

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

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25

26 **Abstract**

27 Contaminated chicken/egg products are major sources of human salmonellosis
28 yet the strategies used by *Salmonella* to colonize chickens are poorly understood. We
29 applied a novel two-step hierarchical procedure to identify new genes important for
30 colonization and persistence of *S. Typhimurium* in chickens. A library of 182 *S.*
31 *Typhimurium* mutants each containing a targeted deletion of a group of contiguous
32 genes (for a total of 2069 genes deleted) was used to identify regions under selection at
33 1, 3, and 9 days post- infection in chicks. Mutants in 11 regions were under selection at
34 all assayed times ('colonization' mutants) and mutants in 15 regions that were under
35 selection only at day 9 ('persistence' mutants). Second, we assembled a pool of 92
36 mutants, each deleted for a single gene, representing nearly all genes in nine regions
37 under selection. 12 single gene deletion mutants were under selection in this assay, and
38 we confirmed 6 of 9 of these candidate mutants via competitive infections and
39 complementation analysis in chicks. *STM0580*, *STM1295*, *STM1297*, *STM3612*,
40 *STM3615*, and *STM3734* are needed for *Salmonella* to colonize and persist in chicks
41 and were not previously associated with this ability. One of these key genes, *STM1297*
42 (*seID*), is required for anaerobic growth and supports the ability to utilize formate in
43 these conditions, suggesting that metabolism of formate is important during infection.
44 We report a hierarchical screening strategy to interrogate large portions of the genome
45 during infection of animals, using mutant pools of low complexity. Using this strategy we
46 have identified 6 genes not previously known to be needed during infection in chicks,
47 one of which (*STM1297*) suggests an important role for formate metabolism during
48 infection.

49

50

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

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

79 Several genetic screens have been performed to identify *Salmonella* genes
80 necessary for colonization in poultry [22-24]. Earlier screens performed in chicks
81 employed 'signature-tagged mutagenesis' (STM) [22-24], a method to screen a pool
82 random transposon (Tn) insertion mutants during infection [25]. Tens of thousands of Tn
83 mutants are required to ensure near complete coverage of the genome for genetic
84 screening. Yet, random loss from such complex pools due to biological 'bottlenecks' [26,
85 27] can create a high false positive discovery rate. An alternative to this approach is to
86 generate pools containing fewer independent Tn insertions, but if the total number of Tn
87 mutants to be screened remains constant, a greater number of animals is needed.
88 Compounding this issue is the fact that each pool should be screened in multiple
89 animals to ensure that the mutants identified as under selection are not specific to an
90 individual. To have sufficient confidence in the identification of true positive mutants
91 under selection in such screens, the use of random Tn insertions and the necessity for
92 biological replicates drive up the number of animals needed for such studies, often to
93 prohibitive levels.

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

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

110 We further explored the molecular role of selenophosphate synthetase (*selD*),
111 one of the genes we confirm as needed in chicks, during *Salmonella* growth. Using
112 selenide and ATP, SelD synthesizes selenophosphate, a precursor needed for the
113 formation of selenocysteine, a modified amino acid that can be incorporated into protein
114 and tRNA. In *Salmonella* and *E. coli*, selenocysteine is incorporated into formate
115 dehydrogenases (FDH), forming the catalytically active redox center of this enzyme
116 [31]. Produced from pyruvate during anaerobic growth, formate is metabolized by FDHs

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

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

128

129 **Materials and Methods**

130 Ethics Statement

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

133

134 Bacterial strains and growth conditions

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

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

145

146 Generation of mutant pools

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

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

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

163

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 μ l 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-

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

206

207 Chick hatching and screening of pools

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

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

222 Thirty specific pathogen free (SPF) White Leghorn chicks were orally infected
223 with 1×10^9 CFU of the 'input pool' in 100 μ l at 4 days post-hatch. Animals were
224 monitored twice daily for signs of infection. On days 1, 3, and 9 post-infection, groups of
225 10 chicks were humanely euthanized. The cecal contents, cecal tissue, cecal tonsil,
226 spleen, liver, and bursa were excised and homogenized in phosphate buffered saline (1
227 ml PBS for cecal tonsil, 3 ml for all other organs), serially diluted, and plated on three
228 different media, LB plates containing either kanamycin, chloramphenicol or streptomycin
229 to determine colony forming units (CFU) of the input MGD pool and each of the spiking
230 strains. The remainder of each homogenate was grown overnight in LB broth

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

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

239

240 Fluctuation Test

241 In order to monitor biological bottlenecks during animal infection, two strains of
242 ATCC14028 Nal^{R} that carry different antibiotic resistance markers in neutral location, in
243 the *phoN* gene, were generated using the lambda red recombination method (HA530,
244 $\Delta\text{phoN}::\text{Cm}^{\text{R}}$, and HA697, $\Delta\text{phoN}::\text{Strep}^{\text{R}}$) [21, 28]. Our screening 'input' pool was
245 spiked with these two strains at different ratios, 1:200 using the chloramphenicol
246 resistant strain HA530, and 1:1000 using the streptomycin resistant isolate HA697. The
247 ratio of each spiking strain to the total 'input' pool was compared to the ratio at which
248 these spiking strains were recovered from the 'output' at each time point, and from each
249 organ collected, to monitor for biological bottlenecks.

250

251 Nimblegen array use for comparison of the input and output pools

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

254 14028s genome (GEO platform GPL14855 at <http://www.ncbi.nlm.nih.gov/geo/>) was
255 used for detection of mutants in input and output pools. Preparation of labeled RNA and
256 hybridization conditions were as previously described [29]. Briefly, total DNA of input or
257 output mutant pools was sonicated, poly-A tailed, and PCR amplified with a primer
258 targeting the shared portion of each mutant and a primer including oligo dT at the 3'
259 end. The PCR product was subjected to reverse transcription from a T7 RNA
260 polymerase promoter located inside each mutant and a mixture of NTPs that included a
261 fluorescently labeled UTP. The RNA was purified using the RNeasy Mini Kit (Qiagen),
262 and approximately 4 micrograms of labeled RNA was hybridized to the array at 42°C for
263 16 hours, as per manufacturer's recommendations. The arrays were washed according
264 to the manufacturer's protocol, and scanned using a GenePix 4000B laser scanner
265 (Molecular Devices, Sunnyvale, California) at 5 µm resolution. Data was uploaded into
266 WebArrayDB [37, 38] and data analyzed for peak height in the DNA directly
267 downstream of each mutant location. The relative signal of each mutant in the pool
268 harvested from the chicks was compared to the relative signal in a corresponding array
269 of the same pool prior to selection. A custom-built R script was used to automatically
270 estimate the ratio of the signal strength at the expected nucleic acid positions for each
271 mutant before and after selection. Representative automatic results were visually
272 verified in graphs obtained during analysis in WebarrayDB.

273

274 Array data analysis and identification of candidate mutants

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

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

283

284 Testing growth *in vitro*

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

294

295 Competitive infections and complementation analysis in chicks

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

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

312

313 Anaerobic growth of *Δse/D* mutant

314 To assess bacterial growth without oxygen, strains were grown overnight in an
315 anaerobic chamber. Bacteria were collected by centrifugation, transferred into the
316 anaerobic chamber with internal atmosphere of 5% H₂, 5% CO₂, and 90% N₂ (Bactron I,
317 ShellLab) and resuspended in LB broth pre-equilibrated for at least 16 hours in the
318 anaerobic chamber. The resulting bacterial cultures were used to inoculate LB-broth, or
319 LB-broth supplemented with 40 mM sodium formate or 40 mM sodium fumarate at
320 1:100 dilution. Aliquots were collected hourly, serially diluted, and plated on LB agar for
321 CFU enumeration. All experiments were performed on at least three separate
322 occasions. Bacterial generation number was calculated using the following equation:
323 $[\log_{10}(\text{CFU final}) - \log_{10}(\text{CFU start})]/\log_{10}(2)$.

324

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

327 The ability of strains of interest mutant to produce active FDH_N was tested on
328 MacConkey nitrate medium plates [39]. Plates were equilibrated in the anaerobic
329 chamber with internal atmosphere of 5% H₂, 5% CO₂, and 90% N₂ (Bactron I, ShellLab)
330 for at least 16 hours. Wild type, $\Delta seID$ (HA1557), complemented $\Delta seID$ (HA1472) and
331 $\Delta fdhE$ (HA1558) strains were grown aerobically overnight, cells were collected by
332 centrifugation, transferred to the anaerobic chamber and streaked on MacConkey
333 nitrate plates. Plates were incubated at 37°C overnight in anaerobic chamber. Colonies
334 lacking active FDH_N are red due to accumulation of formate [40]. In parallel
335 experiments, strains were streaked on MacConkey nitrate plates and incubated
336 overnight aerobically at 37°C. All plates were imaged after 20 hours of incubation.

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

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

348

349 **Results**

350 **Colonization of four-day old chicks**

351 In order to screen pools of mutants to identify those under selection in chicks, we
352 needed a robust model for subclinical *Salmonella* colonization. Age is one of the
353 important factors that influence the level of colonization and disease in chicks infected
354 with *Salmonella* [42, 43]. Newly hatched chicks younger than 3 days post-hatch are
355 highly sensitive to low dose infection with *Salmonella* Typhimurium and they develop
356 systemic disease with high mortality [42, 43]. When infected after 3 days of age, chicks
357 are heavily colonized with *Salmonella* but do not develop clinical disease. To screen for
358 mutants unable to colonize and persist in the intestinal tract of chicks, we developed a
359 model for subclinical colonization of *Salmonellae* using four-day old chicks. In our
360 experiments, chicks were heavily colonized with *Salmonella* Typhimurium ATCC14028
361 in the intestinal tract from day 1 to day 9 post-infection (13 days of age) (Figure 1A), the
362 latest time point before the rapidly growing White Leghorn broiler chicks become too
363 large to house in our facility. Cecal contents in these chicks are most highly colonized
364 with *Salmonella* as previously reported [44] containing $\sim 1 \times 10^8$ CFU throughout the
365 duration of infection. Systemic organs of four-day old chicks were lightly colonized after
366 oral infection (Figure 1), yet these sites are more heavily colonized in chicks infected at
367 four days post hatch than chicks infected at seven days post hatch [21].

368

369 **Monitoring of biological bottlenecks of mutant pool during chick infection**

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

378 Evaluation of bottlenecks by measuring the fluctuation in representation of
379 mutants in a pool used to infect an animal, in each infected animal and from each niche
380 to be evaluated, increases the probability of successful identification of mutants under
381 selection. We evaluated the fluctuation in the representation of strains used to infect in
382 each animal, in each organ, and at each time point throughout our screen. We spiked
383 our pool of mutants with two strains that have antibiotic resistance markers inserted in
384 the *phoN* gene (HA530 ATCC14028s $\Delta phoN::Cm^R$ and HA697 ATCC14028s
385 $\Delta phoN::Strep^R$), and evaluated the representation of these strains in our 'input' pool and
386 in the 'output' pools from each niche. HA530 was added to the pool at a 1:200 ratio,
387 approximately the proportion of each individual mutant in our pool of 182 mutants, while
388 HA697 was added to the pool at a five-fold lower concentration; 1:1000.

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

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

397

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

399 An overview of our two-step screening strategy is shown (Figure 2). The pool of
400 182 MGD mutants in *Salmonella* Typhimurium ATCC 14028 was used to inoculate four-
401 day old chicks by gavage. Chicks were humanely euthanized at days 1, 3, and 9 post-
402 infection and output pools were collected from several organs. A total of seventeen
403 samples, derived from four different organs (cecal contents, cecal tissue, bursa of
404 Fabricius, and spleen) from nine different chicks, were analyzed by microarray for each
405 time point. By looking at samples under selection in multiple niches in the host, we
406 hypothesized that we could identify mutants that were most consistently under
407 selection.

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

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

417 We identified 11 'colonization' MGD mutants, of which 8 regions contain genes
418 previously reported to be candidates for colonization of chicks [22, 23, 46, 47].
419 Furthermore, 8 of the 15 'persistence' MGD mutants are deleted for genomic regions
420 containing at least one gene predicted to be necessary for colonization of chicks [22,
421 23, 46, 47]. Thus, our screening strategy identifies genomic regions already predicted to
422 be necessary for colonization of the chicken host and identifies novel regions needed
423 for both 'colonization' and 'persistence' within this host.

424

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

426 We tested the growth of 26 MGD mutants identified in our screen in chicks during
427 growth in both LB and M9 minimal broth at 41°C, the normal body temperature of the
428 chickens. All 26 MGD mutants grew similarly to wild type (HA431) in LB broth, but this
429 was not the case in M9 minimal broth (Supplementary Figure S1). Of the candidate
430 'colonization' mutants assayed, three of the eleven ($\Delta STM0002-0011$, $\Delta STM3603-3651$,
431 and $\Delta STM4416-4467$) had growth defects in M9 minimal media at 41°C (Supplementary
432 Figure S1, panel A). Similarly, three of the fifteen candidate 'persistence' mutants
433 ($\Delta STM1165-1156$, $\Delta STM2434-2450$, $\Delta STM2667-2672$) had noticeable growth defects in
434 M9 minimal media at 41°C (Supplementary Figure S1, panel B). Growth characteristics
435 similar to those noted at 41°C were observed in both LB broth and M9 minimal media
436 during growth at 37°C (data not shown) excluding an effect of temperature on our
437 observed growth phenotypes. We excluded from further analysis the six MGD mutants

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

440

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

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

452 In this second round of screening, we identified twelve SGD mutants under
453 selection in chicks (Figure 3). These genes map to seven of the original MGD regions
454 that we identified as under selection (Table 2). Six mutants ($\Delta STM0580$, $\Delta STM1297$,
455 $\Delta STM3472$, $\Delta STM3615$, $\Delta STM3734$, and $\Delta STM4290$) were under selection only on day
456 9 post-infection. We did not identify any SGD mutants with phenotypes in chicks that
457 mapped to two MGD regions, *STM0102-0092* and *STM3626-3650*.

458

459 **Confirmation of candidate phenotypes and complementation analysis**

460 We retested nine of the twelve SGD mutants under selection in individual
461 competition infections with wild type, HA431 (ATCC14028 $\Delta phoN::Kan^R$) in chicks. Six
462 SGD mutants ($\Delta STM0580$, $\Delta STM1295$, $\Delta STM1297$, $\Delta STM3612$, $\Delta STM3615$, and
463 $\Delta STM3734$) had significantly reduced colonization in ceca (Figure 4). Colonization by
464 the $\Delta STM0580$, $\Delta STM1295$, $\Delta STM1297$, and $\Delta STM3615$ mutants was significantly
465 reduced in ceca beginning from day 1 post-infection. The $\Delta STM3612$ and $\Delta STM3734$
466 mutants had significantly reduced colonization in ceca beginning from day 3 post-
467 infection (Figure 4). We also observed reduced colonization by four mutants
468 ($\Delta STM0580$, $\Delta STM1295$, $\Delta STM1297$, and $\Delta STM3734$) in cecal tonsil, bursa of
469 Fabricius, spleen and liver (data not shown). We were unable to confirm the phenotype
470 predicted by the screen for three mutants ($\Delta STM3472$, $\Delta STM3616$, and $\Delta STM3942$
471 Supplementary Figure S2).

472 We performed complementation of six confirmed SGD mutants ($\Delta STM0580$,
473 $\Delta STM1295$, $\Delta STM1297$, $\Delta STM3612$, $\Delta STM3615$, and $\Delta STM3734$) using competition
474 assays in chicks for each deletion mutant containing an intact copy of the corresponding
475 gene, against derivatives of the wild type (either HA877 (HA431 + pWSK29) or HA431
476 (ATCC14028 $\Delta phoN::Kan^R$)). For each deletion mutant that we attempted to
477 complement, we successfully restored colonization to levels similar to wild type,
478 definitively linking each of these genes to the phenotypes we observed during infection
479 (Figure 5).

480

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

483 We selected one of our mutants *STM1297 (selD)*, confirmed to be under
484 selection in chicks, for more detailed study. *selD* encodes selenophosphate synthetase
485 [39] [48], an enzyme required for the generation of selenocystine and thus for the
486 incorporation of selenium into proteins and tRNA (Figure 6A) [48]. In enterobacteria
487 formate dehydrogenase (FDH) is an important enzyme needed for metabolism during
488 anaerobic growth that requires selenocysteine for activity (at position 194 in FDH_N,
489 position 140 in FDH_H, and position 196 in FDH_O). Thus, we were interested in whether
490 *selD* mutants could grow in anaerobic conditions [49]. Mutants lacking *selD* had a
491 growth defect compared to wild type when strains were grown anaerobically (Figure
492 6B). The growth of mutants lacking SelD could not be rescued by the addition of
493 exogenous formate (Figure 6C), suggesting that *selD* mutants cannot utilize this
494 metabolite because *selD* mutants cannot produce active FDH. In contrast, when media
495 were supplemented with fumarate, an alternate energy source, the growth of the $\Delta selD$
496 mutant was restored to mirror the growth of the wild type (Figure 6D). These data
497 suggest that the mutant lacking *selD* fails to grow in anaerobic conditions because it
498 cannot metabolize formate specifically, rather than having a more generalized metabolic
499 defect.

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

506 similar to an FHD-deficient $\Delta fdhE$ 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 *seID* *in cis* (Figure 6F) [40]. Thus, in the *seID* 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 $\Delta seID$ 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 $\Delta seID$ mutant strain
516 remained colorless similar to a formate dehydrogenase-deficient strain lacking $\Delta fdhD$.
517 Formate dehydrogenase activity was restored in the $\Delta seID$ mutant complemented *in cis*
518 (Figure 6H). These combined results suggest that deletion of *seID* abolishes the activity
519 of both FDH_N and FDH_H . Finally, these data support the hypothesis that colonization
520 and persistence defect of $\Delta seID$ 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

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

556 We developed a four-day old chick model for screening of the MGD library to
557 identify mutants that are important for colonization by *S. Typhimurium*. Chicks younger
558 than three days post-hatch develop systemic disease with high mortality upon infection
559 with *Salmonella* Typhimurium [42, 43]. Several previous studies have used this model of
560 acute systemic disease to screen for mutants under selection [22, 24, 47]. Other studies
561 have reported that 1-week old chicks infected with *Salmonella* Typhimurium do not
562 become as heavily colonized in the intestine, making these older animals less useful for
563 screening [21]. When infected at four days of age, chicks were colonized reliably with
564 $\sim 1 \times 10^8$ CFU (total number) of bacteria in intestinal sites (Figure 1). Thus, our four-day
565 old chick model is well suited for screening of a library to identify mutants under
566 selection with high level of colonization throughout the duration of infection.

567 The 'colonization' or 'persistence' phenotypes from our MGD mutant screen were
568 largely shared by SGD mutants from the corresponding MGD region. Thus, the majority
569 of individual mutants identified as under selection at early time points (i.e. 'colonization'
570 mutants) identified in the second screen, mapped to MGD regions identified as required
571 for colonization. However, the SGD mutants (in $\Delta STM3472$, $\Delta STM3615$, and
572 $\Delta STM3734$) located within MGD regions that appeared to be under selection at all times
573 we tested were determined to be defective only at later time points during infection. One
574 potential explanation for this observation is that the combined effect of loss of multiple

575 genes from a given MGD region caused a more severe phenotype in the corresponding
576 MGD than can be observed in mutants in any individual gene in the same region. Of the
577 mutants we confirmed in individual competitive infection, four mutants ($\Delta STM0580$,
578 $\Delta STM1295$, $\Delta STM1297$, and $\Delta STM3734$) colonized poorly in ceca (Figure 4) as well as
579 in cecal tonsil, bursa of Fabricius, spleen, and liver (data not shown). The remaining two
580 mutants, $\Delta STM3612$, and $\Delta STM3615$, had significant defects only in the ceca (Figure
581 4).

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

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

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

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

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

621 pathway is an alternative series of reactions for carbon metabolism and is known to be
622 present in the diverse group of organisms ranging from archaea to eubacteria to
623 eukaryotes [55]. Recent microarray studies for gene expression profiling of *Salmonella*
624 in macrophages showed that intracellular *Salmonella* appear to use the ED pathway to
625 metabolize gluconate and related sugars as a carbon source within macrophages [56,
626 57]. In addition, the ED pathway is essential for colonization of mammalian intestine by
627 *E. coli* [57, 58]. Even though the relevance of these observations to *in vivo* metabolism
628 is not clear, it is plausible that the ED pathway is important for intracellular growth of
629 *Salmonella* in chicks.

630 Another gene we confirmed to be needed for colonization of the chick intestine is
631 *STM3615*. Some Tn insertions in *STM3615* are reported to be under selection in chicks,
632 although these findings have not been confirmed with individual infections or
633 complementation analysis [23]. *STM3615* encodes a putative protein that contains
634 several consensus regions including a signal peptide, a GGDEF domain, an EAL
635 domain, and a HAMP domain [59]. GGDEF (amino acids 213-369) and EAL domains
636 (amino acids 385-623), are found in the di-guanylate cyclases and phosphodiesterases
637 that are key in the metabolism of the bacterial second messenger c-di-GMP [60-62]. In
638 other proteins where both of these domains occur, one of these domains frequently
639 lacks enzymatic activity but may retain binding activity for the cyclic di-nucleotide ligand
640 [63] [64] [62]. Although the sequence of the EAL domain of *STM3615* is in strong
641 agreement with the PFAM consensus sequence for these phosphodiesterase domains,
642 the GGDEF domain has poor agreement with PFAM consensus sequences for domains
643 possessing the diguanylate cyclase activity [59]. This observation suggests that

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

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

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

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

669 We showed that *seID* mutant lacks formate dehydrogenase activity (FDH_N and
670 FDH_H)(Figure 6F-H). Both FDH_N and FDH_H are required for utilization of formate during
671 anaerobic growth [74], and activity of these enzymes strongly depends on the presence
672 of selenium in the catalytic center [31]. We linked the observed growth defect of $\Delta seID$
673 in anaerobic conditions with inability to use formate (Figure 6C). Formate is a major
674 product of mixed-acid fermentation and accounts for approximately a third of the carbon
675 generated from glucose [32, 75]. Formate is present in the ceca of 18-weeks chickens
676 [76]. Furthermore, the use of microencapsulated formate (at ~46 mM) in feed promotes
677 colonization and systemic spread of *S. Enteritidis* in chicks [76]. Supplementation of
678 media with additional formate worsen previously noticed growth defect of $\Delta seID$ in
679 anaerobic conditions and was reversed by complementation (Figure 6D). We further
680 show that growth defect of $\Delta seID$ in anaerobic conditions is specific to formate
681 metabolism because supplementation of growth media with fumarate, an alternative
682 electron acceptor [77], improved growth of the mutant strain to the level of wild type
683 strain (Figure 6E). The significance of formate metabolism during infection of chicks has
684 not been explored previously.

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

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

699

700

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709

710 **Figure Legends**

711

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

714 (A) Thirty 4 day-old chicks were orally infected with 1×10^9 CFU of pool of our multi-gene
715 deletion (MGD) library. On days 1, 3, and 9 post-infection animals were euthanized and
716 total CFU were enumerated in cecal contents (closed diamond), cecal tissue (open
717 square), cecal tonsil (closed triangle), bursa of Fabricius (open circle), spleen (open
718 diamond) and liver (closed square). Data points represent the mean \pm SEM.

719 (B) The representation of marked strains HA530 to the total pool in the output was
720 compared to the same ratio from the input pool. Data are shown as fold change (ratio in
721 organ divided by ratio in input pool) from input for each organ in each individual animal.
722 Data points represent an individual animal with horizontal bars indicating median. The
723 horizontal dotted line indicates a 5-fold change in mutant representation.
724 ceca (C), cecal tonsil (CT), bursa (B), spleen (S), and liver (L).

725

726 **Figure 2. Overview of two-step screening procedure**

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

734

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

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

747

748 **Figure 4. The phenotypes of six single gene deletion mutants are confirmed in**
749 **competitive infection with the wild type during oral infection of chicks.**

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

757

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

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

773
774 **Figure 6. The $\Delta seID$ (*STM1297*) mutant is defective in anaerobic growth in the**
775 **presence of formate.** (A) Overview of formate metabolism in *Salmonella* Typhimurium.
776 (B-D) The inability to metabolize formate results in growth defect in anaerobic
777 conditions. Overnight cultures of wild type (filled square), $\Delta seID$ (open circles), $\Delta seID$
778 strain complemented *in cis* (filled circles) and $\Delta fdhD$ (open triangles) were subcultured
779 in LB broth (B), LB broth supplemented with 40 mM formate (C), or 40 mM fumarate (D)
780 and grown in anaerobic chamber at 37 °C. Aliquots were collected hourly, serially
781 diluted and plated for CFU enumeration. Data points represent the mean of number of
782 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 $\Delta se/D$ 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 $\Delta se/D$ mutant is
787 grown anaerobically it does not display formate dehydrogenase (FDH_H) activity using
788 the benzyl viologen overlay assay.
789

790 Table 1. List of multi-gene deletion (MGD) mutants under selection in four-day old
 791 chicks
 792

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

793 ¹ Selected from outside the lower endpoint of 85% confidence interval

794 ² MGD mutant consistently under selection during all time points (Day 1-Day 9).

795 ³ MGD mutant under selection only on Day 9.

796 * MGD mutant containing gene(s) previously shown to be needed for colonization of the chick in other
 797 studies [22, 23, 46, 47]

798 Bold represents the MGD regions from which SGD mutants were selected for inclusion in the second
 799 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	<i>ramR</i>	Putative regulatory protein	STM0572-0580	D9	-7.8
STM1295	<i>sppA</i>	Protease IV, a signal peptide peptidase	STM1291-1302	D1	-3.0
STM1297	<i>selD</i>	Selenophosphate synthase	STM1291-1302	D9	-4.6
STM3472	<i>ppiA</i>	Peptidyl-prolyl <i>cis-trans</i> isomerase A	STM3462-3472	D9	-3.3
STM3610	<i>yhjG</i>	Putative inner membrane protein	STM3607-3625	D1	-1.9
STM3612	<i>kdgK</i>	ketodeoxygluconokinase	STM3607-3625	D1	-3.6
STM3615	<i>yhjK</i>	Putative diguanylate cyclase/phosphodiesterase	STM3607-3625	D9	-5.8
STM3616	<i>yhjL</i>	Putative TPR- repeat containing protein	STM3607-3625	D1	-1.6
STM3733	<i>pyrE</i>	Orotate phosphoribosyltransferase	STM3732-3739	D1	-4.2
STM3734	<i>rph</i>	RNase PH	STM3732-3739	D9	-6.9
STM3942		Putative cytoplasmic protein	STM3940-3944	D1	-2.4
STM4290	<i>proP</i>	MFS family, low affinity proline transporter	STM4290-4295	D9	-3.3

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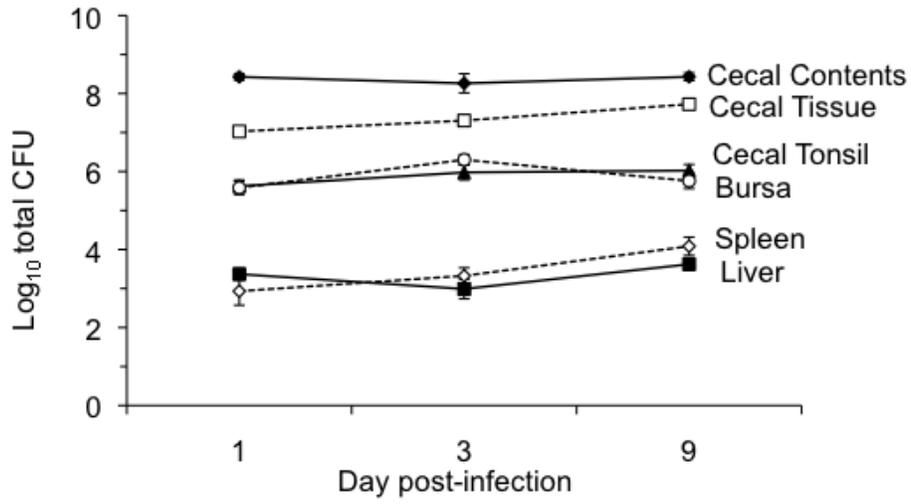
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1067

Figure 1.

A.



B.

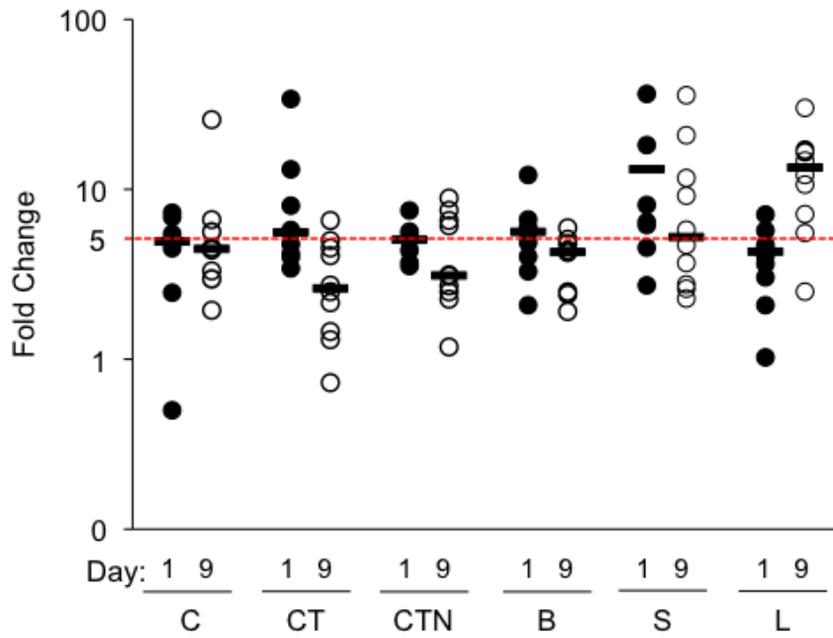


Figure 2.

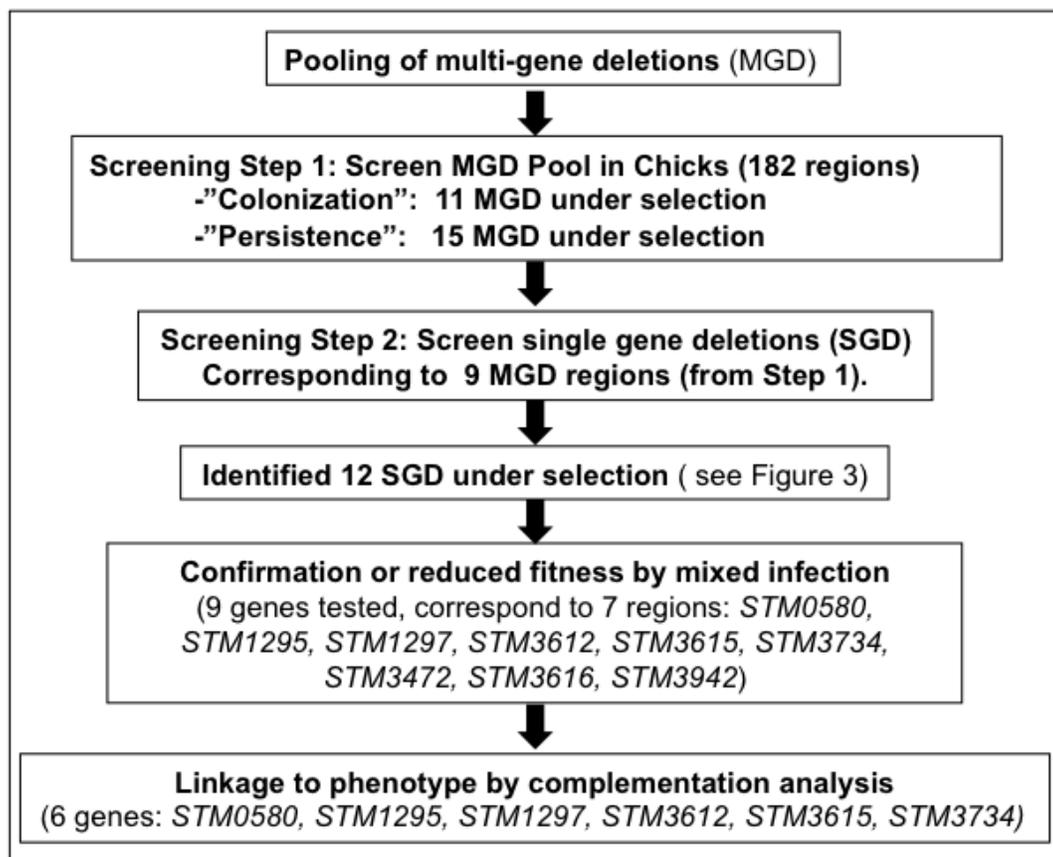


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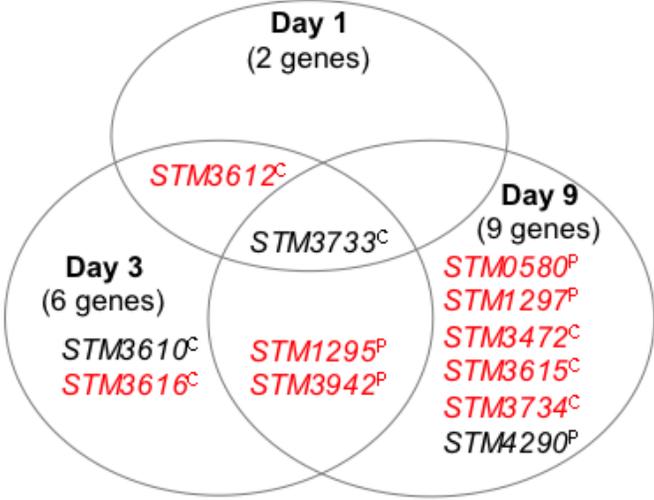


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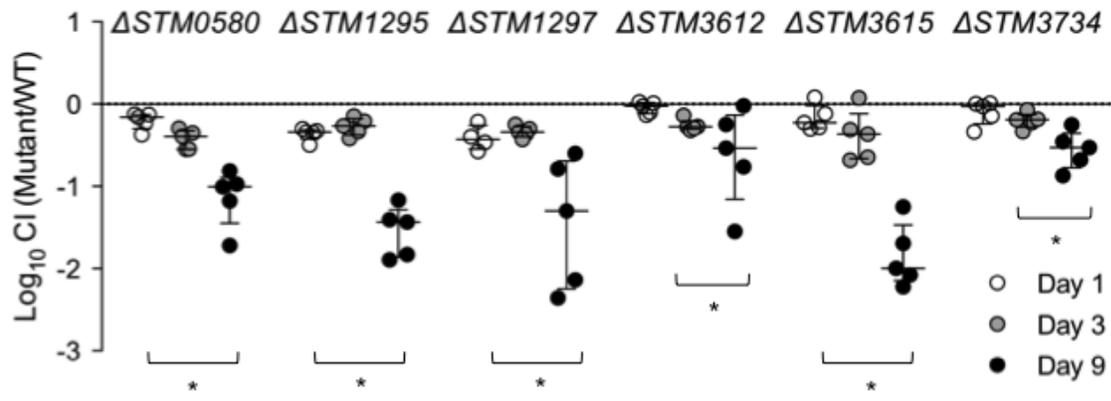


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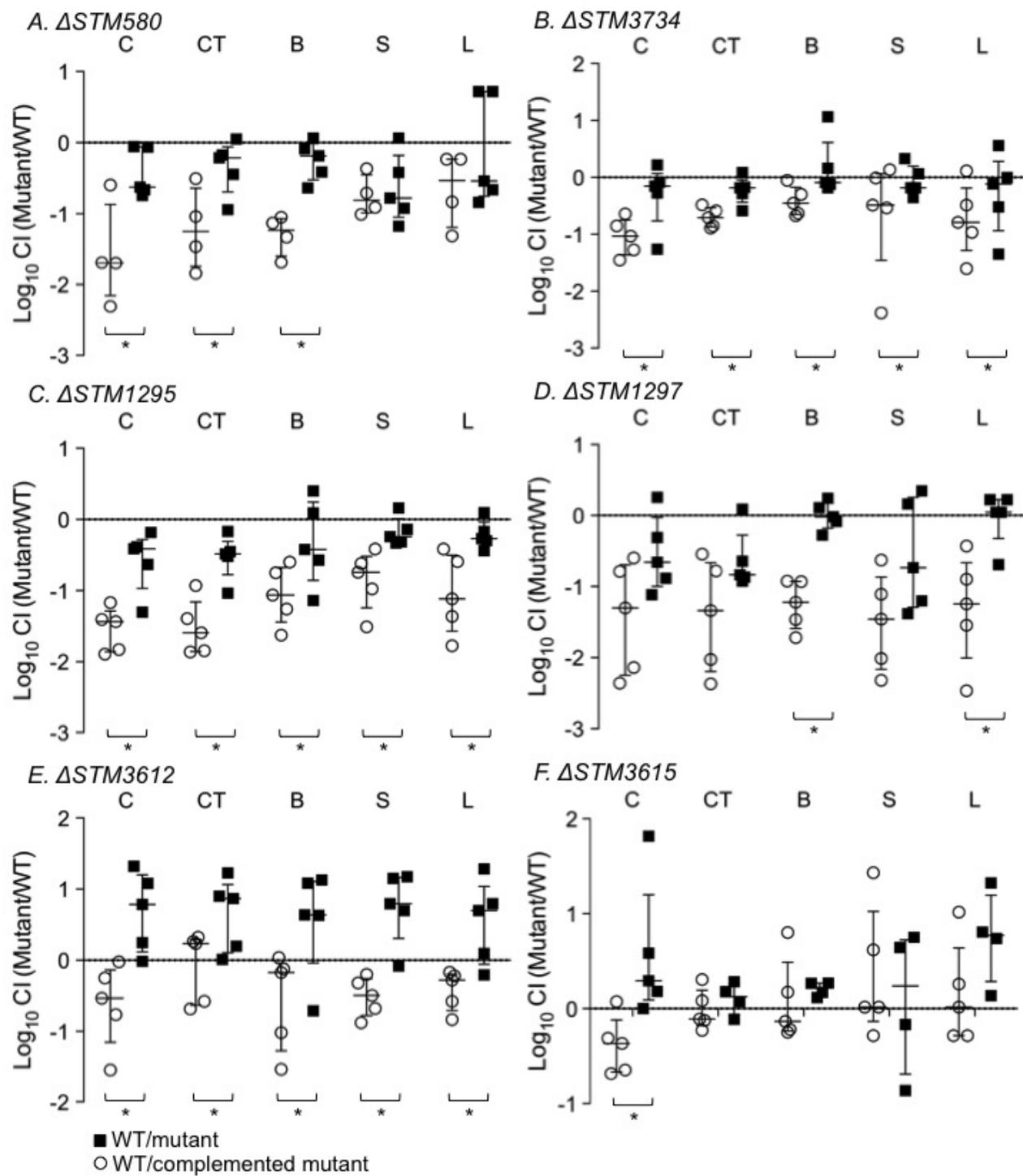


Figure 6.

