the MGD library to identify mutants that are important for colonization by *S.* Typhimurium. Chicks younger than three days post-hatch develop systemic disease with high mortality upon infection with *Salmonella* Typhimurium [42, 43]. Several previous studies have used this model of acute systemic disease to screen for mutants under selection [22, 24, 47]. Other studies have reported that 1-week old chicks infected with *Salmonella* Typhimurium do not become as heavily colonized in the intestine, making these older animals less useful for screening [21]. When infected at four days of age, chicks were colonized reliably with ~1x10^8 CFU (total number) of bacteria in intestinal sites (Figure 1). Thus, our four-day old chick model is well suited for screening of a library to identify mutants under selection with high level of colonization throughout the duration of infection.

The 'colonization' or 'persistence' phenotypes from our MGD mutant screen were largely shared by SGD mutants from the corresponding MGD region. Thus, the majority of individual mutants identified as under selection at early time points (i.e. 'colonization' mutants) identified in the second screen, mapped to MGD regions identified as required for colonization. However, the SGD mutants (in *ΔSTM3472, ΔSTM3615*, and *ΔSTM3734)* located within MGD regions that appeared to be under selection at all times we tested were determined to be defective only at later time points during infection. One potential explanation for this observation is that the combined effect of loss of multiple

genes from a given MGD region caused a more severe phenotype in the corresponding MGD than can be observed in mutants in any individual gene in the same region. Of the mutants we confirmed in individual competitive infection, four mutants *(ΔSTM0580, ΔSTM1295, ΔSTM1297, and ΔSTM3734)* colonized poorly in ceca (Figure 4) as well as in cecal tonsil, bursa of Fabricius, spleen, and liver (data not shown). The remaining two mutants, *ΔSTM3612,* and *ΔSTM3615,* had significant defects only in the ceca (Figure 4).

We identified genes affecting fitness in the chick from seven of the nine regions we tested, in some cases identifying more than one gene necessary for fitness in a given region, for a total of twelve individual genes. There are several potential reasons for our inability to identify individual genes under selection from the remaining two MGD regions. This finding may be due to the absence of key SGD mutants in the second pool, the need for deletion of more than one gene in the region, or that the phenotype observed maps to a region that was not deleted among our SGDs, such as an intergenic region.

Of the 26 MGD regions under selection in our first round of screening, 16 contained genes previously implicated as candidates important for in colonization of chickens (Supplementary Tables 5 and 6) [22, 23]. Three of these regions contained genes shown to have increased expression in the chick cecum [47]. Four regions contained genes previously reported to be necessary for growth *in vitro* at 42°C [46]. These overlapping data show that our screening strategy identifies genomic regions under selection that contain genes that have previously been implicated in colonization of the chick.

We identified, confirmed and complemented six new genes needed during infection of chicks. One of these genes, *STM0580 (ramR),* encodes a putative regulatory protein of the TetR family and is found upstream and in the opposite orientation of *ramA,* a known positive regulator of the AcrAB multi-drug efflux pump [50]. Previous studies reported that the *ramR* gene product plays a role in local repression of *ramA,* and inactivation of *ramR* resulted in increased expression of *ramA*, increased expression of AcrAB, and increased multi-drug resistance [50, 51]. *S.* Typhimurium strains with deletion of *acrB* have previously been shown to colonize chicks poorly in long-term experiments [52]. We show that a Δ*ramR* mutant colonizes the chick intestine poorly, but whether this is related to documented over-expression of AcrAB in *ramR* mutants remains to be investigated.

A further gene we identified, *STM3734 (rph*), encodes ribonuclease PH, the first gene of the bicistronic operon of *rph-pyrE*. The *rph* gene encodes a 3'-5' exoribonuclease and tRNA nucleotydyltransferase involved in tRNA processing. In *E. coli*, mutations in *rph* can lead to polar effects on *pyrE* and to pyrimidine starvation [53]. However, our Δ*rph* mutant did not have reduced growth in M9 minimal media, as would be expected for a pyrimidine auxotroph (data not shown). Furthermore, our positive complementation data are strong evidence that our *rph* deletion is not simply polar on *STM3733.* A recent study reported that *rph* plays a novel role in degradation of structured RNA in *E. coli* [54]. Any potential role that this function may play during infection is an exciting area of further investigation.

STM3612 (kdgK) encodes ketodeoxygluconokinase (KDG kinase EC 2.7.1.45), a key enzyme in the modified Entner-Doudoroff (ED) pathway. The Entner-Doudoroff

pathway is an alternative series of reactions for carbon metabolism and is known to be present in the diverse group of organisms ranging from archaea to eubacteria to eukaryotes [55]. Recent microarray studies for gene expression profiling of *Salmonella* in macrophages showed that intracellular *Salmonella* appear to use the ED pathway to metabolize gluconate and related sugars as a carbon source within macrophages [56, 57]. In addition, the ED pathway is essential for colonization of mammalian intestine by *E. coli* [57, 58]. Even though the relevance of these observations to *in vivo* metabolism is not clear, it is plausible that the ED pathway is important for intracellular growth of *Salmonella* in chicks.

Another gene we confirmed to be needed for colonization of the chick intestine is *STM3615*. Some Tn insertions in STM3615 are reported to be under selection in chicks, although these findings have not been confirmed with individual infections or complementation analysis [23]. *STM3615* encodes a putative protein that contains several consensus regions including a signal peptide, a GGDEF domain, an EAL domain, and a HAMP domain [59]. GGDEF (amino acids 213-369) and EAL domains (amino acids 385-623), are found in the di-guanylate cyclases and phosphodiesterases that are key in the metabolism of the bacterial second messenger c-di-GMP [60-62]. In other proteins where both of these domains occur, one of these domains frequently lacks enzymatic activity but may retain binding activity for the cyclic di-nucleotide ligand [63] [64] [62]. Although the sequence of the EAL domain of STM3615 is in strong agreement with the PFAM consensus sequence for these phosphodiesterase domains, the GGDEF domain has poor agreement with PFAM consensus sequences for domains possessing the diguanylate cyclase activity [59]. This observation suggests that

STM3615 is may have phosphodiesterase activity for cyclic-di-nucleotides, although this remains to be shown directly.

STM3615 also contains a HAMP domain, an approximately 50 amino acid alpha helical region named for its presence in histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins, and phosphatases [59, 65]. HAMP domain proteins in bacteria are usually integral membrane proteins and may be part of two-component regulatory systems, and these proteins may possess one or more additional conserved domains including EAL, GGDEF, 2C-like domain and others. These conserved domains are present in many bacteria signal transduction proteins, and are hypothesized to function in intramolecular communication between different signal domains of a single protein [66, 67], such as from a periplasmic ligand binding domain to a cytoplasmic methyl-acceptor domain. In vitro, STM3615 has been implicated in the development of biofilms, as mutants lacking this gene and the periplasmic oxidoreductase system encoded by dsbA/dsbB have delayed expression of the rdar (red dry and rough) morphotype [68]. However, links between the role of STM3615 during infection of chicks and its role in the rdar morphotype *in vitro,* remain to be established.

Mutants with deletions in two additional genes, *STM1295* and *STM1297,* also colonize the chick ceca poorly. *STM1295* encodes SppA, a signal peptide peptidase that cleaves remnant signal peptides clearing them from the membrane [69, 70]. *STM1297* (*selD*) encodes selenophosphate synthetase, an enzyme that utilizes ATP and selenium to generate selenophosphate [71, 72]. SelD is a critical enzyme in both the selenocysteine decoding and selenouridine utilizing machinery in prokaryotes [73]. The only selenoproteins in *Salmonella* Typhimurium and *E. coli* are formate

dehydrogenases [31]. Neither of these genes has been previously implicated in the pathogenesis of *Salmonella* or any other enteric pathogen.

We showed that *selD* mutant lacks formate dehydrogenase activity (FDH_N and FDH_H)(Figure 6F-H). Both FDH_N and FDH_H are required for utilization of formate during anaerobic growth [74], and activity of these enzymes strongly depends on the presence of selenium in the catalytic center [31]. We linked the observed growth defect of *ΔselD* in anaerobic conditions with inability to use formate (Figure 6C). Formate is a major product of mixed-acid fermentation and accounts for approximately a third of the carbon generated from glucose [32, 75]. Formate is present in the ceca of 18-weeks chickens [76]. Furthermore, the use of microencapsulated formate (at ~46 mM) in feed promotes colonization and systemic spread of *S*. Enteritidis in chicks [76]. Supplementation of media with additional formate worsen previously noticed growth defect of *ΔselD* in anaerobic conditions and was reversed by complementation (Figure 6D). We further show that growth defect of *ΔselD* in anaerobic conditions is specific to formate metabolism because supplementation of growth media with fumarate, an alternative electron acceptor [77], improved growth of the mutant strain to the level of wild type strain (Figure 6E). The significance of formate metabolism during infection of chicks has not been explored previously.

To summarize, we have developed a novel screening strategy using a two-step hierarchical approach using low complexity mutant pools. We used this strategy to identify genes that are important for *Salmonella* colonization and persistence in a four-day old chick model. We identified eleven colonization MGD mutants under selection and fifteen persistence MGD mutants under selection in first step of screening. We

assembled a small pool of SGD deletion mutants from most genes mapping to these nine MGD regions under selection, for a second round of screening. We identified twelve SGD mutants under selection and confirmed six of nine SGD mutants as important for *Salmonella* colonization in the chick model by competitive infections with wild type. All six of those genes were linked directly to the observed phenotypes by complementation analysis. One of the genes we identified, *selD* is required to support fitness of *Salmonella* during anaerobic growth in the presence of formate. Thus our new screening strategy using low complexity mutant pools can be successfully used to identify new genes needed by a pathogen to colonize and persist within a host.

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Figure Legends

Figure 1. The complexity of the multi-gene deletion pool is maintained during infection of four-day old chicks.

(A) Thirty 4 day-old chicks were orally infected with $1x10^9$ CFU of pool of our multi-gene deletion (MGD) library. On days 1, 3, and 9 post-infection animals were euthanized and total CFU were enumerated in cecal contents (closed diamond), cecal tissue (open square), cecal tonsil (closed triangle), bursa of Fabricius (open circle), spleen (open diamond) and liver (closed square). Data points represent the mean +/- SEM.
(B) The representation of marked strains HA530 to the total pool in the output was compared to the same ratio from the input pool. Data are shown as fold change (ratio in organ divided by ratio in input pool) from input for each organ in each individual animal. Data points represent an individual animal with horizontal bars indicating median. The horizontal dotted line indicates a 5-fold change in mutant representation.
ceca (C), cecal tonsil (CT), bursa (B), spleen (S), and liver (L).

Figure 2. Overview of two-step screening procedure

One hundred eighty two (MGD) mutants of *Salmonella* Typhimurium were pooled with strains bearing antibiotic resistance cassettes in neutral locations (HA530, HA697). The first step of screening was performed using a pool of this MGD library to infect four-day old chicks. The second pool of single gene deletion (SGD) mutants, mapping to the MGD regions under selection, was assembled and screened in a second group of chicks. Candidate SGDs under selection were identified and confirmed by individual competitive infection with wild type in chicks.

Figure 3. Twelve single-gene deletion mutants are under selection in a screen of a mutant pool in chicks.

A pool was assembled containing deletion mutants in single genes (SGD) mapping to nine MGD regions under selection from step one. This pool was screened in four-day old chicks. Representation of each mutant in the output organ was compared to that in input pool to identify mutants in individual genes as candidates under selection during infection. Individual candidate genes that appeared to be under selection are shown in this figure. Individual genes under selection mapping to MGD regions originally identified as needed for colonization are denoted by a superscript C, while those mapping to MGD regions needed for persistence are denoted by a superscript P. Genes shown in red were those that were randomly chosen for confirmation in individual competitive infections.

Figure 4. The phenotypes of six single gene deletion mutants are confirmed in competitive infection with the wild type during oral infection of chicks. Chicks were infected with 10^9 CFU of a 1:1 mixture of single gene deletion mutant and wild type. Chicks were euthanized on days 1 (white bars), 3 (grey bars), and 9 (black bars) post-infection and ceca (and additional organs, data not shown) were collected for enumeration of CFU. Each data point represents data from a single animal with median and interquartile range indicated. Statistical significance was determined using a Student's 2-tail *t* test. Asterisks indicate significant difference ($P < 0.05$) in the ratio of mutant/WT in ceca as compared with that of the inoculum.

Figure 5. Complementation reverses the phenotype of each single gene deletion mutant tested in competitive infection in chicks.

(A-B) A wild type copy of *STM0580* (A) and *STM3734* (B) placed on pWSK29 and returned to the corresponding deletion mutant complements each mutant in four-day old chicks. Chicks were infected with an equal mixture of wild type (HA877) and each mutant containing either the empty vector (grey bars) or a wild type copy of each gene (white bars). (C-F) A wild type copy of *STM1295* (C), *STM1297* (D), *STM3612* (E), and *STM3615* (F) returned to the chromosome of the corresponding deletion mutant complements each mutant *in cis*. Competitive infections were performed with wild type, (HA431) and each deletion mutant (grey bars) or wild type (HA877) and each complemented deletion mutant (white bars) On day 9 post-infection, ceca (C), cecal tonsil (CT), bursa (B), spleen (S), and liver (L) were collected to enumerate CFU. Each data point represents data from a single animal with median and interquartile range indicated. Statistical significance was determined using a Student's 2-tail *t* test. Asterisk indicates significant difference ($P < 0.05$) in competitive index between infection groups.

Figure 6. The *ΔselD* (*STM1297*) mutant is defective in anaerobic growth in the presence of formate. (A) Overview of formate metabolism in *Salmonella* Typhimurium. (B-D) The inability to metabolize formate results in growth defect in anaerobic conditions. Overnight cultures of wild type (filled square), *ΔselD* (open circles), *ΔselD* strain complemented *in cis* (filled circles) and *ΔfdhD* (open triangles) were subcultured in LB broth (B), LB broth supplemented with 40 mM formate (C), or 40 mM fumarate (D) and grown in anaerobic chamber at 37 ^{o}C. Aliquots were collected hourly, serially diluted and plated for CFU enumeration. Data points represent the mean of number of generations calculated as described in Materials and Methods and standard deviations

783 of at least three independent experiments. Statistical significance determined by t-test
784 with *p<0.05. (E-G) The *ΔselD* mutant accumulates formate during growth on
785 McConkey nitrate agar due to the loss of active formate dehydrogenases (FDH_N) in
786 anaerobic conditions (F) but not in aerobic conditions (E). (G) When the *ΔselD* mutant is
787 grown anaerobically it does not display formate dehydrogenase (FDH_H) activity using
788 the benzyl viologen overlay assay.
789

Table 1. List of multi-gene deletion (MGD) mutants under selection in four-day old chicks

MGD Mutant Under Selection[1]	Score
Colonization MGD mutant[2]	
STM0002-0011*	-2.9
STM1222-1231*	-3.1
STM1673-1653*	-5.3
STM2647-2651*	-3.7
STM3462-3472	-4.2
STM3603-3651*	-4.2
STM3607-3625*	-4.2
STM3732-3739	-3.6
STM4204-4232*	-4.4
STM4416-4467*	-2.1
STM4565-4579*	-3.7
Persistence MGD mutant[3]	
STM0102-0097*	-3.3
STM0572-0580	-3.3
STM0863-0866	-2.9
STM1165-1156*	-6.5
STM1606-1648*	-3.6
STM1291-1302	-2.1
STM2148-2154*	-3.6
STM2388-2392	-3.0
STM2399-2406*	-3.7
STM2434-2450*	-3.0
STM2667-2672*	-1.3
STM3541-3564*	-2.7
STM3626-3650*	-3.4
STM3940-3944*	-2.1
STM4290-4295*	-2.2

[1] Selected from outside the lower endpoint of 85% confidence interval
[2] MGD mutant consistently under selection during all time points (Day 1-Day 9).
[3] MGD mutant under selection only on Day 9.
* MGD mutant containing gene(s) previously shown to be needed for colonization of the chick in other studies [22, 23, 46, 47]
Bold represents the MGD regions from which SGD mutants were selected for inclusion in the second pool.

800 Table 2. Candidate single-gene deletion (SGD) mutants under selection in four-day old
801 chicks.
802
803

LT2 annotation	Gene	Function	Map to MGD region under selection	Under selection at Day post-infection	Score
STM0580	*ramR*	Putative regulatory protein	STM0572-0580	D9	-7.8
STM1295	*sppA*	Protease IV, a signal peptide peptidase	STM1291-1302	D1	-3.0
STM1297	*selD*	Selenophosphate synthase	STM1291-1302	D9	-4.6
STM3472	*ppiA*	Peptidyl-prolyl *cis-trans* isomerase A	STM3462-3472	D9	-3.3
STM3610	*yhjG*	Putative inner membrane protein	STM3607-3625	D1	-1.9
STM3612	*kdgK*	ketodeoxygluconokinase	STM3607-3625	D1	-3.6
STM3615	*yhjK*	Putative diguanylate cyclase/phosphodiestrase	STM3607-3625	D9	-5.8
STM3616	*yhjL*	Putative TPR- repeat containing protein	STM3607-3625	D1	-1.6
STM3733	*pyrE*	Orotate phosphoribosyltransferase	STM3732-3739	D1	-4.2
STM3734	*rph*	RNase PH	STM3732-3739	D9	-6.9
STM3942		Putative cytoplasmic protein	STM3940-3944	D1	-2.4
STM4290	*proP*	MFS family, low affinity proline transporter	STM4290-4295	D9	-3.3

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Figure 1.

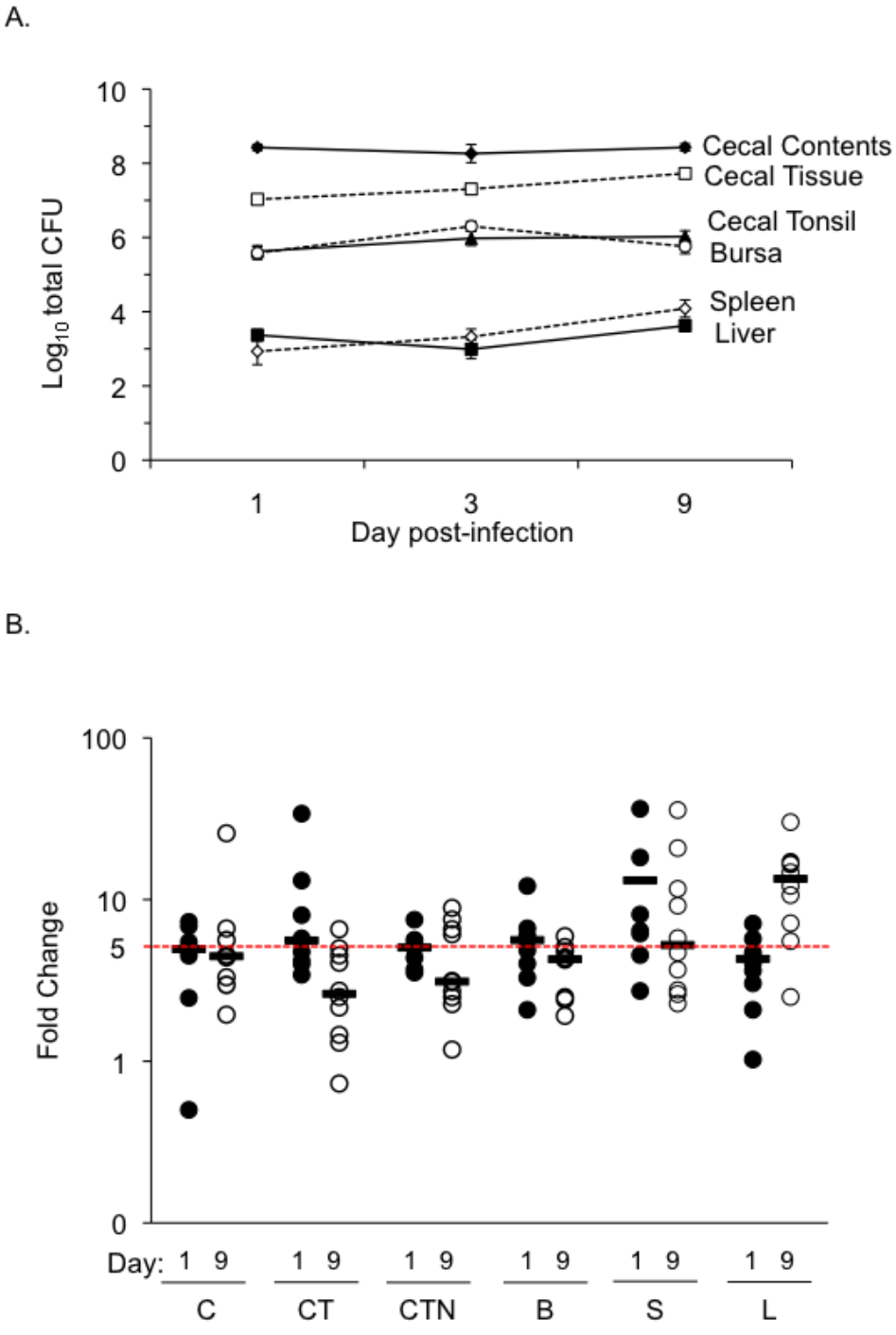
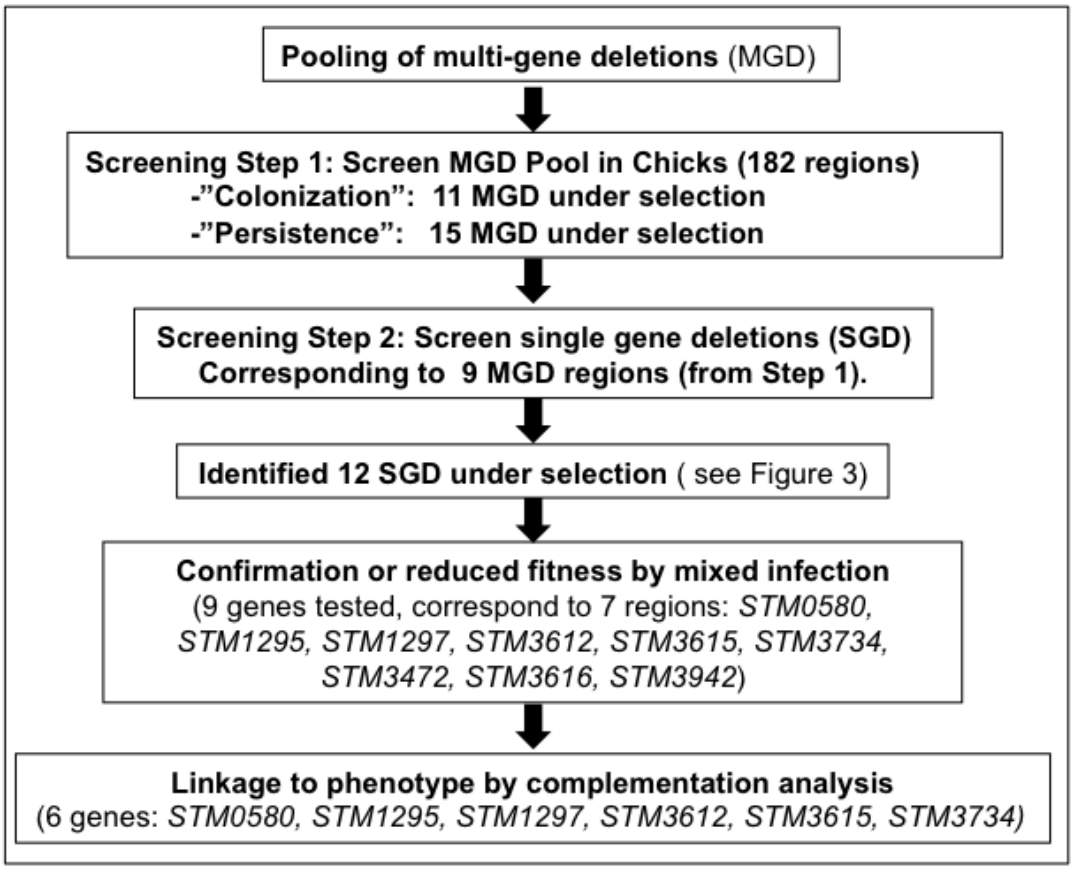


Figure 2.



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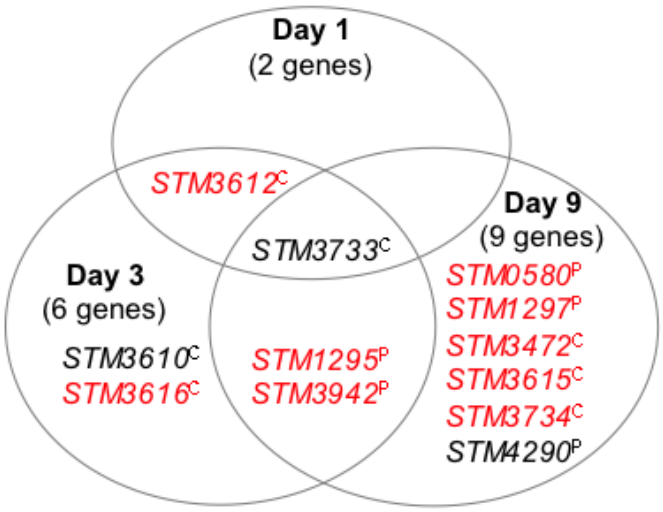

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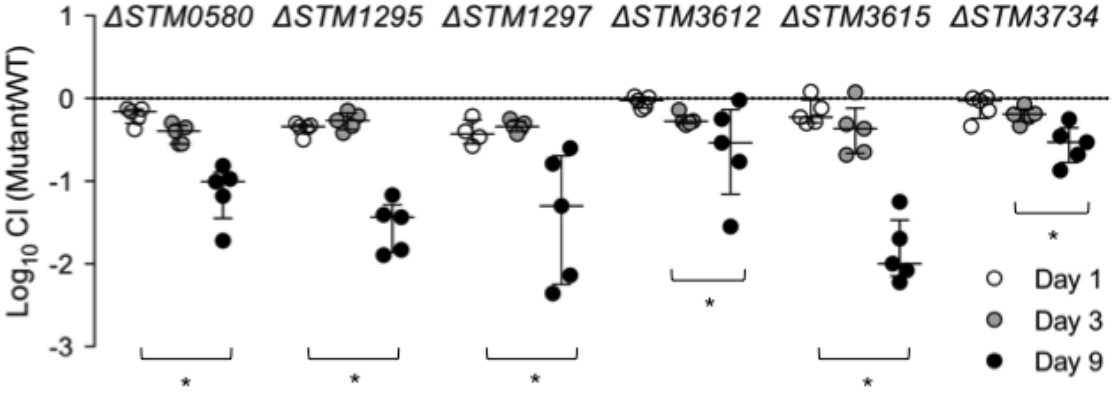

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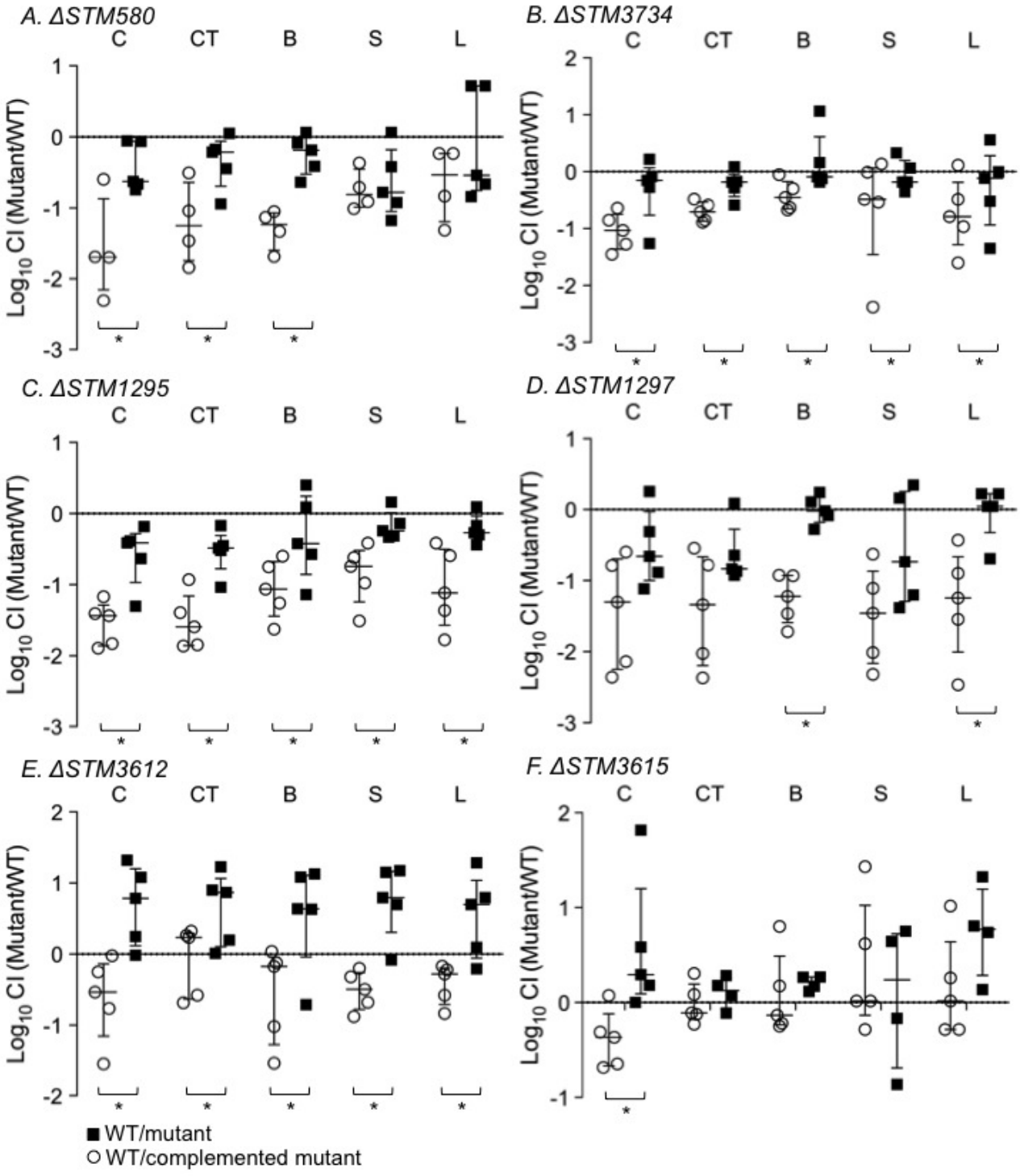
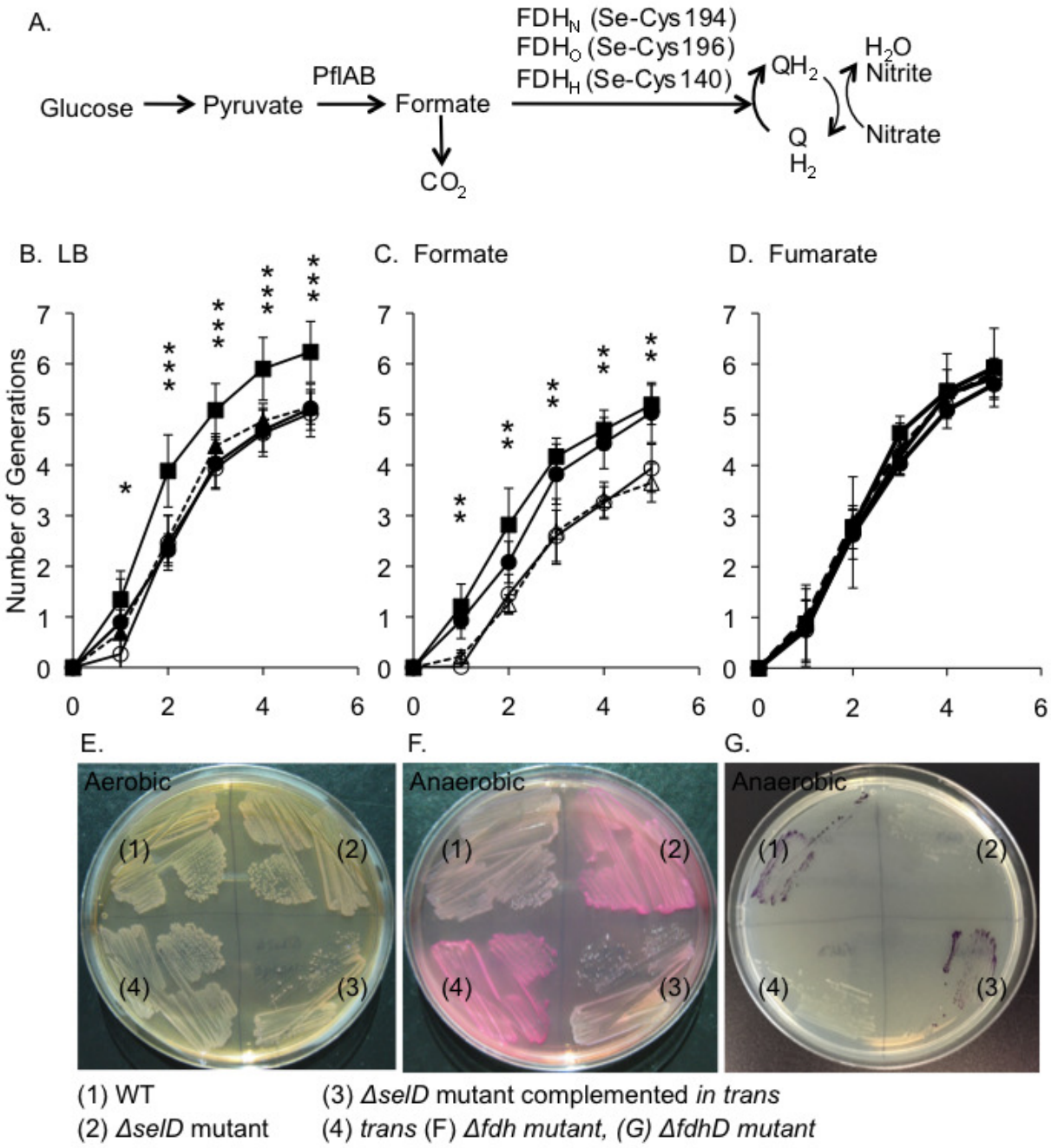


Figure 6.



(1) WT
(2) *ΔselD* mutant
(3) *ΔselD* mutant complemented *in trans*
(4) *trans* (F) *Δfdh mutant, (G) ΔfdhD mutant*