Long-term experimental evolution in *Escherichia coli*. XII. DNA topology as a key target of selection

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ABSTRACT

The genetic bases of adaptation are being investigated in twelve 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 2,000 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.
INTRODUCTION

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 more than 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 large fitness gains 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; Cooper et al. 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; Cooper et al. 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 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 only by 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 to Ara-6, were founded from the ancestor that is Ara− (unable to use arabinose as a carbon source), and six others, Ara+1 to 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 twelve populations at generations 2,000, 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) 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 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 (BioRad, 200 ohms, 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$_{600}$ = 2 (or in DM25 to the end of exponential growth). Cells were harvested and pUC18 was extracted using the QIAPrep
Spin Miniprep Kit (Qiagen). 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 h 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 chloroquine concentration 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 slower, 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 ΔLk is the difference in the linking number (i.e., difference in average topoisomer value) between two clones and Lk0 for pUC18 is 2,686 bp / 10.45 bp / turn = 257. The σ value for the ancestor was expressed relative to the mid-point 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 mid-point 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 $\sigma$ 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 $\sigma$ of 0.0039. The resulting $\sigma$ 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 hours of incubation at 37°. (Under these conditions, the duration of the lag phase was about 0.75 hour, and after about 5 hours 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 (BioRad) and with bovine serum albumin as a standard. Equal amounts of protein samples were subjected to 12% SDS-polyacrylamide gel electrophoresis, and the separated proteins were then electrotransferred onto polyvinylidene difluoride membranes (Amersham Pharmacia). Prestained protein standards (Amersham) 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 NBT/BCIP (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 about 500 bp of adjacent sequences on each side of the mutation was cloned first 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 non-replicative 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 NmuCI (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 4-fold, with competitions running for one day or, with 1:100
daily serial transfer, six days (longer duration allows detection of smaller fitness effects) in the
same medium as the evolution experiment. Both one-day and six-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 h 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, 1,000, 1,500, and 2,000 were subjected to PCR using primers
ODS177 (5’GACTACGTGGTGAAATCCAG3’) and ODS178 (5’ACCAGGCAACACTTCATAGTG3’). The presence or absence of the evolved topA mutation was determined by NmuCI digestion of the PCR products. The evolved fis allele does not change any restriction sites. Therefore, PCR products obtained with primers ODS65 (5’GTACCGAATTGCACGTAAAC3’) and ODS141 (5’CACCGTGAATAATGGTCTTAG3’) for many clones isolated from 2,000, 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 (three clones from each of the 12 long-term populations for each of three time points: 2,000, 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 three 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, ten 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 nine populations showing changes by 2,000 generations. These initial 2,000 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 $\sigma$ value difference of about 12% and in two others of about 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 2,000 generations. In Ara+3, supercoiling initially increased but became 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 2,000 and 20,000 generations in this population. Finally, Ara-6 showed no change in supercoiling at 10,000 generations, whereas it was increased at 2,000 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 2,000 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 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 $\sigma$ value by about 12%, whereas the fis mutation increased it by about 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 2,000 in population Ara-1, while the combined effects of the topA and fis mutations matches 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 2,000 generations, with the substitution of the fis mutation sometime later. Sequencing topA in a 2,000-generation clone confirmed the presence of the same evolved allele as found at 20,000 generations. Replacing the evolved topA allele in the same 2,000-generation clone with the ancestral allele restored supercoiling to the ancestral level (Figure 2). This result also precludes the presence at 2,000
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 2,000-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 the strains to be distinguished. As shown in prior studies
(Lensi 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 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, 1,000, 1,500, and 2,000. The topA mutation was present in 22/45 clones at 500 generations and in 19/45 clones at 1,000 generations, and it was present in 45/45 clones at both 1,500 and 2,000 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 2,000 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 2,000 generations, and it induces the ~12% change seen at that time in the Ara-1 population. The *fis* mutation, absent at 2,000 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 1,000 generations. Based on 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 were present at the same time that had similar fitness but lacked the evolved *topA* allele. Recall that the focal population at generation 1,000 had two
subpopulations: one had the evolved topA allele, 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 six-fold 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 1,000 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). Ten of 12 independent populations underwent changes in DNA topology such that the level of supercoiling was higher (more negative) than 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 the ones 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; Cooper *et al.* 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 four bases upstream of the ATG translational start codon, and it
reduced the level of Fis protein (Figure 3). Fis represses transcription of \textit{gyrAB} and reduces the activity of DNA gyrase (Schneider \textit{et al.} 1999), which may explain the increased level of supercoiling caused by the evolved \textit{fis} allele.

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 \textit{et al.} 1993; Gmuender \textit{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 \textit{et al.} 1981; Free and Dorman, 1994; Figueroa-Bossi \textit{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 \textit{et al.} 1994; Cooper \textit{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 \textit{et al.} 1998). A seemingly contradictory result to that hypothesis is the observed decline in the level of Fis, which activates rRNA transcription (Ross \textit{et al.} 1990). However, the loss of \textit{fis in vivo} has no obvious effect on transcriptional activity of rRNA operons owing to a compensatory mechanism (Ross \textit{et al.} 1990), which has been suggested to involve an increase in the superhelical density of the DNA after \textit{fis} mutation (Auner \textit{et al.} 2003).

In addition to \textit{topA} and \textit{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 about 50% from 500 to 1,000 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 1,000 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 out-competed as additional beneficial mutations arose in the subpopulation carrying the beneficial evolved *topA* allele. All these results show 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 now 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 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.

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LITERATURE CITED


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FIGURE LEGENDS

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 2,000, 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 $\sigma$ value for the ancestor clone (-0.066) was obtained relative to the mid-point 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 to +6 (Ara+1 to Ara+6) and 1 to 6 (Ara-1 to Ara-6). White, hatched, and black bars represent clones isolated at 2,000, 10,000, and 20,000 generations, respectively. The $\sigma$ 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, small bars indicate the absence of any change in supercoiling.

FIGURE 2.—Changes in DNA supercoiling generated by evolved topA and fis mutations. The
changes in $\sigma$ values relative to the ancestral level (-0.066) are shown for various strains. 606 is the ancestral strain; 2K and 20K are clones isolated from population Ara-1 at 2,000 and 20,000 generations, respectively. All others are isogenic constructs made by replacing ancestral alleles with evolved alleles (denoted by superscript $e$), or 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 $fis^A$), after 1 and 1.5 h of exponential growth in LB medium. Western blots and immunodetection assays are shown for an anti-Fis antibody (upper panel) and, as a control, an anti-RpoA antibody (middle panel). The lower panel shows the ratio of Fis to RpoA levels in the same strains, based on 3 replicate experiments with error bars indicating standard deviations. The ratio is arbitrarily set to 1 for the ancestral strain after 1 h of exponential growth. No Fis protein was detected in the control strain with the fis gene deleted.

**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). 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: 2,000 (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).
Figure 1

(A) - Image showing a gel with a marker indicating -0.066.

(B) - Graph showing σ values compared to the ancestor (-0.066) across different populations for 2,000, 10,000, and 20,000 generations.
Figure 2

σ values compared to the ancestor (-0.066)

Genotype
Figure 4

The bar graph shows the relative fitness of different genotypes. The x-axis represents the genotypes, and the y-axis represents relative fitness. The genotypes include 606, 606 top4<sup>+</sup>, 606 fis<sup>+</sup>, 606 top4<sup>+</sup> fis<sup>+</sup>, 2K, 10K, and 20K. The relative fitness values vary across these genotypes, with some showing higher relative fitness than others.