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ORIGINAL ARTICLE

Evolutionary loss of the rdar morphotype in *Salmonella* as a result of high mutation rates during laboratory passage

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Rapid evolution of microbes under laboratory conditions can lead to domestication of environmental or clinical strains. In this work, we show that domestication due to laboratory passage in rich medium is extremely rapid. Passaging of wild-type *Salmonella* in rich medium led to diversification of genotypes contributing to the loss of a spatial phenotype, called the rdar morphotype, within days. Gene expression analysis of the rdar regulatory network demonstrated that mutations were primarily within *rpoS*, indicating that the selection pressure for scavenging during stationary phase had the secondary effect of impairing this highly conserved phenotype. If stationary phase was omitted from the experiment, radiation of genotypes and loss of the rdar morphotype was also demonstrated, but due to mutations within the cellulose biosynthesis pathway and also in an unknown upstream regulator. Thus regardless of the selection pressure, rapid regulatory changes can be observed on laboratory timescales. The speed of accumulation of *rpoS* mutations during daily passaging could not be explained by measured fitness and mutation rates. A model of mutation accumulation suggests that to generate the observed accumulation of σ^{38} mutations, this locus must experience a mutation rate of approximately 10^{-4} mutations/gene/generation. Sequencing and gene expression of population isolates indicated that there were a wide variety of σ^{38} phenotypes within each population. This suggests that the *rpoS* locus is highly mutable by an unknown pathway, and that these mutations accumulate rapidly under common laboratory conditions.
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Introduction

Evolution is the result of the interplay between mutation rates, stochastic events and selection. The short generation times and small genomes of microbes make them ideal model organisms for examining the combined impact of mutation rates and selection on genome evolution. Furthermore, the availability of high throughput and detailed gene expression data allows for an unparalleled examination of the effects of mutation and selection on gene regulatory network function. Members of the genus *Salmonella* develop a pattern formation phenotype that is hypothesized to be an important survival strategy that provides a resting stage comparable to spore formation in Gram positives (White *et al.*, 2006). This phenotype has been termed the rdar morphotype, and is characterized by the formation of red, dry and rough spreading colonies when grown on agar containing Congo red. Though widely conserved among wild isolates of the Salmonellae, the morphotype has been lost in some commonly used laboratory strains, suggesting that it is sensitive to loss due to common laboratory mutations such as deletion or attenuation mutations in *rpoS* (Römling *et al.*, 1998; White and Surette, 2006). White and Surette (2006) demonstrated that 90% of isolates representing the seven subgroups of *Salmonella* were capable of forming rdar colonies; however, among the frequently used strains of the SARC16 subset (Boyd *et al.*, 1996), the prevalence was only 31%. Some strains impaired in rdar formation had lost the function of *rpoS*, which codes for the sigma factor σ^{38} that regulates the expression of numerous genes involved in stationary-phase stress response (O'Neal *et al.*, 1994), and is required for rdar formation (Römling *et al.*, 1998). However, this

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was not the only affected pathway observed White and Surette (2006).

Rdar formation is the result of coordinated expression of genes required for biosynthesis of fimbriae (Collinson *et al.*, 1993, 1996; Römling and Rohde, 1999, 2000), cellulose (Römling *et al.*, 2000; Zogaj *et al.*, 2001) and additional polysaccharides (Gibson *et al.*, 2006). σ^{38} is involved in the gene regulatory network governing the rdar phenotype; in early stationary phase, it activates the transcription of a key regulator in rdar formation, *agfD*. AgfD (CsgD) is a transcriptional regulator that initiates transcription in the *agfBAC* operon, which encodes the main structural proteins for fimbrial biosynthesis (Collinson *et al.*, 1993, 1996; Römling *et al.*, 1998, 2000; White and Surette, 2006). *agfD* is also regulated by the transcriptional regulators OmpR, MlrA and CpxR-P (Gerstel and Römling, 2003; White and Surette, 2006). σ^{38} not only directly regulates *agfD*, but also feeds into its activation via positive regulation of the transcriptional regulator *mlrA*, (Brown *et al.*, 2001). Expression of *agfD* is initiated in late exponential phase with a peak in early stationary phase, which results in initiation of transcription of the rest of the fimbrial biosynthetic genes, and other genes in the regulon including *adrA*, (Römling *et al.*, 2000) which is involved in regulating cellulose biosynthesis.

AdrA is a member of the GGDEF family of proteins that is involved in modulating c-di-GMP levels; specifically, its diguanate cyclase activity generates c-di-GMP (Römling, 2005; Römling *et al.*, 2005; Simm *et al.*, 2007). Cellulose biosynthesis is partially regulated by intracellular concentrations of c-di-GMP, which interacts with the (Bacterial Cellulose Synthesis) BCS proteins located in the inner membrane to activate cellulose biosynthesis (Zogaj *et al.*, 2001; Römling, 2005). Low levels of c-di-GMP are associated with motile behaviors such as swimming and swarming, whereas higher levels of c-di-GMP are associated with sessile adaptations including the synthesis and export of extracellular components (Römling, 2005; Römling *et al.*, 2005). In addition, the rdar morphotype also includes the capsular biosynthesis and aggregative pathways (White and Surette, 2006). This morphotype is the result of complex regulation involving numerous major biosynthetic pathways and is highly conserved among *Salmonella*.

σ^{38} null or attenuation mutations are common in experimental evolution of *Escherichia coli* (*E. coli*)(King *et al.*, 2004; Zinser and Kolter, 2004; Ferenci, 2005; Maharjan *et al.*, 2006). σ^{70}, the exponential-phase σ factor, is responsible for the transcription of housekeeping genes involved in cell maintenance and growth. As the culture approaches stationary phase, σ^{38} increases in concentration and competes with σ^{70} for binding with the core polymerase (Ishihama, 2000). This leads to competing priorities for bacteria: regulation via σ^{38} provides protection against multiple stresses, particularly low pH (Farrell and Finkel, 2003; King *et al.*, 2006), whereas regulation via σ^{70} provides improved nutrient scavenging (Notley-McRobb *et al.*, 2002). Consequently, null or attenuation mutations of *rpoS* are common in laboratory conditions where improved scavenging can provide a selective advantage.

In this work, we demonstrate how laboratory passage leads to loss of the rdar phenotype, and use transcriptional profiling to identify impaired regulatory pathways. As expected, laboratory passage resulted in rapid loss of σ^{38} function; however, selection in a different regimen led to mutations in other parts of the pathway governing rdar formation. In both selection regimens, populations underwent radiation leading to population level heterogeneity in genotypes, several of which contributed to the loss of the rdar phenotype. The rapid loss of rdar formation during daily passage may be the result of high mutation rates at the *rpoS* locus.

Methods and materials

Bacterial strains, media and growth conditions

Salmonella enterica serovar Typhimurium strain ATCC 14028 (ST 14028) was used as the reference strain for all experiments. Strains were grown overnight at 37 °C with agitation (200 r.p.m.) in Miller's Luria-Bertani (Invitrogen Canada, Burlington Ontario) (LB) broth for all competition and passaging experiments. For expression assays, strains were grown overnight at 37 °C with agitation in 1% Tryptone (Difco, Mississauga, Ontario) (T) broth. Reference strain ST 14028 was frozen from an rdar$^+$ ST 14028 isolate. Due to the possibility of the rapid development of rdar$^-$ mutants, all experiments were started from overnight cultures taken directly from this frozen isolate.

Experimental evolution

Frozen *Salmonella* was inoculated into 5 ml LB and incubated at 37 °C with agitation. After 24 h, 50 µl of culture was inoculated into 5 ml fresh medium and incubated at 37 °C with agitation; this was repeated for a total of 14 days, providing approximately 5.6 generations of growth per day. Passaging experiments were done in quadruplicate. To determine the fraction of rdar and non-aggregative (smooth) colonies present in the culture each day, subsamples from 24 h-old cultures were normalized to 1.0 OD_{600} and 100 µl aliquots of a 10^{-6} dilution were plated onto media containing 1% tryptone (Difco) and 1.5% agar (Difco) (T agar) and incubated at 28 °C for 2–4 days. To passage cultures in exponential phase, 50 µl aliquots were removed after 6 h growth at 37 °C with agitation and used to inoculate fresh LB broth; this was repeated for a total of 84 h, providing between 5 and 6 generations ($\log_2$(number of doublings)) per cycle, depending on exact starting

and finishing densities. Isolates were selected from the daily passaging regimen (DPI-1, DPI-2), and from the exponential-phase passaging regimen (EPI-1, EPI-2).

We also tested whether a functional rdar phenotype contributed to the frequency of non-aggregative mutations in experimental evolution cultures. ST 14028 and Δ*agfD* were grown at 37 °C with agitation, and passaged daily for a total of 7 days. To determine the fraction of *rpoS* mutants in each culture at day 7, the σ^{38} responsive reporter sig38H4 (White and Surette, 2006) (described below) was transformed into the population of cells. Forty-eight colonies from each population were picked into a 96-well plate, and the σ^{38}-responsive expression was measured over 24 h. We calculated the frequency of σ^{38} mutants in each population and tested the null hypothesis that these frequencies were equivalent using a two-sample *t*-test assuming equal variances.

Construction of deletion mutant

Deletion mutants for Δ*agfD* and Δ*rpoS* have been described previously (White *et al.*, 2006). An in-frame deletion of 691 bp in *mlrA* (encoding amino acids 3–232 in MlrA) was generated using overlap extension PCR with primers mlrAFor1, mlrARev1, mlrAFor2 and mlrARev2 (Table 1). PCR products were purified, digested with *BamH*I and *Pst*I and ligated into pHSG415. Chromosomal deletions in *mlrA* were introduced into ATCC 14028 following established procedures (White *et al.*, 1999, 2007) and were confirmed by PCR and phenotypic analysis.

Phenotypic characterization and sequencing of mutants

To determine which pathways were affected by experimental evolution leading to the loss of rdar morphotype, deletion mutants Δ*agfD*, Δ*rpoS* and Δ*mlrA* and evolved isolates DPI-1, DPI-2, EPI-1 and EPI-2 were characterized for various phenotypic traits specific to rdar formation. Colony morphology was characterized after growth on T agar for 2–4 days at 28 °C, and confirmed for experimental isolates by further purification and recharacterization. Production of cellulose was monitored by incubation on LB agar (Invitrogen Canada Inc., Burlington, ON, Canada) supplemented with 200 μg ml^{-1} calcufluor white (fluorescent brightener 28, Sigma-Aldrich, Oakville, Ontario, Canada) and visualization of the colonies under UV light. Cellulose production is characterized by blue fluorescence when viewed under broad-spectrum UV light. Fimbrial production was detected by production of a dark red colony when grown on T agar supplemented with 100 μg ml^{-1} Congo red. σ^{38} function was tested by catalase assay (Notley-McRobb *et al.*, 2002), or flooding the plates with gaseous iodine (Hengge-Aronis and Fischer, 1992; Notley-McRobb *et al.*, 2002). For the iodine assay, T agar plates containing *Salmonella* colonies were incubated overnight at 4 °C, then at 37 °C for an hour before being exposed to iodine gas. Functional σ^{38} is required for glycogen storage, and iodine gas turns wild-type colonies dark brown (Notley-McRobb *et al.*, 2002). The catalase assay involves dropping hydrogen peroxide on the colony; colonies with functional σ^{38} produce vigorous bubbling (Notley-McRobb *et al.*, 2002). Colonies on all plates obtained from one daily passaging trial and one exponential passaging trial were tested for σ^{38} function using the catalase/iodine assays.

Sequences of the *rpoS*, *agfD*, *mlrA* and *adrA* coding and intergenic regions were sequenced with the primers rpoScloneFOR and rpoScloneREV, agfD1 and agfD2 (White *et al.*, 2006), mlrAcloneFOR and mlrAcloneREV, adrA01 and adrA03, respectively (Table 1).

Table 1 Primers used in this study

Primer name	*Sequence*
adrA01	TGACTCGAGCAAGTTTATGAGCGC
adrA03	AGTCCATCCTGAAGCCCGGCTGGA
mlrAcloneFor	GTCGGATCCCCAGATTAAACTCGTACATAC
mlrAcloneRev	GTCGGATCCTCTGTTTAAACGCCAAGG
rpoScloneFor	GCCGAATTCCAGGTCTGCACAAAATTC
rpoScloneRev	GCCAAGCTTGACAAGGGTACTTACTCGC
mlrAFor1	GCCGGATCCACCTTCAGCGCGGTAGCAGA
mlrARev1	*CGTCCCTGGGCGCCATCGTT*TCACCCTT,
mlrAFor2	*AACGATGGCGCCCAGGGACG*CGACATTCAT
mlrARev2	GCCCTGCAGAGGAGAGTTTTGCCGCCCTC

Restriction sites are underlined (mlrAFor1: *BamH*I, mlrARev2: *Pst*I). Italicized sequences correspond to regions of identity between mlrARev1 and mlrAFor2.

Gene expression assays

The expression of genes associated with the rdar morphotype was monitored during growth of evolved strains and deletion mutants at 28 °C in T broth. Development of *lux*-based reporters containing promoter regions cloned into a low-copy luciferase vector have been previously described for *mlrA* (White and Surette, 2006), *agfD*, *agfB*, *adrA* (White *et al.*, 2006) and σ^{70} (Stocki *et al.*, 2007).

Each reporter was transformed into Δ*agfD*, Δ*mlrA* and Δ*rpoS*, as well as DPI-1, DPI-2, EPI-1, EPI-2 and ST 14028. Overnight cultures supplemented with 50 μg/ml kanamycin were diluted 1:600 into 150 μl 1% T broth in individual wells of a 96-well clear-bottom black plate (9520 Costar; Corning Inc., Lowell, MA, USA). The wells were covered with 50 μl mineral oil to reduce evaporation. Cultures were incubated at 28 °C with agitation (2 mm orbital shake, 90 s every half hour) assayed for luminescence (1 s) and optical density (620 nm, 0.1 s) every half hour for 48 h in a Wallac Victor^2 (Perkin Elmer Life Sciences, Boston, MA, USA). The maximum expression of each reporter in each strain relative to wild type was calculated from at least three independent experiments with multiple replicates

in each experiment. Because maximum expression was observed during stationary phase where optical density is not an accurate reflection of cell number, the expression values were not normalized by OD. With few exceptions, the magnitude of expression from assay to assay was comparable; therefore replicates were averaged across assays. The average and 95% confidence interval of maximum expression were calculated for each reporter strain.

Growth and fitness assays

For growth assays, triplicate overnight cultures of ST 14028 and DPI-1, DPI-2, EPI-1 and EPI-2 were diluted 1/1000 into 25 ml Luria-Bertani broth and grown at 37 °C for 24 h. At 1 h intervals until 12 h, 0.2–1.0 ml aliquots were taken from each culture and the optical density was measured at 600 nm (OD_{600}). Final readings were taken after 24 h growth.

Fitness of evolved and deletion mutants was determined by direct competition with ST 14028. Evolved mutants DPI-1, DPI-2 and deletion mutants Δ*agfD*, Δ*mlrA* and Δ*rpoS* were diluted in quadruplicate into 5 ml fresh LB and incubated at 37 °C with agitation. After co-ordinated growth in LB for 24 h, 50 μl from each strain was combined with an equal volume of ST 14028 into 5 ml of LB. To determine the exact starting ratios of rdar and non-aggregative colonies, an aliquot of this initial mixture was immediately diluted 5×10^{-5} and plated in duplicate onto T agar, then incubated at 28 °C for 2–4 days. Mixed cultures were grown for 24 h at 37 °C with agitation. At the end of each competition, cultures were diluted 5×10^{-7} and grown on T agar for >48 h to determine the final ratio of rdar and non-aggregative colonies.

The competitive fitness (W) of each strain was calculated as the log ratio of the realized growth rates (Lenski *et al.*, 1991; Lenski, 2004). The fitness of each evolved isolate and deletion mutant relative to the wild type was calculated for at least four replicate trials; in the case of Δ*agfD*, seven replicates were required to determine if there was a significant fitness difference from the wild type.

To determine the fitness of isolate EPI-2 relative to wild type during exponential phase, coordinated overnight cultures of EPI-2 and ST 14028 were mixed 50:50, diluted 200-fold into fresh LB medium and grown at 37 °C. After 6 h, 100 μl of the mixed culture was transferred into 5 ml of fresh LB medium, and a 50 μl aliquot was removed, diluted and grown on T agar at 28 °C to determine initial frequencies of each strain. This was repeated after an additional 6 h growth to determine frequencies of each strain after exponential phase growth. The fitness of EPI-2 relative to wild type was calculated as the log realized growth rate over 6 h as above. To control for the possibility of appearance of new NAS mutants from ST 14028 during the competition, we grew cells from aliquots of the ST 14028 culture used for competition at the beginning and end of both overnight and exponential phase trials to determine whether novel NAS mutants appeared *de novo*. We documented the appearance of one *de novo* NAS mutant during one competition. The results from this competition were not included in the analysis.

The non-aggregative mutants EPI-1 and EPI-2 did not reach the same frequency during exponential-phase passaging as DPI-1 and DPI-2 did in the daily passaging regimen; therefore, we performed two additional competitions. First, we tested whether the competitive ability of EPI-2 was density dependent. Exponential-phase competitions were performed as above, but with starting densities of 10:90, 50:50 and 90:10 ST 14028:EPI-2. Secondly, we tested whether the evolved wild type had a different competitive ability than the ancestral wild type. EPI-2 was competed as above against a wild-type isolate obtained from the end of the exponential-phase passaging experiment (ST*).

For all competitions, the average and s.d. of the fitness coefficient W was calculated. The significance of the fitness of each strain relative to the ancestor was tested by repeated one-tailed Student's *t*-test (h_0: $W=1$, h_1: $W>1$ for all strains, except for *agfD*, h_0: $W=1$, h_1: $W<1$) with a Bonferroni corrected *P*-value (0.05/7) to correct for the increased probability of positive outcomes with multiple tests.

Model of mutational sweep

To determine how selective advantage contributed to the accumulation of non-aggregative colonies in the daily and exponential-phase passaging experiments, we used the model developed by De Gelder *et al.* (2004). Briefly, bacterial growth is modeled as a non-linear autoregressive process:

$$m_{(t)} = 2^{1+\sigma} m_{(t-1)} + 2\lambda n_{(t-1)} \quad (1)$$

where $m_{(t)}$ = number of mutants at time t, $n_{(t)}$ = number of wild type at time t, σ = selection coefficient, or $W-1$ and λ = mutation rate (De Gelder *et al.*, 2004). If the average fraction of mutants in the population is $\beta_{(t)}$, given the initial pool of mutants $\beta_{(0)}$, selection coefficient σ and mutation rate λ, then $\beta_{(t)}$ can be calculated by the following:

$$\beta_t(\beta_0, \sigma, \lambda) = m_{(t)}/(m_{(t)} + n_{(t)}) \quad (2)$$

The recursion can be solved for k cycles with l generations per cycle with the following equation:

$$\beta_{lk}(\beta_0, \sigma, \lambda) = \frac{2^{lk\sigma}\beta_0(2^\sigma - (1-\lambda)) + \lambda(1-\beta_0)(2^{lk\sigma} - (1-\lambda)^{lk})}{2^{lk\sigma}\beta_0(2^\sigma - (1-\lambda)) + \lambda(1-\beta_0)(2^{lk\sigma} - (1-\lambda)^{lk}) + (1-\lambda)^{lk}(1-\beta_0)(2^\sigma - (1-\lambda))} \quad (3)$$

Data of the frequency of non-aggregative colonies in daily and exponential-phase passaging were fitted to the above model using maximum likelihood using the program optim in R 2.4.1 (Team, 2006), with the Nelder–Mead algorithm (program provided online at http://www.webpages.uidaho.edu/~joyce/Labpage/Evo-x.html) to estimate the initial pool of mutants (β_0), mutation rate (λ) and selection coefficient (σ) that explain the rise in frequency of non-aggregative mutants during the course of the passaging experiments. 95% confidence intervals were calculated by parametric bootstrap, and goodness of fit was determined by a Likelihood Ratio Statistic that tests against the null hypothesis that the data are Poisson distributed with a mean $D_k\beta_{lk}$ (where D is sample size) versus the alternative hypothesis that the data are Poisson distributed with a different mean (De Gelder *et al.*, 2004).

Additionally, the mutation rate during daily passaging was estimated fitting the following model to times in generations (G) until half the population is taken over by the mutant:

$$G = \log_2(1/\beta_0)/\sigma \quad (4)$$

(Cooper *et al.*, 2001).

Where G = generations until mutant frequency equals half the population, β_0 = frequency of mutants in initial pool and σ = selection coefficient ($W-1$). We could not solve for exponential-phase passaging because the fraction of non-aggregative mutants did not reach 50% before the end of the experiment. Assuming that the dominant process is selection, we can solve for the initial pool of mutants and calculate the mutation rate required (Cooper *et al.*, 2001). Assuming an initial frozen inoculum of 2 µl, we can solve for frequency of mutants in the initial pool and estimate the mutation rate required to get this fraction in 11.28 generations ($\log_2 2500$), we get:

$$\lambda = (1/2^{G\sigma})/11.28 \quad (5)$$

To fit the model to data of accumulation of non-aggregative isolates in the daily passaging regimen, we used the average fitness of DPI-1 and DPI-2. The times in generations until the non-aggregative mutants reached a frequency of 0.5 were estimated from data of fraction of non-aggregative mutants per day fitted to a simple logarithmic function. The mutation rate was estimated from the four daily passaging trials at 37 °C, and the mean, s.d. and 95% confidence intervals were calculated.

Determining mutation rates in evolved ST 14028 cultures

Rifampicin-sensitive single colonies of isolates DPI-1, DPI-2, EPI-1, EPI-2 and ST 14028 were resuspended in phosphate-buffered saline, and approximately 100–1000 cells were aliquoted into 28 wells of a 96-well plate with 100 µl LB. Subsamples from 21 to 24 wells were plated to determine starting numbers of cells. The plate was incubated at 37 °C with agitation. After 24 h of growth, the entire contents of each well were plated on LB agar supplemented with 100 µg ml^{-1} rifampicin to determine the number of mutants that arose during growth. Subsamples from 25 to 28 wells were plated to determine the number of bacteria in the population. The number of rifampicin-resistant clones within each population was counted, and the results used to calculate mutation frequency according to the MSS Maximum Likelihood Method (Rosche and Foster, 2000).

RpoS activity in cell populations during evolution

To measure the activity of σ^{38} in multiple isolates within evolved populations, two populations from daily passaging (trial 2, day 8 and trial 3, day 7) and ST 14028 were transformed with the σ^{38}-responsive reporter sig38H4 by electroporation (GenePulser 2.0; Bio-Rad Laboratories Inc.). Approximately 1000 transformants from each strain were picked and transferred to LB medium in clear 384-well microtiter plates (Whatman Inc., Florham Park, NJ, USA) using a colony-picking robot (Norgren Systems, Palo Alto, CA, USA) and incubated at 37 °C before storage in 20% glycerol at −70 °C. To assay σ^{38} activity, clones were transferred into 80 µl of T broth in black, clear bottom 384-well microtiter plates (3711 Costar; Corning Inc., Corning, NY, USA) using a 384-pin manual plate replicator (catalog no. VP 386; V&P Scientific, San Diego, CA, USA) and incubated at 28 °C for 48 h with agitation. Luminescence (0.1 s) and optical density (620 nm, 0.1 s) measurements were taken at 24, 26, 28, 30, 32, 34 and 48 h in a Wallac Victor^2 (Perkin Elmer Life Sciences, Boston, MA, USA) Wells that did not grow ($OD_{600} < 0.1$) were removed from the analysis, and the percent frequency histogram of maximum fluorescence was calculated for each time point. Clones were also stamped onto LB agar and incubated at 28 °C for 24 h prior to imaging with a Kodak Image Station 2000MM camera system.

Results

Sweep of non-aggregative mutants in different passaging regimens

Wild-type isolates of pathogenic bacterial species are known to adapt quickly to laboratory growth conditions (Fux *et al.*, 2005). We observed previously that many strains in a well-characterized *Salmonella* reference collection (SARC; (Boyd *et al.*, 1996)) had lost the aggregative colony morphology known as the rdar morphotype, and hypothesized that this was caused by laboratory passage (White and Surette, 2006).

To determine whether daily laboratory passaging could lead to the loss of the rdar morphotype, ST 14028 was grown in LB medium at 28 or 37 °C and transferred daily into fresh medium for a total of 14

days. The relative number of rdar and non-aggregative, smooth (NAS) colonies in 24 h-old cultures was determined after each day. In each experiment, takeover by NAS mutants was extremely rapid and reproducible, with near complete takeover of the population by approximately 56 generations at 37 °C (7 days) and by approximately 79 generations (11 days) at 28 °C (Figure 1a).

During the daily passaging experiments, cells spent considerable time in stationary phase. To determine if stationary-phase survival was contributing to takeover by the NAS mutants, ST 14028 was passaged in LB medium while maintaining the cells in exponential phase. Under these growth conditions, non-aggregative colonies were not observed until approximately 50 generations and the frequency did not reach 50% of the population (Figure 1b). Clones were isolated from the daily passaging regimen (DPI-1, DPI-2) and the exponential-phase passaging regimen (EPI-1, EPI-2) for further analysis. This result was also highly reproducible among all four parallel lines.

We determined that non-aggregative mutants from the daily passaging regimen were deficient in σ^{38} function (see below: Phenotype of isolated non-aggregative mutants). We tested whether a functional rdar phenotype contributed to the accumulation of non-aggregative mutations in the daily passaging experiment by comparing the frequency of *rpoS*$^-$ mutants in the populations of ST 14028 and Δ*agfD* (NAS) after 7 days of passaging in LB at 37 °C. The frequency of *rpoS*$^-$ mutants in ST 14028 was 50.35% ($n=4$, s.d. = 18.37), and in Δ*agfD* was 62.34% ($n=3$, s.d. = 6.16). A two-sample *t*-test assuming equal variances failed to reject the null hypothesis that the frequencies were different (*t*-statistic = 2.57, d.f. = 5, $P=0.35$).

Phenotype of isolated non-aggregative mutants

Four non-aggregative mutants were isolated from the daily passaging or exponential passaging regimens at 37 °C (Figure 2; DPI-1, DPI-2, EPI-1 and EPI-2, respectively). The phenotypes of these mutants were compared to *agfD*, *mlrA* or *rpoS* deletion mutants, all known to have NAS morphology (Table 2; (Römling *et al.*, 1998; Brown *et al.*, 2001)). Among these three control strains, only Δ*rpoS* exhibited a lack of peroxidase activity and lack of iodine staining (Table 2). Morphology of strains DPI-1 and

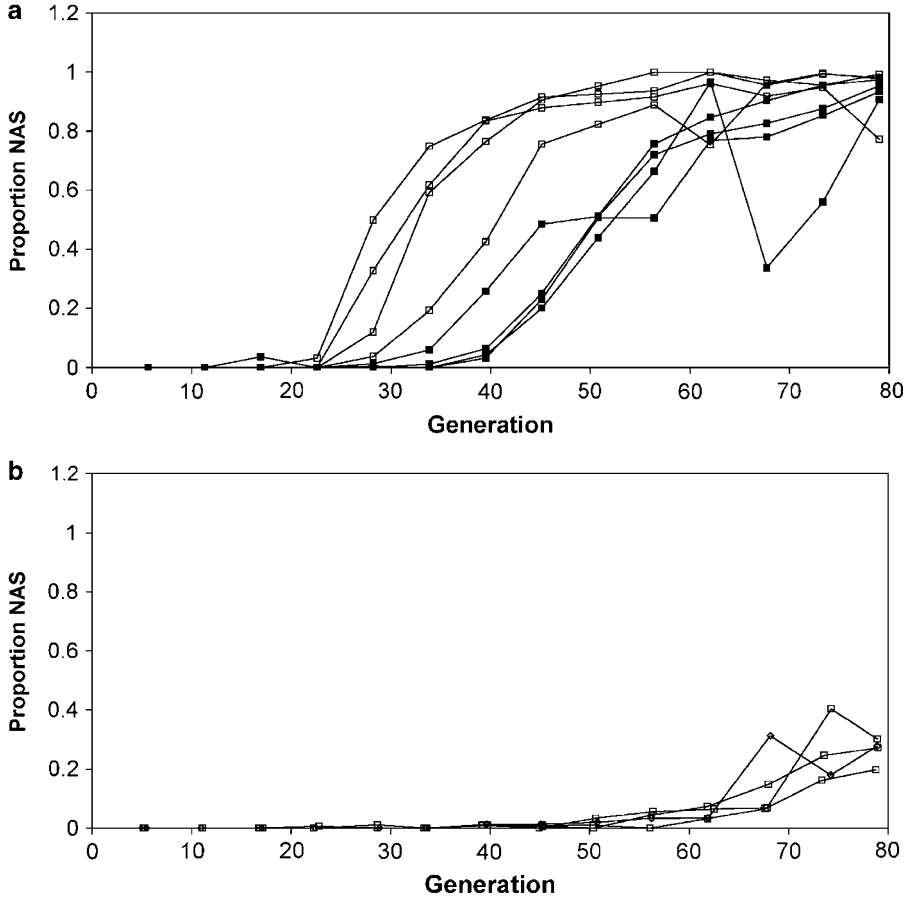

Figure 1 Proportion non-aggregative and smooth (NAS) colonies per generation of experimental evolution. Each line represents an individual trial. (**a**) Daily passaging at 37 °C (open squares) and at 28 °C (filled squares), (**b**) Exponential phase passaging at 37 °C. Generations calculated as the $\log_2$ of the observed number of doublings for each passage.

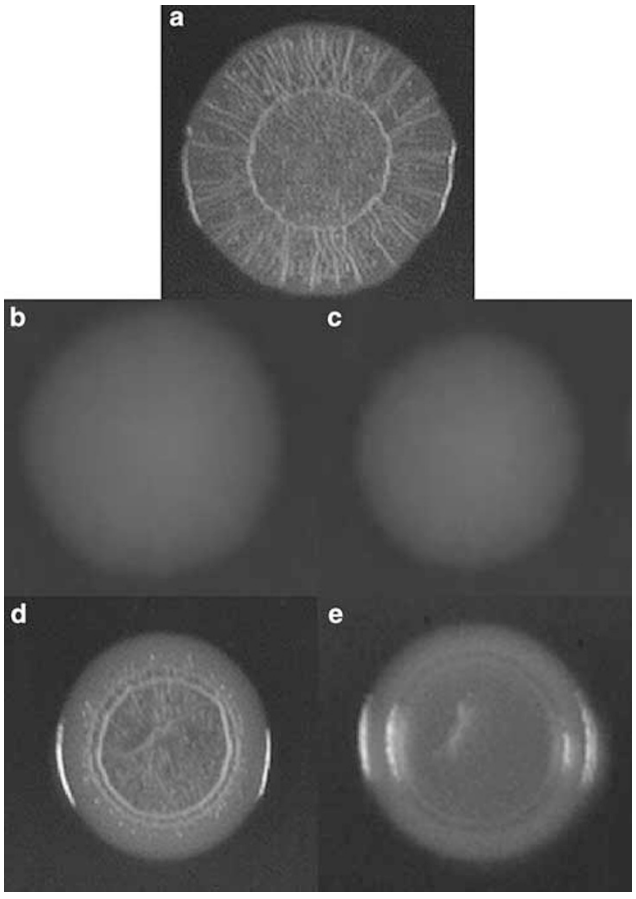

Figure 2 Colony morphology of *Salmonella enterica* serovar Typhimurium strains. 1 μl of cells from overnight cultures of ST 14028 (**a**), DPI-1 (**b**), DPI-2 (**c**), EPI-1 (**d**) or EPI-2 (**e**) were inoculated on T agar and grown at 28 °C for 72 h.

DPI-2 on Congo red- or calcofluor-containing media indicated that each was negative for fimbrial and cellulose production (Table 2). DPI-1 and DPI-2 were also negative for iodine staining, and had reduced peroxidase activity compared with ST 14028 (Table 2), indicating that these strains were likely defective in σ^{38} activity. In contrast, EPI-1 and EPI-2 exhibited a colony morphology that was intermediate between DPI-1 and ST 14028, with a small amount of pattern formation in the centre of the colony (Figure 2). Strains EPI-1 and EPI-2 exhibited positive peroxidase activity and iodine staining, indicating that they were not impaired in σ^{38} activity and were also both positive for fimbrial production as indicated by red colony formation on Congo red (Table 2). Isolate EPI-2 was negative for cellulose production, as judged by a lack of calcofluor binding (Table 2), indicating impairment in the cellulose biosynthesis pathway.

We tested all NAS mutants that appeared during one daily passaging trial and one exponential-phase passaging trial for σ^{38} function by both iodine staining and catalase. In the daily passaging trial, all NAS colonies showed reduced or loss of σ^{38} function. In the exponential phase passaging trial, all NAS colonies had functional σ^{38} according to the peroxidase assay, though EPI-1 exhibited lower σ^{38} activity in expression assays.

Gene expression assays

To characterize the pathways in which the evolved mutants were compromised, we measured the expression of genes involved in rdar formation in

Table 2 Observed phenotypes of evolved and deletion mutants

Colony isolate	*Morphology*[a]	*Congo red*[b]	*Calcofluor*[c]	*Peroxidase/ iodine*[d]	*Source*
ST 14028	RDAR	+	+	++	*Salmonella enterica* serovar typhimurium
DPI-1	NAS	−	−	−	Isolated from daily passaging
DPI-2	NAS	−	−	+	Isolated from daily passaging
EPI-1	Intermediate—some pattern formation but only in middle of colony	+	+	++	Isolated from exponential phase passaging
EPI-2	NAS—faint pattern formation in middle of colony	+	−	+	Isolated from exponential phase passaging
ST*	RDAR—not as well developed as WT	NA	NA	NA	Isolated from exponential phase passaging
Δ*mlrA*	NAS	−	−	++	This paper
Δ*rpoS*	NAS	−	−	−	(White and Surette, 2006)
Δ*agfD*	NAS	−	−	++	(White and Surette, 2006)

[a]Morphology described by RDAR (red, dry and rough when grown on agar medium containing Congo red), or NAS (non-aggregative and smooth).
[b]Congo red phenotypes were either purple (+, fimbrial positive), or unstained (−, fimbrial negative).
[c]Calcofluor, either fluorescent (+, cellulose positive) or non-fluorescent (−, cellulose negative).
[d]Peroxidase/iodine, bubbling, and dark brown (++, RpoS positive), slight bubbling, light brown (+, RpoS intermediate) or non-bubbling and unstained (−, RpoS negative). NA—test was not performed.

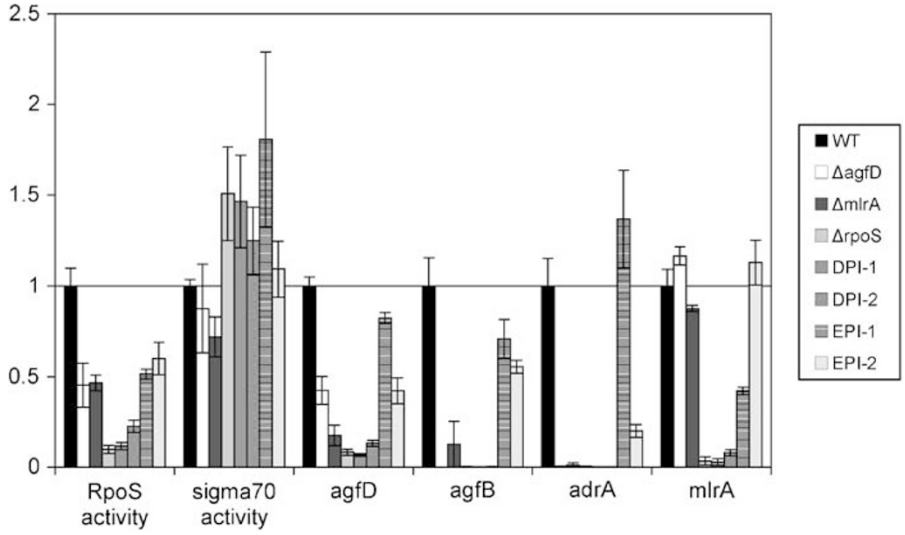

Figure 3 Expression of genes of the rdar regulatory network in *S. enterica* serovar Typhimurium strains. Bars represent the ratio of maximum gene expression (c.p.s.) in each strain compared to ST 14028 (wt) expression levels. Error bars represent 95% confidence intervals.

ST 14028, evolved mutants DPI-1, DPI-2, EPI-1 and EPI-2, as well as Δ*agfD*, Δ*rpoS*, Δ*mlrA* (Figure 3). Every strain had reduced σ^{38} activity compared to ST 14028, including those strains judged to have a functional σ^{38} in phenotypic assays. These mutants formed a group including EPI-1, EPI-2, Δ*mlrA* and Δ*agfD*, which expressed approximately half the native σ^{38} activity. We considered this group to be still within the range of functional σ^{38} activity as judged by their normal expression of σ^{38}-dependent phenotypes of peroxidase activity and glycogen storage. As expected, DPI-1 exhibited very low σ^{38} activity that was equal to the Δ*rpoS* mutant. DPI-2 exhibited σ^{38} activity intermediate between Δ*rpoS* and the functional σ^{38} group, indicating that these isolates were an *rpoS*$^{-}$ null and impaired function mutant (*rpoS*^{imp}) respectively. The mutant EPI-1 exhibited approximately half of *wt mlrA* expression, but was not impaired for the other genes analyzed. EPI-2 exhibited low expression in *adrA*, consistent with its impairment in cellulose production (Figure 3, Table 2).

DNA sequencing to determine chromosomal mutations in evolved strains

DNA sequencing showed that both the exponential phase isolates had native *rpoS* sequences, whereas DPI-2 had a single base-pair change causing an amino acid change (N→S at position 125). Long range PCR of DPI-1 indicated a 3482 bp deletion from STM2922 to *pcm*, encompassing the entire *rpoS* coding sequence (corresponding to positions of 3064362–3067844 of the *Salmonella typhimurium* LT2, complete genome NC_003197.1). We sequenced two additional isolates from the daily passaging regimen. One isolate (DPI-3) obtained from a later day of the DPI-1 passage contained the same deletion as DPI-1, indicating that this particular strain was involved in takeover of the *wt* population. The other isolate (DPI-4) analyzed had a 3202 bp deletion from *surE* to *rpoS*, encompassing *pcm*, *nlpD* and the majority of the *rpoS* coding sequence (corresponding to positions of 3065984–3069187 of the *Salmonella typhimurium* LT2, complete genome NC_003197.1). This analysis confirmed that each of the daily passage isolates contained *rpoS* mutations.

Gene expression assays suggested that EPI-1 had a similar expression profile to Δ*mlrA* (Figure 3); however, no mutations in the *mlrA* coding sequence or promoter region were identified. EPI-2 had impaired *adrA* expression (Figure 3) and was negative for cellulose production (Table 2), but no mutations were detected in the *adrA* coding sequence or promoter region. Cellulose production by EPI-2 was restored when complemented with constitutively expressed *adrA*, indicating that EPI-2 had functional *bcs* operons (Solano *et al.*, 2002).

Growth and fitness assays

We measured the optical density of ST 14028 and evolved strains in LB, the medium of the evolution experiments and the competitions, during 24 h of growth. DPI-1 and DPI-2 grew slower than ST 14028, though DPI-2 was intermediate between DPI-1 and ST 14028. Neither DPI-1 nor DPI-2 reached the same density as ST 14028 during stationary phase. We were unable to determine whether these strains had an advantage coming out of lag phase (Figure 4). However, when grown in minimal media with different sugar substrates in every case Δ*rpoS*, DPI-1, DPI-2 and EPI-1 came out of lag phase faster than ST 14028 (data not shown). EPI-2 was the only mutant to grow faster than ST 14028, with a faster

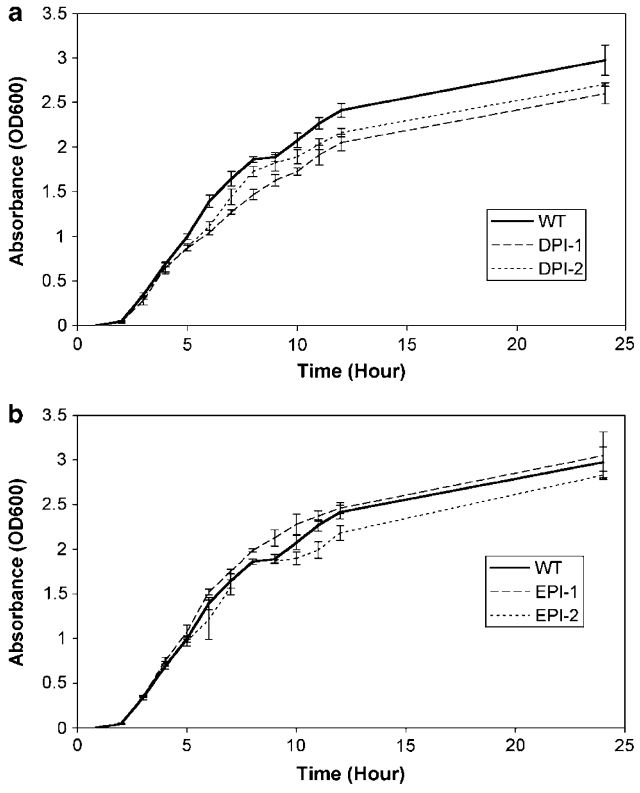

Figure 4 Measured optical density (OD_{600}) of growth of cultures in LB broth over 24 h. (**a**) WT, DPI-1 and DPI-2, (**b**) WT, EPI 1, EPI-2. Error bars represent 95% confidence intervals. Initial observations were on undiluted culture, but dilutions were performed as density increased to ensure that optical density was not saturating.

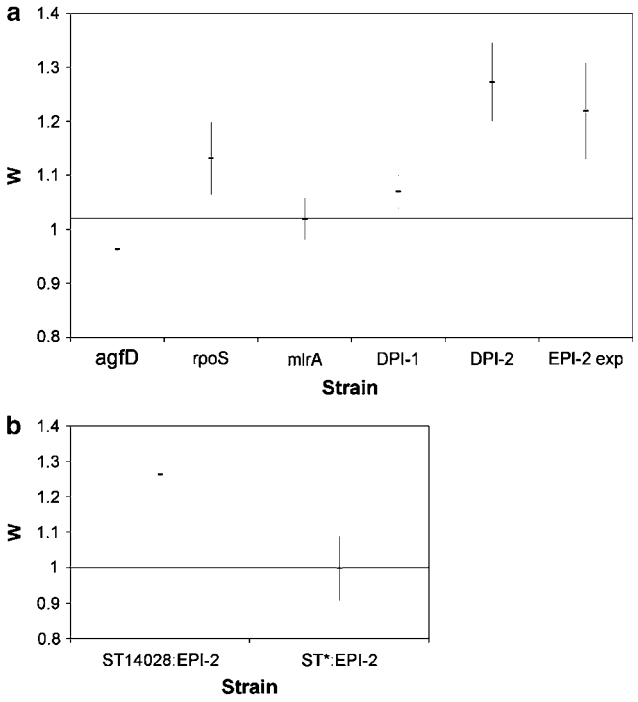

Figure 5 Competitive fitness (W) of evolved strains and deletion mutants relative to ST14028. (**a**) 95% confidence interval of competitive fitness (W) of deletion mutants Δ*agfD*, Δ*rpoS*, Δ*mlrA* and evolved isolates DPI-1, DPI-2, and EPI-2 relative to ST14028. All competitions were performed at 37 °C for 24 h, except for EPI-2. Competitions for this strain were performed for 24 h (ON) and 6 h (Exp) (**b**) 95% confidence interval of competitive fitness (W) of EPI-2 relative to ST 14028 and relative to isolate scored as wild type from end of exponential phase screen (ST*). For all competitions, $n=4$, except for Δ*agfD*, ($n=7$), and EPI-2 (exp) vs ST 14028 ($n=9$).

growth during exponential phase, however, this advantage diminished as the cultures neared stationary phase (Figure 4b). EPI-1 did not exhibit any advantage in growth (Figure 4b).

We measured the competitive fitness of evolved isolates DPI-1, DPI-2 and EPI-2 and Δ*agfD*, Δ*rpoS* and Δ*mlrA* mutants relative to ST 14028. Competitions allow a determination of selective advantage and are much better at distinguishing subtle differences in growth; values of $W>1$ indicated stronger competitive fitness of the evolved or deletion mutant relative to ST 14028. All evolved isolates had values of W higher than one and were significant according to an adjusted *P*-value of 0.007 with the exception of DPI-1, ($P=0.01$, $n=4$, $W_{avg}=1.070$) and Δ*rpoS* ($P=0.016$, $n=4$, $W_{avg}=1.13$). The strongest competitor was DPI-2 ($W_{avg}=1.273$, $n=4$) followed by EPI-2 in the exponential regimen ($W_{avg}=1.219$, $n=13$). Δ*mlrA* did not exhibit any difference in competitive fitness relative to ST 14028 ($P=0.205$, $n=4$, $W_{avg}=1.019$), and Δ*agfD* was significantly less fit than its ST 14028 ancestor ($P=0.00703$, $n=7$, $W_{avg}=0.9635$).

In competitions between EPI-2 and ST 14028, EPI-2 was shown to be a strong competitor, similar to DPI-2 (Figure 5a). However, NAS mutants in the exponential passaging regimen did not accumulate as rapidly as NAS mutants in the daily passaging regimen despite the similar fitness of isolated mutants (Figure 1). To test whether this was a result of density dependence or evolution of the wild type, we tested EPI-2 against ST 14028 in a number of starting ratios, and EPI-2 against a wild-type isolate obtained from the end of the exponential phase passaging experiment (ST*). There was no difference between different density treatments ($F=0.853$, d.f. $=2$, $P=0.474$); however, there was a strong difference between the fitness of EPI-2 relative to ST 14028 and ST* (Figure 5b) (*t*-statistic $=3.46$, d.f. $=18$, $P=0.001386$).

Predicted mutation rates

Fluctuation analysis of the appearance of rifampicin resistant mutants in 24 h of growth in LB showed that the mutation rates in both ST 14028 and evolved isolates varied between 9.84×10^{-10} and 6.19×10^{-9} mutations/gene/generation. The mutation rates were similar between isolates (Table 3).

Using a model of mutation accumulation as a function of fitness and mutation rates (De Gelder *et al.*, 2004), we examined the contributions of

measured fitness to accumulation of *rpoS* mutants in the daily passaging regimen. This autoregressive model examines the combined influence of mutation and selection on the accumulation of mutants in a population. Because all NAS mutants in one daily passaging trial had a loss of σ^{38} function, and because the sequences of four daily passaging isolates showed mutations in *rpoS*, we assumed that all NAS mutants observed during daily passaging were due to mutations at this locus. Using published mutation rates (4.96×10^{-7} (Drake, 1991)) and the observed selection coefficients, the model predicts that the *rpoS* mutants would not have reached fixation during the course of the experiment (Figure 6, upper left panel).

We then fit the De Gelder model to the data of frequency of non-aggregative mutants during the daily passaging and exponential phase passaging experiments. The test of goodness of fit indicated that the model did not fit the data well for either the daily passaging or exponential-phase passaging experiments (H_0: $x = D_k\beta_{lk}$, daily passaging, $P = 0.630$, exponential-phase passaging, $P = 0.935$). A lack of power may be partially responsible for poor fit, especially in the exponential-phase passaging where mutant frequencies did not surpass 0.4 before the end of the experiment. But more likely this is due to overdispersion of the data, meaning that there are sources of variability in the data that are unaccounted for in the model. For example, growth may be variable throughout the experiment if there are multiple mutants appearing in the population with different competitive fitnesses (personal communication, Jose Ponciano). Instead, we compared model predictions given known fitnesses and increasing mutation rates. We found that at a mutation rate of 4.96×10^{-4} mutations/gene/generation and fitness (W) of 1.27 predicted a sweep of mutations similar to what was measured (Figure 6, lower right panel).

By solving Equation (5) for frequencies of non-aggregative mutants in four repeated trials of daily passaging at 37 °C, we determined the mutation rate λ to be 1.96×10^{-3} mutations/gene/generation ($n = 4$, 95% CI $= 9.76 \times 10^{-4} \leqslant \lambda \leqslant 2.62 \times 10^{-3}$). Varying the estimate of initial inoculum size between 1 and 10 µl has minimal effects on the calculation of mutation rate by this equation (data not shown).

Table 3 Mutation rates of ST 14028 and evolved isolates

Isolate	*Mutation rate (mutations/gene/generation)*	*95% CI lower limit*	*95% CI upper limit*
ST 14028	2.74×10^{-9}	7.20×10^{-10}	1.61×10^{-9}
DPI-1	9.84×10^{-10}	1.08×10^{-10}	4.70×10^{-10}
DPI-2	1.75×10^{-9}	3.77×10^{-10}	9.72×10^{-9}
EPI-1	6.19×10^{-9}	2.28×10^{-9}	4.06×10^{-9}
EPI-2	1.87×10^{-9}	3.87×10^{-10}	1.03×10^{-9}

Mutation rates calculated from fluctuation assay testing rifampicin resistance, MSS Maximum Likelihood algorithm (Rosche and Foster, 2000 and references therein).

RpoS responsive expression in evolved populations

DNA sequencing revealed that there were three unique *rpoS* mutations present amongst four DPI isolates. This indicated that there may be a variety of *rpoS* mutations within the evolved populations from daily passaging. To test this hypothesis, we transformed the σ^{38}-dependent sig38H4 luciferase reporter (White *et al.*, 2006) into the evolved populations (after 7 and 8 days of passage, respectively), and into ancestral ST 14028, and measured the σ^{38} activity in ~1000 isolates. If the evolved populations consist of a variety of *rpoS* mutant

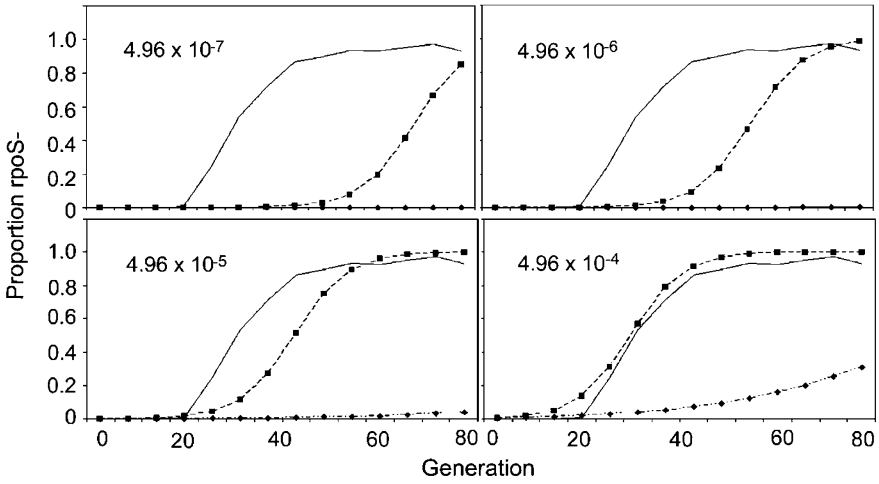

Figure 6 Predicted and measured mutational sweep of *rpoS* mutants in the daily passaging regimen according to De Gelder model (De Gelder *et al.*, 2004) for different values of λ. Solid line: observed frequency of *rpoS* mutants in daily passaging at 37 °C. Dashed line with squares: predicted frequency of *rpoS* mutants in daily passaging given fitness of $W = 1.27$, corresponding to DPI-2. Dashed line with diamonds: predicted frequency of *rpoS* mutants in daily passaging give fitness of $W = 1.07$, corresponding to DPI-1.

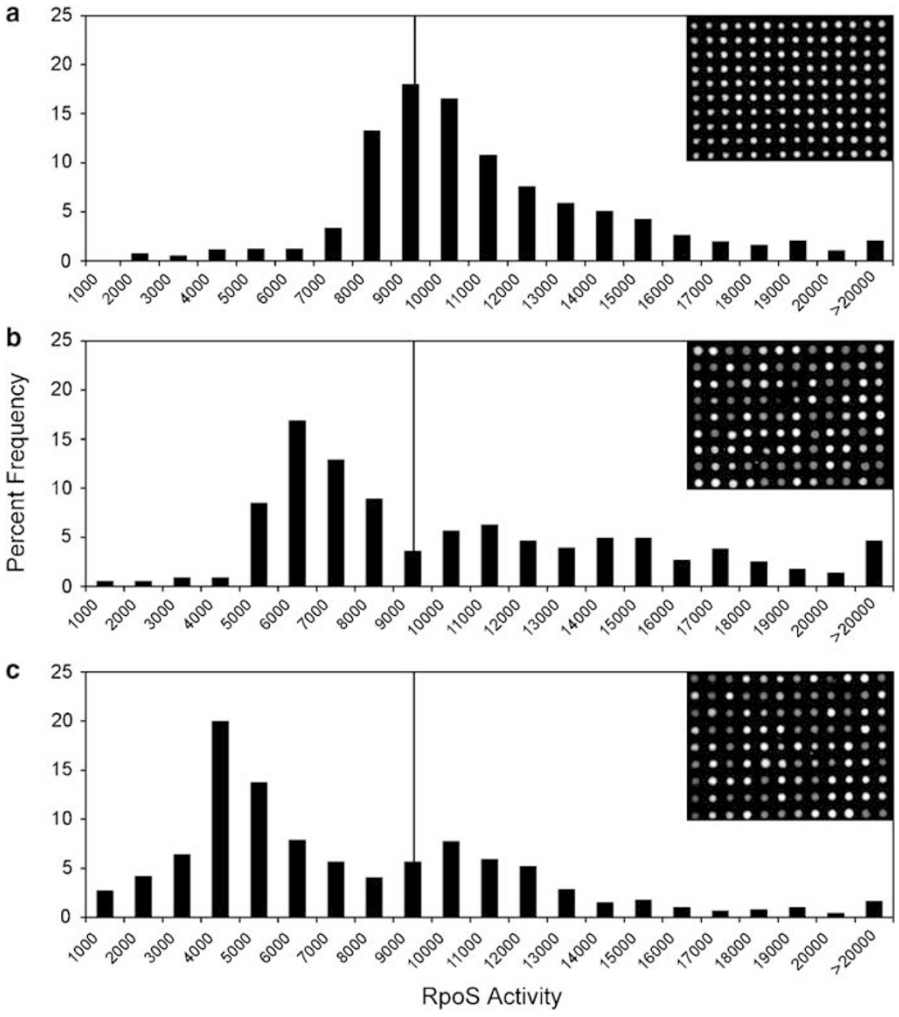

Figure 7 Frequency histogram of expression of RpoS responsive promoter sig38H4 in evolved and ancestral populations. (**a**) ST14028, (**b**) Trial two, day eight, (**c**) Trial three, day seven, 34 h postinduction. Inset: solid phase expression of subset same populations 24 h postinduction. Vertical line indicates wild-type level of expression.

strains we would expect a wide distribution of σ^{38}-expression phenotypes. The ST 14028 population exhibited a peak of expression at 9000–10 000 c.p.s., and the solid-phase expression was largely uniform (Figure 7). However, the two evolved populations exhibited peaks of σ^{38} activity at lower levels, with the population from trial two, day 8 having a lower peak at 4000 c.p.s., and the population from trial three, day 7 at 6000 c.p.s., and both exhibiting a large proportion of isolates with extremely low σ^{38} activity. Interestingly, both evolved populations contained a small fraction of colonies that exhibited a higher than normal σ^{38} activity. Similar to the liquid-phase expression, the evolved populations also exhibited more variability in σ^{38} expression in the solid phase (Figure 7).

Discussion

We analyzed evolutionary loss of the rdar morphotype during passage of *S. enterica* serovar Typhimurium ATCC 14028 in standard laboratory media. In both exponential phase and daily passaging experiments the increase in frequency of non-aggregative and smooth mutants was highly reproducible. In the daily passaging regimen, the takeover of mutants was much faster than the exponential-phase regimen. Analysis of the rdar morphotype in *Salmonella* strains from the SARC reference collection (Boyd *et al.*, 1996) has shown that the morphotype has been lost in 69% of this commonly used subset of strains (White and Surette, 2006). The results of this experiment suggest that the loss of rdar morphotype was partially due to loss of σ^{38} function as a result of laboratory passaging. However, not all of the mutations uncovered in this work were *rpoS* mutations. Exponential-phase passaging produced different mutations that remain unidentified, which demonstrates that the rdar phenotype is sensitive to loss due to multiple types of laboratory-acquired mutations. Furthermore, there was no significant difference in the accumulation of *rpoS* mutations in 7 days of passaging between ST 14028

and Δ*agfD*, a rdar morphotype-negative strain, indicating that the loss of the phenotype was not a consequence of rdar morphotype expression but was more a function of nutritional scavenging. Thus the acquired mutations confer a benefit in a regimen under which rdar is not expressed. This has implications in the study of this organism; repeated laboratory passage will likely result in the loss of this highly regulated phenotype. Loss of the phenotype due to passaging has been reported (Römling *et al.*, 1998, 2003), but the rapidity by which mutations accumulate in the population is surprising. It is conceivable that common laboratory practice of streaking from frozen culture and selecting isolated colonies may result in the occasional selection of an *rpoS* mutant.

The phenotypic and expression characterization demonstrated that the isolates from the daily passaging regimen, DPI-1 and DPI-2, were an *rpoS* deletion and impaired function mutant respectively. *rpoS* mutations are common in many selection regimens (Zambrano *et al.*, 1993; Finkel and Kolter, 1999; Notley-McRobb and Ferenci, 2000; Chen *et al.*, 2004; Zinser and Kolter, 2004; Mandel and Silhavy, 2005; Maharjan *et al.*, 2006). In *Escherichia coli*, as growth rate of the culture declines, the concentration of σ^{38} within the cell reaches 30% the level of σ^{70} (Jishage and Ishihama, 1995; Ihssen and Egli, 2004). At this point, σ^{38} becomes an effective competitor with σ^{70} for the core RNA polymerase and begins to drive various stress–response genes (Jishage and Ishihama, 1995). The competition between the drive for protection against stress via a functioning σ^{38}, and improved scavenging via increased σ^{70} leads to varied mutations in *rpoS* (Notley-McRobb *et al.*, 2002; Chen *et al.*, 2004; King *et al.*, 2004; Ferenci, 2005; Mandel and Silhavy, 2005; Maharjan *et al.*, 2006). *rpoS* mutations have been identified in clinical isolates of *S. enterica* serovar Typhi (Robbe-Saule *et al.*, 2003), though are rare, and are not demonstrated in serovar Typhimurium, thus it is unclear whether these mutants truly exist in clinical settings. This study emphasizes how easy it is to generate *rpoS* mutations in the laboratory and care must be taken in clinical studies to ensure that *rpoS*$^-$ mutants are not selected by accident from an *rpoS*$^+$ ancestor.

The exponential passage isolates had different phenotypic profiles than the daily passage isolates. Although isolate EPI-1 was positive for catalase activity and possessed the *wt rpoS* sequence, it was slightly impaired in σ^{38} activity as indicated by lower expression of sig38H4. This suggests that an unidentified regulator of *rpoS* could be impaired in function. The regulation of σ^{38} transcription, translation and overall activity is among the most complicated of all bacterial pathways (Jishage and Ishihama, 1995; Hengge-Aronis, 1999; Venturi, 2003; Hirsch and Elliott, 2005), and it is possible that σ^{38} activity was impaired by mutation within this pathway in EPI-1. Isolate EPI-2, in contrast, had normal σ^{38} activity, but was impaired in cellulose biosynthesis and yielded a unique expression and phenotypic profile. The expression analysis showed that transcription of *agfD*::*lux* in this strain was lower than wild type, similar to Δ*agfD*, and that *adrA*::*lux* transcription was reduced. *adrA* transcription is partially dependent on σ^{38} and can be dependent on AgfD, although evidence suggests that at least in *E. coli* cellulose can be produced independently of AgfD (Da Re and Ghigo, 2006). However, EPI-2 did not possess mutations in either *adrA* or *agfD*, therefore it is unclear how transcription of *adrA* was impaired in this strain. It is likely that the alteration of upstream regulation resulted in the lower expression of this gene.

The growth and competition assays illustrated how new mutants competed relative to the ancestor. Both Δ*rpoS*, and DPI-1 (*rpoS*$^-$) and DPI-2 (*rpoS*^{imp}) demonstrated considerably slower exponential growth than wild type and did not reach the same finishing density. It is unclear then how these strains maintained the high competitive advantage demonstrated by the competition experiments. However, the competitions indicated a strong competitive fitness of all three strains relative to the ancestor. In particular, DPI-2 (*rpoS*^{imp}) demonstrated the highest competitive fitness. *rpoS*^{imp} mutants often have an advantage over both *rpoS*$^-$ and *rpoS*$^+$ strains in environments that may present a pH challenge (Farrell and Finkel, 2003; King *et al.*, 2004, 2006). In this study, cells would have encountered high pH when they reached stationary phase in LB.

Competition assays can detect subtle growth differences and the effects of competitive interactions between strains that may not be apparent in simple growth curves. σ^{38} mutations have been demonstrated to confer an advantage in stationary phase (Zambrano *et al.*, 1993), and growth in minimal medium supplemented with sugars and casamino acids demonstrated that the σ^{38} mutants had a growth advantage in coming out of lag phase relative to wild type (data not shown), though this was not demonstrated in rich medium. It is likely that the σ^{38} mutation conferred an advantage during the stationary phase of the daily growth curve.

The EPI-2 isolate had a clear growth advantage in exponential phase, reaching stationary phase before the other isolates. Maintaining the population in exponential phase selected for faster growth and this advantage translated to a much higher competitive fitness relative to the ancestor in the exponential growth regimen. The difference in growth curves for EPI-1 and EPI-2, which were isolated from the exponential-phase passaging experiment, is intriguing. In rich medium, EPI-1 increased in optical density (OD_{600}) in the same manner as ST 14028. Unfortunately, we were unable to compete EPI-1 against ST214028 to determine whether it had a competitive advantage relative to wild type.

The competition assays indicated a strong competitive fitness of all three evolved isolates in these experimental conditions relative to the ancestor. Despite the demonstrated fitness of EPI-2 relative to ST 14028, the accumulation of this mutant in the exponential phase passaging population was not as rapid as the *rpoS*$^{-}$ and *rpoS*^{imp} mutants isolated in the daily passaging regimen. This may be due to parallel evolution of cells later scored as wild type, as competition experiments demonstrated that the ST* was a better competitor relative to EPI-2 than ST 14028. Exponential phase passaging demonstrated that there were at least three identifiable evolved mutants at the end of 50 generations, EPI-1, EPI-2 and ST*, and it is likely that other mutants existed that were not detected by our selection. This indicates radiation of the population in this regimen, over a very short time period, much as cultures in glucose-limited chemostats exhibited increased genomic and phenotypic variability (Maharjan *et al.*, 2006). However, even accounting for clonal interference, the discrepancy between accumulations of mutants in either regimen cannot be explained. In particular, the De Gelder model showed that the accumulation of σ^{38} mutations was far too rapid to be explained by published mutation rates, or the mutation rate measured by fluctuation analysis in DPI-1 and DPI-2.

Using published mutation rates and the measured competitive fitness, the De Gelder model does not reflect the speed by which the non-aggregative mutants swept the population during the daily passaging regimen. When a higher mutation rate of 4.96×10^{-4} mutations/gene/generation was incorporated into this model, the predicted sweep of mutations was similar to what was observed in our experiments. Furthermore, when we calculated the mutation rate using the approach of Cooper and Lenski (Cooper *et al.*, 2001), we obtained an estimate of 1.96×10^{-3} mutations/gene/generation ($n = 4$, 95% CI $= 9.76 \times 10^{-4} \leqslant \lambda \leqslant 2.62 \times 10^{-3}$).

Stationary phase culture can lead to high genome-wide rates of mutation (Loewe *et al.*, 2003), without induction of known mutator pathways. Mutator phenotypes have also been identified in clinical strains of *E. coli* and *S. enterica* with a prevalence of over 1% (LeClerc *et al.*, 1996) and in long-term experimental evolution (Sniegowski *et al.*, 1997). However, fluctuation assays showed that mutation rates under these experimental conditions remained low and were comparable between ancestral ST 14028 and all evolved isolates, including those carrying σ^{38} mutations. Therefore, it is unlikely that a mutator phenotype arose and contributed σ^{38} mutations during the timelines of our experiment. The De Gelder model predicts the accumulation of a mutation phenotype. Because the isolates from daily passaging we sequenced all had mutations at *rpoS*, we assumed that the NAS isolates appearing in this regimen are solely due to mutations in this gene. If the mutations in the populations measured in Figure 7 are due to mutations in other parts of the pathway, then this elevated mutation rate cannot be attributed to mutation at the *rpoS* locus alone. However, the *mutS–rpoS* intergenic region has been demonstrated to be variable in enteric bacteria (Herbelin *et al.*, 2000; Kotewicz *et al.*, 2002, 2003), which suggests a history of duplications and deletions in this region. This suggests that this elevated mutation rate may apply primarily at the *rpoS* locus. A deletion of the *rbs* operon in long-term evolution lines of *E. coli* was demonstrated to be the result of an insertion element which contributed to a mutation rate of 5.4×10^{-5} mutations/gene/generation at that locus (Cooper *et al.*, 2001). However, we did not identify an IS element in the region of *rpoS*, the deletions did not share a common start or end point, and these mutations arose with an estimated order or two orders of magnitude higher frequency compared to mutations caused by insertion elements leading to loss of ribose catabolism (Cooper *et al.*, 2001). Furthermore, although deletions in *rpoS* have been demonstrated in other systems (Chen *et al.*, 2004), they are less frequent than single base-pair changes (Zambrano *et al.*, 1993); an IS element would not explain the presence of single base pair changes such as in DPI-2.

The distribution of σ^{38}-responsive expression phenotypes supports the hypothesis that loss of σ^{38} function was being driven by the accumulation of many different types of mutations. The two populations from which the DPI isolates were taken showed a wide variety of σ^{38} expression phenotypes, in contrast with the ST 14028 ancestor, which showed a normal distribution of σ^{38} expression.

These experiments demonstrate that domestication in laboratory conditions is extremely rapid, and depending on selection regimens may affect numerous pathways regulating the development of a highly conserved phenotype. The de Gelder model and fluctuation analysis suggest also that there may be higher than published mutation rates at the *rpoS* locus, which is consistent with the hypothesis that during evolutionary history this locus has been the site of numerous insertions and deletions.

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