DOI: 10.1534/genetics.104.035717

Long-Term Experimental Evolution in *Escherichia coli*. XII. DNA Topology as a Key Target of Selection

Estelle Crozat,* Nadège Philippe,* Richard E. Lenski,† Johannes Geiselmann* and Dominique Schneider*,[1]

**Laboratoire Adaptation et Pathogénie des Microorganismes, Université Joseph Fourier, Institut Jean Roget, CNRS UMR 5163, F-38041 Grenoble, France and †Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, Michigan 48824*

Manuscript received September 3, 2004
Accepted for publication October 29, 2004

ABSTRACT

The genetic bases of adaptation are being investigated in 12 populations of *Escherichia coli*, founded from a common ancestor and serially propagated for 20,000 generations, during which time they achieved substantial fitness gains. Each day, populations alternated between active growth and nutrient exhaustion. DNA supercoiling in bacteria is influenced by nutritional state, and DNA topology helps coordinate the overall pattern of gene expression in response to environmental changes. We therefore examined whether the genetic controls over supercoiling might have changed during the evolution experiment. Parallel changes in topology occurred in most populations, with the level of DNA supercoiling increasing, usually in the first 2000 generations. Two mutations in the *topA* and *fis* genes that control supercoiling were discovered in a population that served as the focus for further investigation. Moving the mutations, alone and in combination, into the ancestral background had an additive effect on supercoiling, and together they reproduced the net change in DNA topology observed in this population. Moreover, both mutations were beneficial in competition experiments. Clonal interference involving other beneficial DNA topology mutations was also detected. These findings define a new class of fitness-enhancing mutations and indicate that the control of DNA supercoiling can be a key target of selection in evolving bacterial populations.

EVOLUTION experiments allow one to study the dynamics and genetic bases of microbial evolution, and this approach has become increasingly widespread in recent years (Elena and Lenski 2003). The longest-running evolution experiment involves 12 populations of *Escherichia coli* founded from the same ancestral strain, which have been serially propagated in the same glucose-limited medium for >20,000 generations (Lenski *et al.* 1991; Lenski and Travisano 1994; Cooper and Lenski 2000; Lenski 2004). The populations improved their performance as demonstrated by the large fitness gains seen when the evolved bacteria compete against their ancestor in the same environment (Cooper and Lenski 2000). Many genetic changes have been found in these evolved lines including point mutations, deletions, inversions, and insertions of insertion sequence elements (Sniegowski *et al.* 1997; Papadopoulos *et al.* 1999; Schneider *et al.* 2000; Cooper *et al.* 2001a, 2003; Lenski *et al.* 2003). To date, mutations at two loci (*rbs* and *spoT*) have been conclusively demonstrated to be beneficial in the evolutionary environment (Cooper *et al.* 2001a, 2003), but their effects explain only a small fraction of the total fitness improvement. Both loci are mutated in most or all of the independently derived lineages, indicating parallel adaptive evolution.

In the experiment cited above, each day the evolving populations experienced a lag phase followed by exponential growth, then depletion of the limiting glucose, and finally stationary phase (until transfer into fresh medium the next day). Such transitions are known to influence DNA topology (Hatfield and Benham 2002; Reyes-Dominguez *et al.* 2003). DNA supercoiling is dynamically modified during many environmental challenges, including the transition from growth to stationary phase (Balke and Gralla 1987), nutrient upshift (Reyes-Dominguez *et al.* 2003), anaerobiosis (Bhriain *et al.* 1989), thermal stress (Goldstein and Drlica 1984), oxidative stress (Weinstein-Fischer *et al.* 2000), osmotic stress (Higgins *et al.* 1988), and acid stress (Karem and Foster 1993). Phenotypic acclimation by bacteria to these challenges requires rapid changes in expression of many genes, and one important acclimatory response is the transient modification of supercoiling, which can produce genome-wide changes in rates of transcription (Jovanovich and Lebowitz 1987; Pruss and Drlica 1989; Steck *et al.* 1993; Gmuender *et al.* 2001). The topology of DNA therefore helps to coordinate the gene regulatory networks of bacteria in response to varying environments. The maintenance of

[1]*Corresponding author:* Laboratoire Adaptation et Pathogénie des Microorganismes, Facultés Médecine/Pharmacie, Université Joseph Fourier, Institut Jean Roget, 38700 La Tronche, France.
E-mail: dominique.schneider@ujf-grenoble.fr

proper DNA topology is also required for cell viability, since it affects many processes in bacteria including replication, repair, transcription, recombination, and transposition. The level of DNA supercoiling is tightly regulated in the cell by the combined activities of topoisomerases (Champoux 2001) and histone-like proteins (Dorman and Deighan 2003). In *E. coli*, three main topoisomerases are involved in maintaining DNA supercoiling: topoisomerases I (Wang 1971) and IV (Zechiedrich *et al.* 2000) relax DNA, while DNA gyrase introduces negative supercoils (Gellert *et al.* 1976). Histone-like proteins constrain the supercoiling level by binding to DNA and regulating the expression of the topoisomerase-encoding genes (Dorman and Deighan 2003).

In this study, we sought to determine whether the level of DNA supercoiling might have changed during the evolution experiment, because the populations experienced daily challenges of nutrient upshift and exhaustion. If so, such changes would suggest candidate loci for further study by sequencing, genetic manipulation, and analyses of phenotypic effects.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions: Twelve populations of *E. coli* B were started from two genotypes that differed by only a single neutral marker (arabinose utilization), and they were propagated for 20,000 generations at 37° in a glucose-limited defined medium (Lenski *et al.* 1991; Lenski and Travisano 1994; Cooper and Lenski 2000; Lenski 2004). Six populations, designated Ara−1–Ara−6, were founded from the ancestor that is Ara^- (unable to use arabinose as a carbon source) and six others, Ara+1–Ara+6, from an Ara^+ revertant of the ancestor. The arabinose utilization phenotype serves as a marker in competition experiments and was shown to be neutral under these conditions (Lenski *et al.* 1991). We used three clones sampled at random from each of the 12 populations at generations 2000, 10,000, and 20,000 (Cooper and Lenski 2000). Population Ara−1 served as the focal population in this study and was the source of the evolved *topA* and *fis* alleles. Strain 606 fis^{Δ} has an in-frame *fis* deletion and was constructed (D. Schneider, unpublished data) using the suicide-plasmid pKO3 (Link *et al.* 1997). This deletion construct is presumed to be otherwise isogenic to the ancestor (REL606) and was used as a control in immunoblot analyses.

We used plasmids pUC18 (Yanisch-Perron *et al.* 1985) for DNA supercoiling measurements, pKO3 (Link *et al.* 1997) for allele replacements, and pCRII-Topo (Invitrogen, San Diego) for cloning experiments.

All experiments were performed by growing strains either in the same Davis glucose (25 μg/ml) minimal medium (DM25) that was used in the evolution experiment (Lenski *et al.* 1991) or in rich Luria broth (LB) medium (Sambrook *et al.* 1989). Ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), or kanamycin (50 μg/ml) were added as needed.

DNA supercoiling measurements: Topological changes during evolution were measured by analyzing the relative abundance of different topoisomers of a reporter plasmid. The pUC18 plasmid was introduced to various strains by electrotransformation (Bio-Rad, Richmond, CA, 200 ohm, 25 μF, 2.5 kV). Electrocompetent cells were prepared as described elsewhere (Sambrook *et al.* 1989). It should be noted that the way we measured DNA topology allowed us to assess changes only in the activity of the nicking-closing enzymes (*i.e.*, topoisomerases). The superhelix density *in vivo* is also affected by other DNA-binding proteins (Lilley 1986; Bliska and Cozzarelli 1987).

Bacteria containing pUC18 were grown in LB to an $OD_{600nm} = 2$ (or in DM25 to the end of exponential growth). Cells were harvested and pUC18 was extracted using the QIAprep Spin Miniprep Kit (QIAGEN, Chatsworth, CA). The same amount of plasmid DNA (200 ng) from each strain was loaded onto 1% agarose gels containing 1.5 μg/ml chloroquine. The gels were run overnight at 4° at 3 V/cm. Under these conditions, more highly supercoiled topoisomers migrate faster (Higgins *et al.* 1988). Chloroquine was washed from the gels by soaking for 4 hr in water, and photographs were taken under UV light after ethidium bromide staining. Because such gels are sensitive to buffer conditions, we checked that negative supercoils occur under the choloroquine concentration that we used. It is known that the relative mobility of the different topoisomers varies with chloroquine concentrations (Higgins *et al.* 1988); at a concentration of 25 μg/ml, more supercoiled topoisomers migrate more slowly, while more relaxed ones migrate faster. As expected, at that higher concentration, we obtained the symmetrical outcomes compared to those obtained at a chloroquine concentration of 1.5 μg/ml (data not shown).

Quantitative comparisons of DNA topology between each evolved clone and the ancestor were performed by scanning the relevant gels. Densitometric analyses were performed to obtain the relative abundance of all of the plasmid topoisomers for each clone, and the average topoisomer value was computed from these data for each clone, including the ancestor. To estimate the level of DNA supercoiling, mean σ-values were calculated according to the formula $\sigma = \Delta Lk/Lk_0$, where Δ Lk is the difference in the linking number (*i.e.*, difference in average topoisomer value) between two clones and Lk_0 for pUC18 is 2686 bp/10.45 bp/turn = 257. The σ-value for the ancestor was expressed relative to the midpoint topoisomer of plasmid that was completely relaxed by using calf-thymus topoisomerase I and assigned $\sigma = 0$. Comparing the topoisomer distribution of plasmid DNA from the ancestor with the completely relaxed plasmid control yielded an estimate of $\sigma = -0.066$ for the ancestor. We emphasize that the midpoint topoisomer of the completely relaxed plasmid was used only to establish a baseline value. All comparisons between evolved clones and the ancestor and between the various isogenic constructs used the complete distribution of topoisomers obtained by densitometry. The σ-value for each evolved clone or isogenic construct was obtained by adding or subtracting its average topoisomer value to that of the ancestor for more supercoiled or more relaxed molecules, respectively. For conversion, one negative supercoil difference corresponds to a difference in σ of −0.0039. The resulting σ-value for each evolved clone and isogenic construct was then compared to that of the ancestor. These analyses thus allowed a precise quantitative comparison of the distribution of plasmid topoisomers between each clone and the ancestor.

Changes in DNA topology were measured after growing the various clones in LB medium. However, the populations had evolved in a glucose-limited minimal medium. Therefore, we also measured the supercoiling of the reporter plasmid after growth in the same minimal medium as used in the evolution experiment. Cell densities and molecular yields are lower in the minimal medium, but similar changes in DNA topology were observed in minimal and LB media (data not shown). All subsequent experiments on supercoiling were performed using cells grown in LB medium.

Electrophoresis and immunoblot analyses of proteins: Triplicate cultures of the ancestor carrying the ancestral *fis* allele,

an evolved *fis* allele, or a complete deletion of *fis* were prepared in LB, and aliquots were sampled after 1, 1.5, 2.5, and 5 hr of incubation at 37°. (Under these conditions, the duration of the lag phase was ~0.75 hr, and after ~5 hr the cultures enter into stationary phase.) Cell pellets were resuspended in lysis buffer (20 mM HEPES pH 7.4, 1 mM EDTA, 10% glycerol, 1 mM DTT) before sonication. After centrifugation at 10,000 × *g* for 30 min at 4°, total protein concentrations were determined using the Bradford protein assay kit (Bio-Rad) and with bovine serum albumin as a standard. Equal amounts of protein samples were subjected to 16% SDS-polyacrylamide gel electrophoresis, and the separated proteins were then electrotransferred onto polyvinylidene difluoride membranes (Amersham Pharmacia). Prestained protein standards (Amersham, Buckinghamshire, UK) were used for molecular weight estimation. Immunoblot analyses were performed with antibodies against Fis and RpoA (courtesy of A. Ishihama and M. Cashel, respectively). The blots were developed using ECL (Amersham Pharmacia) or nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Sigma) systems, and the staining intensity was quantified using the ImageGauge (Fujifilm) software.

Strain construction: The *topA* and *fis* mutations, alone and in combination, were introduced into the ancestral genetic background by using the suicide plasmid pKO3 (Link *et al.* 1997). Briefly, a PCR product containing ~500 bp of adjacent sequences on each side of the mutation first was cloned into pCRII-Topo and then subcloned into pKO3. After electrotransformation of the ancestor with the appropriate plasmid, chloramphenicol-resistant cells with a copy of this nonreplicative plasmid that had integrated into the chromosome were selected at high temperature. Subsequent selection on 5% sucrose-LB plates without NaCl (Blomfield *et al.* 1991) allows detection of plasmid loss, because the plasmid carries *sacB*, which renders cells sensitive to killing by sucrose. These plasmid-free cells were then screened for the presence of the evolved *topA* allele by PCR/restriction fragment length polymorphism (PCR/RFLP) using *Nmu*CI (Fermentas) to distinguish between ancestral and evolved alleles or for the presence of the *fis* allele by direct sequencing. As a further control for the precision of these manipulations, constructed strains were "deconstructed" (the introduced evolved alleles were replaced again by their ancestral counterparts, using pKO3) to check for unintended secondary mutations (Cooper *et al.* 2003); in all cases, the deconstructed strains were confirmed to have reversed the phenotypic effects of the introduced *topA* and *fis* evolved alleles. The same approach was used to replace the evolved alleles by the ancestral ones in some evolved clones.

Fitness assays: The relative fitness of two strains was estimated by directly competing them, as described elsewhere (Lenski *et al.* 1991). Briefly, isogenic ancestral strains with the introduced evolved *topA* allele, the evolved *fis* allele, or both were allowed to compete against the ancestor carrying the opposite neutral Ara marker (Lenski *et al.* 1991). Each pairwise competition was replicated at least fourfold, with competitions running for 1 day or, with 1:100 daily serial transfer, for 6 days (longer duration allows detection of smaller fitness effects) in the same medium as the evolution experiment. Both 1-day and 6-day competitions include all the same phases of population growth that occur in the serial-transfer regime, including a lag-phase prior growth, exponential growth, depletion of the limiting glucose, and stationary phase (Vasi *et al.* 1994). In both cases, competitors were separately acclimated to the same regime, then transferred together from stationary-phase cultures into fresh medium (with 1:200 dilution each, thus 1:100 combined). Samples of the competition assays were taken to measure the abundance of the competitors; these samples were always taken in stationary phase (24 hr after the previous transfer), such that only the duration of the competitions varied and not the physiological state of the cells or the time they spent in various states. Fitness was calculated as the ratio of the realized (net) population growth rates obtained for the two strains while they competed with one another; *t*-tests were performed to evaluate whether measured fitness values differed significantly from the null hypothetical value of one. The same basic protocol was also used to assess fitness variation among clones sampled from the evolution experiment; a one-way ANOVA was then performed to test whether there was significant variation in fitness among the clones.

Evolutionary dynamics of mutation substitutions: PCR/RFLP strategies were used to track the origin and spread of the mutations discovered in the focal population, using clones isolated from samples frozen during the evolution experiment. For the *topA* locus, numerous clones from generations 500, 1000, 1500, and 2000 were subjected to PCR using primers ODS177 (5′-GACTACGTGGTGAAATCCAG-3′) and ODS178 (5′-ACCAGG CAACACTTCATAGTG-3′). The presence or absence of the evolved *topA* mutation was determined by *Nmu*CI digestion of the PCR products. The evolved *fis* allele does not change any restriction sites. Therefore, PCR products obtained with primers ODS65 (5′-GTACCGAATTGCACGTAAAG-3′) and ODS141 (5′-CACCGTGAATAATGGTCTTAG-3′) for many clones isolated from 2000, 10,000, and 20,000 generations were directly sequenced using primer ODS65.

RESULTS

DNA topology changed during experimental evolution: To investigate DNA supercoiling, we examined reporter plasmid DNA that was introduced into, and then isolated from, 108 evolved clones (3 clones from each of the 12 long-term populations for each of three time points: 2000, 10,000, and 20,000 generations; Cooper and Lenski 2000). All measures were compared to the ancestor clone harboring the same reporter plasmid. The ancestor and evolved clones were grown in rich medium and, upon reaching stationary phase, plasmid DNA was extracted, quantified, and loaded onto chloroquine-agarose gels to separate topoisomers and visualize their distribution. In all cases, the 3 evolved clones from the same population and same generation gave identical results.

Three main trends emerge from the levels of DNA supercoiling observed in the various evolved clones relative to that measured in the ancestor (Figure 1). First, 10 of the 12 populations at generation 20,000 show increased supercoiling relative to their ancestor. Second, alterations of the DNA topology began early in the evolution experiment, with 9 populations showing changes by 2000 generations. These initial 2000 generations were also the period of most rapid fitness improvement (Lenski and Travisano 1994; Cooper and Lenski 2000). Third, three populations showed continued increases in supercoiling over time, in one case reaching a final σ-value difference of ~12% and in two others of ~17% relative to the ancestral state.

Only a few exceptions to these general trends were observed (Figure 1). In population Ara+1, the change in supercoiling was seen only transiently at 2000 generations. In Ara+3, supercoiling initially increased but be-

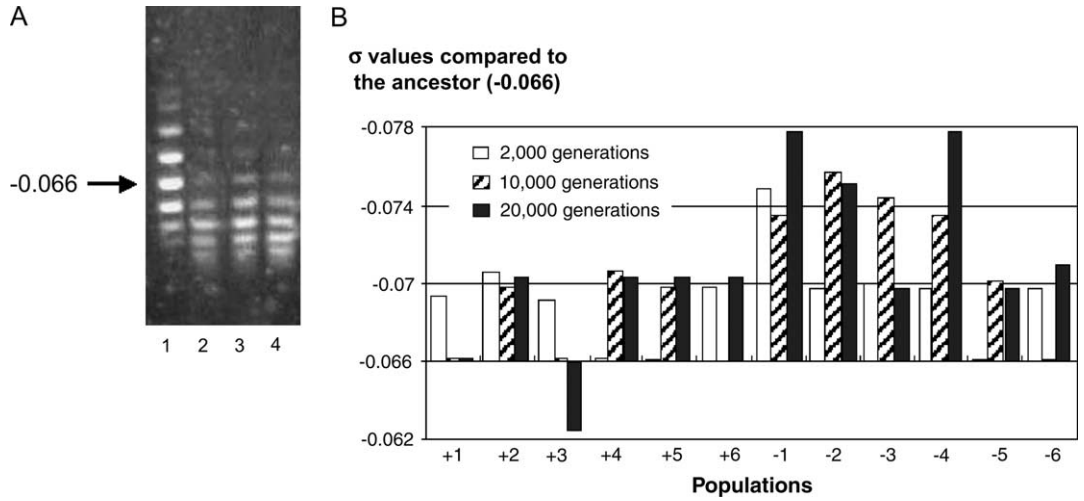

FIGURE 1.—Parallel changes in DNA topology during experimental evolution. Plasmid DNA was isolated from ancestral and evolved cells carrying reporter plasmid pUC18 and analyzed by electrophoresis on gels that allow visualization of the topoisomer distribution, with more tightly supercoiled topoisomers migrating faster (HIGGINS *et al.* 1988). (A) Gel for population Ara−1 with pUC18 extracted from the ancestor clone (lane 1) and evolved clones from 2000, 10,000, and 20,000 generations (lanes 2–4, respectively). Quantitative comparisons between each evolved clone and the ancestor are based on densitometric analyses of the gels (see MATERIALS AND METHODS). The indicated σ-value for the ancestor clone (−0.066) was obtained relative to the midpoint topoisomer of plasmid completely relaxed with calf-thymus topoisomerase I (see MATERIALS AND METHODS). (B) The change in supercoiling level is shown by bars for each evolved population, labeled as +1–+6 (Ara+1–Ara+6) and −1 to −6 (Ara−1–Ara−6). Open, hatched, and solid bars represent clones isolated at 2000, 10,000, and 20,000 generations, respectively. The σ-values calculated in evolved clones are compared to the ancestral level (−0.066), such that higher absolute values show increased supercoiling and lower absolute values indicate relaxation of DNA. The absence of a bar for +6 at 10,000 generations indicates that no measurement could be obtained (see text). Otherwise, short bars indicate the absence of any change in supercoiling.

came more relaxed than the ancestral state at 20,000 generations. In population Ara+6, the supercoiling level could not be measured for the 10,000-generation clones, because they grew slowly under the ampicillin selection required for maintenance of the reporter plasmid, and no plasmid DNA could be recovered. However, increased supercoiling was seen at 2000 and 20,000 generations in this population. Finally, Ara−6 showed no change in supercoiling at 10,000 generations, whereas it was increased at 2000 and 20,000 generations. During the course of the evolution experiment, four populations became mutators (SNIEGOWSKI *et al.* 1997; COOPER and LENSKI 2000), including Ara+3, Ara+6, Ara−2, and Ara−4. There is no apparent association of the evolved changes in DNA topology with mutator status.

Summarizing to this point, our results show consistent and parallel changes in the DNA supercoiling level in most evolved populations, with many changes already seen by 2000 generations. Rapid and parallel changes across independent lineages are indicative of adaptive evolution (BULL *et al.* 1997; COOPER *et al.* 2001a; NOSIL *et al.* 2002). These data therefore strongly suggest that the parallel changes in DNA topology are adaptive. To test this hypothesis further, we sought to identify the mutations responsible for increased supercoiling among the various genes involved in the control of DNA topology. We focused on changes in population Ara−1 for two reasons. First, this population has served as a focal lineage in several other studies of the long-term lines (PAPADOPOULOS *et al.* 1999; COOPER and LENSKI 2000; SCHNEIDER *et al.* 2000; COOPER *et al.* 2003). Second, the supercoiling level in this population increased in at least two successive steps, implicating the involvement of at least two mutations (Figure 1).

Identification of mutations altering DNA topology: By sequencing candidate loci in a clone isolated at 20,000 generations from population Ara−1, we found two mutations. One mutation is in *topA*, which encodes topoisomerase I, which in turn relaxes DNA (WANG 1971). The other mutation occurs in *fis*, which encodes the Fis protein that controls the level of DNA supercoiling (SCHNEIDER *et al.* 1999). The mutation in *topA* is a C-to-T substitution, leading to the change of the histidine 33 residue of topoisomerase I into a tyrosine. The mutation in *fis* is an A-to-C substitution, which lies four nucleotides upstream of the ATG translational start codon.

To analyze precisely the phenotypic effects of these two mutations, we constructed a set of isogenic strains by allelic replacements. We moved each mutation alone and in combination into the ancestral chromosome, replacing the corresponding ancestral allele. We also replaced the evolved alleles with their ancestral counterparts in some evolved clones. These isogenic strains, with

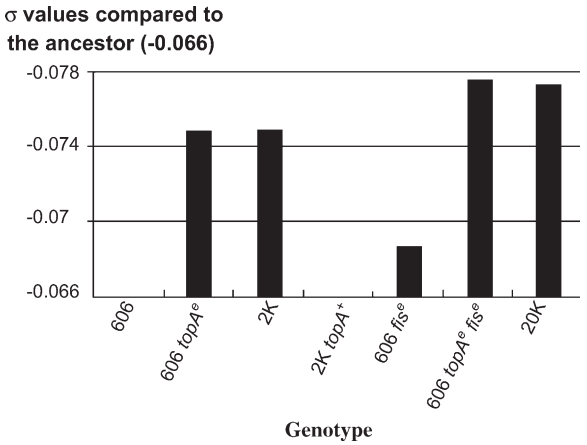

FIGURE 2.—Changes in DNA supercoiling generated by evolved *topA* and *fis* mutations. The changes in σ-values relative to the ancestral level (−0.066) are shown for various strains. The strain 606 is the ancestral strain; 2K and 20K are clones isolated from population Ara−1 at 2000 and 20,000 generations, respectively. All others are isogenic constructs made by replacing ancestral alleles with evolved alleles (denoted by superscript *e*) or by replacing an evolved allele with its ancestral counterpart (superscript +).

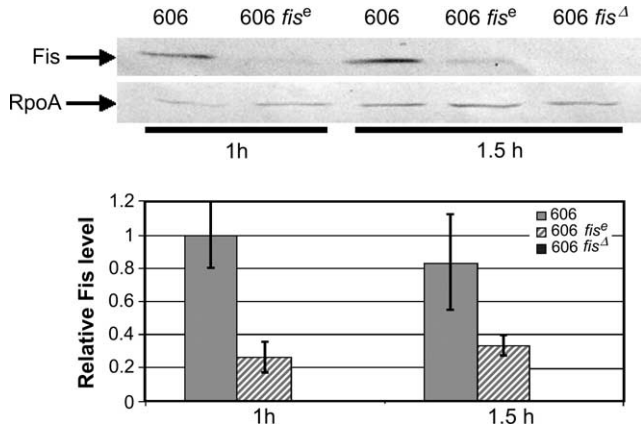

FIGURE 3.—Relative levels of Fis protein. Extracts were prepared from the ancestral strain (606), an isogenic strain with the evolved *fis* allele (606 fis^e), and a *fis*-deleted derivative of the ancestor (606 fis^Δ) after 1 and 1.5 hr of exponential growth in LB medium. Western blots and immunodetection assays are shown for an anti-Fis antibody (top) and, as a control, an anti-RpoA antibody (middle). (Bottom) The ratio of Fis to RpoA levels in the same strains, based on three replicate experiments with error bars indicating standard deviations. The ratio is arbitrarily set to 1 for the ancestral strain after 1 hr of exponential growth. No Fis protein was detected in the control strain with the *fis* gene deleted.

and without the *topA* and *fis* mutations, were then used to examine the effects of the evolved alleles on DNA supercoiling and on competitive fitness.

Both mutations contribute to altered DNA topology: The reporter plasmid was introduced into the isogenic strains, which were grown in LB, their plasmids extracted, and DNA topology measured as before. As shown in Figure 2, both the *topA* and *fis* mutations, when introduced separately into the ancestral chromosome, caused increases in DNA supercoiling. The *topA* mutation increased the σ-value by ∼12%, whereas the *fis* mutation increased it by ∼5%. When combined in the ancestral background, the two mutations had an additive effect, leading to a net increase of 17% relative to the ancestor. The effect of the *topA* mutation reproduces the DNA supercoiling level measured in clones from generation 2000 in population Ara−1, while the combined effects of the *topA* and *fis* mutations match the supercoiling observed in the 20,000-generation clones (Figure 2). Thus, the two mutations are sufficient to explain the observed changes in DNA topology in this population.

These changes suggest the substitution of the *topA* mutation before 2000 generations and the substitution of the *fis* mutation sometime later. Sequencing *topA* in a 2000-generation clone confirmed the presence of the same evolved allele as found at 20,000 generations. Replacing the evolved *topA* allele in the same 2000-generation clone with the ancestral allele restored supercoiling to the ancestral level (Figure 2). This result also precludes the presence at 2000 generations of compensatory mutations in the *gyrAB* genes, which have been found elsewhere to compensate for certain mutations in *topA* (DiNardo *et al.* 1982; Pruss *et al.* 1982). The absence of mutations in *gyrAB* was confirmed by sequencing the entirety of these genes in the 2000-generation clone. These data also imply a reduction of the DNA-relaxing activity of topoisomerase I in the evolved clones, because this protein otherwise reduces supercoiling.

To analyze the effect of the evolved *fis* mutation, we performed Western blot experiments and immunodetection using an antibody raised against Fis, with an antibody raised against RpoA serving as a control (Figure 3). We used three variants of the ancestral strain that carried the ancestral *fis* allele, the evolved *fis* allele, or a deletion of the *fis* gene. As expected (Mallik *et al.* 2004), the Fis level peaked during exponential growth in the ancestor (data not shown). The evolved *fis* allele had somewhat less than half the ancestral level of Fis protein, while the *fis*-deletion strain produced no detectable Fis (Figure 3). The level of the control protein did not differ between these strains.

Beneficial fitness effects of *topA* and *fis* mutations and their dynamics: To evaluate the fitness effects of the mutations responsible for the altered DNA topology in the focal population, we performed competitions between the ancestral strain with the evolved *topA* allele only, the evolved *fis* allele only, both evolved alleles, or both ancestral alleles, under the same culture conditions that prevailed during the long-term evolution experiment (Figure 4). In each case, the genotype of interest competed against a variant of the ancestor bearing a neutral marker (arabinose utilization) that allowed

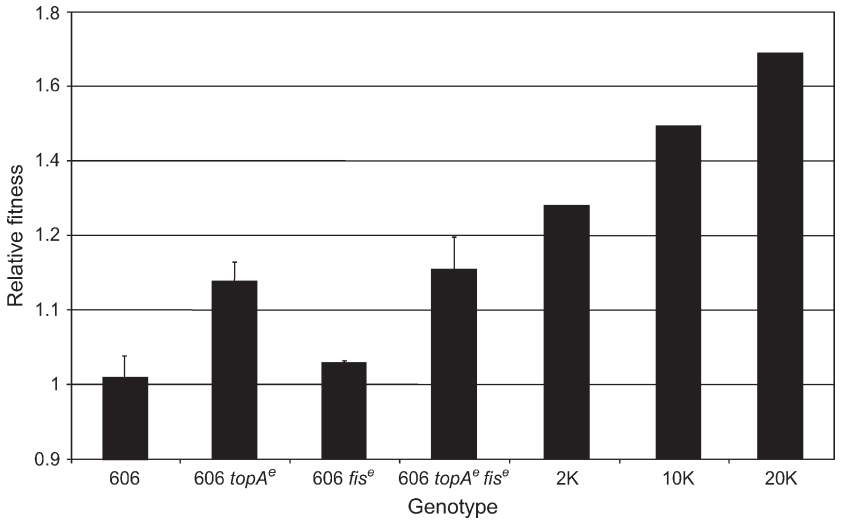

FIGURE 4.—Fitness effects of the evolved DNA topology-altering mutations in the ancestral genetic background. Competition experiments were performed in the same medium used in the long-term evolution. Error bars are 95% confidence intervals based on 16, 11, 4, and 6 replicate competition assays for each genotype (left to right). The strain 606 is the ancestor; *topA*^{e} and *fis*^{e} are alleles that evolved in focal population Ara−1 and then were moved, alone or in combination, into the ancestral chromosome. For comparison, we also show the mean fitness for population Ara−1 at three time points: 2000 (2K), 10,000 (10K), and 20,000 (20K) generations. The value shown for 2K is the average of values obtained by LENSKI *et al.* (1991) and by LENSKI and TRAVISANO (1994); the value for 10K is the average of values obtained by LENSKI and TRAVISANO (1994) and by COOPER and LENSKI (2000); and the value for 20K is from COOPER and LENSKI (2000).

the strains to be distinguished. As shown in prior studies (LENSKI *et al.* 1991), this marker had no discernible effect on fitness ($H_0 = 1$, *t*-test, $t_s = 0.4293$, $P = 0.6738$). Each evolved allele was beneficial by itself, with fitness relative to the ancestral allele of 1.133 for the *topA* mutation ($H_0 = 1$, *t*-test, $t_s = 10.58$, $P < 0.0001$) and 1.029 for the *fis* mutation ($H_0 = 1$, *t*-test, $t_s = 41.14$, $P < 0.0001$). Combining the two mutations gave a relative fitness of 1.155, which does not differ significantly from the fitness predicted by a simple additive model (13.3% + 2.9% = 16.2%). Also, these gains were eliminated when each evolved allele was replaced again by the ancestral allele (data not shown), confirming that the gains were caused by the *topA* and *fis* mutations rather than by some hypothetical secondary mutations that might have accidentally occurred during the strain constructions. In addition, replacing each evolved allele with the corresponding ancestral allele in evolved clones reduced their fitness relative to the otherwise isogenic evolved clones, as would be expected if these mutations were beneficial (data not shown). Thus, increasing DNA supercoiling had a beneficial effect during evolution in the experimental culture regime.

To examine in detail the temporal dynamics of the appearance and substitution of the evolved *topA* allele in population Ara−1, we performed PCR/RFLP experiments (see MATERIALS AND METHODS) using a total of 180 evolved clones isolated at generations 500, 1000, 1500, and 2000. The *topA* mutation was present in 22/45 clones at 500 generations and in 19/45 clones at 1000 generations, and it was present in 45/45 clones at both 1500 and 2000 generations. Thus, it contributed to the early large gain in fitness, existed as a polymorphism for some time (probably indicating clonal interference from other beneficial mutations: see below), and then reached fixation. Another beneficial mutation, in the *spoT* gene, was recently shown to have arisen somewhat later in the same population (COOPER *et al.* 2003).

We performed similar analyses for the evolved *fis* allele. This allele was not found in any of 50 clones isolated at 2000 generations, but was present in 15/37 clones at 10,000 generations. Therefore, the evolved *fis* allele contributed to the later, slower increase in fitness. The dynamics of the evolved *topA* and *fis* alleles match the observed changes in supercoiling (Figures 1 and 2). The *topA* allele was substituted by 2000 generations, and it induced the ~12% change seen at that time in the Ara−1 population. The *fis* mutation, absent at 2000 generations and substituted later, induces an additional ~5% change, leading to the ~17% net change in supercoiling seen at 20,000 generations.

Clonal interference and DNA topology as a key target of selection: Given the large fitness benefit of the *topA* mutation and its presence in about half the clones at 500 generations, one might have expected its complete fixation by 1000 generations. On the basis of its fitness advantage of >10% (Figure 4), the *topA* mutation, if unimpeded, would require <500 generations to reach 100% frequency (LENSKI *et al.* 1991). However, competition between subpopulations carrying different beneficial mutations—known as "clonal interference"—can prolong the time required for substitution (GERRISH and LENSKI 1998). To examine this possibility, we sought to determine whether other clones present at the same time had similar fitness but lacked the evolved *topA* allele. Recall that the focal population at generation 1000 had two subpopulations: one had the evolved *topA* allele, and the other retained the ancestral allele. We isolated two clones of each class from this sample; none of them had the previously characterized *spoT* mutation (COOPER *et al.* 2003), another beneficial mutation that

arose somewhat later in the same population. We then measured fitness and DNA supercoiling for these four clones. Competitions between each clone and the ancestor were performed with sixfold replication and gave similar fitness values for all four clones with no significant variation among them (one-way ANOVA, $F_{3,20} = 1.956$, $P = 0.1531$). The finding that co-occurring clones that retained the ancestral *topA* allele had similar fitness to those bearing the evolved allele supports the hypothesis that clonal interference impeded the substitution of the evolved allele. Moreover, and surprisingly, all four clones had approximately the same 12% increase in supercoiling compared with the ancestor. In other words, even clones from generation 1000 without the evolved *topA* allele had an altered DNA-topology phenotype similar to those carrying that allele. Therefore, the subpopulation that retained the ancestral *topA* allele acquired some other topology-altering and fitness-enhancing mutation, although the identity of this mutation is not known at present. By showing that similar beneficial mutations arose in parallel even within the same population (as well as across many replicate populations), these data further demonstrate that DNA topology was a key target of selection in the environment of the evolution experiment.

DISCUSSION

We describe the discovery of a new class of fitness-enhancing mutations that were substituted in evolving populations of *E. coli* during 20,000 generations in a culture environment in which cells experienced daily fluctuations between glucose abundance and depletion (Lenski *et al.* 1991; Lenski and Travisano 1994; Cooper and Lenski 2000; Lenski 2004). Of 12 independent populations, 10 underwent changes in DNA topology such that the level of supercoiling was higher (more negative) than that in the ancestor. We closely examined one focal population that showed a two-step increase in the level of DNA supercoiling. Two mutations were found in candidate genes known to affect DNA topology, *topA* and *fis*. The two mutations were moved, alone and in combination, into the chromosome of the ancestor. We measured supercoiling levels as well as relative fitness in these isogenic strains and found that both mutations were beneficial and that they contributed additively to the increased DNA supercoiling (Figures 2 and 4). By using numerous clones sampled from the focal population at various time points, we showed that the *topA* mutation was substituted prior to the *fis* mutation, as was likely given its larger fitness effect (Figure 4). We also found that the substitution of the evolved *topA* allele was impeded by competition with other clones that evidently had acquired other beneficial mutations that similarly affected DNA topology. The parallel phenotypic changes across the replicate populations as well as the spread of multiple mutations affecting DNA topology within at least the focal population demonstrate that the level of DNA supercoiling was an important target of selection during the evolution experiment.

The changes in DNA topology were assessed by measuring the distribution of topoisomers of a reporter plasmid. This method is widely used to compare superhelical density between different strains or under different environmental conditions. Zechiedrich *et al.* (2000) compared the effect of inhibiting topoisomerase I and topoisomerase IV on the superhelical level of a reporter plasmid and on the "effective" supercoiling level in the cell. The latter was estimated by the level of site-specific recombination by the bacteriophage λ integrase (Boles *et al.* 1990). Loss of the activity of both topoisomerases led to DNA topology changes of the reporter plasmid that reflected the supercoiling changes in the cell (Zechiedrich *et al.* 2000). In another study, changes in negative supercoiling of the *E. coli* chromosome in two cell-cycle mutants, *mukB* and *seqA*, were investigated by direct measurement of the superhelicity of membrane-free nucleoids using titration of the intercalating agent ethidium bromide (Weitao *et al.* 2000). The two mutants have opposing effects on the chromosome topology. Measurements of superhelicity for a reporter plasmid and for the chromosome using ethidium bromide titration showed the same qualitative effects of the mutations, with the magnitude of the effects measured for the chromosome somewhat greater than that measured with the plasmid (Weitao *et al.* 2000). These results therefore strongly support the interpretation that the DNA topology changes detected using a reporter plasmid qualitatively reflect what is happening *in vivo* for the chromosome.

Beneficial mutations play a central role in evolution, but they are only a tiny fraction of all mutations (Fisher 1930; Kimura 1983) and are therefore difficult to identify and investigate. Evolution experiments with bacteria and other microorganisms offer the opportunity to study the adaptive process in the laboratory (Elena and Lenski 2003). Even so, sequencing dozens of 500-bp regions chosen at random after 20,000 generations in the long-term evolution experiment with *E. coli* revealed almost no mutations (Lenski *et al.* 2003). By contrast, parallel phenotypic changes in the replicate evolving population clones led to the discovery of two loci (*rbs* and *spoT*) in which beneficial mutations occurred in most or all of the populations (Cooper *et al.* 2001a, 2003). Here, we discovered parallel changes in DNA topology, and we used this information to identify two loci, *topA* and *fis*, that acquired beneficial mutations in a focal population.

The *topA* mutation replaced the histidine at position 33, which is a conserved polar residue (Chen and Wang 1998), with a tyrosine. Although it was previously shown that an H33A mutant topoisomerase I is unaffected in its relaxation catalytic activity (Chen and Wang 1998),

our data indicate that the H33Y mutant protein causes an increase in DNA supercoiling, probably reflecting a conformational change. Moreover, the H33 residue lies in domain I in the structure of the 67K N-terminal fragment of topoisomerase I (Lima *et al.* 1994) and, together with other conserved residues, it surrounds the core of residues that cluster around the active-site tyrosine. Domain I resembles a nucleotide-binding domain and may interact with one end of the cut DNA (Lima *et al.* 1994). Our H33Y mutant probably retains some activity because complete inactivation of *topA* is lethal unless compensatory mutations in the *gyrAB* genes decrease supercoiling (DiNardo *et al.* 1982; Pruss *et al.* 1982), and we found no such compensatory mutations. The *fis* mutation lies 4 bases upstream of the ATG translational start codon, and it reduced the level of Fis protein (Figure 3). Fis represses transcription of *gyrAB* and reduces the activity of DNA gyrase (Schneider *et al.* 1999), which may explain the increased level of supercoiling caused by the evolved *fis* allele. We have also found mutations in *topA*, *fis*, or both in some other evolved populations in addition to the focal population (D. Schneider, C. L. Winkworth, E. Crozat, H. Geiselmann, M. A. Riley and R. E. Lenski, unpublished data). A full survey and characterization of the mutations substituted at these loci in all 12 populations is now in progress, and the identity of these other mutations may provide further insight into the molecular basis of the adaptive changes at these loci.

Why does an increase of DNA supercoiling enhance fitness of the bacteria? The exact nature of the physiological link may be difficult to decipher, because changes in DNA topology simultaneously influence the expression of many genes (Jovanovich and Lebowitz 1987; Pruss and Drlica 1989; Steck *et al.* 1993; Gmuender *et al.* 2001). One interesting possibility is that transcription initiation from the promoters synthesizing stable RNAs is highly dependent on the level of DNA supercoiling (Oostra *et al.* 1981; Free and Dorman 1994; Figueroa-Bossi *et al.* 1998). An increase in supercoiling would facilitate transcription of the rRNA operons. A higher rate of rRNA synthesis could be advantageous because the evolved lines have substantially higher exponential growth rates than does the ancestor (Vasi *et al.* 1994; Cooper *et al.* 2001b). Cells growing faster may need more rRNA than cells growing slowly, and the ratio of RNA to DNA appears to have increased in the evolved lines (Lenski *et al.* 1998). A seemingly contradictory result to that hypothesis is the observed decline in the level of Fis, which activates rRNA transcription (Ross *et al.* 1990). However, the loss of *fis in vivo* has no obvious effect on transcriptional activity of rRNA operons, owing to a compensatory mechanism (Ross *et al.* 1990), which has been suggested to involve an increase in the superhelical density of the DNA after *fis* mutation (Auner *et al.* 2003).

In addition to *topA* and *fis* mutations that were substituted in the focal population, another mutation that increased DNA supercoiling also reached high frequency but was eventually lost. This mutation was discovered by examining the time course of the spread of the *topA* mutation, which became "stuck" at a frequency of ~50% from 500 to 1000 generations, despite its large selective advantage measured in direct competition between isogenic strains. This dynamic suggested clonal interference (Gerrish and Lenski 1998), which was confirmed by finding that co-occurring clones from generation 1000 that lacked the evolved *topA* allele nonetheless had similarly high fitness. Moreover, these co-occurring clones had increased DNA supercoiling, indicating that they, too, carried a mutation affecting some topology-related gene. The identity of that other mutation is not known, and it was presumably eventually outcompeted as additional beneficial mutations arose in the subpopulation carrying the beneficial evolved *topA* allele. All these results show that DNA supercoiling was a very important aspect of fitness in the focal population, providing one of the earliest and largest adaptive changes.

The identification of *topA* and *fis* mutations gives a total of four confirmed beneficial mutations that were substituted in the focal population Ara−1, with the others being a deletion of the *rbs* operon (Cooper *et al.* 2001a) and a point mutation in *spoT* (Cooper *et al.* 2003). Assuming that the measured fitness effects of these four mutations were additive, they would collectively increase fitness by ~28%, which is still well below the overall improvement of ~67% measured after 20,000 generations (Figure 4; Cooper and Lenski 2000). Evidently, some beneficial mutations remain to be discovered, which is consistent with the estimate that each evolved population would have substituted between 10 and 20 beneficial mutations (Lenski 2004). The eventual identification of all the beneficial mutations will allow the reconstruction of the entire adaptive evolutionary history of this focal population by successively introducing the various beneficial mutations into the ancestral background.

We thank Akira Ishihama (Nippon Institute for Biological Science) and Michael Cashel (National Institutes of Health) for the generous gifts of anti-Fis and anti-RpoA antibodies, respectively. We thank M. Cashel for critical reading of the manuscript. This work was supported by grants from the French Centre National de Recherche Scientifique, Rhône-Alpes Region, and Université Joseph Fourier and from the National Science Foundation (to R.E.L.).

LITERATURE CITED

Auner, H., M. Buckle, A. Deufel, T. Kutateladze, L. Lazarus *et al.*, 2003 Mechanism of transcriptional activation by FIS: role of core promoter structure and DNA topology. J. Mol. Biol. **331:** 331–344.

Balke, V. L., and J. D. Gralla, 1987 Changes in the linking number of supercoiled DNA accompany growth transitions in *Escherichia coli*. J. Bacteriol. **169:** 4499–4506.

Bhriain, N. N., C. J. Dorman and C. F. Higgins, 1989 An overlap

between osmotic and anaerobic stress responses: a potential role for DNA supercoiling in the coordinate regulation of gene expression. Mol. Microbiol. **3:** 933–942.

Bliska, J. B., and N. R. Cozzarelli, 1987 Use of site-specific recombination as a probe of DNA structure and metabolism *in vivo*. J. Mol. Biol. **194:** 205–218.

Blomfield, I. C., V. Vaughn, R. F. Rest and B. I. Eisenstein, 1991 Allelic exchange in *Escherichia coli* using the *Bacillus subtilis sacB* gene and a temperature-sensitive pSC101 replicon. Mol. Microbiol. **5:** 1447–1457.

Boles, T. C., J. H. White and N. R. Cozzarelli, 1990 Structure of plectonemically supercoiled DNA. J. Mol. Biol. **213:** 931–951.

Bull, J. J., M. R. Badgett, H. A. Wichman, J. P. Huelsenbeck, D. M. Hillis *et al.*, 1997 Exceptional convergent evolution in a virus. Genetics **147:** 1497–1507.

Champoux, J. J., 2001 DNA topoisomerases: structure, function, and mechanism. Annu. Rev. Biochem. **70:** 369–413.

Chen, S.-J., and J. C. Wang, 1998 Identification of active site residues in *Escherichia coli* DNA topoisomerase I. J. Biol. Chem. **273:** 6050–6056.

Cooper, T. F., D. E. Rozen and R. E. Lenski, 2003 Parallel changes in gene expression after 20,000 generations of evolution in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **100:** 1072–1077.

Cooper, V. S., and R. E. Lenski, 2000 The population genetics of ecological specialization in evolving *Escherichia coli* populations. Nature **407:** 736–739.

Cooper, V. S., D. Schneider, M. Blot and R. E. Lenski, 2001a Mechanisms causing rapid and parallel losses of ribose catabolism in evolving populations of *Escherichia coli* B. J. Bacteriol. **183:** 2834–2841.

Cooper, V. S., A. F. Bennett and R. E. Lenski, 2001b Evolution of thermal dependence of growth rate of *Escherichia coli* populations during 20,000 generations in a constant environment. Evolution **55:** 889–896.

DiNardo, S., K. A. Voelkel, R. Sternglanz, A. E. Reynolds and A. Wright, 1982 *Escherichia coli* DNA topoisomerase I mutants have compensatory mutations in DNA gyrase genes. Cell **31:** 43–51.

Dorman, C. J., and P. Deighan, 2003 Regulation of gene expression by histone-like proteins in bacteria. Curr. Opin. Genet. Dev. **13:** 179–184.

Elena, S. F., and R. E. Lenski, 2003 Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. Nat. Rev. Genet. **4:** 457–469.

Figueroa-Bossi, N., M. Guérin, R. Rahmouni, M. Leng and L. Bossi, 1998 The supercoiling sensitivity of a bacterial tRNA promoter parallels its responsiveness to stringent control. EMBO J. **17:** 2359–2367.

Fisher, R. A., 1930 *The Genetical Theory of Natural Selection*. Oxford University Press, Oxford.

Free, A., and C. J. Dorman, 1994 *Escherichia coli tyrT* gene transcription is sensitive to DNA supercoiling in its native chromosomal context: effect of DNA topoisomerase IV overexpression on *tyrT* promoter function. Mol. Microbiol. **14:** 151–161.

Gellert, M., K. Mizuuchi, M. H. O'Dea and H. A. Nash, 1976 DNA gyrase: an enzyme that introduces superhelical turns into DNA. Proc. Natl. Acad. Sci. USA **73:** 3872–3876.

Gerrish, P. J., and R. E. Lenski, 1998 The fate of competing beneficial mutations in an asexual population. Genetica **102/103:** 127–144.

Gmuender, H., K. Kuratli, K. Di Padova, C. P. Gray, W. Keck *et al.*, 2001 Gene expression changes triggered by exposure of *Haemophilus influenzae* to novobiocin or ciprofloxacin: combined transcription and translation analysis. Genome Res. **11:** 28–42.

Goldstein, E., and K. Drlica, 1984 Regulation of bacterial DNA supercoiling: plasmid linking numbers vary with growth temperature. Proc. Natl. Acad. Sci. USA **81:** 4046–4050.

Hatfield, G. W., and C. J. Benham, 2002 DNA topology-mediated control of global gene expression in *Escherichia coli*. Annu. Rev. Genet. **36:** 175–203.

Higgins, C. F., C. J. Dorman, D. A. Stirling, L. Waddell, I. R. Booth *et al.*, 1988 A physiological role for DNA supercoiling in the osmotic regulation of gene expression in *Salmonella typhimurium* and *Escherichia coli*. Cell **52:** 569–584.

Jovanovich, S. B., and J. Lebowitz, 1987 Estimation of the effect of coumermycin A1 on *Salmonella typhimurium* promoters by using random operon fusions. J. Bacteriol. **169:** 4431–4435.

Karem, K., and J. W. Foster, 1993 The influence of DNA topology on the environmental regulation of a pH-regulated locus in *Salmonella typhimurium*. Mol. Microbiol. **10:** 75–86.

Kimura, M., 1983 *The Neutral Theory of Molecular Evolution*. Cambridge University Press, Cambridge, UK.

Lenski, R. E., 2004 Phenotypic and genomic evolution during a 20,000-generation experiment with the bacterium *Escherichia coli*. Plant Breed. Rev. **24:** 225–265.

Lenski, R. E., and M. Travisano, 1994 Dynamics of adaptation and diversification: a 10,000-generation experiment with bacterial populations. Proc. Natl. Acad. Sci. USA **91:** 6808–6814.

Lenski, R. E., M. R. Rose, S. C. Simpson and S. C. Tadler, 1991 Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. Am. Nat. **138:** 1315–1341.

Lenski, R. E., J. A. Mongold, P. D. Sniegowski, M. Travisano, F. Vasi *et al.*, 1998 Evolution of competitive fitness in experimental populations of *E. coli*: What makes one genotype a better competitor than another? Antonie van Leeuwenhoek **73:** 35–47.

Lenski, R. E., C. L. Winkworth and M. A. Riley, 2003 Rates of DNA sequence evolution in experimental populations of *Escherichia coli* during 20,000 generations. J. Mol. Evol. **56:** 498–508.

Lilley, D. M. J., 1986 Bacterial chromatin. A new twist to an old story. Nature **320:** 14–15.

Lima, C. D., J. C. Wang and A. Mondragon, 1994 Three-dimensional structure of the 67K N-terminal fragment of *E. coli* DNA topoisomerase I. Nature **367:** 138–146.

Link, A. J., D. Phillips and G. M. Church, 1997 Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. J. Bacteriol. **179:** 6228–6237.

Mallik, P., T. S. Pratt, M. B. Beach, M. D. Bradley, J. Undamatla *et al.*, 2004 Growth phase-dependent regulation and stringent control of *fis* are conserved processes in enteric bacteria and involve a single promoter (*fis* P) in *Escherichia coli*. J. Bacteriol. **186:** 122–135.

Nosil, P., B. J. Crespi and C. P. Sandoval, 2002 Host-plant adaptation drives the parallel evolution of reproductive isolation. Nature **417:** 440–443.

Oostra, B. A., A. J. van Vliet, G. Ab and M. Gruber, 1981 Enhancement of ribosomal ribonucleic acid synthesis by deoxyribonucleic acid gyrase activity in *Escherichia coli*. J. Bacteriol. **148:** 782–787.

Papadopoulos, D., D. Schneider, J. Meier-Eiss, W. Arber, R. E. Lenski *et al.*, 1999 Genomic evolution during a 10,000-generation experiment with bacteria. Proc. Natl. Acad. Sci. USA **96:** 3807–3812.

Pruss, G. J., and K. Drlica, 1989 DNA supercoiling and prokaryotic transcription. Cell **56:** 521–523.

Pruss, G. J., S. H. Manes and K. Drlica, 1982 *Escherichia coli* DNA topoisomerase I mutants: increased supercoiling is corrected by mutations near gyrase genes. Cell **31:** 35–42.

Reyes-Dominguez, Y., G. Contreras-Ferrat, J. Ramirez-Santos, J. Membrillo-Hernandez and M. C. Gomez-Eichelmann, 2003 Plasmid DNA supercoiling and gyrase activity in *Escherichia coli* wild-type and *rpoS* stationary-phase cells. J. Bacteriol. **185:** 1097–1100.

Ross, W., J. F. Thompson, J. T. Newlands and R. L. Gourse, 1990 *Escherichia coli* Fis protein activates ribosomal RNA transcription in vitro and in vivo. EMBO J. **9:** 3733–3742.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Plainview, NY.

Schneider, D., E. Duperchy, E. Coursange, R. E. Lenski and M. Blot, 2000 Long-term experimental evolution in *Escherichia coli*. IX. Characterization of insertion sequence-mediated mutations and rearrangements. Genetics **156:** 477–488.

Schneider, R., A. Travers, T. Kutateladze and G. Muskhelishvili, 1999 A DNA architectural protein couples cellular physiology and DNA topology in *Escherichia coli*. Mol. Microbiol. **34:** 953–964.

Sniegowski, P. D., P. J. Gerrish and R. E. Lenski, 1997 Evolution of high mutation rates in experimental populations of *Escherichia coli*. Nature **387:** 703–705.

Steck, T. R., R. J. Franco, J. Y. Wang and K. Drlica, 1993 Topo-

isomerase mutations affect the relative abundance of many *Escherichia coli* proteins. Mol. Microbiol. **10:** 473–481.

Vasi, F., M. Travisano and R. E. Lenski, 1994 Long-term experimental evolution in *Escherichia coli*. II. Changes in life-history traits during adaptation to a seasonal environment. Am. Nat. **144:** 432–456.

Wang, J. C., 1971 Interaction between DNA and an *Escherichia coli* protein omega. J. Mol. Biol. **55:** 523–533.

Weinstein-Fischer, D., M. Elgrably-Weiss and S. Altuvia, 2000 *Escherichia coli* response to hydrogen peroxide: a role for DNA supercoiling, topoisomerase I and Fis. Mol. Microbiol. **35:** 1413–1420.

Weitao, T., K. Nordström and S. Dasgupta, 2000 *Escherichia coli* cell cycle control genes affect chromosome superhelicity. EMBO Rep. **1:** 494–499.

Yanisch-Perron, C., J. Vieira and J. Messing, 1985 Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:** 103–119.

Zechiedrich, E. L., A. B. Khodursky, S. Bachellier, R. Schneider, D. Chen *et al.*, 2000 Roles of topoisomerases in maintaining steady-state DNA supercoiling in *Escherichia coli*. J. Biol. Chem. **275:** 8103–8113.

Communicating editor: G. R. Smith