Divergence Involving Global Regulatory Gene Mutations in an *Escherichia coli* Population Evolving under Phosphate Limitation

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Accepted: 18 June 2010

Abstract

Many of the important changes in evolution are regulatory in nature. Sequenced bacterial genomes point to flexibility in regulatory circuits but we do not know how regulation is remodeled in evolving bacteria. Here, we study the regulatory changes that emerge in populations evolving under controlled conditions during experimental evolution of *Escherichia coli* in a phosphate-limited chemostat culture. Genomes were sequenced from five clones with different combinations of phenotypic properties that coexisted in a population after 37 days. Each of the distinct isolates contained a different mutation in 1 of 3 highly pleiotropic regulatory genes (*hfq*, *spoT*, or *rpoS*). The mutations resulted in dissimilar proteomic changes, consistent with the documented effects of *hfq*, *spoT*, and *rpoS* mutations. The different mutations do share a common benefit, however, in that the mutations each redirect cellular resources away from stress responses that are redundant in a constant selection environment. The *hfq* mutation lowers several individual stress responses as well the small RNA–dependent activation of *rpoS* translation and hence general stress resistance. The *spoT* mutation reduces ppGpp levels, decreasing the stringent response as well as *rpoS* expression. The mutations in and upstream of *rpoS* resulted in partial or complete loss of general stress resistance. Our observations suggest that the degeneracy at the core of bacterial stress regulation provides alternative solutions to a common evolutionary challenge. These results can explain phenotypic divergence in a constant environment and also how evolutionary jumps and adaptive radiations involve altered gene regulation.

Key words: *Escherichia coli* genomics, experimental evolution, stress responses.

Experimental evolution studies combined with genomics are beginning to reveal not only the mechanistic aspects of evolutionary change but also the underlying principles (Barrick et al. 2009). An inherent but poorly understood feature of evolution is the tendency to diversify. Divergence is evident from the richness of the biosphere and can also be studied in experimental populations of microbes (Rainey and Travisano 1998; Zhong et al. 2004; Barrett and Bell 2006; Maharjan et al. 2006; Kinnersley et al. 2009; Rozen et al. 2009). Ecological interactions have been proposed to contribute to population heterogeneity: examples include divergence in structured environments (Rainey and Travisano 1998), with alternative resources (Zhong et al. 2004; Barrett and Bell 2006), cross-feeding, and cheating between population members (Treves et al. 1998) and seasonal specialization and niche construction (Rozen et al. 2009).

Another source of co-persistence of diverged forms is from alternative adaptations intrinsic to the organism. An example is the coexistence of yeasts with two different modes of energy metabolism but equal competitive fitness (Gudelj et al. 2007). Parallel metabolic and transport adaptations were also proposed to explain diversity in *Escherichia coli* evolving under glucose limitation (Maharjan et al. 2006, 2007). Multiple alternative metabolic pathway solutions can also result in diversity with the same selection condition (Portnoy et al. 2008). Based on these indications, the hypothesis we test is that inherent degeneracy in cellular processes leads to divergent means of getting fit within the same environment without ecological partitioning. Biological degeneracy occurs at many levels (Edelman and Gally 2001); here, we demonstrate that alternative solutions to reorienting gene regulation can provide degenerate paths to fitness. Furthermore, we show that when mutations beneficially affect global regulatory genes, considerable phenotypic divergence can be rapidly achieved with a few alternative mutational steps, setting the scene for adaptive radiations.

Evolutionary jumps often involve altered patterns of gene regulation (Wittkopp et al. 2004; Hunter 2008) and regulatory gene changes correlate with adaptive radiations (Barrier et al. 2001). The annotation of transcriptional regulatory networks indicates that bacterial regulation is also diverse within and between bacterial species (Lozada-Chavez et al. 2006). Regulatory changes have been noted in evolving experimental populations: Altered global gene expression in yeast (Gresham et al. 2008) and *E. coli* (Philippe et al. 2007) are accompanied by altered epistatic interactions with other global regulators (Cooper et al. 2008). Still untested, however, is whether alternative, parallel paths to fitness also arise within the same population in a constant environment.

Divergence is evident in chemostats under controlled conditions (Zhong et al. 2004; Maharjan et al. 2006; Kinnersley et al. 2009). Here, we use limitation for inorganic phosphate (Pi) as the simplest possible nutritional selection condition, with glucose as the carbon source. The utilization of Pi by *E. coli* involves accumulation followed by incorporation into adenosine triphosphate (Torriani 1990). Nevertheless, continued Pi-limitation involves more than induction of the *pho* regulon (Torriani 1990) responsible for Pi scavenging because the reduced growth rate forced by limitation turns on global hunger and starvation responses (Ferenci 1999). Pi-limitation induces the general stress response regulated by RpoS or sigma factor σ^S (Bougdour et al. 2006), which controls expression of 10% of the genome (Weber et al. 2005). Pi-limitation also elevates the intracellular level of ppGpp (Spira et al. 1995), in turn inducing the stringent response that affects as many as 500 genes (Durfee et al. 2008). The reported experiments used a specific growth rate of 0.1 per hour, in which these responses are triggered (Ferenci 2007). RpoS and ppGpp are central to redirecting transcription between vegetative and stress/starvation states of *E. coli* and other bacteria, as summarized in figure 1. These nutrient limitation settings in global regulation are central to understanding the evolutionary selection conditions and set the scene for the experimental results below.

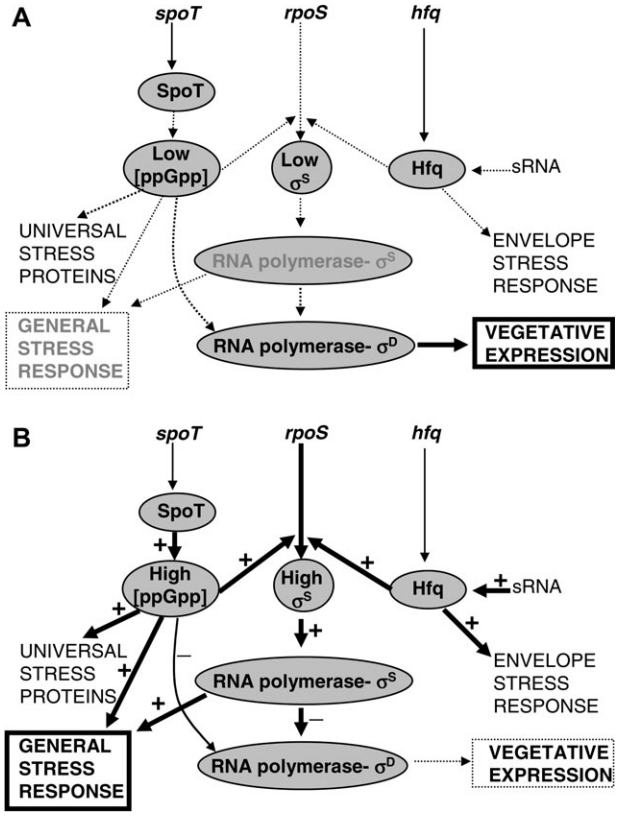

Fig. 1.—Regulation in the induction of stress responses in *Escherichia coli*. In unstressed, vegetatively growing bacteria (*A*), RNA polymerase mainly initiates transcription by complexing with the sigma factor σ^D, and stress responses are uninduced. Under stressed conditions or when the growth rate is limited in chemostats (*B*), stress responses are activated. The *rpoS* gene encodes the RpoS protein, the sigma factor σ^S that interacts with core RNA polymerase to express several hundred genes involved in the general stress response (Hengge-Aronis 2002). Elevated levels of σ^S result in reduced transcription by σ^D and decreased expression of transporters, ribosomes, and metabolic functions (Ferenci 2005). *spoT* encodes a bifunctional enzyme controlling the level of the signal molecule ppGpp in the cell (Potrykus and Cashel 2008). Under slow growth and starvation conditions, SpoT is involved in elevating ppGpp levels, which in turn has a positive role in *rpoS* expression. ppGpp is also involved in modulating RNA polymerase activity at certain promoters (dotted arrow) and a role in induction of other proteins, for example, universal stress proteins (dashed arrow, [Magnusson et al. 2005]). Hfq in the presence of small RNAs is important in the translational control of *rpoS* expression as well as in other stress responses governed by other small RNAs, for example, the envelope stress response (dashed arrow, [Gottesman 2004]). Mutations in any of the *spoT*, *rpoS*, or *hfq* genes results in a shift of expression away from stress responses and toward vegetative functions transcribed by σ^D. The + and – in the figure denote activation or inhibition of steps, respectively. The thickness of the line denotes the contribution of the signals under the given conditions.

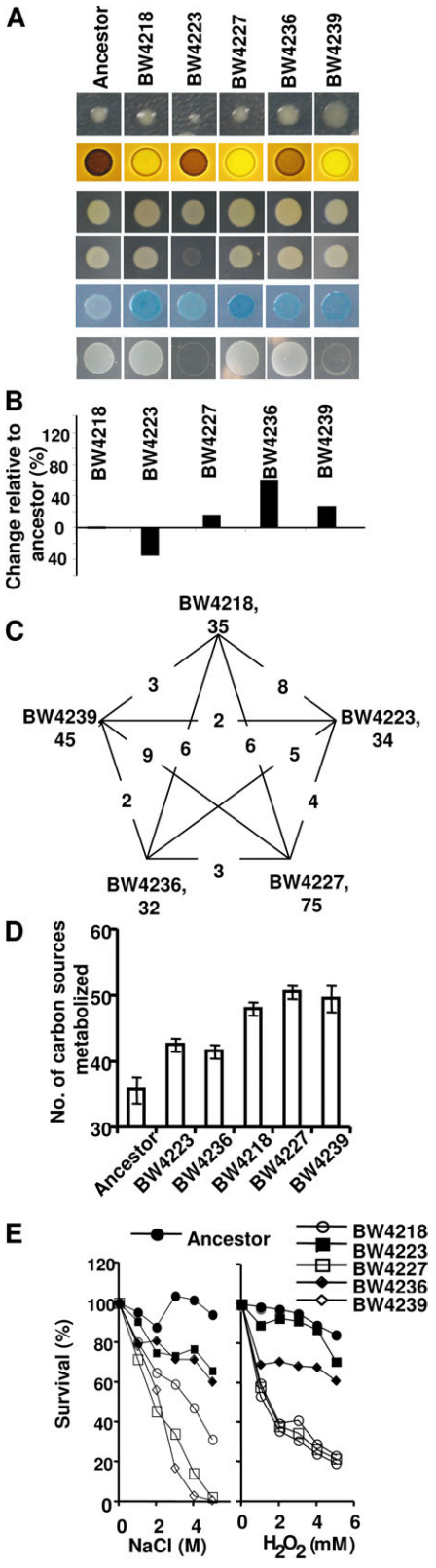

Fig. 2.—Phenotypic and proteomic diversity in five *Escherichia coli* clones coevolved from the parental strain MC4100TF. (*A*) The phenotype of the five isolates from day 37 was characterized in five different ways (from top to bottom): colony morphology after growth on LB; staining with iodine for RpoS status; sensitivity to 1% methyl α-glucoside on glycerol plates; staining with X-P (5-bromo-4-chloro-3-indolylphosphate) for AP activity; and sensitivity to 3% SDS on L-agar plates. (*B*) The growth yield of isolates in Pi-limited chemostats at dilution rate 0.1 per hour (% change relative to ancestor) measured as a mean of four estimations is shown. (*C*) The number of protein changes under Pi-limitation detected by proteomics (more than 1.5-fold in three replicates relative to ancestor) are shown by the number next to each strain, which reflects the number of proteomic differences to ancestral levels. The numbers on lines indicate the number of changes shared by any pair of strains. (*D*) The number of C-sources metabolized was measured with 95 substrates in replicate 96-well Biolog plates as previously described (Maharjan et al. 2007). (*E*) Stress survival of isolates was compared with osmotic and oxidative stress by viable counts at increasing concentrations of NaCl and hydrogen peroxide.

Phenotypic Differences between Coexisting Isolates from Pi-Limited Chemostats

A Pi-limited population growing at a dilution rate of 0.1 per hour (or ~7-h doubling time) changed considerably over 37 days of continuous propagation. By day 37, the population exhibited considerable colony heterogeneity. Five isolates with different combinations of the traits shown in figure 2*A* were chosen for detailed analysis.

To define the global extent of regulatory divergence, proteomic analysis of the five strains was undertaken (detailed results in supplementary table S1, Supplementary Material online). There were >30 protein changes relative to ancestor in each strain but only a minority were shared by isolates (fig. 2*C*). These results suggest divergent regulatory patterns under identical growth conditions. The genomic basis of divergence was investigated by complete high-coverage assembly of the five genomes and comparison with ancestor (Ferenci et al. 2009). Multiple mutations were found in all isolates (table 1): here, we focus mainly on the astonishing result that distinct global regulatory mutations were found in each of the five isolates.

We found 2–6 mutations in the strains shown in table 1, with BW4239 containing 6 but the others 2–3 mutations. In total, there were 13 mutations, and most were single nucleotide polymorphisms. No mutator mutations were found in the sequenced genomes. Three of the clones shared two mutations (in *valS* and intergene between *insC-isrC*) not obviously contributing to fitness; presumably, these mutations arose in the population before the different regulatory mutations.

Fitness through *hfq*, *rpoS*, and *spoT* Mutations

A single *hfq* mutation (Q52H substitution in the RNA chaperone Hfq) was found in BW4223. Hfq is essential for the regulatory effects of dozens of small RNAs involved in

Table 1
DNA Changes in the Evolved Genomes

Strain	Region/Gene	Product	Genome Position	Type	Nucleotide	Codon	Amino acid Change
BW4218	ig (*insC-isrC*)	NA	1961811	SNP	A -> G	NA	NA
	ig (*rpoS* leader)	Transcript involved in translational control of *rpoS*	2751440	SNP	C -> A	NA	NA
	valS	Valyl-tRNA synthetase	4418389	SNP	C -> T	GCG -> GTG	R736L
BW4223	***hfq***	RNA-binding protein	4337201	SNP	G -> T	CAG -> CAT	Q52H
	lysZ	tRNA (lys)	683353–683559	Deletion	NA	NA	NA
BW4227	ig (*insC-isrC*)		1961811	SNP	A -> G	NA	NA
	rpoS	RNA polymerase (σ^S)	2750861	Indel	C -> *	Frameshift	NA
	valS	Valyl-tRNA synthetase	4418389	SNP	C -> T	GCG -> GTG	R736L
BW4236	ig (*insC-isrC*)	NA	1961811	SNP	A -> G	NA	NA
	spoT	Bifunctional (p)ppGpp synthetase II	3710130	SNP	A -> T	AAC -> TAC	N459Y
	valS	Valyl-tRNA synthetase	4418389	SNP	C -> T	GCG -> GTG	R736L
BW4239	*menD*	Bifunctional 2-oxoglutarate decarboxylase	2261978	SNP	G -> T	CGC -> CTC	A370E
	gabD	Succinate-semialdehyde dehydrogenase I	2675241	SNP	G -> A	ATG -> ATA	M45I
	rpoS	RNA polymerase (σ^S)	2751015	SNP	T -> C	TTG -> TCG	N124S
	trkH	Potassium transporter	3921075	SNP	T -> A	CTG -> CAG	L80Q
	ig (*purH-rrsE*)	NA	4095306	SNP	C -> T	NA	NA
	trkG	Potassium transporter subunit	1314085	Insertion (IS2)	NA	NA	NA

NOTE.—ig, intergene; *, absent at given position; NA, not applicable; SNP, single nucleotide polymorphism. Genes involved in global regulation are in bold. Genome positions are based on genome sequence of *Escherichia coli* K-12 strain BW2952 (Ferenci et al. 2009).

regulation in *E. coli* (Gottesman 2004), including stress responses through control of RpoS translation (fig. 1). The phenotypic effects described below indicate that Q52H affects several regulatory roles.

This mutation is sufficient to confer fitness under Pi-limitation when the Q52H allele is transferred to ancestor, as shown in figure 3*A*. A remarkably diverse set of effects were caused by *hfq* in BW4223 but explicable from the known pleiotropy (Tsui et al. 1994) and diverse roles of Hfq in regulation: alterations in glucose uptake (Vanderpool 2007) leading to sensitivity to methyl-α–glucoside, decreased RpoS levels (Hengge-Aronis 2002) and increased outer membrane permeability (causing elevated detergent sensitivity) (Valentin-Hansen et al. 2007) are all present (figs. 2*A* and 3*D*). The decrease in RpoS was 18% but significantly different to wild type (*t*-test, $P = 0.0017$). All these properties were evident when the regulatory mutation was transferred to a clean ancestral background without the other mutations in the isolate (fig. 3*A–F*). Thus, the *hfq* mutation was largely responsible not only for fitness but also the multiple property changes of BW4223, and indeed, the isolate in which the *hfq* mutation was replaced by the wild-type allele had ancestral properties as shown in figure 3*A–F*. This suggests that the *lysZ* mutation in BW4223 does not significantly contribute to the evolved properties.

Three different *rpoS* mutations were found by genomic sequencing of BW4218, BW4227, and BW4239: two intragenic and causing a null phenotype and one upstream of *rpoS* in the transcribed sequence before the *rpoS* gene (table 1). The upstream mutation is in a region involved in mRNA secondary structure formation that is involved in the translational control of *rpoS* expression (Hengge-Aronis 2002). The mutation may prevent the activation of the RNA transcript (removal of the secondary structure) and hence decrease *rpoS* translation.

All three mutations provide a strong fitness benefit under Pi-limitation and in BW4218 or BW4227, the *rpoS* mutation was the dominant fitness determinant in the isolate (fig. 3*A*). The established benefit of *rpoS* mutations at slow growth rates is in the relief of repression of vegetative genes through competing sigma factors (fig. 1, [Ferenci 2005]). In BW4239, other mutations besides *rpoS* contributed to fitness, and replacement of the *rpoS*4239 mutation with the wild-type allele only partly reduced the selection coefficient of the isolate. The nature of the benefit conferred by the other mutations in BW4239 is not yet known but will be investigated in the future.

Another evolved regulatory difference in the same population was a *spoT* mutation in BW4236 (table 1). The positive selection coefficient of BW4236 was almost entirely due to the *spoT* mutation (fig. 3*A*). SpoT is a bifunctional enzyme that can either degrade or synthesize ppGpp, a stress alarmone in *E. coli* (Gentry and Cashel 1996). Under starvation conditions, the elevation of ppGpp is due to SpoT involvement. The most direct effect of the N459Y substitution was to reduce ppGpp levels relative to ancestor (from 0.092 ± 0.004 to 0.052 ± 0.001 relative units). Unsurprisingly, the *spoT* mutation in BW4236 also changed multiple properties, including reduced RpoS consistent with the ppGpp–RpoS relationship (Spira et al. 2008) and a markedly increased growth yield (fig. 2*B*). The other mutations in BW4236 did not contribute to fitness or phenotypes (fig. 3*A–F*).

The N459Y substitution has not been previously described in SpoT, but mutations close to this site were found in the Lenski experimental populations increasing fitness in glucose culture (Philippe et al. 2007). In contrast to our results, they did not find decreased ppGpp, and the benefit was in the increased growth yield, which we also observe in batch culture (fig. 3*C*). Under Pi-limitation, the RpoS reduction is also a likely benefit as discussed above.

Phosphate Transport and *pho* Regulation in Evolved Isolates

Previous studies showed that *pho* genes are negatively regulated by RpoS (Taschner et al. 2004), so relief of this repression is a likely benefit either through *rpoS* mutations or perhaps indirectly through *hfq* and *spoT* mutations. As shown in figure 4, the isolates and strains with the regulatory mutations all exhibited somewhat elevated transport rates when measured with ^{32}Pi. However, the increase relative to ancestor was highest in BW4239, in which fitness (fig. 3*A*) and transport (fig. 4*E*) are both boosted by mutations other than the *rpoS* mutation. The regulatory mutations in isolation all increase transport, in line with a common reduction in negative regulation by RpoS. The *hfq* mutation has the lowest positive effect (fig. 4*B*), and this is mirrored by the minor increase in *phoA* gene expression shown in figure 3*B*. These small increases in transport-related functions are in contrast to glucose-limited populations, in which evolved transport rates are boosted up to 8-fold (Maharjan et al. 2006, 2007).

A Shared Fitness Trade-off through *hfq*, *spoT*, and *rpoS* Mutations

A shared consequence of the regulatory mutations was the reduction in RpoS levels, as shown in figure 2*A* and figure 3*F*. In confirmation of the staining data, even in the non-*rpoS* mutants BW4223 and BW4236, the RpoS protein levels dropped by 21% and 32%, respectively when measured by quantitative immunoblotting (Spira et al. 2008). In this respect, the *rpoS*, *spoT*, and *hfq* mutations were convergent in resetting the stress protection and nutritional competence (SPANC) balance, the trade-off between SPANC determined by RpoS (fig. 1, [Ferenci 2005]). Each mutation changed the SPANC balance toward better nutrition not only under Pi-limitation but also in general; all the regulatory mutations permitted more carbon sources to be metabolized, as tested with 95 Biolog substrates (fig. 2*D*). So the effect of the mutations was evident in unselected traits like acetate utilization. Conversely, the common cost of the adaptations was decreased stress resistance, as seen with high osmolarity or peroxide stress (fig. 2*E*). Altogether, these results demonstrate that the strategy of improving nutritional capability has degenerate mutational solutions. The changes to Pi uptake observed in figure 4 are also part of the global regulatory change toward better nutrition through reduced negative control by RpoS.

Conclusions

These results have broad implications for evolutionary diversification in general and regulatory divergence in particular.

1. Our results demonstrate that there is sufficient internal regulatory flexibility in a living organism to simultaneously undertake divergent adaptive pathways in the same environment. The importance of regulatory mutations was supported by our observation that each of the five distinct regulatory mutations was integral to the fitness of the isolates under Pi-limitation (fig. 3*A*). Hence, reordering of the regulatory network at the core of bacterial transcriptional control shown in figure 1 was essential in adapting to Pi-limitation. Even if the different regulatory adaptations do not persist to fixation, the transitory coexistence of the different types allows parallel exploration of the mutational possibilities for further evolution in the different backgrounds.
2. The data indicate that the stress/nutrition trade-off is a driver of diversification. This is consistent with results on natural isolates of the species *E. coli* as well as with *E. coli* populations in infections (Levert M, Zamfir O, Clermont O, Bouvet O, Lespinats S, Hipeaux MC, Branger C, Picard B, Saint-Ruf C, Norel F, Balliau T, Zivy M, Le Nagard H, Cruvellier S, Chane-Woon-Ming B, Nilsson S, Gudelj I, Phan K, Ferenci T, Tenaillon O, Denamur E, unpublished data; Spira B, Galbiati HF, Betteridge T, Phan K, Ferenci T, unpublished data). Hence, changing of the SPANC balance in members of the species extends to nonlaboratory situations. Our ancestral strain has endogenous levels of ppGpp and RpoS toward the higher end for natural isolates (King et al. 2004), which accentuates the cost of stress resistance. Nevertheless, RpoS/ppGpp levels are still higher in several naturally stress-resistant isolates and in some pathogenic lineages of *E. coli*, and these carry an even greater nutritional cost than in our ancestor. Trade-offs are generally important in life-history traits such as between fecundity and survival (Stearns 1992), so the regulatory rebalancing we describe has broader implications in explaining the role of trade-offs in evolution.
3. The indications are that biological degeneracy is important in evolution not simply to add complexity (Edelman and Gally 2001) but also to increase evolvability (Lenski et al. 2006). We find that degeneracy contributes to adaptation: the possibility of three alternative adaptations contributes to the capacity to evolve and indeed an increased capacity for risk hedging. In the specific examples studied here, the different *rpoS*, *hfq*, and *spoT* mutations may have similar benefits under Pi-limitation but very different fitness profiles in other environments. For example, as shown in figure 2, osmotically stressful environments counterselect more against the *rpoS* null mutations

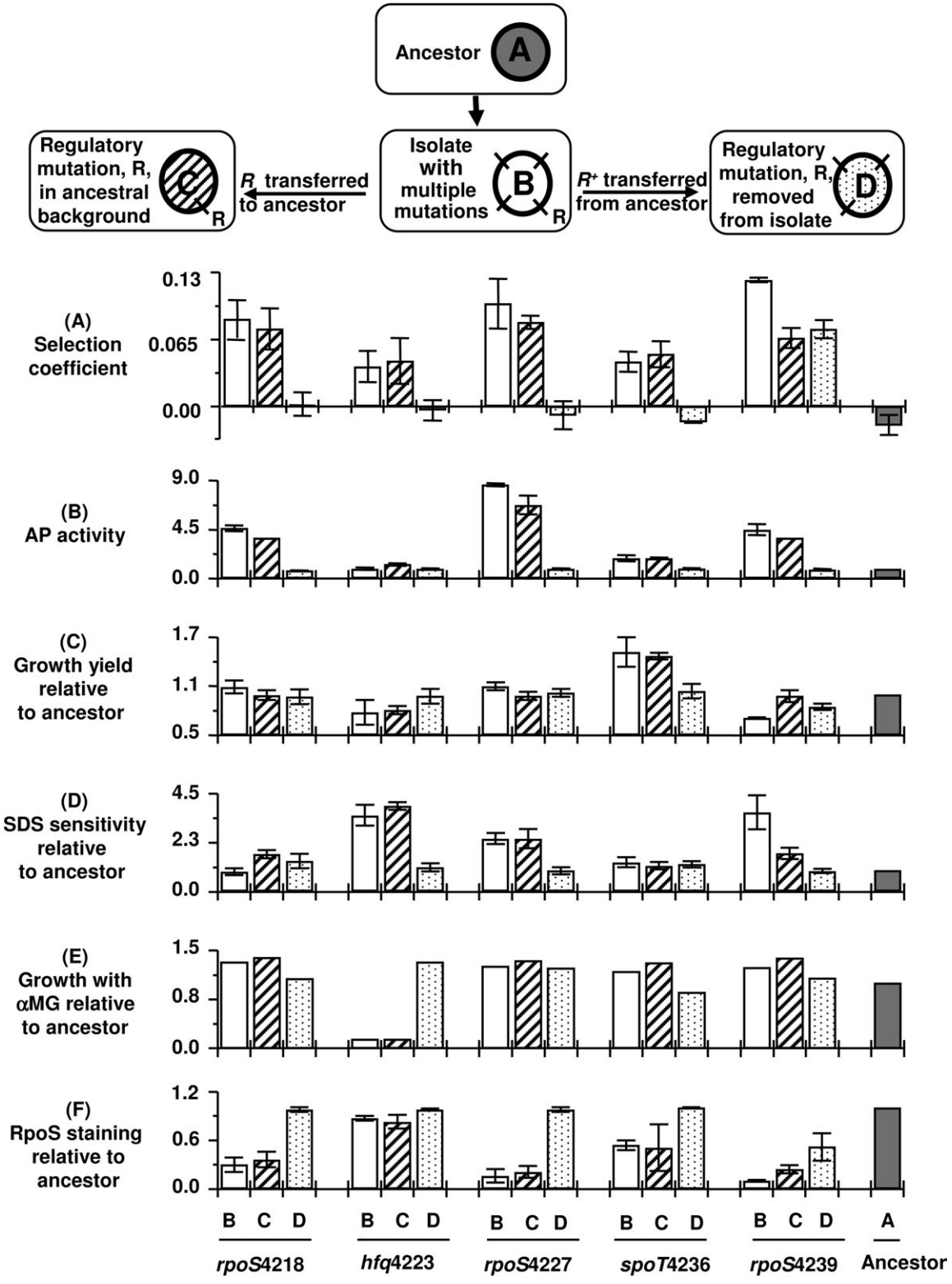

Fig. 3.—Fitness and phenotypes in strains with altered regulatory genes. We compared the five evolved isolates (Type B in the schematic) and two classes of derivative strains manipulated for each regulatory allele by cotransduction with linked markers (see Materials and Methods). In one class, each mutated regulatory gene (*R*) was transferred into a clean ancestral background (Type C). In the other class, the regulatory mutation present in each isolate was replaced by the ancestral regulatory gene (Type D) while retaining the other evolved mutations. (*A*) Competitive fitness was measured for Type B, C, and D strains in Pi-limited chemostats. Competitions were all against a reference strain BW3454 containing a *metC::Tn10* countable marker;

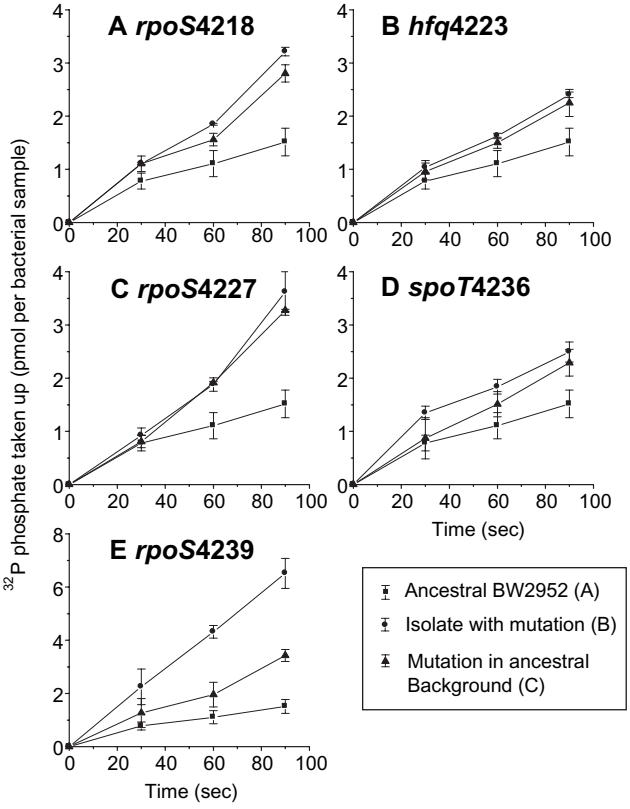

Fig. 4.—The effect of regulatory mutations on transport rates measured with ^{32}Pi. Pi-limited chemostat cultures were cultured for 30 h to establish limitation and then assayed for transport rates when measured with a low concentration (1 μM) ^{32}Pi. In each panel, the ancestral BW2952 strain (■) is compared with the chemostat isolate containing the mutation shown (●) as well as the type C strain from figure 3 in which the regulatory mutation is in a clean ancestral background (□). Each assayed sample contained 5×10^7 bacterial cells.

but membrane-damaging conditions such as detergents are more detrimental for *hfq* strains. In effect, functional and regulatory degeneracies provide an increased scope for gaining fitness, just as duplicated genes allow increased evolutionary options.

4. A conclusion from this study is that the emergence of alternative adaptations does not require distinct niches, confirming conclusions from other studies (Maharjan et al. 2006, 2007; Gudelj et al. 2007; Portnoy et al. 2008). Furthermore, our results suggest that sympatric divergence (in the biogeographical sense, in the same environment, see [Fitzpatrick et al. 2008]) may be the result of internal subspecialization of degenerate regulatory capabilities. Our experiments provide a case of sympatric divergence where there is no question of allopatry, whereas in natural populations it is extremely difficult to exclude allopatry through fine-grained subdivision of the habitat (Fitzpatrick et al. 2008). More speculatively, our results indicate how sympatric divergence can arise in a population biology sense (where interbreeding is reduced in the same environment [Fitzpatrick et al. 2008]). If highly global regulatory specialization is common, then this itself could cause reduced compatibility for interbreeding. The high frequency of major regulatory changes in evolution (Wittkopp et al. 2004; Hunter 2008) may provide the basis for sympatric speciation.

Materials and Methods

Growth Media and Strains

Bacteria were cultured in T-salts minimal medium supplemented with glucose (0.2% w/v) plus 30 μM KH_2PO_4 for chemostats or 1 mM KH_2PO_4 for batch culture (Spira et al. 1995). Bacteria for phenotypic tests were grown or minimal medium A or L-broth (as described by Miller [1972]). All growth was at 37 °C. For long-term chemostats, MC4100TF was grown overnight in T-salts and inoculated into an 80-ml chemostat containing T-salts, 0.2% glucose and 30 μM KH_2PO_4 as described (Spira and Ferenci 2008). The bacterial concentration in the chemostat was stable through 37 days, between 1.5 and 2.5×10^8 bacteria/ml.

The strains used in this study are described in table 2. The different alleles of *rpoS*, *hfq*, and *spoT* were transferred from evolved strains into ancestral strain or from ancestral strain into evolved strains by P1 transduction as described in Miller (1972). For the transfer of the *spoT* mutation, *zib563*::Tn*10* was used as the linked selection marker. For the transfer of *rpoS* and *hfq* from evolved isolates into ancestral strain or from ancestral into evolved isolates, we first constructed *cysD::amp* and *purA::tet* strains using the protocol described in Yu and Court (1998). The proximity of *cysD::Amp* locus to *rpoS* and *purA::tet* locus to *hfq* allowed cotransduction (>90% cotransduction in both cases). The transductants were tested for alleles by sequencing.

Detection of *rpoS* Status

The level of RpoS was assessed by staining glycogen with iodine; the intensity of the brown color varies according

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this strain is marginally fitter than ancestor. Competing strains were mixed 50:50 after 16 h individual acclimatization in chemostats. Selection coefficients (Dykhuizen and Hartl 1983) were calculated from changes in population proportions. The mean and standard deviations were obtained from 3 to 5 replicates. (*B*) The alkaline phosphatase was measured using ONP-Pi as substrate after growth in media containing 30 μM KH_2PO_4. (*C*) The growth yield of strains was measured from the absorbance in L-broth after overnight growth to steady state and shown relative to ancestor. (*D*) Sensitivity to 3% SDS was measured by growth in microtitre plates, with densities divided by that of the ancestor shown. (*E*) Sensitivity to 1% methyl α-glucoside (α-MG) was measured in patches on glycerol plates. Patches were scanned densitometrically, and density relative to ancestor is shown. (*F*) The RpoS level was estimated by glycogen staining as in figure 2 and scanning densities relative to that of ancestor.

Table 2
Strains Used in the Study

Strains	Relevant Genotype	Reference or Origin
MC4100TF	F-*araD139 D(argF-lac)U169 rspL150 deoCl relA1 thiA ptsF25 flb5301 rbsR*	Spira et al. (2008)
BW4218	Chemostat evolved isolate	This study
BW4223	Chemostat evolved isolate	This study
BW4227	Chemostat evolved isolate	This study
BW4236	Chemostat evolved isolate	This study
BW3454	MC4100TF *metC162*::Tn*10*	Notley-McRobb and Ferenci (1999)
BW4239	Chemostat evolved isolate	This study
BW5151	DY330 *purA*:: Tn*10*	This study
BW5153	MC4100TF *purA*:: Tn*10*	This study
BW5166	MC4100 *hfq*4223	This study
BW6006	BW4223 *hfq*4100	This study
BW5197	BW4236 *spoT*4100TF	This study
BW5199	MC4100 *spoT*4236	This study
BW5200	MC4100TF *zib563*::Tn*10*	Spira et al. (2008)
BW6007	DY330 *cysD*::*amp*	This study
BW6008	MC4100TF *cysD*::*amp*	This study
BW6009	BW4218 *cysD*::*amp*	This study
BW6010	BW4227 *cysD*::*amp*	This study
BW6011	BW4239 *cysD*::*amp*	This study
BW6012	MC4100 *rpoS*4218	This study
BW6013	MC4100 *rpoS*4227	This study
BW6014	MC4100 *rpoS*4239	This study
BW6015	BW4218 *rpoS*4100	This study
BW6016	BW4227 *rpoS*4100	This study
BW6017	BW4239 *rpoS*4100	This study
DY330	W3110 Δ*lacU169* gal490 λcl857 Δ(*cro-bioA*)	Yu et al. (2000)

to the level of σ^S in the cell (Notley-McRobb et al. 2002). For quantitation, photographs were scanned densitometrically across 2-μl spotted patches on L-agar using Image J software and densities related to ancestor values. For blots, bacterial cultures were grown overnight in LB medium at 37 °C. Proteins from 2×10^9 cells were resolved by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis in a 12.5% gel, and RpoS in blots detected with diluted monoclonal anti-RpoS antibodies (NeoClone). The Super Signal West Pico kit (Pierce) was used to detect the RpoS bands as recommended by the manufacturer. The signal intensities on autoradiograms were scanned and computed using the Image J software. At least three replicate cultures were used and tested for statistical significance.

Alkaline Phosphatase Assay

p-nitrophenyl-phosphate (p-NPP) was used as substrate as described (Spira et al. 1995), and AP activity units are defined as the increase in absorbance at 410 nm/min. Optical cell density at 600/nm.

SDS Susceptibility Assay

Sensitivity to SDS was assayed from overnight cultures grown in L-broth by spotting of cultures (2 μl) onto L-agar plates containing 3% (w/v) SDS. Liquid cultures containing 3% SDS were followed by measuring absorbance of 6-fold replicates of strains in L-broth in microtitre plates.

Methyl α-glucoside (α-MG)

To assay sensitivity to α-MG, culture (2 μl) was spotted onto minimal medium A 0.2% glycerol agar plate with or without 1% α-MG. For quantitation, photographs were scanned densitometrically across the growth patches using Image J software and densities related to ancestor values.

Growth Yields

The yields were determined by measuring optical density of the cultures at 600 nm.

Biolog Assay

The catabolism of the starting strain and the chemostat isolates with 95 substrates were determined using the commercially available Biolog GN2 (Biolog) as previously described (King et al. 2004).

Stress Resistance Assays

Bacteria from overnight cultures in L-broth were washed twice and diluted in 0.9% NaCl to a density of $\sim 4 \times 10^3$ cells/ml. For oxidative stress, freshly diluted H_2O_2 was added to 1 ml culture to final concentrations of 1, 2, 3, 4, and 5 mM and held at room temperature for 30 min. For osmolarity, suspensions of 4×10^3 cells/ml were incubated in 1, 2, 3, 4, 5 M NaCl for 1 h at room temperature.

Pi Uptake Assay

For transport assays, 500 μl bacteria from 30-h-old Pi-limited chemostat cultures were mixed with 5 μl of 100 μM KH_2PO_4 and 10 μl of 1μCi ^{32}P/μl (MP Biomedicals). Samples (100 μl) taken at time points were filtered through pore size 0.45-μm filters, washed immediately with 5-ml washing solution (T-salt plus 100 μM KH_2PO_4). The uptake rates were determined by measuring the scintillation of ^{32}P in the 5×10^7 cells on the filters.

ppGpp Assay

Cells growing exponentially in T-salts/glucose were supplemented with and 0.25 mM ^{32}P-KH_2PO_4 (100 μCi/ml) at an $OD_{600} = 0.2$. Samples were harvested after 70, 80, and 90 min. The labeled samples were analyzed as in Spira et al. (2008).

Fitness Experiments in Chemostats

For fitness comparisons, a tetracycline-resistant derivative of MC4100TF carrying a *met*C::Tn10 insertion was used,

and medium was supplemented with 4 μg/ml methionine. Chemostat competitions were as previously described (Maharjan et al. 2006) and the selection coefficients based on the equations in Dykhuizen and Hartl (1983).

Proteomics and genomics details and strategies are described in the supplementary tables S1 and S2 (Supplementary Material online) and associated legends.

Supplementary Material

Supplementary tables S1 and S2 are available at *Genome Biology and Evolution* online (http://www.oxfordjournals.org/our_journals/gbe/).

Acknowledgments

We thank Alex Uren and Anna Yeung for characterizing isolates in chemostats. R.P.M. and C.M. were supported by Australian Research Council grants to T.F. and P.R.R. B.S. was supported by the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP—Brazil). L.W. and L.F. were partly supported by the National Natural Science Foundation of China (NSFC) Key Programs Grant 30530010, the Chinese National Science Fund for Distinguished Young Scholars (30788001), NSFC General Program Grants 30870070, 30870078, and 30771175, National 863 Program of China Grants 2006AA020703 and 2006AA06Z409, National 973 Program of China Grant 2009CB522603, and National Key Programs for Infectious Diseases of China 2008ZX1004-002, 2008ZX1004-009, and 2009ZX10004-108.

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Associate editor: Emmanuelle Lerat