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A Physiological Role for DNA Supercoiling in the Osmotic Regulation of Gene Expression in S. typhimurium and E. coli

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Summary

The *proU* locus encodes an osmotically inducible glycine betaine transport system that is important in the adaptation to osmotic stress. We present evidence that DNA supercoiling plays a key role in the osmotic induction of *proU* transcription. An increase in extracellular osmolarity increases in vivo DNA supercoiling, and the expression of *proU* is highly sensitive to these changes. Furthermore, *topA* mutations can mimic an increase in osmolarity, facilitating *proU* expression even in media of low osmolarity in which it is not normally expressed. Selection for *trans*-acting mutations that affect *proU* expression has yielded only mutations that alter DNA supercoiling, either in *topA* or a new genetic locus, *osmZ*, which strongly influences in vivo supercoiling. Mutations in *osmZ* are highly pleiotropic, affecting expression of a variety of chromosomal genes including *ompF*, *ompC*, *fimA*, and the *bgl* operon, as well as increasing the frequency of site-specific DNA inversions that mediate fimbrial phase variation.

Introduction

Chromosomal DNA from bacterial cells is negatively supercoiled. Several enzymes can modify chromosomal supercoiling, of which DNA gyrase and topoisomerase I are the best characterized (for reviews, see Drlica, 1984, 1987; Wang, 1985). DNA gyrase is composed of two subunits encoded by the *gyrA* and *gyrB* genes, and introduces negative supercoils in an energy-dependent process. Under certain circumstances, DNA gyrase can also relax DNA. In contrast, topoisomerase I is a relaxing enzyme and removes negative supercoils by an energy-independent mechanism. Topoisomerase I is encoded by the *topA* gene, which is located near the *trp* operon on the E. coli and S. typhimurium chromosomes. The absolute level of in vivo DNA supercoiling is determined, at least in part, by a balance between the opposing actions of topoisomerase I and DNA gyrase, and is regulated by the homeostatic modulation of *topA* and *gyr* gene expression in response to changes in chromosomal supercoiling (Menzel and Gellert, 1983; Tse-Dinh, 1985). Other less well characterized enzymes, such as topoisomerase III (Dean et al., 1983; Srivenugopal et al., 1984), may also be involved in linking number determination. However, it is now becoming clear that the in vivo superhelix density is significantly lower than that measured for the DNA once extracted from the cell (Lilley, 1986; Bliska and Cozzarelli, 1987), despite the fact that the linking number of the DNA is unaltered during extraction. Thus the in vivo level of DNA supercoiling is significantly influenced by factors other than the nicking-closing enzymes. These factors are not well understood but presumably include proteins such as the HU proteins which, upon binding to DNA, can alter its superhelix density (Broyles and Pettijohn, 1986; Drlica and Rouviere-Yaniv, 1987).

The use of mutants defective in the *gyrA*, *gyrB*, and *topA* genes, and of specific inhibitors of DNA gyrase, has identified a wide variety of cellular processes that are sensitive to changes in DNA supercoiling, including transposition, chromosome replication, recombination, and transcription (Drlica, 1984, 1987; Wang, 1985). Many promoters are sensitive to DNA supercoiling in in vitro systems (Wood and Lebowitz, 1984; Borowiec and Gralla, 1985), and artificial perturbation of in vivo supercoiling by introducing mutations in the *gyr* or *topA* genes, or by using specific gyrase inhibitors, profoundly influences the expression of a number of genes (Sanzey, 1979; Drlica, 1984, 1987; Menzel and Gellert, 1987). However, it is not yet clear whether changes in superhelix density play a role in the regulation of gene expression in response to normal environmental stimuli. That is, can in vivo DNA supercoiling vary in response to a given environmental stimulus, and if so, is this alteration in superhelix density directly responsible for the specific induction of gene expression in response to that stimulus? It has been suggested that anaerobicity can affect chromosomal supercoiling (Yamamoto and Droffner, 1985), and it is possible that these changes play a role in regulating gene expression in response to anaerobiosis. In this paper we provide evidence of a role for DNA supercoiling in the osmotic regulation of gene expression. Changes in the osmolarity of the growth medium are shown to alter the linking number of intracellular DNA, and these changes in superhelicity appear to be responsible for the specific induction of at least one genetic locus (*proU*) that plays a role in adaptation to growth at high osmolarity.

Most cells, whether prokaryotic or eukaryotic, respond to osmotic stress in a similar way: they accumulate high intracellular concentrations of a compatible solute to balance external osmolarity and restore turgor. One of the most commonly adopted compatible solutes is glycine betaine (N,N,N-trimethyl glycine), which plays an important osmoprotective function in plants, animals, and bacteria (Yancey et al., 1982; LeRudulier et al., 1984; Higgins et al., 1987a; Booth et al., 1987). Accumulated glycine betaine

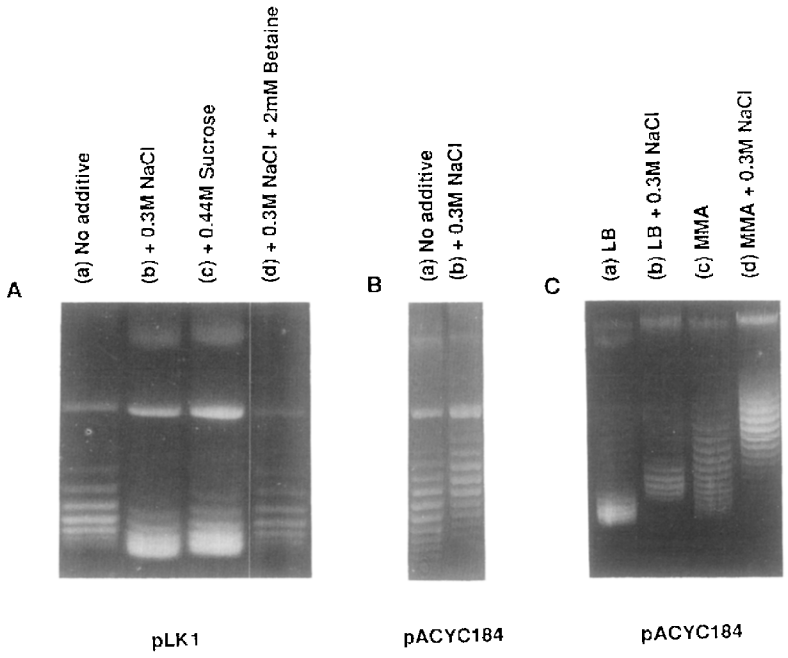

Figure 1. Effect of Osmolarity on In Vivo Plasmid Supercoiling

Plasmid DNA from strains grown under the indicated conditions was isolated and the topoisomers separated by electrophoresis in a chloroquine agarose gel. (A) Plasmid pLK1 from the wild-type S. typhimurium strain LT2 grown in LB (lane a), LB plus 0.3 M NaCl (lane b), LB plus 0.44 M sucrose (lane c), or LB plus 0.3 M NaCl plus 2 mM glycine betaine (lane d). The gel contained 1.5 μg ml^{-1} chloroquine. Under these conditions the more highly supercoiled topoisomers migrate more rapidly. 0.3 M NaCl and 0.44 M sucrose are iso-osmotic concentrations of the two solutes. (B) Plasmid pACYC184 DNA isolated from the wild-type S. typhimurium strain LT2 grown in MMAA (lane a) or MMAA plus 0.3 M NaCl (lane b). The gel contained 25 μg ml^{-1} chloroquine, at which concentration the more highly supercoiled topoisomers migrate more slowly. (C) Plasmid pACYC184 from E. coli MC4100 grown in LB (lane a), LB plus 0.3 M NaCl (lane b), MMA (lane c), or MMA plus 0.3 M NaCl (lane d). Gel conditions were as in (B).

not only balances external osmolarity but also serves to protect intracellular proteins against denaturation by high ionic strength (Pollard and Wyn Jones, 1979; Arakana and Timasheff, 1983). In the Gram-negative bacteria E. coli and S. typhimurium, glycine betaine is only accumulated under conditions of osmotic stress. In some E. coli strains, glycine betaine can be synthesized from exogenously supplied choline (Landfald and Strom, 1986). More generally, however, the accumulation of glycine betaine is a result of increased uptake from the extracellular medium.

The pathways for glycine betaine uptake are essentially identical in E. coli and S. typhimurium. Two genetically distinct transport systems are encoded by the *proP* and *proU* loci (Cairney et al., 1985a, 1985b; May et al., 1986). The *proP* gene encodes a low-affinity uptake system that transports both glycine betaine and proline. In contrast, *proU* encodes a specific, high-affinity, binding protein–dependent transport system with an affinity for glycine betaine of about 1.0 μM (Cairney et al., 1985a; May et al., 1986; Higgins et al., 1987b). Expression of *proU* is very tightly regulated by medium osmolarity. Studies using *proU-lacZ* fusions have shown that there is essentially no expression of *proU* when cells are grown at low osmolarity, yet transcription is increased more than 100-fold by an increase in extracellular osmolarity (Cairney et al., 1985a; Dunlap and Csonka, 1985; Gowrishankar, 1985; Barron et al., 1986). The final level of *proU* expression attained is finely tuned to reflect medium osmolarity. We have recently presented evidence that the intracellular signal for *proU* induction is K^+ ions (Sutherland et al., 1986; Higgins et al., 1987a). The rapid uptake of potassium appears to be the cell's primary response to osmotic upshock, and to a first approximation, intracellular potassium concentrations increase in proportion to external osmolarity (Epstein and Schultz, 1965; Laimins et al., 1981). Accumulated K^+ ions are then apparently responsible for the induction of *proU* transcription, as well as for other secondary responses to high osmolarity. Two distinct mechanisms can be envisaged by which intracellular K^+ ions might influence transcription from the *proU* promoter. Potassium might induce a conformational change in a specific positive or negative regulatory protein, altering its interaction at the *proU* promoter/operator. Alternatively, an increase in intracellular K^+ might directly influence the interactions between RNA polymerase and the *proU* promoter, possibly via an alteration of DNA structure or topology. In this paper we present evidence for this latter model, and demonstrate that osmotically induced changes in DNA supercoiling play an important role in the regulation of *proU* transcription. Furthermore, we have identified a new gene, *osmZ*, which plays an important role in determining the in vivo level of DNA supercoiling. Mutations in *osmZ* are highly pleiotropic, increasing the frequency of site-specific recombination events and affecting the expression of a variety of different chromosomal genes.

Results

Growth at High Osmolarity Alters In Vivo DNA Supercoiling

In order to probe the effects of extracellular osmolarity on DNA supercoiling in vivo, we monitored reporter plasmid DNA isolated from cells grown under various conditions. Two different reporter plasmids were used, pACYC184 and pLK1, that differ in size, sequence, and origin of replication. These plasmids were separately introduced into the parental S. typhimurium and E. coli strains, LT2 and MC4100. Figure 1 shows chloroquine-agarose gels of

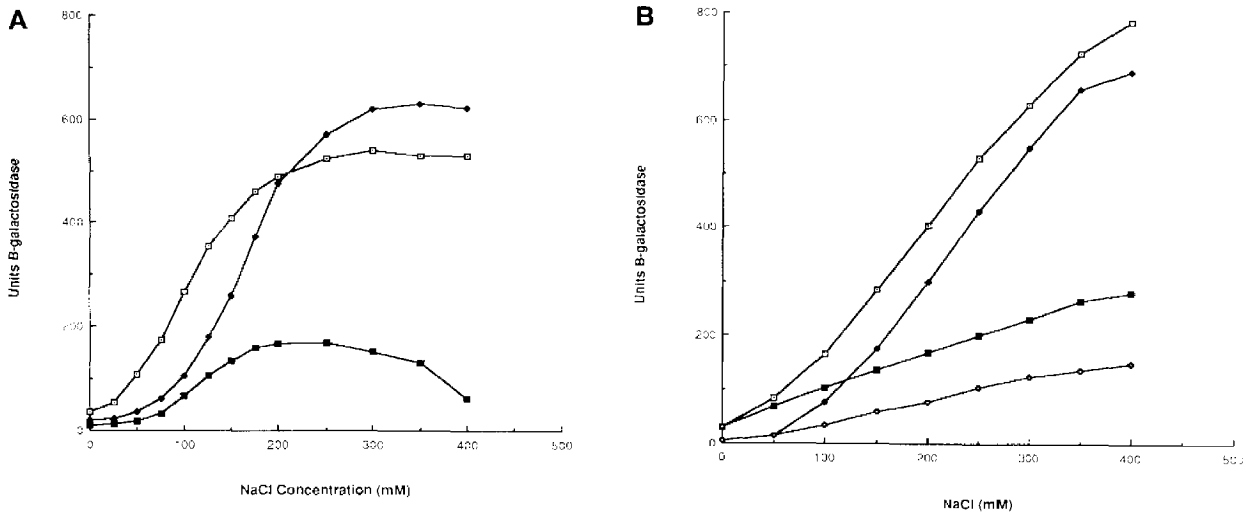

Figure 2. Effects of the *topA2770*, Δ*topA2771*, and *osmZ200* Mutations on the Osmotic Response of *proU-lacZ* Expression

(A) Cells of CH1565 (*topA*$^+$; diamond shapes), CH1568 (*topA2770*; open squares), and CH1631 (Δ*topA2771*; solid squares) were grown to mid-log in MMAA containing the indicated amount of NaCl, and β-galactosidase activity was assayed. Results similar to those for CH1631 were obtained for CH1641 (Δ*topA2762*). Each point is an average of three independent determinations. (B) Cells of GM37 (*osmZ*$^+$; open and solid diamonds) and BRE2071 (*osmZ200*; open and solid squares) were grown overnight in MMA containing the indicated amounts of NaCl, and β-galactosidase activity was assayed. Assays were in the absence (open squares, solid diamonds) or the presence (solid squares, open diamonds) of 1 mM glycine betaine.

plasmid DNA isolated from these strains after growth in different media. An increase in the osmolarity of the growth medium resulted in considerable oversupercoiling of the plasmid DNA. This effect was independent of the particular plasmid used, and a similar increase in supercoiling was observed whether NaCl (0.3 M) or an isoosmotic concentration of sucrose (0.44 M) was used to increase medium osmolarity.

When the osmoprotectant glycine betaine is added to the growth medium of cells under osmotic stress it reverses many of the effects of osmolarity on cell physiology, including the osmotic induction of *proU* expression (Roth et al., 1985; Barron et al., 1986; Sutherland et al., 1986). (The effect of glycine betaine on *proU* expression is also shown in Figure 2B.) This is thought to be due to the preferential uptake of glycine betaine as an intracellular osmolyte, reducing the intracellular potassium pool (Sutherland et al., 1986). The effects of glycine betaine on DNA supercoiling were therefore examined. For cells grown at high osmolarity (0.3 M NaCl), the addition of 2 mM glycine betaine restored DNA supercoiling to a level similar to that of cells grown at low osmolarity. Thus there is a good correlation between the effects of osmolarity and glycine betaine on DNA supercoiling and their effects on *proU* expression.

Gyrase Inhibitors and *gyr* Mutations Reduce *proU* Expression

If the increase in DNA supercoiling in response to growth at high osmolarity plays a role in the osmotic induction of *proU* expression, inhibitors of DNA gyrase, which reduce the negative supercoiling of DNA, might be expected to inhibit the osmotic induction of *proU*. Expression of *proU* was monitored in strains harboring chromosomal *proU-lacZ* fusions from which β-galactosidase activity accurately reflects transcription from the *proU* promoter (Sutherland et al., 1986; May et al., 1986). Gyrase inhibitors were used at concentrations that were not significantly inhibitory to cell growth. Both novobiocin and nalidixic acid, which inhibit the activities of the GyrB and GyrA subunits of DNA gyrase, respectively, caused a substantial reduction in *proU-lacZ* expression in S. typhimurium grown at high osmolarity (Table 1). Novobiocin also reduced the very low basal level of *proU-lacZ* expression seen in cells grown at low osmolarity. Similar results were obtained for E. coli, although higher concentrations of novobiocin were required to inhibit *proU* expression in this species. These data imply that transcription from the chromosomal *proU* promoter is sensitive to the degree of DNA supercoiling; inhibitors that decrease supercoiling decrease *proU* expression.

We also examined the effects of gyrase mutations on *proU* expression. We have previously described a series of S. typhimurium strains harboring well-defined *topA* and *tos* mutations (Richardson et al., 1984). *tos* mutations decrease DNA supercoiling in a well-defined manner and some, if not all, are mutations in the *gyr* genes (Richardson et al., 1984). The level of plasmid supercoiling in each of these *topA tos* strains is different such that together, they cover a wide range of in vivo supercoiling levels both above and below that of the wild type. A chromosomal *proU-lacZ* fusion was transduced into strains CH589, CH590, CH591, and CH593 harboring different *tos* (putative *gyr*) mutations, and β-galactosidase activity was assayed (Table 2). The *tos* mutations each decreased the level of DNA supercoiling below that of the wild type, but to different extents (Table 2). In all cases, the *tos* mutations decreased expression of *proU*. Furthermore, there was

Table 1. Effect of Gyrase Inhibitors on *proU* Expression

Strain	Species/Relevant Genotype	Medium	Gyrase Inhibitor	Units β-Galactosidase	
				– NaCl	+ NaCl (0.3 M)
CH1301	S. typhimurium φ(*proU-lacZ*)	LB	–	57	283
CH1301	S. typhimurium φ(*proU-lacZ*)	LB	Novobiocin (50 μg ml^{-1})	18	33
CH1301	S. typhimurium φ(*proU-lacZ*)	MMAA	–	8	320
CH1301	S. typhimurium φ(*proU-lacZ*)	MMAA	Novobiocin (50 μg ml^{-1})	10	52
CH1301	S. typhimurium φ(*proU-lacZ*)	MMAA	Nalidixic acid (4 μg ml^{-1})	8	69
GM37	E. coli φ(*proU-lacZ*)	MMAA		53	1,362
GM37	E. coli φ(*proU-lacZ*)	MMAA	Novobiocin (200 μg ml^{-1})	21	574

An overnight culture of cells was diluted 1:40 in the appropriate medium, with antibiotic where indicated, and grown to mid-log; then β-galactosidase activity was assayed. Each value is an average of at least three independent determinations. The concentrations of antibiotics used were sublethal.

Table 2. Effects of *topA* and *tos (gyr)* Mutations on *proU* Expression in S. typhimurium

Strain	Relevant Genotype	Superhelix Density[a]	Medium	Units β-Galactosidase	
				– NaCl	+ NaCl (0.3 M)
CH1566	*proU1707*::MudJ	– 0.056	NB	17	256
CH1633	*proU1707*::MudJ *tos-1*[b]	– 0.055	NB	8	225
CH1635	*proU1707*::MudJ *tos-2*	– 0.054	NB	2	176
CH1637	*proU1707*::MudJ *tos-3*	– 0.050	NB	0	163
CH1639	*proU1707*::MudJ *tos-4*	– 0.047	NB	0	158
CH1641	*proU1707*::MudJ Δ*topA2762*	– 0.064	NB	12	21
CH1642	*proU1707*::MudJ Δ*topA2762 tos-1*	– 0.061	NB	26	22
CH1643	*proU1707*::MudJ Δ*topA2762 tos-2*	– 0.057	NB	31	19
CH1644	*proU1707*::MudJ Δ*topA2762 tos-3*	– 0.055	NB	23	24
CH1645	*proU1707*::MudJ Δ*topA2762 tos-4*	– 0.054	NB	22	24
CH1565	*proU1708*::MudJ	–	LB	65[c]	289
CH1568	*proU1708*::MudJ *topA2770*	–	LB	177	250
CH1631	*proU1708*::MudJ Δ*topA2771*	–	LB	12	66
CH1568/F'123	*proU1708*::MudJ *topA2770* /*F'123(topA*$^{+}$)		LB	61	297

Cells were grown to mid-log in the indicated medium and β-galactosidase activity was assayed. Each value is an average of at least three independent determinations.

[a] The superhelix density of plasmid pLK1 DNA in these strains is taken from our previous data (Richardson et al., 1984).

[b] *tos* mutations were isolated as topoisomerase I suppressors (Richardson et al., 1984); some, if not all, are *gyrA* or *gyrB* mutations.

[c] There is a basal level of induction of *proU* expression in LB compared with NB because of the relatively high osmolarity of this medium. On addition of 0.3 M NaCl, induction is less than in MMA because proline/betaine in LB partly restores turgor and reduces *proU* expression (Sutherland et al., 1986).

good correlation between the degree to which each *tos* mutation reduced *proU* expression and the effect of that mutation on supercoiling. Although none of the *tos* mutations had as great an effect as did nalidixic acid or novobiocin, this was not unexpected as they are missense mutations that by no means completely eliminate gyrase activity. Thus either mutations or specific inhibitors that reduce DNA supercoiling also reduce *proU* transcription; the greater the reduction in supercoiling, the greater the inhibition of *proU* expression.

topA Mutants Express *proU* at Low Osmolarity

If *proU* expression is controlled by an osmotically induced increase in DNA supercoiling, then *proU* would be expected to be expressed at low osmolarity in strains harboring mutations that increase in vivo DNA supercoiling (e.g., in *topA*). In S. typhimurium, *topA* mutations can readily be selected as suppressors of the *leu-500* promoter mutation, restoring leucine prototrophy, as described in Experimental Procedures. We selected a Leu^{+} derivative of strain CH1565 (*leu-500 proU-lacZ trp-1016*::Tn*10*) and showed the suppressor mutation to be linked to the *trp* operon and, therefore, presumably a *topA* mutation. This suppressor mutation (in strain CH1568) conferred two phenotypes in addition to restoring leucine prototrophy: it was osmotically sensitive, unable to grow on MacConkey-lactose plates containing 0.3 M NaCl, and it was altered in the regulation of *proU* expression. In strain CH1568, β-galactosidase was expressed from the *proU-lacZ* fusion, even in media of low osmolarity in which *proU* is normally repressed (Table 2). When assayed at a range of osmolarities, the mutation was found to shift the osmotic induction profile for *proU* (Figure 2A) rather than simply causing constitutive expression, suggesting an alteration in osmotic sensing rather than a defect in a classical repressor protein.

Strain CH1568 was analyzed genetically (see Experimental Procedures for details) and shown to contain just a single mutation, linked to the *trp* operon, which is responsible for all three phenotypes: suppression of the *leu-500* mutation, altered osmoregulation of *proU*, and osmotic sensitivity on MacConkey plates. The fact that the

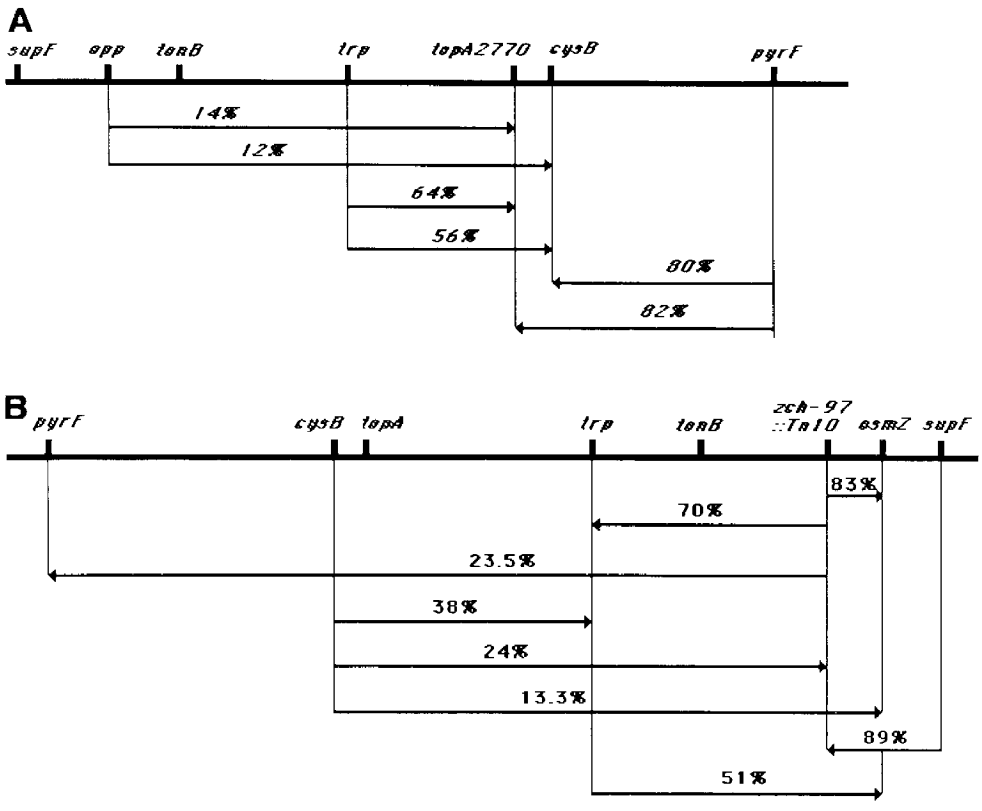

Figure 3. Genetic Organization of the *trp* Region of the S. typhimurium and E. coli Chromosomes

The *trp* region is at 34 min on the S. typhimurium chromosome and 27 min on the E. coli chromosome, within the region inverted between the two species. Thus, while gene order within this region is the same in the two species, the overall region is inverted. (A) Mapping the *topA2770* mutation. The map shows gene order at 34 min on the S. typhimurium chromosome. The P22 cotransduction frequencies (from 100 transductants screened) from two point crosses are indicated. The donor strain is indicated by the base of the arrow; the recipient, by the arrowhead. The *pyrF696*::Tn*10*, *oppB255*::Tn*10*, *trp-1012*::Tn*10*, and *cysB517* alleles, from strains CH50, CH56, CH57, and CH273, respectively, were used for mapping purposes (Higgins et al., 1983). To locate unambiguously the *topA2770* mutation, three point crosses were carried out with CH1648 (*pyrF*::Tn*10 cysB*) and CH1649 (*trp*::Tn*10 cysB*) as donors, and CH1559 (*topA2770*) as recipient. (B) Mapping the *osmZ* locus. The map shows gene order at 27 min on the E. coli chromosome. The percentage cotransduction frequencies, and relative locations of each gene, are indicated. For each cross at least 100 transductants were screened. For two point crosses the base of the arrow is the selected marker, the arrowhead the recipient. The *trpB114*::Tn*10* insertion was used to determine the cotransduction frequency between *trp* and *osmZ*. To map the *zch-97*::Tn*10* insertion relative to *supF*, the Tn*10* insertion was introduced into the *supF*$^+$ strain MBM7007 and used as recipient with the *supF* strain MBM7014 as donor, selecting for suppression of the amber mutations of the recipient and screening for simultaneous loss of Tet^r. The gene order *supF-osmZ-trp-pyrF* was unambiguously confirmed by a three factor cross, with strain GM131 (*zch-97*::Tn*10*) as donor and PLK831 (*trpE pyrF*) as recipient, and a four factor cross, selecting for CysB$^+$ transductants, with strain GM128 (*osmZ200 zch-97*::Tn*10*) as donor and GM161 (*osmZ*$^+$ *trpE cysB*) as recipient.

mutation in CH1568 suppressed *leu-500* and was closely linked to the *trp* operon implied that it was an allele of *topA*. (It has been suggested previously that mutations which map near *topA* might influence the regulation of *proU*; DiBlasio and Vinopal, 1986, ASM abstract K123). This was confirmed by detailed genetic mapping that unambiguously positioned the mutation between *trp* and *pyrF*, very closely linked to *cysB* (Figure 3A). Further evidence that the mutation is in *topA* comes from the finding that reporter plasmid DNA isolated from strains harboring the lesion is oversupercoiled (Figure 4A). When F'123, which contains the E. coli *topA* gene, was introduced into strain CH1568 (*topA2770*), all three phenotypes of the *topA2770* mutation (suppression of *leu-500*; altered *proU* regulation; osmotic sensitivity) were complemented (Table 2). Thus the *topA2770* mutation is recessive. Finally, the lesion was complemented by plasmid pLN48 (Louarn et al., 1984), which encodes the intact *topA* gene with little adjacent sequence (data not shown). Thus the various phenotypes of strain CH1568 are due to a single mutation in *topA* (designated *topA2770*).

The finding that *topA* mutants can express *proU* at low osmolarity implies that it should be possible to isolate mutations in the *topA* gene by screening directly for altered osmoregulation of *proU*. Cells of strain CH1565 (*leu-500 proU-lacZ*) were diluted appropriately and plated onto MacConkey-lactose indicator plates. On these plates the osmolarity is sufficiently low that *proU* is not normally expressed, and the colonies are white (Lac$^-$). Spontaneously arising pink (Lac$^-$) papillae were selected, purified, and screened for leucine prototrophy (suppression of *leu-500*). One Lac$^+$ Leu$^+$ colony was characterized further

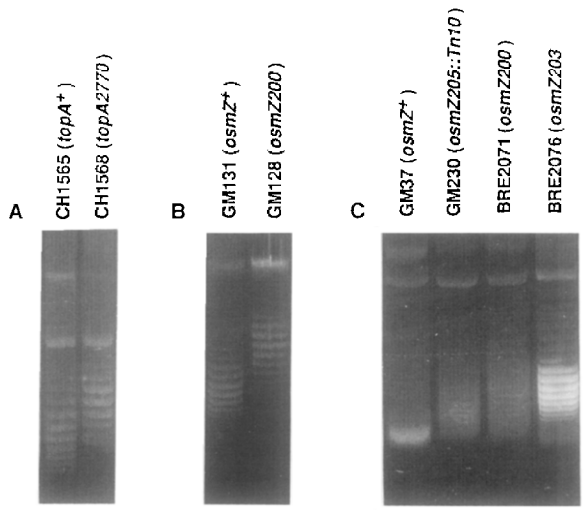

Figure 4. The *osmZ* and *topA2770* Mutations Alter DNA Supercoiling

pACYC184 plasmid DNA isolated from the indicated strains was separated on a 25 μg ml^{-1} chloroquine-agarose gel. At this chloroquine concentration, more relaxed topoisomers migrate more rapidly through the gel. (A) Strains CH1565 (*topA*$^+$) and CH1568 (*topA2770*). (B) Strains GM131 (*osmZ*$^+$) and GM128 (*osmZ200*). (C) Strains GM37 (*osmZ*$^+$), GM230 (*osmZ205*::Tn*10*), BRE2071 (*osmZ200*), and BRE2076 (*osmZ203*).

and found to contain a mutation genetically and phenotypically indistinguishable from the *topA2770* mutation described above. It should be pointed out that not all Lac$^-$ papillae contained *topA* mutations; most remained Leu$^-$ and contained either a *proU* promoter mutation or a mutation in the *osmZ* gene (see below). Nevertheless, *topA* mutations can be selected by screening for altered regulation of *proU*.

The Effect of *topA* Deletions on *proU* Expression

The *topA2770* mutation described above may be a missense mutation that alters topoisomerase I activity rather than completely abolishing the function of this enzyme. Indeed, this was implicit in the finding that suppression of the *leu-500* mutation and the reduction in growth rate was not as marked as for previously isolated *topA* deletions (data not shown). To examine the effects of *topA* deletions on *proU* expression, the *proU-lacZ* fusion from CH1301 was transduced into strain CH582 (Δ[*trp-topA-cysB*]*2762*). The *topA2762* deletion is well defined, and strain CH582 carries no compensating mutations (Richardson et al., 1984). As for the *topA2770* mutation, the *topA* deletion was found to confer osmotic sensitivity on MacConkey-lactose plates. Somewhat unexpectedly, however, the *topA2762* deletion had a rather different effect on *proU* expression than did the *topA2770* mutation; *proU* was not expressed to any significant extent as low osmolarity, and only poorly at high osmolarity (strain CH1641; Table 2). When the induction of *proU* over a range of osmolarities was examined, the difference between these two *topA* mutations could be clearly seen (Figure 2A). It was important to show that no secondary mutation, which might account for these differences, had arisen upon transduction of the *proU-lacZ* fusion into the *topA* deletion strain during construction of CH1641. We therefore isolated new *topA* deletions in strain CH1565 (*leu-500 proU-lacZ trp-1016*::*Tn10*), which already contained a *proU-lacZ* fusion, as described in Experimental Procedures. Each of these newly isolated *topA* deletions (e.g., Δ*topA2771*; CH1631) affected expression of the *proU-lacZ* fusion in a manner indistinguishable from that of the *topA2762* deletion (Table 2). Thus *topA* deletions do affect *proU* expression differently from the *topA2770* mutation, and there is a degree of allele specificity.

It is not yet clear why *topA* deletions express *proU* very poorly. It is possible that *topA* deletions oversupercoil *proU* promoter DNA more extensively than *topA2770*, to such an extent that transcription is inhibited (Borowiec and Gralla, 1987). Although we did not detect major differences between the effects of the *topA2770* and Δ*topA2771* mutations on DNA supercoiling of a reporter plasmid, there may be undetected differences at the chromosomal level. We therefore examined the effect on *proU* expression of compensating (*tos*) mutations, which restore DNA supercoiling to levels approaching those of the wild type (Richardson et al., 1984). If the *topA* deletion simply affects *proU* expression by virtue of its effects on DNA supercoiling, then the compensating mutations would be expected to restore expression of *proU*. However, this was not the case. Even in Δ*topA* strains carrying compensating *tos* mutations (CH1642–1645), *proU* was only poorly induced by high osmolarity (Table 2). In contrast, compensating *tos* mutations suppressed the sensitivity of *topA* deletion strains to 0.3 M NaCl on MacConkey-lactose plates; this phenotype, at least, seems to be a direct consequence of changes in DNA supercoiling. The most probable explanation for these data is that the level of supercoiling of a reporter plasmid does not truly reflect chromosomal topology. Other factors, such as proteins bound to the DNA, may affect the local environment of the *proU* gene on the chromosome, suppressing or enhancing the effects of *topA* and *gyr* mutations. There are indications that reporter plasmids may not always provide a true reflection of the chromosomal situation (Lamond, 1985; Pruss and Drlica, 1986).

osmZ: A New Locus That Alters *proU* Expression

A powerful means of elucidating the mechanisms of osmotic control of *proU* expression is to isolate mutations in genes that alter this regulation. We devised several selection regimes that enabled us to isolate mutations that fail to induce *proU* at high osmolarity. However, all such mutations were linked to the *proU* locus, and unlinked mutations could not be isolated (our unpublished data). An alternative strategy is to isolate mutants expressing *proU-lacZ* fusions at low osmolarity. We showed above that such a selection facilitated isolation of *topA* mutations. To exploit this selection further, cells of E. coli strain GM37 [Φ *proU-lacZ*)hyb2] were streaked onto a MacConkey-lactose plate. On these plates the osmolarity is sufficiently low that *proU* is not normally expressed and the colonies are white (Lac$^-$). After 48 hr of incubation, many Lac$^+$ (deep red) papillae appeared: six such papillae were

Table 3. Effect of *osmZ* Mutations on *proU-lacZ* Expression in E. coli

Strain	Relevant Genotype	Type of Fusion[a]	β-Galactosidase Activity	
			NaCl	+ NaCl (0.3 M)
GM37	*osmZ*$^+$	Protein	5.9	1,209
GM230	*osmZ205*::Tn*10*	Protein	91.9	–[b]
BRE2071	*osmZ200*	Protein	103.7	1,636
BRE2072	*osmZ201*	Protein	165.9	1,653
BRE2073	*osmZ202*	Protein	97.8	1,724
BRE2074[c]	*osmZ*$^+$ *proU601*	Protein	160.0	1,730
BRE2076	*osmZ203*	Protein	165.9	–[b]
BRE2080	*osmZ204*	Protein	68.2	1,825
GM50	*osmZ*$^+$	Operon	8.9	518
GM152	*osmZ200*	Operon	91.9	616
GM284	*osmZ203* F'123 (osmZ$^+$)	Protein	7.9	1,186

Cells were grown overnight in MMA, with or without 0.3 M NaCl as indicated, and β-galactosidase activity was determined. These data are the mean values of at least five independent experiments.

[a] In all cases, the protein fusion used was φ(*proU-lacZ*)hyb2, and the operon fusion was φ(*proU-lacZ*$^+$)3.

[b] Activity at high osmolarity could not be determined for this strain as it is osmotically sensitive.

[c] BRE2074 contains a *proU* promoter mutation and not an *osmZ* lesion. See text for further details.

picked, single colony purified, and characterized further. When they were assayed for β-galactosidase activity, expression of the *proU-lacZ* fusion was found to be at least 10-fold higher than that of the parental strain (GM37) at low osmolarity (Table 3). However, osmotic induction was still apparent and, indeed, *proU* expression in these mutants could be induced to a higher level than in the parental strain. It should be noted that the six mutations affected *proU-lacZ* expression to different extents, indicating that they are either in different genes or that they are different alleles of the same gene. Additional evidence that the mutations are nonidentical comes from the finding that one of the mutants (BRE2076) is osmotically sensitive in minimal medium, as well as from their Bgl phenotypes (see below).

These six independent mutants were analyzed genetically. The *proU-lacZ* fusion was transduced with phage P1 into the parental strain MC4100, selecting for the Kan^r marker carried by the λp*lac* Mu prophage, and the Lac phenotype of the Kan^r transductants screened on MacConkey-lactose plates. For one of the mutants (BRE2074), 63 out of 68 Kan^r transductants showed the mutant phenotype (Lac$^+$ at low osmolarity), while the remaining transductants were as the wild type (Lac$^-$ at low osmolarity but osmotically inducible). The osmoregulatory mutations in BRE2074 is therefore closely linked to the *proU-lacZ* fusion. Further analysis showed it to be *cis*-acting and recessive, and when it was crossed into a wild-type (*proU*$^+$) background, the resultant strain showed increased glycine betaine transport at low osmolarity and an increased amount of the periplasmic glycine betaine binding protein (data not shown). This mutation is presumably a *proU* promoter mutation similar to the S. typhimurium mutations recently described by Druger-Liotta et al. (1987), and was not analyzed further.

The other five mutations (in strains BRE2071, BRE2072, BRE2073, BRE2076, BRE2080) were genetically unlinked to the *proU-lacZ* fusion, since when the fusion was rescued from these strains by transduction (as described above), 100% of the transductants showed wild-type regulation. To facilitate mapping and further analysis of these mutations, a *Tn10* transposon 83% linked to the mutation in BRE2071 was isolated as described in Experimental Procedures. This transposon (*zch-97*::Tn*10*) was found to be similarly linked to each of the other four regulatory mutations (in strains BRE2072, BRE2073, BRE2076, BRE2080), implying that the mutations are all at the same genetic locus. We name this locus *osmZ*. When strain GM128 (*osmZ200 zch-97*::Tn*10*) was used as donor to transduce the parental *proU-lacZ* fusion strain (GM37) to Tet^r, 76% of the transductants showed the mutant *proU* osmoregulatory phenotype. Thus, a single mutation, unlinked to *proU*, is responsible for the altered osmoregulation of *proU*. Furthermore, for BRE2076 (*osmZ203*) there was 100% linkage between the altered osmotic control of *proU-lacZ* expression and osmosensitivity, showing that the same mutational event is responsible for both phenotypes.

The effect of the *osmZ200* mutation on the osmotic regulation of *proU-lacZ* expression was analyzed in some detail. Cells were grown overnight in MMA containing increasing concentrations of NaCl (Figure 2B). The osmotic response curve of the mutant was shifted compared with that of the wild type, as if the cell sensed a salt concentration approximately 50 mM higher than that actually provided. When the same experiment was carried out in the presence of 1 mM glycine betaine, a strong reduction in *proU-lacZ* expression was observed, even in the mutant strain (Figure 2B). We have shown previously that glycine betaine reduces *proU* expression in the wild type by virtue of its effects on intracellular potassium pools (Sutherland et al., 1986).

The above results were obtained with a translational (protein) fusion between *proU* and *lacZ*, and could reflect either transcriptional or translational control. We therefore constructed a strain (GM152) containing the *osmZ200* allele and the Φ(*proU-lacZ*$^+$)3 operon fusion. As for the protein fusion, the *osmZ200* mutation caused an increase in expression of the *proU-lacZ* operon fusion at low osmolar-

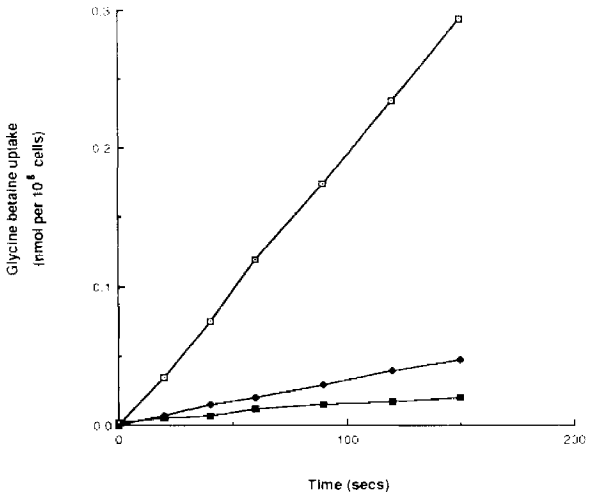

Figure 5. Effect of the *osmZ200* Mutation on Glycine Betaine Transport

Cells were grown in MMA containing 75 mM NaCl, and transport of glycine betaine was assayed as described in Experimental Procedures. An osmolarity of 75 mM NaCl was selected, as this osmolarity gives the maximum difference in *proU-lacZ* expression between *osmZ*$^+$ and *osmZ*$^-$ strains (see Figure 2B). The data are mean values of duplicate experiments. Strains used were as follows: MC4100 (*proU*$^+$ *osmZ*$^+$), solid diamonds; GM125 (*proU*$^-$ *osmZ200*), open squares; GM37 (*proU*$^-$ *osmZ*$^+$), solid squares. At the glycine betaine concentration used (1.4 μM), uptake through the low-affinity glycine betaine uptake system (ProP) is negligible, and essentially all measurable uptake is via ProU.

ity (Table 3), demonstrating that this mutation affects *proU* expression at the transcriptional level. To obtain more direct evidence that expression of *proU* is really increased in the *osmZ* mutants, and that the above results are not due to a fusion artifact, we measured glycine betaine uptake in a strain carrying the *osmZ200* mutation but that was wild type for the *proU* locus (Figure 5). These assays were carried out at 75 mM NaCl, the osmolarity at which there is the greatest difference in *proU* expression between the wild type and the *osmZ200* mutant (Figure 2B). An excellent correlation was found between the effects of the *osmZ200* mutation on expression of the *proU-lacZ* fusion and its effects on glycine betaine transport. In addition, the levels of the periplasmic glycine betaine–binding protein were increased in strains carrying the *osmZ200* mutation (data not shown). Thus it is clear that *osmZ* is a *trans*-acting locus that affects the osmotic control of *proU* expression at the transcriptional level.

Mapping the *osmZ* Gene

The *osmZ200* mutation was shown by Hfr mapping to be closely linked to the *trp* operon at 27 min on the E. coli chromosome (data not shown). Introduction of F'123, which carries the *trp* region of the chromosome, complemented the *osmZ* mutations, providing further evidence that the mutations are in this region of the chromosome (Table 3). Because the *topA* gene is also located near the *trp* operon (Wang and Becherer, 1983) and because *topA* mutations can similarly affect *proU* expression, at least in S. typhimurium (see above), it was crucial to establish whether or not *osmZ* mutations were alleles of *topA*. The fact that *osmZ* is entirely distinct from *topA* was established in several different ways. First, the *osmZ200* mutation was accurately mapped with respect to adjacent markers, by phage P1 transduction (Figure 3B). These data demonstrate the gene order *pyrF-cysB-trp-zch-97*::Tn*10-osmZ200-supF*. Three point genetic crosses unambiguously placed *osmZ* between *trp* and *supF*, whereas the *topA* gene is on the opposite (*cysB*) side of *trp*. Second, the *osmZ* mutations confer the same phenotypes as mutations in the *bglY*, *cur*, and *pilG* genes, and the three genes appear to be allelic (see below). The *pilG* and *bglY* mutations have been independently mapped to the *supF* side of the *trp* operon (Defez and DeFelice, 1981; Spears et al., 1986). Third, unlike *topA* mutations, mutations in *osmZ* do not reduce the growth rate substantially. *osmZ* mutations can readily be transduced from strain to strain, in complete contrast to *topA* mutations in E. coli, which cannot be transduced into a "clean" genetic background in the absence of a compensating mutation in DNA gyrase (DiNardo et al., 1982; Pruss et al., 1982). Transduction is possible not only for point mutations in *osmZ*, but also for an *osmZ*::Tn*10* insertion. The *zch-96*::Tn*10* insertion was originally isolated on the basis of its linkage to the *tonB* gene (K. Hantke, personal communication). Fortuitously, this insertion was found to confer the OsmZ phenotype as well as osmotic sensitivity. The Tn*10* insertion was 100% linked (300 transductants screened) to the OsmZ phenotype, and must therefore be in the *osmZ* locus. We redesignate this insertion *osmZ205*::Tn*10*. This insertion could readily be transduced into the wild-type parental strain (GM37), a finding incompatible with the insertion being in *topA*. Fourth, the *osmZ200* mutation (despite being complemented by F'123; Table 3) is not complemented by the TopA$^+$ plasmid pLN48 (data not shown), although this plasmid fully complements E. coli and S. typhimurium *topA* mutations. Fifth, the *osmZ* mutations described above were isolated in E. coli. Using a similar screen we have also isolated similar mutations in S. typhimurium that confer very similar phenotypes (data not shown). Indeed, all S. typhimurium mutations we have been able to isolate by this selection are either in *osmZ* or in *topA*, or are closely linked to the *proU* locus itself. Three point mapping with phage P22 has shown unambiguously that, in this species, *osmZ* and *topA* are located on opposite sides of the *trp* operon. Thus there is no doubt that the *osmZ* gene is entirely distinct from *topA*.

Mutations in *osmZ* Are Highly Pleiotropic

Three regulatory loci, *pilG*, *bglY*, and *cur*, have previously been mapped to the same chromosomal region as *osmZ*. *pilG* mutations were selected as increasing the frequency of the site-specific DNA inversion event responsible for fimbrial (*fimA*) phase variation (Spears et al., 1986), while *bglY* mutations activated expression of the cryptic *bgl* operon (Defez and DeFelice, 1981). *cur* mutations affect expression of the metastable *flu* genes, which control surface properties of E. coli (Diderichsen, 1980a, 1980b). Because *osmZ*, *pilG*, *cur*, and *bglY* all affect expression of

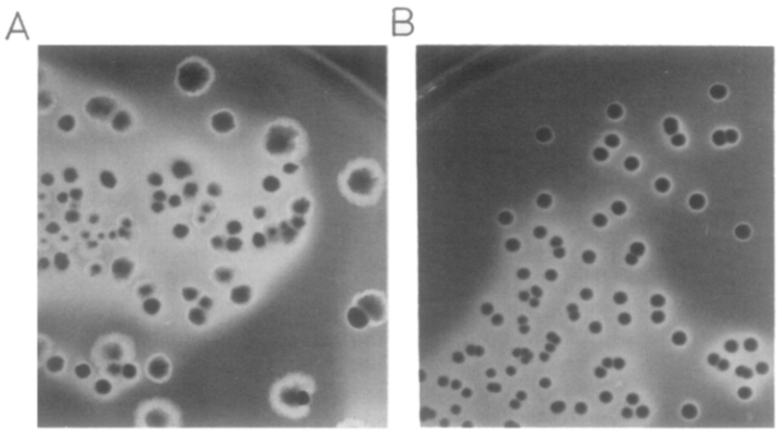

Figure 6. Effect of the *osmZ200* Mutation on Fimbrial Phase Variation

Shown are colonies growing on MacConkey-lactose indicator plates. Dark colonies are Lac$^+$, light colonies are Lac$^-$. (A) Strain CH1647 [φ(*fimA-lacZ*)]. Cells were grown from a single colony and plated. Switching between Lac$^-$ and Lac$^-$ colonies, which occurs at a frequency of about 10^{-3} due to inversion of the *fimA* promoter DNA, can be clearly seen. (B) Strain CH1646 [φ(*fimA-lacZ*)*osmZ200*]. The *osmZ* (*pilG*) mutation increases the DNA inversion rate such that any single colony contains an approximately equal mixture of "on" and "off" variants and hence appear homogeneously Lac$^+$. When assayed for β-galactosidase activity, these *osmZ* derivatives are intermediate in activity between the "on" and "off" variants (see text). Further details are in Dorman and Higgins (1987).

genes that are located elsewhere on the chromosome, it seemed possible that they might be allelic. This was shown to be the case. When the *osmZ200* mutation was transduced into strain VL386, carrying a *fimA::lacZ* fusion, the frequency of DNA inversion at the *fimA* promoter increased dramatically (Figure 6). When *fimA-lacZ* expression was assayed in the presence of an *osmZ* mutation, expression was found to be intermediate between the values obtained for the "on" and "off" variants of the OsmZ$^+$ strains (1536 units compared with 1930 and 414 units, for strains CH1646 (*osmZ200*), CH1647 (*osmZ$^+$*) "on," and CH1647 (*osmZ$^+$*) "off," respectively). The intermediate values are due to the very rapid switching between "on" and "off" in the *osmZ* strain, homogenizing the population (see Dorman and Higgins, 1987; Spears et al., 1986, for further details). The phenotypic effects of the *osmZ200* mutation on fimbrial phase variation and on *proU-lacZ* expression could not be separated genetically.

To ascertain whether *osmZ* and *bglY* are allelic, strains carrying the various *osmZ* mutations were assayed for β-glucoside uptake and hydrolysis (Table 4). The *osmZ$^+$* parental strains (GM37 and GM131) were Bgl$^-$, as expected, while strains harboring the five *osmZ* mutants, or the *osmZ205*::Tn*10* insertion, were Bgl$^+$. It should be noted that the different *osmZ* mutations induce β-glucosidase activity to different extents, suggesting that they are nonidentical missense mutations (as was also implicit in their different effects on *proU-lacZ* expression; see above). To confirm that the same mutational event was responsible for both the Bgl and ProU phenotypes, the *osmZ* mutations were transduced into the parental strain GM37, taking advantage of their linkage to the *zch-97::Tn10* insertion. All of the Tet^r transductants that were altered in the control of *proU* expression also became Bgl$^+$; conversely, those Tet^r transductants that showed normal regulation of *proU* remained Bgl$^-$. Finally, F'123 complemented each of the *osmZ* mutants, restoring the Bgl$^-$ phenotype of the wild type (data not shown). Thus there is no doubt that *bglY* and *osmZ* are alleles of the same genetic locus. Finally, certain alleles of *osmZ* cause flocculation, as do *cur* mutations that have been mapped to the same region of the chromosome (Diderichsen, 1980a, 1980b), and *osmZ* mutations also affect osmotic regulation of the outer membrane porins, OmpC and OmpF (our unpublished data). Thus *osmZ* is a highly pleiotropic regulatory locus affecting the expression of a number of unlinked and apparently unrelated genes.

Table 4. Effect of *osmZ* Mutations on β-Glucosidase Activity

Strain	Relevant Genotype	Units β-Glucosidase
GM37	*osmZ$^+$*	8
GM230	*osmZ205*::Tn*10*	36
BRE2071	*osmZ200*	238
BRE2072	*osmZ201*	87
BRE2073	*osmZ202*	349
BRE2076	*osmZ203*	321
BRE2080	*osmZ204*	38
GM128	*osmZ200 zch-97*::Tn*10*	98
GM131	*osmZ$^+$ zch-97*::Tn*10*	7

Cells were grown in MMAA medium with succinate as carbon source, and β-glucosidase activity was assayed as described in Experimental Procedures. Each value is an average of at least three separate determinations. The values for GM230 and BRE2080 are significantly above background, and the Bgl$^+$ phenotype of these strains, compared with the Bgl$^-$ phenotype of GM37 and GM131, can also be seen in p-nitrophenylglucoside plate tests (data not shown).

osmZ Mutations Alter DNA Supercoiling

Because *osmZ* mutations are highly pleiotropic, affecting a variety of genes that are believed to be sensitive to supercoiling (see Discussion), and because we have previously shown that mutations which alter DNA supercoiling can affect *proU* expression, the effects of *osmZ* mutations on DNA supercoiling were analyzed. Figure 4 shows that *osmZ* mutations increase the level of supercoiling of a reporter plasmid. Again, it should be noted that the various mutations are not identical and that they affect the level of supercoiling to different extents.

Discussion

It is well established that promoter function in vivo and in vitro can be strongly influenced by the level of DNA supercoiling (for reviews, see Drlica, 1984, 1987; Wang, 1985). Perturbation of DNA supercoiling by introducing mutations in the *topA* or *gyr* genes, or by using specific inhibitors of gyrase activity, affects the expression of a number of genes (e.g., Sanzey, 1979; Sternglanz et al., 1981; Menzel and Gellert, 1987; Rudd and Menzel, 1987). However, it is not yet clear whether changes in DNA supercoiling play a normal regulatory role: whether or not in vivo DNA superhelicity changes in response to a normal environmental stimulus and is then responsible for the specific induction or repression of gene expression in response to that stimulus. In this paper we present evidence for such a regulatory mechanism in the induction of transcription of the *proU* loci of E. coli and S. typhimurium in response to osmotic stress.

Transcription of the *proU* locus is very sensitive to fluctuations in medium osmolarity (Cairney et al., 1985a; Dunlap and Csonka, 1986; May et al., 1986). Several groups, including our own, have attempted to identify putative positive or negative regulatory proteins involved in osmoregulation by a variety of genetic means, but without success (Druga-Liotta et al., 1987; our unpublished data). Considering the different selections employed this is somewhat surprising, and suggests that regulation may not be mediated by a classical regulatory protein. It is, of course, possible that such regulatory mutations are lethal, although nonlethal missense mutations should have been detected. In addition, *proU* itself is nonessential and can be deleted, and available evidence does not implicate a global osmotic regulon (Higgins et al., 1987a). An alternative model is that specific regulatory proteins are not involved and that osmolarity directly influences the interactions between RNA polymerase and the *proU* promoter. This could be achieved by modifying RNA polymerase activity. Alternatively, a change in DNA structure or topology at the *proU* promoter could enhance the productive interaction of RNA polymerase. In this paper we present evidence for the latter model. First, increased extracellular osmolarity increases in vivo DNA supercoiling. Second, transcription from the *proU* promoter is exquisitely sensitive to changes in DNA supercoiling. Transcription is reduced by inhibitors of DNA gyrase or by *gyr* mutations that reduce the level of supercoiling. Furthermore, *proU* is expressed, even under normally noninducing conditions, in strains carrying mutations in *topA* or in a newly identified gene, *osmZ*, that increases DNA supercoiling. It is interesting to note that different *topA* alleles have different effects on *proU* expression. While certain missense mutations mimic conditions of high osmolarity, deletions of *topA* almost eliminate *proU* expression even in the presence of compensating *tos* mutations. This may indicate a more specific role for the TopA protein in the regulation of *proU* expression. Third, *topA* mutations confer osmotic sensitivity. Fourth, the only *trans*-acting regulatory mutations affecting *proU* expression that we have been able to isolate are in genes whose products affect DNA supercoiling. Finally, the *proU* locus encodes a glycine betaine transport system that plays an important role in the cell's ability to adapt to osmotic stress. Thus regulation of *proU* expression by DNA supercoiling is not simply fortuitous but serves an important physiological role.

Only three genes are known that, when mutated, alter the in vivo level of DNA supercoiling: *topA*, *gyrA*, and *gyrB*, encoding topoisomerase I and the two subunits of DNA gyrase, respectively. While topoisomerase I and DNA gyrase are clearly of central importance, there is evidence that the activities of these two enzymes alone are not sufficient to determine the in vivo level of DNA supercoiling (Raji et al., 1985; Pruss et al., 1986; Bliska and Cozzarelli, 1987). We have identified a new gene, designated *osmZ*, that when mutated increases DNA supercoiling. It should be pointed out that we have not measured chromosomal supercoiling directly, but only that of a reporter plasmid. However, the finding that *osmZ* mutations affect a variety of different chromosomal functions provides a strong indication that *osmZ* also influences chromosomal topology.

Mutations in *osmZ* were isolated as *trans*-acting mutations that allow *proU* transcription in media of low osmolarity. Subsequently, we found that these mutations also affect the expression of several other genes that are dispersed around the chromosome, including *ompF* and *ompC*, the *bglCSB* operon, and *fimA*. Indeed, *osmZ* appears to be identical to three previously identified regulatory genes, *pilG*, *cur*, and *bglY*, that were originally identified as influencing expression of the *fimA*, *flu*, and *bglCSB* operons, respectively. The fact that *osmZ* mutations influence the expression of several unrelated genes, most of which play no obvious role in osmoprotection, makes it unlikely that the *osmZ* gene product is a classical positive or negative osmoregulatory protein. Furthermore, the phenotypes of cells carrying an *osmZ* mutation imply an alteration in osmotic sensing; *osmZ* mutations do not express *proU* constitutively, but appear to perceive a higher external osmolarity than that to which they are actually exposed. This is also apparent when the effects of *osmZ* mutations on porin expression are examined. Normally, OmpF is expressed at low osmolarity and OmpC at high osmolarity. In *osmZ* mutants, OmpF synthesis is reduced and OmpC increased, again as if the cell perceives a higher osmolarity than that to which it is actually exposed. These phenotypes are not compatible with inactivation of a classical regulatory protein but suggest that *osmZ* mutants are defective in osmosensing.

Significantly, DNA supercoiling has been implicated in the expression of the various *osmZ*-dependent genes. In this paper we have demonstrated that transcription of *proU* is highly supercoiling dependent; the same is true for the *ompF* and *ompC* porin genes (our unpublished data). The *bglCSB* operon, required for β-glucoside utilization (Schnetz et al., 1987; Mahadevan et al., 1987), is normally cryptic, and can be activated by an IS insertion or point mutation immediately upstream from the promoter (Reynolds et al., 1981). The *bglCSB* promoter is known to be sensitive to supercoiling, and it has been suggested that activation by upstream IS elements might be a result of altered DNA topology at the *bglCSB* promoter

(Reynolds et al., 1985). It should be noted, however, that *bgl* is activated by gyrase mutations that decrease superhelix density, as well as by increased negative supercoiling in *osmZ* mutants. *pilG* mutations increase the frequency of site-specific DNA inversions controlling expression of the phase-variable *fimA* gene, and *pilG* has been proposed to encode a negative inhibitor of recombination (Spears et al., 1986). The enzymes that mediate this DNA inversion event are related to the phage λ int protein (Dorman and Higgins, 1987), which is highly dependent on DNA topology (Craig, 1985). *fimA* inversion is also IHF-dependent (Dorman and Higgins, 1987; Eisenstein et al., 1987), again implicating an important role for DNA topology (Craig and Nash, 1984; Bliska and Cozzarelli, 1987). Similarly, although the *flu* locus is not well characterized, it is metastable and probably regulated by a site-specific DNA inversion event (Diderichsen, 1980b). Presumably, *flu* regulation will be highly topologically dependent, as are most other site-specific recombination processes (Krasnow and Cozzarelli, 1983; Boocock et al., 1986; Johnson et al., 1987). Thus the common link between the diverse genes whose expression is affected by *osmZ* mutations seems to be that all are influenced by DNA supercoiling. As *osmZ* mutations cause an alteration in DNA supercoiling, this is presumably the primary defect. The other phenotypes of *osmZ* mutations can best be explained as secondary consequences of changes in DNA supercoiling.

We can only speculate as to how *osmZ* mutations might influence DNA supercoiling. We have shown unambiguously that *osmZ* is distinct from the structural genes encoding topoisomerase I and DNA gyrase. Possibly, *osmZ* encodes an additional topoisomerase. There is indirect evidence that another topoisomerase (relaxing) activity is involved in determining in vivo supercoiling levels (Mirkin et al., 1984; Pruss et al., 1986). A candidate enzyme is topoisomerase III, an activity that has been purified but whose genetic and biological functions remain unknown (Dean et al., 1983; Srivenugopal et al., 1984). Perhaps significantly, topoisomerase III activity is dependent on K^+ ions. A second possibility is that the *osmZ* gene product regulates topoisomerase I or DNA gyrase, either directly at the level of enzyme activity or indirectly by influencing expression of the *topA* or *gyr* genes. A final possibility is that the *osmZ* gene product does not alter nicking-closing activity directly, but instead binds to DNA and alters its apparent superhelicity without introducing strand breaks. It is well established that proteins bound to DNA, such as the HU proteins, can alter topology and linking number (Broyles and Pettijohn, 1986). It is also apparent that the linking deficit of DNA in vivo is rather less than that of DNA isolated from the cell, implying that proteins bound to DNA affect topology in vivo (Lilley, 1986; Bliska and Cozzarelli, 1987). A change in apparent supercoiling due to bound proteins would presumably be compensated for in vivo by topoisomerase and gyrase activity such that DNA isolated from the cell has an increased superhelix density. At present we cannot distinguish between these various possibilities, although the finding that *osmZ* mutations affect two entirely different reporter plasmids, as well as genes located at diverse points on the chromosome, implies that the *osmZ* gene product has broad specificity.

How might extracellular osmolarity influence intracellular DNA supercoiling? Again, there are a number of possibilities, although one model consistent with the available data is that the effects of osmolarity are mediated by K^+ ions. Potassium is the major cationic species in the cell, and a rapid accumulation of K^+ ions is the cell's primary response to osmotic upshock; to a first approximation, intracellular K^+ concentrations increase in proportion to extracellular osmolarity (Epstein and Schultz, 1965; Sutherland et al., 1986). Other osmotic responses, including the induction of *proU* expression, appear to be secondarily induced in response to increased intracellular potassium (Sutherland et al., 1986; Higgins et al., 1987a). Thus it is conceivable that the accumulation of K^+ ions is responsible for altered DNA supercoiling and, hence, the induction of *proU* expression. Consistent with this model is the finding that, when added to cells growing at high osmolarity, glycine betaine restores chromosomal supercoiling to a level similar to that of cells growing at low osmolarity. Glycine betaine is known to replace intracellular K^+, and this is believed to be the mechanism by which it reduces *proU* expression (Sutherland et al., 1986). It is not difficult to envisage a mechanism whereby K^+ ions, accumulated to high intracellular concentrations in response to osmotic shock (as high as 500 mM), might influence DNA topology. K^+ ions are known to influence the activities of topoisomerases I and III, at least in vitro (Wang, 1971; Burrington and Morgan, 1976; Srivenugopal et al., 1984), and K^+ and other ions can affect supercoiling directly (Pollock and Ambremski, 1979; Pollock and Nash, 1983; Brady et al., 1987). Interestingly, osmotic shock has been reported to influence the activities of eukaryotic topoisomerases (Sundin and Varshavsky, 1981). K^+ and other ions can also directly influence the kinetics of structural transitions in DNA such as the extrusion of cruciforms (Sullivan and Lilley, 1987). Interestingly, some, and perhaps many, DNA-binding proteins are buffered against the effects of increasing intracellular K^+. For example, the AraC protein undergoes a conformational change in response to increasing ionic strength (ion compensation) that suppresses the otherwise potentially deleterious effects of increased intracellular salt concentrations on DNA binding and regulation (Martin and Schleif, 1987).

How might changes in DNA supercoiling influence transcription from the *proU* promoter? The factors that determine whether a given promoter is sensitive to the level of DNA supercoiling are not well understood. At the simplest level, negative supercoiling can be seen to provide energy for strand separation and to assist the formation of an open complex (Drew et al., 1985). However, there is no doubt that the situation is actually somewhat more complex. Although transcription generally increases with supercoiling, it eventually reaches an optimum value and then declines at high superhelix densities (Borowiec and Gralla, 1987). Supercoiling also affects DNA bending and looping in promoter regions (Borowiec et al., 1987), and promoter elements outside the melted region have been found to be involved in the response to supercoiling

(Borowiec and Gralla, 1987). Thus, in addition to facilitating strand separation, supercoiling is believed to play a role in recognition by RNA polymerase. Perhaps the sequences at the *proU* promoter make it exquisitely sensitive in this regard. Different promoters are known to show greater or lesser sensitivity to changes in DNA superhelicity. An alternative possibility is that increased supercoiling may induce a structural transition at or near the *proU* promoter. Structural changes, the best characterized of which are the extrusion of cruciforms, are strongly influenced by DNA supercoiling and by other factors, such as temperature and ionic strength (Lilley, 1984; Sullivan and Lilley, 1986, 1987). Such a structural transition might influence the productive binding of RNA polymerase at the *proU* promoter. The sigmoidal increase in *proU* expression over a relatively small change in extracellular osmolarity would be consistent with this model. We have cloned the *proU* promoter and are currently addressing this question.

A final question that needs to be addressed is that of specificity. If chromosomal supercoiling varies with medium osmolarity, how is it that only a small number of genes are osmotically sensitive? A number of points are relevant. First, only about 10% of promoters show more than a 2-fold change in expression upon changes in DNA supercoiling (Drlica, 1987); other genes would not, therefore, be expected to be significantly affected by osmolarity. Second, changes in medium osmolarity do, in fact, affect the expression of a large number of genes (Guitierrez et al., 1987; our unpublished data), although in most cases the effects are only 2- to 3-fold and the genes are not normally considered as osmoregulated functions (our unpublished data). Third, to achieve a large increase in expression with changes in osmolarity, certain promoters (like *proU*) may have a propensity for structural change over just a small change in free energy of supercoiling. This is true, for example, for cruciform extrusion, where a small difference in supercoiling can result in a major structural change (Lilley, 1984). Fourth, the E. coli chromosome is divided into about 40 domains, each of which appears to be independently supercoiled (Worcel and Burgi, 1972; Sinden and Pettijohn, 1981). Osmotic stress may have different effects on different domains, and there may be some specificity of topoisomerases. In eukaryotic cells, topoisomerase I shows marked sequence specificity (Busk et al., 1987). Finally, there is increasing evidence for local topological effects (Ellison et al., 1987). DNA-binding proteins such as IHF or FIS, or even strand separation during transcription, can have local effects on DNA topology and affect promoter function or site-specific recombination events (Pruss and Drlica, 1986; Johnson et al., 1987; Thompson et al., 1987). It is also apparent that several promoters respond differently to DNA supercoiling when cloned onto a plasmid compared with the chromosomal state (e.g., Lamond, 1985), implying a constraining influence of chromosomal structure on topological sensitivity. Our observation that in a *topA* deletion strain *tos* mutations affect plasmid supercoiling, but do not affect chromosomal *proU* expression, is also consistent with this idea. A more complete understanding of the control of chromosomal supercoiling, and the mechanisms whereby supercoiling influences promoter activity, will be necessary before the specificity of regulation can be fully understood.

The present study provides strong evidence that changes in DNA supercoiling are important for the specific induction of *proU* expression in response to osmotic stress. Might other osmoregulated genes be similarly regulated? We have obtained evidence that DNA supercoiling is at least partly responsible for the osmotic control of porin expression, and have shown that porin regulation is modified by *osmZ* mutations. We have also argued elsewhere that a general signal (intracellular K^+) is responsible for the induction of several other osmoregulated genes (Higgins et al., 1987a). It therefore seems a reasonable hypothesis that at least some of these genes (e.g., the *bet* betaine biosynthesis genes) will respond to supercoiling in a manner similar to *proU*. It will be interesting to compare the sequences of the promoters of several such genes. Besides osmolarity, could supercoiling play a role in the response to other environmental stimuli? It has previously been shown that DNA supercoiling changes when cells are shifted to anaerobic growth, and it has been suggested that this change in superhelicity might play a role in regulation of the aerobic–anaerobic switch (Yamamoto and Droffner, 1985). Although certain anaerobically induced genes are sensitive to supercoiling, it now seems unlikely that anaerobically induced changes in supercoiling are responsible for the induction of the majority of anaerobic enzymes (Kranz and Haselkorn, 1986; our unpublished data). Nevertheless, we can demonstrate an interaction between osmolarity and anaerobicity in determining the absolute level of expression of several genes. It therefore seems likely that environmentally induced changes in DNA supercoiling will provide an additional, overlying level of regulation that influences the expression of many genes, in addition to providing the predominant means of control for a subset of osmotically induced genes such as *proU*.

Experimental Procedures

Bacterial Strains and Growth Conditions

All S. typhimurium and E. coli strains used in this study are listed in Table 5. Unless otherwise indicated, the same genetic backgrounds were always used: LT2 and MC4100 for S. typhimurium and E. coli, respectively. Pairs of strains for comparison were isogenic except for the lesion indicated. Cells were grown aerobically at 37°C. The following rich and minimal media were used: Luria broth (LB; Roth, 1970); nutrient broth (NB; Miller, 1972); minimal medium A (MMA; Miller, 1972); minimal medium A plus 0.1% casamino acids (MMAA). Glucose (0.4%) was used as carbon source in minimal media unless otherwise stated. For auxotrophic screening and selection, VBCG plates were used (Roth, 1970) with supplementary amino acids, when required, at 0.4 mM. Solid media were prepared by using 1.5% agar. MacConkey-lactose plates were as described by Miller (1972). When appropriate, the osmolarity of the medium was increased by addition of NaCl to a final concentration of 0.3 M or an iso-osmotic concentration of sucrose (0.44 M). Antibiotics were used at the following concentrations: Kanamycin (Kan), 25 μg ml^{-1}; tetracycline (Tet), 20 μg ml^{-1}; ampicillin (Amp), 25 μg ml^{-1}; chloramphenicol (Cml), 12.5 μg ml^{-1}.

Genetic Techniques

Transductions in S. typhimurium were carried out by using a high transducing derivative of phage P22*int-4* as described by Roth (1970). E. coli transductions were performed by using phage P1*vir* (Silhavy et al.,

Table 5. Bacterial Strains

Strain	Genotype	Source/Construction
S. typhimurium		
CH50	*oppB255*::Tn*10 pro-594*	Higgins et al., 1983
CH56	*trp-1012*::Tn*10 pro-594*	Higgins et al., 1983
CH57	*pyrF696*::Tn*10*	Higgins et al., 1983
CH273	*cysB517*	Higgins et al., 1983
CH340	*leu-500 ara-9 trp-1016*::Tn*10*	Richardson et al., 1984
CH582	*leu-500 ara-9* Δ*topA (trp-cysB) 2762*	Richardson et al., 1984
CH584	*leu-500 ara-9* Δ*topA (trp-cysB) 2762 tos-1*	Richardson et al., 1984
CH585	*leu-500 ara-9* Δ*topA (trp-cysB) 2762 tos-2*	Richardson et al., 1984
CH586	*leu-500 ara-9* Δ*topA (trp-cysB) 2762 tos-3*	Richardson et al., 1984
CH588	*leu-500 ara-9* Δ*topA (trp-cysB) 2762 tos-4*	Richardson et al., 1984
CH589	*leu-500 ara-9 tos-1*	Richardson et al., 1984
CH590	*leu-500 ara-9 tos-2*	Richardson et al., 1984
CH591	*leu-500 ara-9 tos-3*	Richardson et al., 1984
CH593	*leu-500 ara-9 tos-4*	Richardson et al., 1984
CH1301	*proU1705*::MudJ	Sutherland et al., 1986
CH1512	*proU1707*::MudJ	Sutherland et al., 1986
CH1513	*proU1708*::MudJ	Sutherland et al., 1986
CH1559	*leu-500 ara-9 proU1708*::MudJ *topA2770*	Recipient CH1568 to Trp$^+$: donor P22 lysate, LT2
CH1560	*leu-500 ara-9 proU1708*::MudJ	Recipient CH1568 for Trp$^+$ Top$^+$; P22 donor lysate, LT2
CH1565	*leu-500 ara-9 trp-1016*::Tn*10 proU1708*::MudJ	Recipient CH340; P22 donor lysate CH1513
CH1566	*leu-500 ara-9 trp-1016*::Tn*10 proU1707*::MudJ	Recipient CH340; P22 donor lysate, CH1512
CH1568	*leu-500 ara-9 trp-1016*::Tn*10 proU1708*::MudJ *topA2770*	*topA* mutation in CH1565, this study
CH1631	*leu-500 ara-9 proU1708*::MudJ Δ*(topA-trp)2771*	*topA* deletion in CH1565, this study
CH1633	*leu-500 ara-9 proU1707*::MudJ *tos-1*	Recipient CH589; P22 donor lysate, CH1512
CH1635	*leu-500 ara-9 proU1707*::MudJ *tos-2*	Recipient CH590; P22 donor lysate, CH1512
CH1637	*leu-500 ara-9 proU1707*::MudJ *tos-3*	Recipient CH591, P22 donor lysate, CH1512
CH1639	*leu-500 ara-9 proU1707*::MudJ *tos-4*	Recipient CH593, P22 donor lysate, CH1512
CH1641	*leu-500 ara-9* Δ*topA(trp-cysB)2762 proU1707*::MudJ	Recipient CH582, P22 donor lysate, CH1512
CH1642	*leu-500 ara-9* Δ*topA(trp-cysB)2762 proU1707*::MudJ *tos-1*	Recipient CH584, P22 donor lysate, CH1512
CH1643	*leu-500 ara-9* Δ*topA(trp-cysB)2762 proU1707*::MudJ *tos-2*	Recipient CH585, P22 donor lysate, CH1512
CH1644	*leu-500 ara-9* Δ*topA(trp-cysB)2762 proU1707*::MudJ *tos-3*	Recipient CH586, P22 donor lysate, CH1512
CH1645	*leu-500 ara-9* Δ*topA(trp-cysB)2762 proU1707*::MudJ *tos-4*	Recipient CH588, P22 donor lysate, CH1512
CH1648	*cysB517 pyrF696*::Tn*10*	Recipient CH273; donor P22 lysate, CH57
CH1649	*cysB517 trp-1012*::Tn*10*	Recipient CH273; donor P22 lysate, CH56
E. coli		
BRE2071	MC4100 φ*(proU-lacZ)* hyb2 (λ*plac* Mu15) *osmZ200*	This study
BRE2072	MC4100 φ*(proU-lacZ)* hyb2 (λ*plac* Mu15) *osmZ201*	This study
BRE2073	MC4100 φ*(proU-lacZ)* hyb2 (λ*plac* Mu15) *osmZ202*	This study
BRE2074	MC4100 φ*(proU-lacZ)* hyb2 (λ*plac* Mu15) *proU601*	This study
BRE2076	MC4100 φ*(proU-lacZ)* hyb2 (λ*plac* Mu15) *osmZ203*	This study
BRE2080	MC4100 φ*(proU-lacZ)* hyb2 (λ*plac* Mu15) *osmZ204*	This study
BW7622	HfrKL96 (PO44) *trpB114*::Tn*10 thi-1 relA1 spoT1*	Barry Wanner
CH1646	*ara* Δ*(lac-pro) rpsL thi* φ*(fimA-lacZ)* λpl(209) *osmZ200 zch-97*::Tn*10*	Recipient VL386; P1 donor lysate GM128
CH1647	*ara* Δ*(lac-pro) rpsL thi* φ*(fimA-lacZ)* λpl(209) *zch-97*::Tn*10*	Recipient VL386; P1 donor lysate GM128
GM37	MC4100 φ*(proU-lacZ)* hyb2 (λ*plac* Mu15)	May et al., 1986
GM50	MC4100 φ*(proU-lacZ*$^+$)3 (λ*plac* Mu55)	May et al., 1986
GM128	MC4100 φ*(proU-lacZ)* hyb2 (λ *plac* Mu15) *zch-97*::Tn*10 osmZ200*	This study
GM131	MC4100 φ*(proU-lacZ)* hyb2 (λ*plac* Mu15) *zch-97*::Tn*10*	This study
GM152	MC4100 φ*(proU-lacZ*$^+$)3 (λ*plac* Mu55) *osmZ200 srl*::Tn*10*	This study
GM161	*trp-75 cysB93 tfr-8* Δ*(argF-lac)U169* φ*(proU-lacZ)* hyb2 (λ*plac* Mu15)	This study
GM230	MC4100 φ*(proU-lacZ)*hyb2 (λ*plac* Mu15) *osmZ205*::Tn*10*	Recipient GM37; donor *zch-96*::Tn*10* from K. Hantke
MBM7007	*araC*(am) *araD* Δ*(argF-lac)U196 trp*(am) *malB*(am) *rpsL relA thi*	Berman and Beckwith, 1979
MBM7014	*araC*(am) *araD* Δ*(argF-lac)U196 trp*(am) *malB* (am) *rpsL relA thi supF*	Berman and Beckwith, 1979
MC4100	*araD139* Δ*(argF-lac)U196 rpsL150 relA1 deoC1 ptsF25 rbsR flb* B5301	Casadaban, 1976
PLK831	*gal-25 trpE63 pyrF287 fnr-1 rpsL195 iclR7 trpR72*	B. Bachmann
VL386	*ara* Δ*(lac-pro) rpsL thi* φ*(fimA-lacZ)* λpl(209)	Freitag et al., 1985

1984). After transduction of transposons Tn*5*, Tn*10*, Mu, or λ*plac* Mu between strains, the location of the transposon and the presence of just a single copy of the transposon in the transductant were checked by marker rescue. A Tn*10* transposon linked to the *osmZ200* mutation was isolated by the transducing of BRE2071 to Tet^r with a phage P1 lysate grown on a pool of several thousand independent chromosomal Tn*10* insertions, and screening of the Tet^r transductants for the OsmZ$^-$ phenotype on MacConkey-lactose plates. A Tn*10* insertion isolated in this manner (*zch-97::*Tn*10*) was 83% linked to the *osmZ200* mutation by P1 transduction.

Plasmid DNA was transformed into competent E. coli strains as described by Silhavy et al. (1984). Plasmid DNA was introduced into S. typhimurium by transformation into the restriction-deficient strain LR5000, and was subsequently moved between strains by P22-mediated transduction. From complementation studies, F'123 was moved into appropriate recipients, with selection for Trp$^+$ transconjugants performed as described previously (Gibson et al., 1984).

Selection of *topA* Mutations by Suppression of *leu-500*

S. typhimurium *topA* mutations can readily by selected by suppression of the *leu-500* promoter mutation (Mukai and Margolin, 1963; Trucksis et al., 1981; Richardson et al., 1984). *leu-500* is a point mutation in the −10 region of the *leu* operon promoter, and confers leucine auxotrophy (Gemmill et al., 1984). Selection for leucine prototrophs yields true revertants and unlinked pseudorevertants that are in the *topA* gene (originally called *supX*), and is described in detail by Richardson et al. (1984). The *topA* pseudorevertants can be distinguished from true revertants by their small colony size, even on rich medium. Selection for Leu$^+$ derivatives of CH1565 (*leu-500 proU-lacZ trp-1016::*Tn*10*) was on minimal plates supplemented with tryptophan (0.4 mM). Two distinct size classes of Leu$^+$ revertants arose at approximately equal frequencies. The small colonies (presumed *topA* mutants) were picked, purified, and characterized further. All those analyzed contained a suppressor mutation that was genetically unlinked to the *leu* operon but linked to *trp*, as expected for *topA* mutations. Many were found to be Tet^s and Trp$^-$ because of a deletion extending from *trp::*Tn*10* through *topA*. It is well known that Tn*10* insertions excise imprecisely at high frequency, deleting adjacent chromosomal DNA (Kleckner et al., 1979). As the *trp* and *topA* genes are closely linked on the S. typhimurium chromosome, such deletions were not unexpected.

Mapping the *leu-500* Suppressor Mutation to *topA*

Strain CH1568 contains a *leu-500* suppressor that confers two additional phenotypes, osmotic sensitivity and induction of *proU* at low osmolarity. A single mutation in *topA* was shown to be responsible for all three phenotypes. First, when the *proU-lacZ* fusion from CH1568 was transduced into a wild-type background, all transductants (50 out of 50) were regulated normally, confirming that the altered osmotic response is due to a mutation unlinked to the *proU* locus. Second, when phage P22 grown on strain CH1568 was used to transduce CH1560 (*leu-500 proU-lacZ*) to Tet^r, 64% (96/150) of the transductants became Leu$^-$, were osmotically sensitive, and expressed *proU-lacZ* constitutively. The three phenotypes never segregated from each other, showing that they are due to the same, single mutation linked to the *trp::*Tn*10*. Third, when CH1568 was transduced to Trp$^+$ by using a P22 lysate grown on the wild-type strain LT2, 72% (36/50) became Leu$^-$ (i.e., had lost the *leu-500* suppressor). All of these Leu$^-$ derivatives had also lost the ability to express *proU* at low osmolarity as well as their osmotic sensitivity; again, we were unable to separate the three phenotypes genetically.

Enzyme Assays

β-galactosidase activity was measured in SDS-chloroform permeabilized cells at either mid-log or stationary phase, as indicated. The relative activities, expressed as described by Miller (1972), were the same at either stage of growth. β-glucosidase activity was assayed in growing cells by measurement of the ability to transport, phosphorylate, and hydrolyze the analog p-nitrophenyl-β-D-glucoside (2 mM), yielding a yellow chromophore. Cells were grown in MMA with 0.4% succinate as carbon source and the gratuitous inducer β-methyl-D-glucoside at 5 mM (Prasad and Schaefler, 1974). Relative units were calculated as for β-galactosidase.

Measurement of DNA Supercoiling

In vivo supercoiling was monitored by determination of the relative distribution of topoisomers of a reporter plasmid. Two different plasmids were used, pACYC184 (Cml^r Tet^r, 4.0 kb; Chang and Cohen, 1978) and the ColE1-based plasmid pLK1 (Amp^r, 2.1 kb; Richardson et al., 1984). Plasmid DNA was purified from cleared lysates of cells grown to an OD_{600} of 0.8 to 1.0 by cesium chloride density gradient centrifugation as described previously (Birnboim and Doly, 1979; Maniatis et al., 1982). Topoisomers were separated by electrophoresis in 1% agarose gels containing either 1.5 μg ml^{-1} or 25 μg ml^{-1} chloroquine, as indicated. Depending on the chloroquine concentration, the mobility of different topoisomers varies: at 1.5 μg ml^{-1} the more supercoiled topoisomers migrate faster; at 25 μg ml^{-1}, the more relaxed topoisomers migrate faster. Electrophoresis was in 90 mM Tris (pH 8.3), 90 mM borate, 10 mM EDTA, and the appropriate concentration of chloroquine at 3 V per cm^{-1} for 18 hr. Chloroquine was washed from the gel by soaking in distilled water for at least 4 hr before staining with ethidium bromide and photographing under UV light.

Measurement of Glycine Betaine Transport

Cells were grown overnight in MMA containing 75 mM NaCl. The cells were then washed twice and resuspended in MMA lacking a carbon source but containing 300 mM NaCl, the osmolarity at which the ProU transport system functions optimally (Cairney et al., 1985a; May et al., 1986). Cells were equilibrated at room temperature for 10 min, and transport was initiated by mixing 2 ml of cells with [methyl-^{14}C]-glycine betaine (7.1 mCi mmol^{-1}; Amersham). The final concentration of glycine betaine was 1.4 μM, a sufficiently low concentration that essentially all glycine betaine uptake is via the ProU system. Samples (200 μl) were removed at the indicated times, and the cells were collected by filtration through Millipore filters (0.45 μm pore size) and washed with 10 ml of MMA containing 0.3 M NaCl. The filters were then dried, and the radioactivity that had been retained was measured by scintillation counting. The data are mean values of duplicate experiments.

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