Environmentally constrained mutation and adaptive evolution in *Salmonella*

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The relationship between environment and mutation is complex [1]. Claims of Lamarkian mutation [2] have proved unfounded [3–5]; it is apparent, however, that the external environment can influence the generation of heritable variation, through either direct effects on DNA sequence [6] or DNA maintenance and copying mechanisms [7–10], or as a consequence of evolutionary processes [11–16]. The spectrum of mutational events subject to environmental influence is unknown [6] and precisely how environmental signals modulate mutation is unclear. Evidence from bacteria suggests that a transient recombination-dependent hypermutational state can be induced by starvation [5]. It is also apparent that changes in the mutability of specific loci can be influenced by alterations in DNA topology [10,17]. Here we describe a remarkable instance of adaptive evolution in *Salmonella* which is caused by a mutation that occurs in intermediate-strength osmotic environments. We show that the mutation is not 'directed' and describe its genetic basis. We also present compelling evidence in support of the hypothesis that the mutational event is constrained by signals transmitted from the external environment via changes in the activity of DNA gyrase.

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Received: 1 October 1999
Revised: 5 November 1999
Accepted: 5 November 1999

Published: 6 December 1999

Current Biology 1999, 9:1477–1480

0960-9822/99/$ – see front matter

Results and discussion

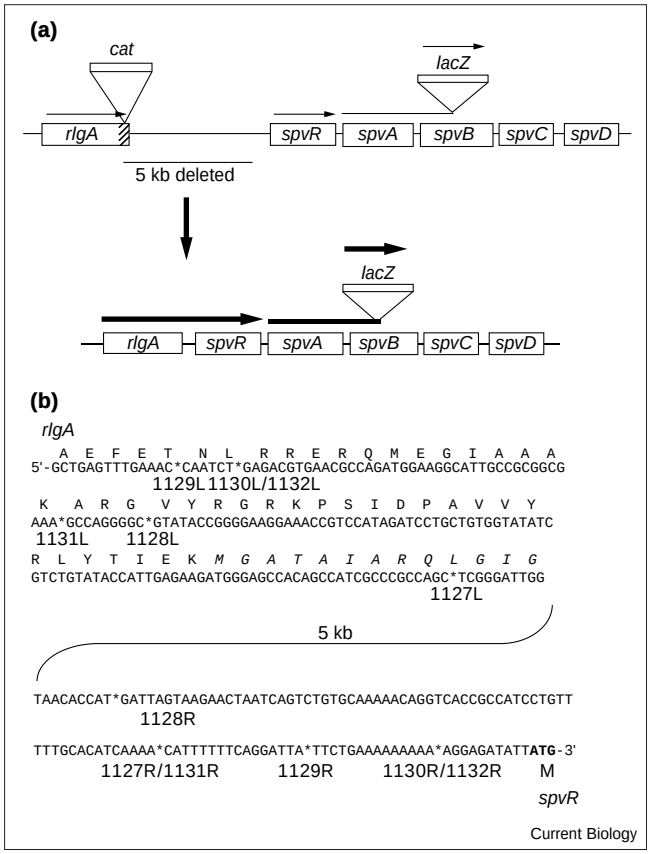
Salmonella typhimurium strain CJD671 contains a *lacZ* operon fusion to the stress-induced *spvB* gene, located on a 90 kilobase (kb) plasmid, which places expression of β-galactosidase under the control of regulatory influences that affect *spvB* transcription [18] (Figure 1a). After overnight growth on MacConkey lactose indicator plates, individual cells of strain CJD671 form pale pink colonies indicative of low levels of *lacZ* expression. After a further 2 days growth spontaneous mutant forms of CJD671 appear repeatedly as dark red papillations within these colonies. Analysis of *lacZ* expression in 10 independently isolated red mutants showed that these produced 70-fold more β-galactosidase activity than the ancestral culture. Red mutants also evolve repeatedly in a broth-based equivalent of MacConkey-lactose (LS-lac; see Materials and methods). Six replicate populations founded from CJD671 all became polymorphic within 48 hours. In each population, red mutants comprised 21.3% (standard deviation (SD) 7.5) of the total number of cells and this increased to 44.8% (SD 5.1) after 72 hours. When a sample from each of the 3-day evolved populations was placed in fresh media and subjected to five serial transfers at 24 hour intervals, the frequency of red mutants remained at 44.4% (SD 5.3).

Among the red mutants, we isolated a class that had undergone a deletion of DNA from the *spv* region of the plasmid. Analysis of six independent mutants of this class (CJD1127–1132) revealed loss of ~5 kb from the region immediately upstream of the *spv* locus that resulted in a transcriptional fusion between *spvR* and an upstream gene, *rlgA* (Figure 1b). These six mutants showed variability in the sequence at the novel junction, although in each instance the deletion resulted in loss of the carboxy-terminal domain of RlgA (Figure 1b), a protein that belongs to the resolvase family of site-specific recombinases (GenBank accession number AF029069). As resolvases often autoregulate their genes and have their DNA-binding domain within the carboxyl terminus [19], we hypothesised that the deletion rendered the truncated RlgA protein incapable of autorepressing the *rlgA* gene. This would generate an unregulated *rlgA–spvR* operon leading to overexpression of the SpvR protein and strong upregulation of the *spvB–lacZ* fusion (Figure 1a). Consistent with this hypothesis, introduction into the six mutants of an intact copy of the *rlgA* gene cloned in a multicopy plasmid caused repression of *spvB–lacZ* expression.

These six mutants were grown in direct competition with the ancestor to quantify the selective advantage of the red mutant in LS-lac [20]. In populations founded with equal numbers of competing genotypes no significant difference was observed between the mean fitness of the mutant and the ancestor (Table 1). This suggested that the fitness of the mutant might depend on its frequency. When competing populations of mutant and ancestral genotypes were founded at a ratio of mutant to ancestor

of 1:100, the red mutant had a highly significant fitness advantage (Table 1). When the ratio of the rare to common genotype was reversed, the mean fitness of the mutant genotype was equal to the mean fitness of the rare (ancestral) genotype. The red mutant and the ancestor were competed (at a mutant to ancestor ratio of 1:100) in LS-lac without NaCl and then in LS supplemented with a range of different carbon sources (Table 1). The data showed that the fitness benefit of the mutant was highly specific to environments containing lactose and was not affected by the osmolarity of the medium. This suggests that the red mutant is favoured by selection as a result of its enhanced capacity to scavenge lactose. The fitness of the mutant is frequency dependent and declines as the mutant becomes common; however, it never decreases below the fitness of the ancestor. The polymorphism is therefore not stable and shows an interesting asymmetry not previously described in evolving microbial populations.

Figure 1



The *spv* locus on the 90 kb plasmid in *S. typhimurium* strain CJD671 and six mutant derivatives. (a) The structural genes *spvABCD* form an operon and transcription of the *spvB–lacZ* fusion is under growth phase control and is activated *in vitro* on entry to the stationary phase of growth. Activation requires the *trans*-acting LysR-like transcription factor SpvR, which is expressed from a gene adjacent to, and immediately upstream of, the *spvABCD* structural gene operon [18]. The *rlgA* gene lies 5 kb upstream of *spvR*. This is also autoregulated and encodes a member of the resolvase family of site-specific recombinases. The hatched portion contains the left-hand deletion end-points in the six independent mutants CJD1127-32. A *cat* gene insertion was made in this DNA segment in strain CJD1280. Thin or thick horizontal arrows represent weak or strong transcription, respectively. The figure is not drawn to scale. (b) Sequence of the left and right deletion end-points in the six independent mutants CJD1127-32. The DNA sequence coding for the carboxyl terminus of the RlgA protein is shown. This corresponds to the hatched area in the *rlgA* gene in (a). The amino-acid sequence is shown in single-letter notation. The italicised amino acids comprise the putative helix-turn-helix DNA-binding motif of the protein. The *rlgA–spvR* 5 kb intergenic region is represented by the curved line. The translation initiation codon of the *spvR* gene is shown in bold type. Deletion end-points are represented by asterisks; a mutant number followed by L or R represents the left or right end-point, respectively, of that deletion.

When the ancestral genotype was propagated for 3 days in the range of media listed in Table 1, the mutant arose only in LS-lac. It was not detected in cultures propagated in LS-lac without NaCl. This was unexpected given that competition experiments showed the fitness of the red mutant to be unaffected by osmolarity (Table 1). This led us to conclude that the genetic event necessary for evolution of all classes of red mutants was constrained by the environment, in this instance the signal appeared to be osmolarity. We found a clear osmolyte dose dependency: more red mutants evolved at intermediate rather than high or low salt concentrations (Figure 2a). When populations of CJD671 were selected in LS-lac in which

Table 1

Effect of frequency and environment on relative fitness of the red mutant.

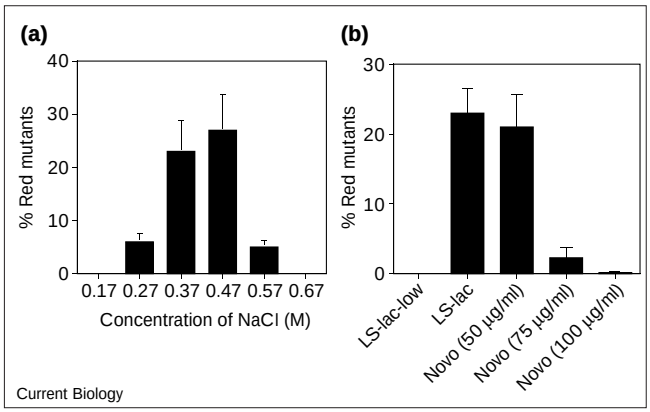
Environment	Initial ratio of competing genotypes (RM:AN)	Relative mean fitness of red mutants
LS-lac	1:1	1.02 (0.87–1.17)
LS-lac	1:100	1.85 (1.78–1.92)***
LS-lac	100:1	1.00 (0.92–1.10)
LS-lac-low	1:100	1.82 (1.74–1.91)***
LS-lac-low	100:1	1.03 (1.00–1.05)
LS	1:100	0.96 (0.89–1.03)
LS-glu	1:100	0.99 (0.93–1.05)
LS-gal	1:100	1.01 (0.94–1.09)
LS-glu-gal	1:100	1.03 (0.97–1.09)
LS-gly	1:100	1.03 (0.98–1.07)
LS-cit	1:100	1.01 (0.94–1.09)
LS-suc	1:100	1.01 (0.94–1.08)

RM, red mutant; AN, ancestor. Data are averages (mean) and 95% confidence intervals based on the *t* distribution with 5 degrees of freedom. ***$p < 0.001$ by ANOVA. LS is Luria broth containing 0.47 M NaCl. Three-letter codes denote carbon sources added to LS to a final concentration of 1% (w/s): lac, lactose; glu, glucose; gal, galactose; gly, glycerol; cit, citrate; suc, sucrose. LS-lac-low is low osmolarity LS-lac, that is, Luria broth containing lactose but no additional NaCl.

an equiosmolar concentration of sucrose was substituted for NaCl, red mutants arose after 2 days growth. Thus, the constraint was a general effect of osmolarity and not specific to NaCl.

Although many examples of constraints on adaptive evolution are known [21,22], genetic understanding of mechanistic detail is typically lacking. The possibility that the homologous recombination pathway contributed to the evolution of the red mutants was ruled out when red mutants were found to arise from a *recA* derivative of strain CJD671 after 2 days of selection in a manner identical to the wild-type strain. Therefore, we looked to the resolvase protein as a possible effector of the deletion event. A disrupted *rlgA*::*cat* gene was introduced into the 90 kb plasmid by allelic exchange. The *cat* insertion had been placed within the 3′ end of the *rlgA* gene at a location that inactivated the gene but left the left-hand deletion end-point intact (Figure 1a). This was important because deletions, should they occur, could still cause the *rlgA–spvR* operon fusion necessary for *spvB–lacZ* overexpression. No mutants of the class described above evolved in LS-lac from CJD671 containing the defective *rlgA* gene, confirming that the 5 kb deletion formation required a functional resolvase. The absence of site-specificity of action of the resolvase at the junction end-points suggests that RlgA did not evolve to act at these sites.

Figure 2



Frequency of red mutant evolution as a function of environmental modulation. (a) The effect of osmolyte concentration. 0.47 M NaCl was the optimal concentration, with no mutants being detected in cultures at low (0.17 M) or very high (0.67 M) concentrations of NaCl. The numbers of bacterial colonies screened were between 1,000 and 1,200 for each salt concentration. Data are means and SD from three replicate experiments. A similar dose-dependent response was seen when sucrose was used as the osmolyte. (b) The effect of sublethal concentrations of the DNA gyrase inhibitor novobiocin (Novo) on the ability of red mutants to evolve in LS-lac (none evolved in the low osmolarity medium, LS-lac-low). At concentrations at or above 75 μg/ml novobiocin, the frequency of red mutant evolution is severely reduced. Data are averages (mean) and SD from three replicate experiments.

Resolvase activity is influenced by DNA topology and by the physiology of the bacterial cell [23]. In high osmolarity environments DNA is more negatively supercoiled [24] and it is possible that under these conditions the RlgA resolvase is able to interact with its DNA substrate in a manner that triggers the deletion. Moreover, DNA structural transitions have been found to drive mutational events in other systems [25]. To test the hypothesis that the osmolyte is an environmental trigger that alters the structure of DNA, leading to deletion of the 5 kb *spvA–spvR* intergenic region, populations of ancestral CJD671 were evolved in LS-lac containing sublethal concentrations of the antibiotic novobiocin (see Supplementary Material). This antibiotic specifically targets the ATP-binding B domain of DNA gyrase, depriving it of energy and resulting in a loss of negative supercoiling activity. Inhibiting gyrase with this antibiotic avoids the pleiotropic effects associated with other gyrase inhibitors such as quinolones. We observed a clear dose-dependent effect, with fewer than 1% red mutants being detected after 48 hours of selection in the presence of 100 μg/ml novobiocin (Figure 2b).

The serendipitous co-location of the resolvase gene with the artificially created *lacZ* fusion allows the high-level *lacZ*-expressing mutants to evolve. The fact that this mutation does not occur in low-osmolarity LS-lac, even though it would be selectively advantageous, provides a striking example of an evolutionary constraint, which although genetic in nature, is directly influenced by the external environment. In this instance the architecture of the DNA appears to be the conduit through which the environmental signal is transduced. Given that DNA transactions, including those that lead to mutation, are sensitive to variations in DNA structure, and that such structural changes occur in response to a variety of environmental stresses, it is probable that the mutations described here represent a specific instance of a general phenomenon. This is supported by the observation that all red mutations were blocked in a low osmolarity environment, and that all were blocked in a high osmolarity environment in which DNA gyrase activity was inhibited. A global role for DNA supercoiling in the regulation of gene expression in response to environmental stress has already been proposed [17]. The data presented here extend this proposal to include mutagenic events. It is probable that environmentally provoked events of this type, in combination with conventional gene regulation, can contribute to the ability of bacteria to adapt to environmental stress.

Finally, this work shows how an environmental factor, lacking any inherent ability to damage DNA, can nonetheless influence specific mutational events. An environmentally determined constraint of this kind has the potential to evolve by selection into an information-transducing mechanism that could affect the rate and nature of random variation

generated. Such a mechanism might serve to channel evolutionary change by specifying what is likely and would hasten the rate and direction of evolution [12,16,26].

Materials and methods

Media and growth conditions

LS is Luria broth (LB [27]) containing 0.47 M NaCl. LS-lac is LS containing 1% (w/v) lactose. For amendments see footnote to Table 1. All cultures were propagated with aeration at 37°C.

Fitness assays

Competitive fitness was determined by direct competition between wild type and mutant strains in 1 ml shaken broth cultures [20]. Relative fitness was calculated as the ratio of the Malthusian parameters of the two strains being compared. Cultures were founded with 10^5 cells of each competitor for 1:1 competitions, or 10^5 and 10^3 for invasion from rare experiments. The ratio of ancestral to red mutant genotypes was determined after 24 h growth by plating on lactose indicator plates [27] and scoring the ratio of red to pale pink colonies.

Bacterial strains and plasmids

Ancestral *S. typhimurium* strain CJD671 is a derivative of the wild-type strain LT-2A containing a MudJ element in the *spvB* gene. The *recA* derivative of strain CJD671, CJD1275, was constructed by electroporating the large plasmid from CJD671 into the otherwise isogenic strain CH23. The *rlgA*::*cat* insertion mutant strain CJD1280 was constructed by standard methods (details available upon request). Genetic manipulations were performed using standard techniques [28]. Details of the oligonucleotide primers, plasmids and other materials used are available upon request.

Red mutant detection

Deletions of DNA in the *rlgA–spvR* intergenic region were detected by Southern blotting [28] and verified by PCR amplification of the 500 base pair (bp) junction fragment. Details of probes, oligonucleotides and techniques are available upon request.

Enzyme assays

β-Galactosidase assays were carried out on SDS/$CHCl_3$-permeabilised cells [27]. Triplicate cultures were each assayed in duplicate. Results are the mean of the six measurements. Standard deviations were less than 10%.

Supplementary material

Supplementary material including a figure and methodological detail of the novobiocin experiments is available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements

We thank Mark Ridley and Paul Sniegowski for comments on the manuscript. This work was supported by grants 041878 from the Wellcome Trust (UK) and 94–95 and 07–98 from the Health Research Board (Ireland) to C.J.D. P.B.R. acknowledges the financial support of the Biotechnology and Biological Sciences Research Council (UK).

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Supplementary material

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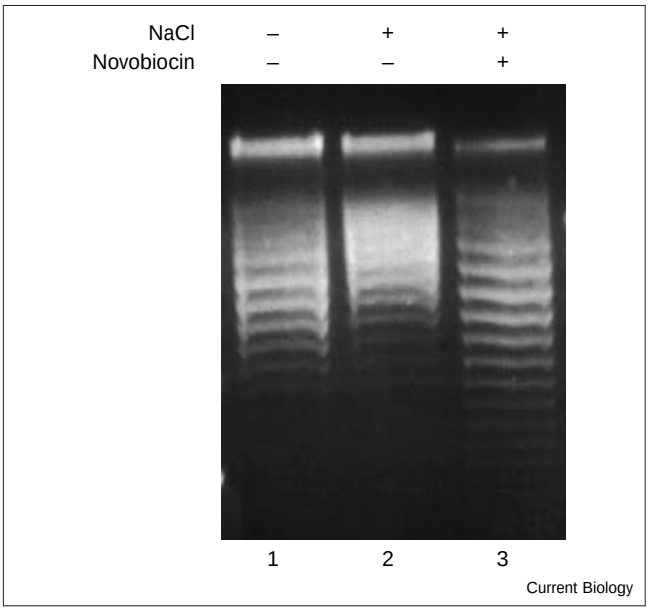
Current Biology 6 December 1999, 9:1477–1480

Figure S1



Salmonella typhimurium strain CJD671 was transformed with the 4 kb cloning vector plasmid pACYC184. This plasmid was used as a reporter of DNA supercoiling in cultures grown in LS-lac-low growth medium (lane 1), the same medium containing 0.47 M NaCl (LS, lane 2), and LS containing 100 μg/ml novobiocin (lane 3). The presence or absence of supplements is shown by a '+' or '–' sign, respectively. Plasmids were isolated and electrophoresed in agarose–chloroquine gels as described previously [S1]. The direction of electrophoresis is from top to bottom; topoisomers that were more negatively supercoiled in the bacteria migrate more slowly and are near the top of the gel, those that were more relaxed migrate faster and are further down the gel. Increased osmolarity resulted in more negatively supercoiled plasmid topoisomers (compare lanes 1 and 2) but novobiocin treatment counteracts the effects of osmolarity and relaxes the DNA (lane 3).

Supplementary references

S1. Higgins CF, Dorman CJ, Stirling DA, Waddell L, Booth IR, May G and Bremer E: A physiological role for DNA supercoiling in the osmotic regulation of gene expression in ***S. typhimurium*** and ***E. coli***. *Cell* 1988, 52: 569-584.