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Authors

Yang, Hee-Jeong
Bogomolnaya, Lydia M
Elfenbein, Johanna R
et al.

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Novel Two-Step Hierarchical Screening of Mutant Pools Reveals Mutants under Selection in Chicks

Hee-Jeong Yang,[a] Lydia M. Bogomolnaya,[a,d] Johanna R. Elfenbein,[a]* Tiana Endicott-Yazdani,[a] M. Megan Reynolds,[b] Steffen Porwollik,[c] Pui Cheng,[c] Xiao-Qin Xia,[c]* Michael McClelland,[c] Helene Andrews-Polymenis[a]

Department of Microbial Pathogenesis and Immunology, College of Medicine, Texas A&M University System Health Science Center, Bryan, Texas, USA[a]; Department of Biochemistry and Biophysics, College of Agriculture and Life Science, Texas A&M University, College Station, Texas, USA[b]; Department of Microbiology and Molecular Genetics, University of California, Irvine, California, USA[c]; Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Russia[d]

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 *Salmonella enterica* serotype 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 2,069 genes deleted) was used to identify regions under selection at 1, 3, and 9 days postinfection in chicks. Mutants in 11 regions were under selection at all assayed times (colonization mutants), and mutants in 15 regions were under selection only at day 9 (persistence mutants). We assembled a pool of 92 mutants, each deleted for a single gene, representing nearly all genes in nine regions under selection. Twelve 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 under 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 pools of mutants of low complexity. Using this strategy, we identified six genes not previously known to be needed during infection in chicks, and one of these (*STM1297*) suggests an important role for formate metabolism during infection.

Nontyphoidal *Salmonella* spp. are the most common cause of bacterial foodborne disease, with over 1 million cases and nearly 500 deaths in the United States annually (1). Contaminated poultry and eggs are a major source of human *Salmonella* infection (2, 3) and are implicated as the cause of more than 50% of *Salmonella* outbreaks in the United States (4). *Salmonella enterica* serotypes Typhimurium and Enteritidis can colonize young chicks and can persist subclinically for the life of the animal (5–7). These carrier birds are a reservoir for this organism, shedding the organism in feces and contaminating the environment and the human food supply. These *Salmonella*-colonized chickens are hard to identify and remove from food production because they frequently do not have clinical disease (8–10).

Despite the importance of chickens in contamination of the food supply, there is a relative paucity of information on the strategies employed by *Salmonella* to colonize and persist within this host. Flagellar motility and lipopolysaccharide biosynthesis are necessary for *Salmonella* to colonize the intestine of both chickens and mammals (11–16). The type 3 secretion system 1 (TTSS-1) encoded on *Salmonella* pathogenicity island 1 (SPI-1) plays a major role and the TTSS-2 a minor role in intestinal colonization of mammalian hosts (17–19). However, the available data suggest that the TTSS-1 and TTSS-2 are both dispensable for intestinal colonization of chicks that have not been seeded with adult intestinal microflora prior to 6 days postinfection (20, 21). Mutants 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 used to screen a pool of random

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Address correspondence to Michael McClelland (for questions about the analysis of the mutant pools), mcclelland.michael@gmail.com, or Helene Andrews-Polymenis (for all other questions), handrews@medicine.tamhsc.edu.

* Present address: Johanna R. Elfenbein, Department of Clinical Sciences, North Carolina State University College of Veterinary Medicine, Raleigh, North Carolina, USA; Xiao-Qin Xia, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China.

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transposon (Tn) insertion mutants during infection (25). Tens of thousands of Tn mutants are required to ensure nearly 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 animal. 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 (multigene deletion [MGD] mutants) in *Salmonella* Typhimurium ATCC 14028s using lambda red recombination (28–30). Collectively, these MGD mutants are deleted for ~2,069 genes, and they can be pooled to identify those mutants under selection during infection. This method allows us to assay approximately half of the nonessential 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 [SGD] mutants) mapping to MGD regions under selection identifies genes needed during infection. Using this strategy in 4-day-old specific-pathogen-free (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 being 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 *Escherichia 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 (see Fig. 6A). The FDH that is active is reliant on the electron acceptor present, among other variables (32, 33). We confirm that under anaerobic conditions, mutants lacking *selD* grow poorly due to an 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 six novel genes needed for colonization or persistence in the chick, an understudied host of *Salmonella* that is a major source of contamination of the human food supply. Finally, we show that one of the genes identified in our study, *STM1297* (*selD*), is required for survival of *Salmonella* under 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 ATCC 14028s (ATCC, Manassas, VA) (see Table S1 in the supplemental material). 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 to stationary phase in LB broth supplemented with the appropriate antibiotics at 41°C with aeration. Antibiotics and other supplements were used at the following concentrations: 20 mg/liter 5-bromo-4-chloro-3-indolyl phosphate (XP), 50 mg/liter kanamycin (Kan), 20 mg/liter chloramphenicol (Cm), 100 mg/liter nalidixic acid (Nal), 100 mg/liter streptomycin (Strep), and 100 mg/liter carbenicillin (Carb).

Generation of mutant pools. Multigene 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 to stationary phase in 1 ml of LB broth supplemented with kanamycin at 37°C with agitation. A single pool of mutants was generated by combining 200 μl of each mutant, and the resulting pool was divided into aliquots stored at −80°C until needed for animal infection. The composition of the MGD mutant pool is listed in Table S3 in the supplemental material.

The SGD mutant pool representing nearly all mutants from each of the 9 regions under selection was generated by growing 1 ml of each SGD mutant in 96-well deep-well dishes and combining 100 μl of each mutant to generate the larger pool. The composition of the SGD mutant pool is listed in Table S4 in the supplemental material.

Plasmid construction. All primers used in this study for the construction of complementing plasmids are listed in Table S2 in the supplemental material. Complementing plasmids carrying intact *STM0580* and *STM3734* genes were generated as follows. Each gene was amplified using primers *STM0580*_HindIII_F and *STM0580*_KpnI_R for *STM0580* and *STM3734*_EcoRI_F and *STM3734*_HindIII_R for *STM3734*. PCR products containing the full-length open reading frame (ORF) with approximately 200 bp upstream and 50 to 100 bp of the downstream sequence were generated by PCR using *Pfu Turbo* DNA polymerase (Agilent Technologies) in a 50-μl volume at an annealing temperature of 58°C for 30 cycles. PCR products were digested with HindIII and KpnI for *STM0580* and EcoRI and HindIII for *STM3734* as per manufacturer instructions (New England BioLabs, Ipswich, MA). Digested PCR products were ligated into pWSK29 previously cut with the same enzymes. Each ligation was transformed into *E. coli* XL1-Blue, and positive clones were selected on LB plates containing carbenicillin. Correct inserts were confirmed by restriction digestion and sequencing.

Complementation of mutants in *cis* containing deletions in *STM1295*, *STM1297*, *STM3612*, and *STM3615* was performed by inserting intact copies of each of these genes into the chromosome disrupting the *phoN* gene. The advantage of placing complementing genes in this site is that such mutations have no effect on colonization or virulence in murine or chick models (35) (data not shown), and we have a simple colorimetric assay for identifying disruptions in the *phoN* gene (35). Inactivation of *phoN*, 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*-positive 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 multicloning 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 a multicloning 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 digestion, and the resulting strains carrying these 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 (see Table S2 in the supplemental material). PCR was carried out using *Ex Taq* polymerase (TaKaRa) in a 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 specific for *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 to 65% 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 prehatch (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 4-day-old chicks was performed as follows. The inoculum for the input pool was prepared by dilution of a 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 test" below).

Thirty SPF White Leghorn chicks were orally infected with 1×10^9 CFU of the input pool in 100 μl at 4 days posthatch. Animals were monitored twice daily for signs of infection. On days 1, 3, and 9 postinfection, 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 (PBS; 1 ml PBS for cecal tonsil, 3 ml PBS for all other organs), serially diluted, and plated on three different media, LB plates containing either kanamycin, chloramphenicol, or streptomycin, to determine the number of CFU of the input MGD mutant 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 mutant pool inoculum, screening of the SGD mutant pool, and analysis of SGD mutant output pools were performed in a similar way to the same procedures used for the MGD mutant pool described in the previous paragraph. We infected groups of 10 chicks and euthanized them at days 1, 3, and 9 postinfection. Mutant representation after infection was determined from organs as described above. Data were analyzed for the MGD mutant pool.

Fluctuation test. In order to monitor biological bottlenecks during animal infection, two strains of ATCC 14028s Nal^r that carry different antibiotic resistance markers in a 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 chloramphenicol-resistant strain HA530 and 1:1,000 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 (Gene Expression Omnibus accession number GPL14855; 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 pool 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 nucleoside triphosphates that included a fluorescently labeled UTP. The RNA was purified using the RNeasy minikit (Qiagen), and approximately 4 μg of labeled RNA was hybridized to the array at 42°C for 16 h, 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, CA) at a 5-μm resolution. Data were uploaded into WebArrayDB (37, 38), and data were 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 or samples (cecal tissue, cecal contents, bursa of Fabricius, and spleen) from 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 for 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 subcultured at a dilution of 1:100 in 5 ml of fresh LB broth supplemented with kanamycin, and subcultures were grown for 24 h 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, subcultured in fresh sterile M9 minimal broth supplemented with kanamycin, and grown for 24 h at 41°C. The number of 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 to 15 chicks. Four-day-old SPF White Leghorn chicks were orally infected with 1×10^9 CFU of a 1:1 mixture of wild-type HA431 (ATCC 14028s Δ*phoN*::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 number of CFU and the exact input ratio of the wild type to the mutant. Groups of five chicks were euthanized on days 1, 3, and 9 postinfection for verification of mutant phenotypes, and at each time point, the cecum, 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 postinfection, and sample collection and processing were performed for verification of mutant phenotype. The numbers of 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 the wild type 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 a *P* value of <0.05.

Anaerobic growth of Δ*selD* 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; Shel Lab), and resuspended in LB broth preequilibrated for at least 16 h 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 a 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: $[\log_{10}(\text{CFU final}) - \log_{10}(\text{CFU start})]/\log_{10}(2)$.

Plate assay for FDH_N and FDH_H activities. The ability of mutant strains of interest to produce active nitrate reductase-linked formate dehydrogenase (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; Shel Lab) for at least 16 h. Wild-type, Δ*selD* (HA1557), complemented Δ*selD* (HA1472), and Δ*fdhE* (HA1558) strains were grown aerobically overnight, and cells were collected by centrifugation, transferred to the anaerobic chamber, and streaked on MacConkey nitrate plates. Plates were incubated at 37°C overnight in the 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 h of incubation.

To test the ability of strains of interest to produce active hydrogen-linked formate dehydrogenase (FDH_H), the wild type, Δ*fdhD* mutant (HA1559), and strains listed in the preceding paragraph were grown anaerobically in LB broth for 4 h 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 h prior to use to remove remaining dissolved oxygen. Inoculated plates were incubated at 37°C for 20 h in the anaerobic chamber. Benzyl viologen (BV) dye overlay agar was prepared as described by Mandrand-Berthelot et al. (41). Within a 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 4-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 posthatch 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 4-day-old chicks. In our experiments, chicks were heavily colonized with *Salmonella* Typhimurium ATCC 14028s in the intestinal tract from day 1 to day 9 postinfection (13 days of age) (Fig. 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 $\sim 1 \times 10^8$ CFU throughout the duration of infection. Systemic organs of 4-day-old chicks were lightly colonized after oral infection (Fig. 1), yet these sites were more heavily colonized in chicks infected at 4 days posthatch than chicks infected at 7 days posthatch (21).

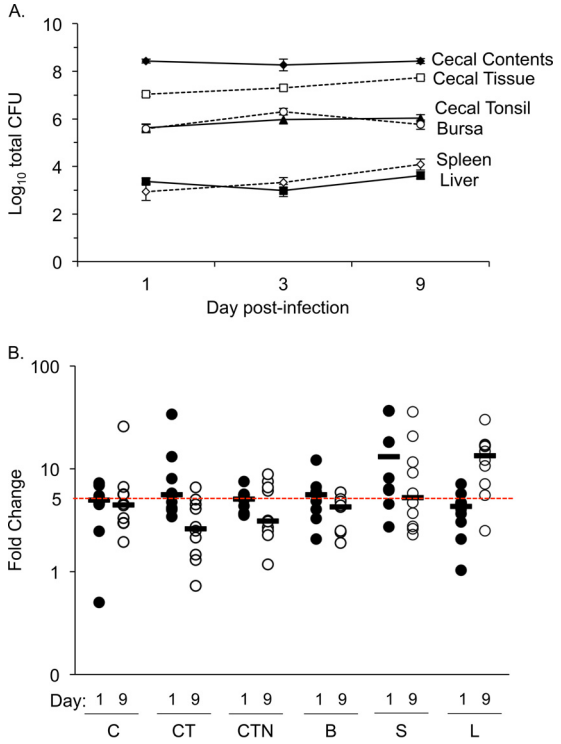

FIG 1 The complexity of the multigene deletion pool is maintained during infection of 4-day-old chicks. (A) Thirty 4-day-old chicks were orally infected with 1×10^9 CFU of a pool of our multigene deletion (MGD) library. On days 1, 3, and 9 postinfection, animals were euthanized and total CFU were enumerated in cecal contents (closed diamonds), cecal tissue (open squares), cecal tonsil (closed triangles), bursa of Fabricius (open circles), spleen (open diamonds), and liver (closed squares). Data points represent the means ± SEMs. (B) The representation of marked strain HA530 to the total pool in the output was compared to the same ratio from the input pool. Data are shown as the fold change (ratio in organ divided by ratio in input pool) from input for each organ in each individual animal. Each data point represents an individual animal, with horizontal bars indicating medians. The horizontal dotted line indicates a 5-fold change in mutant representation. C, ceca; CT, cecal tissue; CTN, cecal tonsil; B, bursa; S, spleen; L, liver.

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

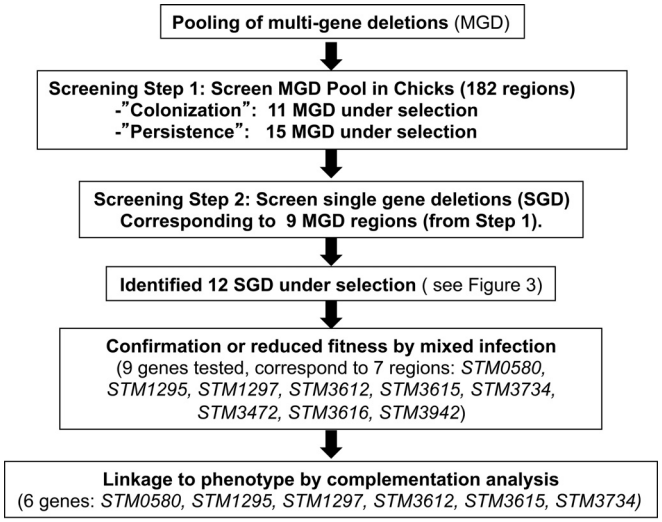

FIG 2 Overview of two-step screening procedure. One hundred eighty-two MGD mutants of *Salmonella* Typhimurium were pooled with strains bearing antibiotic resistance cassettes in neutral locations (HA530 and HA697). The first step of screening was performed using a pool of this MGD library to infect 4-day-old chicks. The second pool of 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 the wild type in chicks.

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 [ATCC 14028s Δ*phoN*::Cm^r] and HA697 [ATCC 14028s Δ*phoN*::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 5-fold lower concentration of 1:1,000.

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 postinfection with only a few exceptions (Fig. 1B). Consistent with previous observations, the representation of HA530 in the output pools from systemic sites fluctuated more severely than from intestinal sites (Fig. 1B, spleen and liver). Samples from each niche where HA530 fluctuated 5-fold or less (Fig. 1B, horizontal dotted line) were used to determine the representation of each mutant in the pool using a NimbleGen tiling array.

Step 1: screening of a pool of targeted multigene deletion mutants in chicks. An overview of our two-step screening strategy is shown in Fig. 2. The pool of 182 MGD mutants in *Salmonella* Typhimurium ATCC 14028s was used to inoculate 4-day-old chicks by gavage. Chicks were humanely euthanized at days 1, 3, and 9 postinfection, and output pools were collected from several organs. A total of 17 samples, derived from four different organs or samples (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 postinfection. We termed these mutants candidate "colonization MGD mutants," as they appeared to be unable to establish an infection from the earliest time points that we measured. The second category of mutants colonized chicks similarly to the wild-type organism early in infection, but these mutants were selected against only at the latest time point we assayed: day 9 postinfection. We termed these mutants candidate "persistence MGD mutants."

We identified 11 "colonization MGD mutants," of which 8 regions contained 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 coloniza-

TABLE 1 List of MGD mutants under selection in 4-day-old chicks

MGD mutant under selection[a]	Score
Colonization MGD mutants[b]	
Δ*STM0002-0011*[c]	−2.9
Δ*STM1222-1231*[c]	−3.1
Δ*STM1673-1653*[c]	−5.3
Δ*STM2647-2651*[c]	−3.7
ΔSTM3462-3472	−4.2
Δ*STM3603-3651*[c]	−4.2
ΔSTM3607-3625[c]	−4.2
ΔSTM3732-3739	−3.6
Δ*STM4204-4232*[c]	−4.4
Δ*STM4416-4467*[c]	−2.1
Δ*STM4565-4579*[c]	−3.7
Persistence MGD mutants[d]	
ΔSTM0102-0097[c]	−3.3
ΔSTM0572-0580	−3.3
Δ*STM0863-0866*	−2.9
Δ*STM1165-1156*[c]	−6.5
Δ*STM1606-1648*[c]	−3.6
ΔSTM1291-1302	−2.1
Δ*STM2148-2154*[c]	−3.6
Δ*STM2388-2392*	−3.0
Δ*STM2399-2406*[c]	−3.7
Δ*STM2434-2450*[c]	−3.0
Δ*STM2667-2672*[c]	−1.3
Δ*STM3541-3564*	−2.7
ΔSTM3626-3650[c]	−3.4
ΔSTM3940-3944[c]	−2.1
ΔSTM4290-4295[c]	−2.2

[a] Selected from outside the lower endpoint of the 85% confidence interval. Bold represents the MGD regions from which SGD mutants were selected for inclusion in the second pool. The mutant designation indicates the range of genes deleted; for example, mutant Δ*STM0002-0011* contains a deletion of genes *STM0002* to *STM0011*.
[b] MGD mutant consistently under selection during all time points (day 1 to day 9).
[c] MGD mutant containing a gene(s) previously shown to be needed for colonization of the chick in other studies (22, 23, 46, 47).
[d] MGD mutant under selection only on day 9.

tion 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 (see Fig. S1 in the supplemental material). Of the candidate colonization mutants assayed, 3 of the 11 (Δ*STM0002* to Δ*STM0011*, Δ*STM3603* to Δ*STM3651*, and Δ*STM4416* to Δ*STM4467*) had growth defects in M9 minimal media at 41°C (see Fig. S1A in the supplemental material). Similarly, 3 of the 15 candidate persistence mutants (Δ*STM1165* to Δ*STM1156*, Δ*STM2434* to Δ*STM2450*, and Δ*STM2667* to Δ*STM2672*) had noticeable growth defects in M9 minimal media at 41°C (see Fig. S1B in the supplemental material). 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 grew poorly in M9 media *in vitro*, although these may still encode genes of relevance to colonization or persistence in chicks.

Step 2: screening of SGD 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 mutant pool that we assembled contained deletion mutants in 92 of the 103 genes located in these nine regions (see Table S3 in the supplemental material), and this pool was screened in chicks using the same methodology described above.

In this second round of screening, we identified 12 SGD mutants under selection in chicks (Fig. 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 postinfection. We did not identify any SGD mutants with phenotypes in chicks that mapped to two MGD regions, *STM0102* to *STM0092* and *STM3626* to *STM3650*.

Confirmation of candidate phenotypes and complementation analysis. We retested 9 of the 12 SGD mutants under selection in individual competition infections with wild-type HA431 (ATCC 14028s Δ*phoN*::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 (Fig. 4). Colonization by the Δ*STM0580*, Δ*STM1295*, Δ*STM1297*, and Δ*STM3615* mutants was significantly reduced in ceca beginning from day 1 postinfection. The Δ*STM3612* and Δ*STM3734* mutants had significantly reduced colonization in ceca beginning from day 3 postinfection (Fig. 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*) (see Fig. S2 in the supplemental material).

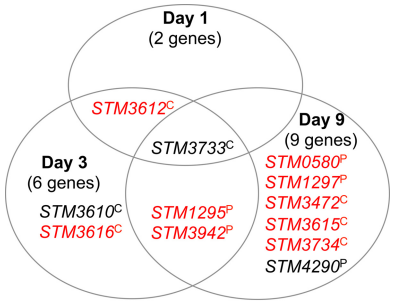

FIG 3 Twelve single gene deletion mutants are under selection in a screen of a mutant pool in chicks. A pool was assembled containing deletion mutants in single genes (SGD) mapping to the nine MGD regions under selection from step 1. This pool was screened in 4-day-old chicks. Representation of each mutant in the output organ was compared to that in the 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. C, individual genes under selection mapping to MGD regions originally identified as needed for colonization; P, individual genes under selection mapping to MGD regions needed for persistence; genes in red, genes that were randomly chosen for confirmation in individual competitive infections.

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 [ATCC 14028s Δ*phoN*::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 (Fig. 5).

Formate metabolism is important for colonization and persistence of *Salmonella* in the chick intestine. We selected one of our mutants, Δ*STM1297* (Δ*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 (Fig. 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 formate dehydrogenase O [FDH_O]). Thus, we were interested in whether *selD* mutants could grow under anaerobic conditions (49). Mutants lacking *selD* had a growth defect compared to wild type when strains were grown anaerobically (Fig. 6B). The growth of mutants lacking SelD could not be rescued by the addition of exogenous formate (Fig. 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 Δ*selD* mutant was restored to mirror the growth of the wild type (Fig. 6D). These data suggest that the mutant lacking *selD* fails to grow under 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 Δ*selD* mutants. When the wild type, a Δ*selD* mutant, a

TABLE 2 Candidate SGD mutants under selection in 4-day-old chicks

LT2 annotation	Gene	Function	MGD region to which the gene mapped under selection	Day postinfection under selection	Score
STM0580	*ramR*	Putative regulatory protein	*STM0572-0580*	9	−7.8
STM1295	*sppA*	Protease IV, a signal peptide peptidase	*STM1291-1302*	1	−3.0
STM1297	*selD*	Selenophosphate synthase	*STM1291-1302*	9	−4.6
STM3472	*ppiA*	Peptidyl-prolyl *cis-trans* isomerase A	*STM3462-3472*	9	−3.3
STM3610	*yhjG*	Putative inner membrane protein	*STM3607-3625*	1	−1.9
STM3612	*kdgK*	Ketodeoxygluconokinase	*STM3607-3625*	1	−3.6
STM3615	*yhjK*	Putative diguanylate cyclase/phosphodiesterase	*STM3607-3625*	9	−5.8
STM3616	*yhjL*	Putative TPR repeat-containing protein	*STM3607-3625*	1	−1.6
STM3733	*pyrE*	Orotate phosphoribosyltransferase	*STM3732-3739*	1	−4.2
STM3734	*rph*	RNase PH	*STM3732-3739*	9	−6.9
STM3942		Putative cytoplasmic protein	*STM3940-3944*	1	−2.4
STM4290	*proP*	MFS family, low-affinity proline transporter	*STM4290-4295*	9	−3.3

Δ*selD* mutant complemented in *cis*, and a Δ*fdhE* mutant strain were streaked on MacConkey nitrate plates and grown aerobically, there was no difference in colony appearance between strains (Fig. 6E) (39). In contrast, when grown anaerobically, the Δ*selD* mutant appeared red on MacConkey nitrate plates, similar to an FHD-deficient Δ*fdhE* strain (Fig. 6F). This color change indicates accumulation of formate in the colony, and it was suppressed by providing an intact copy of *selD* in *cis* (Fig. 6F) (40). Thus, in the *selD* mutant, FDH_N is not active under conditions where this enzyme should normally be induced and active.

FDH_H is known to be able to reduce benzyl viologen (BV) (40). To assess the activity of this enzyme in Δ*selD* mutants, we used a BV dye overlay assay (41). Anaerobically grown colonies with active FDH_H develop deep purple color when overlaid with benzyl viologen-containing top agar, while colonies lacking formate dehydrogenase activity remain colorless. In our BV assay, anaerobically grown wild-type colonies changed color as expected (Fig. 6G). In contrast, colonies of the Δ*selD* mutant strain remained colorless, similar to a formate dehydrogenase-deficient strain lacking Δ*fdhD*. Formate dehydrogenase activity was restored in the Δ*selD* mutant complemented in *cis* (Fig. 6H). These combined results suggest that deletion of *selD* abolishes the activity of both FDH_N and FDH_H. Finally, these data support the hypothesis that colonization and persistence defect of the Δ*selD* mutant (Fig. 4 and 5D) may be due to a growth defect under anaerobic conditions and more specifically due to an inability to utilize formate.

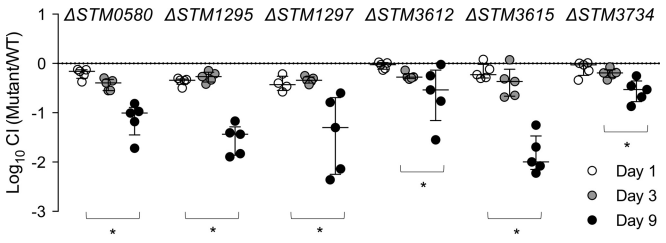

FIG 4 The phenotypes of six single gene deletion mutants are confirmed in competitive infection with the wild type during oral infection of chicks. Chicks were infected with 10^9 CFU of a 1:1 mixture of single gene deletion mutant and wild type. Chicks were euthanized on days 1 (white circles), 3 (gray circles), and 9 (black circles) postinfection and ceca (and additional organs [data not shown]) were collected for enumeration of CFU. Each data point represents data from a single animal, with medians and interquartile ranges indicated by bars and whiskers, respectively. Statistical significance was determined using a Student's 2-tailed *t* test. *, significant difference ($P < 0.05$) in the ratio of the mutant/wild type in ceca compared with that of the inoculum.

DISCUSSION

We report a novel screening methodology to identify genes necessary for a pathogen to colonize an animal host. Our strategy uses two separate pools of low complexity of targeted deletion mutants in a hierarchical process. First, we screened a pool of targeted deletion mutants in multiple adjacent genes (MGD mutants) in oral infection of 4-day-old chicks. Next, we constructed a second pool of targeted deletion mutants in single genes (SGD mutants) that corresponded to genes deleted in MGD mutants identified as under selection from the MGD mutant screen. This pool was screened in oral infection of chicks to identify SGD mutants under selection. Using this screening strategy, we were able to effectively interrogate almost half of the *Salmonella* genome during infection, overcoming a major limitation to currently employed high-density transposon mutant screens.

Our two-step screening strategy allowed us to use a relatively small number of animals for screening while still maintaining high genome coverage and using multiple biological replicates. We used only 60 animals to effectively screen 2,069 genes with at least three biological replicates per time point. A previous study utilized Tn mutants to identify genes under selection in chickens (23). This study provided only two biological replicates and required 180 chickens (23). Our studies provide similar genome coverage yet utilize far fewer animals.

In the first step of our screen, the use of an MGD mutant library allowed us to cover about 50% of the nonessential *Salmonella* Typhimurium genome in a pool with 10-fold fewer clones than genes deleted. Use of this low-complexity pool is advantageous for screening in niches where the pool encounters biological bottlenecks, including after oral infection in live animals (26, 27, 29). We noted that our input pool was sufficiently small that it did not experience significant bottlenecks in intestinal niches. However, as expected, the representation of our neutrally marked strains fluctuated in systemic sites, as transit out of the intestine represents a well-established bottleneck (Fig. 1B). Use of the second screening step allowed us to rapidly narrow down to a handful of individual candidate genes in a single experiment (Fig. 2). This two-step strategy should prove useful to interrogate the genomes of other pathogens when animal models are limited in supply or

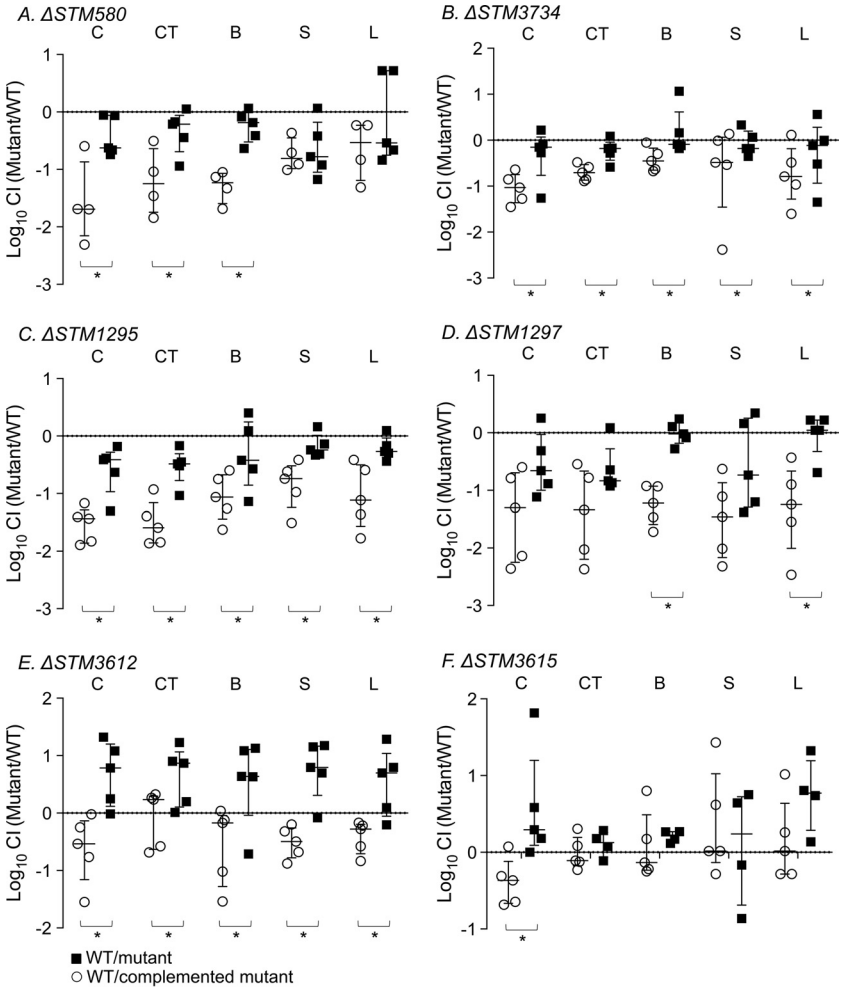

FIG 5 Complementation reverses the phenotype of each single gene deletion mutant tested in competitive infection in chicks. (A and B) Wild-type copies of *STM0580* (A) and *STM3734* (B) carried by pWSK29 were returned to the corresponding deletion mutant and complemented each mutant in 4-day-old chicks. Chicks were infected with an equal mixture of wild type (HA877) and each mutant containing either the empty vector (black symbols) or a wild-type copy of each gene (white symbols). (C to F) Wild-type copies of *STM1295* (C), *STM1297* (D), *STM3612* (E), and *STM3615* (F) that were returned to the chromosome of the corresponding deletion mutant complemented each mutant in *cis*. Competitive infections were performed with wild-type strain HA431 and each deletion mutant (black symbols) or wild-type strain HA877 and each complemented deletion mutant (white symbols). On day 9 postinfection, the 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 medians and interquartile ranges indicated by bars and whiskers, respectively. Statistical significance was determined using a Student's 2-tailed *t* test. *, a significant difference ($P < 0.05$) in the competitive index between infection groups.

too costly to preclude an adequate number of biological replicates for large numbers of mutant pools.

We developed a 4-day-old chick model for screening of the MGD library to identify mutants that are important for colonization by *S.* Typhimurium. Chicks younger than 3 days posthatch 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 4 days of age, chicks were colonized reliably with $\sim 1 \times 10^8$ CFU (total number) of bacteria in intestinal sites (Fig. 1). Thus, our 4-day-old chick model is well suited for screening of a library to identify mutants under selection with a 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) in the second screen mapped to MGD regions identified as required for colonization. However, the SGD mutants (in Δ*STM3472*, Δ*STM3615*, and Δ*STM3734*) located within MGD regions that appeared to be under selection at all times that 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 mutant 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 (Δ*STM0580*, Δ*STM1295*, Δ*STM1297*, and Δ*STM3734*) colonized poorly in ceca (Fig. 4) as well as in cecal tonsil, bursa of

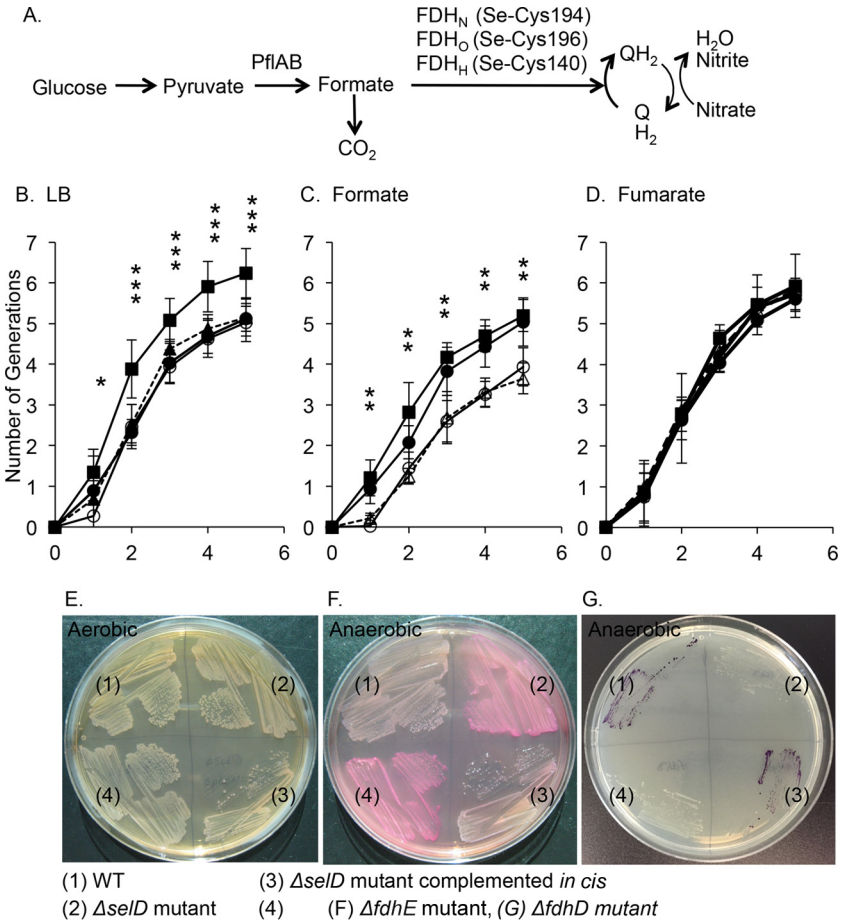

FIG 6 The Δ*selD* (Δ*STM1297*) mutant is defective in anaerobic growth in the presence of formate. (A) Overview of formate metabolism in *Salmonella* Typhimurium. Q, ubiquinone; QH_2, ubiquinol. (B to D) The inability to metabolize formate results in growth defect under anaerobic conditions. Overnight cultures of the wild type (filled squares), Δ*selD* (open circles), Δ*selD* strain complemented in *cis* (filled circles), and Δ*fdhD* (open triangles) were subcultured in LB broth (B), LB broth supplemented with 40 mM formate (C), or 40 mM fumarate (D) and grown in an anaerobic chamber at 37°C. Aliquots were collected hourly (the times [in hours] are indicated on the *x* axis), serially diluted, and plated for CFU enumeration. Each data point represents the mean number of generations calculated as described in Materials and Methods and standard deviations of at least three independent experiments. Statistical significance was determined by *t* test. *, $P < 0.05$ (statistically significant difference in growth of mutant strains compared to wild type); multiple asterisks, more than one mutant strain is significantly different from wild type. (E to G) The Δ*selD* mutant accumulates formate during growth on MacConkey nitrate agar due to the loss of active formate dehydrogenases (FDH_N) under anaerobic conditions (F) but not under aerobic conditions (E). (G) When the Δ*selD* mutant is grown anaerobically, it did not display formate dehydrogenase (FDH_H) activity using the benzyl viologen overlay assay.

Fabricius, spleen, and liver (data not shown). The remaining two mutants, Δ*STM3612* and Δ*STM3615*, had significant defects only in the ceca (Fig. 4).

We identified genes affecting fitness in the chick from seven of the nine regions we tested, in some cases identifying more than 1 gene necessary for fitness in a given region, for a total of 12 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 SGD regions, 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 colonization of chickens (see Tables S5 and S6 in the supplemental material) (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 multidrug 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 multidrug 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

overexpression of AcrAB in *ramR* mutants remains to be investigated.

A further gene we identified, *STM3734* (*rph*), encodes RNase PH, the first gene of the bicistronic operon of *rph-pyrE*. The *rph* gene encodes a 3′-5′ exoribonuclease and tRNA nucleotidyltransferase 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 provide strong evidence that our *rph* deletion did not simply lead to polar effects 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* appears to use the ED pathway to metabolize gluconate and related sugars as a carbon source (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 by 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 to 369) and EAL domains (amino acids 385 to 623) are found in the diguanylate cyclases and phosphodiesterases that are key in the metabolism of the bacterial second messenger cyclic-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 dinucleotide ligand (62–64). Although the sequence of the EAL domain encoded by *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 may have phosphodiesterase activity for cyclic dinucleotides, 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 the EAL domain, the GGDEF domain, a 2C-like domain, and others. These conserved domains are present in many bacterial 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 the *selD* mutant lacks formate dehydrogenase activity (FDH_N and FDH_H) (Fig. 6F to 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 Δ*selD* under anaerobic conditions with inability to use formate (Fig. 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-week-old 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 worsens the previously noticed growth defect of Δ*selD* under anaerobic conditions and was reversed by complementation (Fig. 6D). We further show that growth defect of Δ*selD* under 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 the wild-type strain (Fig. 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 4-day-old chick model. We identified 11 colonization MGD mutants under selection and 15 persistence MGD mutants under selection in the 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 12 SGD mutants under selection and confirmed 6 of 9 SGD mutants as important for *Salmonella* colonization in the chick model by competitive infections with the 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-com-

plexity 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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