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An overlap between osmotic and anaerobic stress responses: a potential role for DNA supercoiling in the coordinate regulation of gene expression

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Summary

The regulation of several genes in response to osmotic and anaerobic stress has been examined. We have demonstrated a clear overlap between these two regulatory signals. Thus, the osmotically induced *proU* and *ompC* genes require anaerobic growth for optimum induction while the anaerobically induced *tppB* gene is also regulated by osmolarity. Furthermore, normal expression of *tppB* and *ompC* requires the positive regulatory protein OmpR, yet this requirement can be partially, or even fully, overcome by altering the growth conditions. Finally, the pleiotropic, anaerobic regulatory locus, *oxrC*, is also shown to affect expression of the osmotically regulated *proU* gene. The *oxrC* mutation is shown to affect the level of negative supercoiling of plasmid DNA and its effects on gene expression can be explained as secondary consequences of altered DNA topology. We suggest that there is a class of 'stress-regulated' genes that are regulated by a common mechanism in response to different environmental signals. Furthermore, our data are consistent with the notion that this regulatory overlap is mediated by changes in DNA supercoiling in response to these environmental stresses.

Introduction

There is now considerable evidence for global regulatory networks in which a given environmental signal causes the coordinate induction or repression of a number of diverse and unlinked genes (Gottesman, 1984). For example, when cells are shifted from aerobic to anaerobic growth conditions the rate of synthesis of over fifty proteins is altered (Smith and Neidhardt, 1983a,b; Spector *et al.*, 1986). Similarly, the expression of many genes is affected by changes in the osmolarity or pH of the growth medium (Higgins *et al.*, 1987; Guitierrez *et al.*, 1987; Aliabadi *et al.*, 1988). Unlike more specific regulatory stimuli, such as the presence or absence of a defined nutrient, these more 'diffuse' environmental changes have often proved less tractable when it comes to identifying regulatory proteins which mediate the cell response. For example, although the FNR protein was identified many years ago as a positive regulator of a number of anerobically induced genes (Newman and Cole, 1978; Lambden and Guest, 1976), it is now clear that many anaerobic genes are FNR-independent. Additional regulatory loci, each affecting a different subset of anaerobically induced genes, have been identified (Jamieson and Higgins, 1984; 1986; Aliabadi *et al.*, 1988). Similarly, selections designed to identify genes responsible for osmotic control of gene expression have failed to identify a global osmotic regulatory protein (Higgins *et al.*, 1988). Although the OmpR and EnvZ proteins are important for the osmotic regulation of porin expression (Hall and Silhavy, 1981a,b), the expression of most osmotically regulated genes is OmpR- and EnvZ-independent (Cairney *et al.*, 1985; May *et al.*, 1986; Guitierrez *et al.*, 1987).

DNA supercoiling is known to play an important role in determining the efficiency with which many promoters are transcribed. Two enzymes, DNA gyrase and DNA topoisomerase I, are primarily responsible for determining the level of DNA supercoiling. DNA gyrase adds negative supercoils in an energy-dependent reaction while topoisomerase I removes supercoils and relaxes DNA. Perturbation of the activities of these enzymes, by mutation or by the use of inhibitors, affects transcription from a variety of promoters (Sanzey, 1979; Drlica, 1984; 1987; Richardson *et al.*, 1984; Menzel and Gellert, 1987; Jovanovich and Lebowitz, 1987) and many promoters are sensitive to DNA supercoiling *in vitro* (Wood and Lebowitz, 1984; Borowiec and Gralla, 1987; Dixon *et al.*, 1988). Recently it has been demonstrated that the supercoiling of cellular DNA is not maintained at a constant level but varies in response to growth conditions. Thus, environmental stresses such as osmolarity or anaerobicity can influence the supercoiling of cellular DNA (Higgins *et al.*, 1988; Dorman *et al.*, 1988). Other factors such as growth phase and carbon limitation also affect DNA topology (Dorman *et al.*, 1988; Balke and Gralla, 1987). Furthermore, these changes in DNA supercoiling appear to be responsible for regulating the expression of a number of specific genes in response to environmental stimuli (Higgins *et al.*, 1988; Dorman *et al.*, 1988). The finding that different environmental stresses

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can have apparently similar effects on DNA supercoiling leads to the prediction that supercoiling-sensitive genes may be regulated in response to multiple stimuli. In this paper we test this prediction. Although our findings are limited to a subset of genes, our results demonstrate a clear overlap between the responses to osmotic stress and to the availability of oxygen. We suggest that there is a class of 'stress-induced' genes whose expression is influenced by a variety of external stimuli and is regulated by a common mechanism. Our data support the model that environmentally induced changes in DNA supercoiling play an important role in the regulation of bacterial gene expression in response to stress. Changes in DNA supercoiling appear to provide an underlying global regulatory network upon which more specific regulatory processes are superimposed.

Results and Discussion

Both increased osmolarity and anaerobic growth can lead to an increase in the degree of negative supercoiling of cellular DNA and, furthermore, these topological changes appear to be responsible for the induction or repression of certain genes (Higgins *et al.*, 1988; Dorman *et al.*, 1988). These findings lead to the prediction that genes induced or repressed by, for example, osmolarity, might also be affected by anaerobic growth, and vice versa. We have therefore examined the effects of both osmolarity and anaerobiosis on the expression of genes that are generally considered to be specifically regulated by only one of these stimuli.

The OmpC porin is anaerobically regulated

The OmpC and OmpF porins are major outer membrane proteins of *Escherichia coli* and *Salmonella typhimurium*. The relative levels of these proteins depend upon growth conditions, the most important of which is generally considered to be osmolarity (Hall and Silhavy, 1981a). At low osmolarity, transcription of *ompF* predominates and *ompC* is repressed, while at high osmolarity, *ompF* is repressed and *ompC* expression is increased.

To assess whether porin expression in *E. coli* is also regulated by oxygen availability, we took advantage of *ompC-lacZ* and *ompF-lacZ* operon fusions (Table 1). At constant osmolarity, the expression of *ompC* was strongly induced by anaerobic growth and was repressed in highly aerated cultures. Induction by osmolarity and by anaerobiosis did not appear to be additive. In contrast, *ompF-lacZ* expression was relatively unaffected by anaerobic incubation; for *ompF*, the osmolarity of the growth medium appears to be the major regulatory factor. Since *ompC-lacZ* fusions are not available in *S. typhimurium*, expression of the porins in this species was monitored by gel electrophoresis (Fig. 1). The anaerobic induction of OmpC was similar to that found for the *E. coli omp-lacZ* fusions.

Table 1. Effect of anaerobiosis on porin expression in *E. coli*.

Strain	$+O_2$	$+O_2$ +0.3M NaCl	$-O_2$	$-O_2$ +0.3M NaCl
MH225 (*ompC-lacZ*)	247	592	1135	1078
CH1145 (*ompC-lacZ ompR*)	11	14	50	44
MH513 (*ompF-lacZ*)	363	185	464	141
CH1147 (*ompF-lacZ ompR*)	4	2	3	2

Cells were grown in minimal glucose medium, aerobically or anaerobically, in the presence or absence of 0.3M NaCl as indicated. β-galactosidase activity was assayed for cells in early-exponential growth.

These results show that anaerobiosis is an important component in the regulation of *ompC* expression. Indeed, the *ompC* gene can be efficiently induced in anaerobic cultures in the absence of any increase in growth-medium osmolarity; *ompC* could be justifiably designated as an anaerobically regulated gene rather than (as is generally the case) as an osmoregulated gene.

proU *expression requires anaerobic growth*

The *proU* gene encodes a glycine-betaine transport system which plays an important role in the cell's adaptation to osmotic stress (Csonka, 1982; Cairney *et al.*, 1985; May *et al.*, 1986). Expression of *proU* is tightly regulated by medium osmolarity and this regulation appears to be mediated by changes in chromosomal supercoiling (Higgins *et al.*, 1988). Figure 2 shows that anaerobiosis also has a considerable influence on *proU* expression. Like *ompC*, expression of *proU* is strongly induced by anaerobic growth although, unlike *ompC*, expression of *proU* shows an absolute requirement for raised osmolarity. The apparent decrease in *proU* expression observed at very high osmolarities (0.45M NaCl) is due to a general inhibition of protein synthesis capacity under these conditions (unpublished results).

Most previous studies on *proU* expression have been carried out in semi-aerated cultures in which shaking is insufficient to achieve complete aeration during growth. Under such conditions we observe an intermediate level of expression, between that found for fully aerated and for fully anaerobic cultures (Fig. 2; Sutherland *et al.*, 1986). Thus, absolute anaerobiosis, such as that achieved with a GasPack, is not required for induction of *proU*. It should also be noted that when growing 'aerated' cultures in test tubes we frequently observe variability in *proU* expression between independent cultures, as a result of differences in

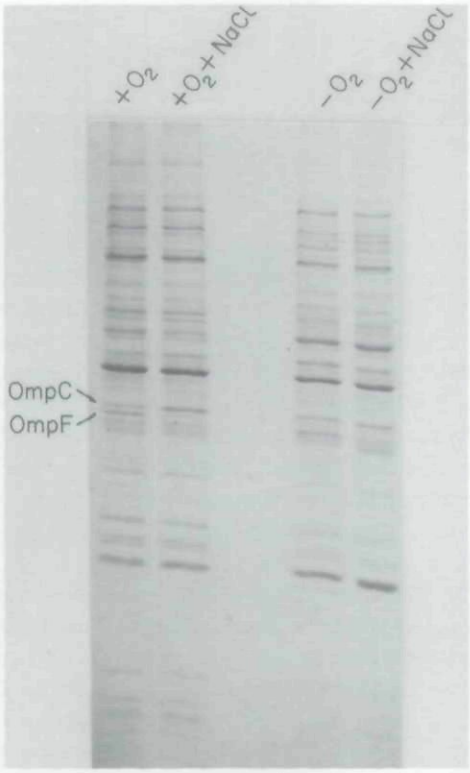

Fig. 1. The effect of anaerobic growth on the expression of OmpC and OmpF in *S. typhimurium*. The wild-type strain LT2 was grown in minimal-glucose medium at 37°C. 0.3 M NaCl was added, where indicated, to increase the osmolarity of the medium. Cells were grown aerobically or anaerobically to mid-exponential phase, harvested, and cell proteins separated by electrophoresis on a 12.5% SDS-polyacrylamide gel. Protein bands were visualized by staining with Coomassie Brilliant Blue. The OmpC and OmpF porins are indicated.

aeration (affected by tube diameter and the density to which the culture was grown; data not shown). This illustrates the care that must be taken to ensure reproducible conditions during the study of promoters such as *proU*, which are affected by several different environmental parameters. Again, the distinction between osmotic and anaerobic genes becomes blurred. Indeed, if *proU* expression had originally been monitored in LB as a function of anaerobiosis the gene could, sensibly, have been designated as an anaerobically regulated gene.

Effect of medium osmolarity on tppB *expression*

The results above show that the expression of two well-characterized, osmotically regulated genes is also strongly influenced by changes in anaerobiosis. We were interested to determine whether the converse might also be true, i.e. whether genes normally considered to be part of the anaerobic response also respond to osmotic stress.

The *tppB* locus encodes the tripeptide permease of *S. typhimurium*. [Note: The latest *Salmonella* genetic map (Sanderson and Roth, 1988) mistakenly designates *tppB* as allelic with *envZ*. This is entirely incorrect. *tppB* encodes the tripeptide permease and is located at 27 min on the chromosome.] Expression of *tppB* is induced by anaerobic growth and by leucine and/or alanine; the effects of these two regulatory signals are additive (Gibson *et al.*, 1984; Jamieson and Higgins, 1984). We previously reported that *tppB* is not osmotically regulated (Gibson *et al.*, 1987). Those experiments were carried out aerobically in rich medium (i.e. with leucine and alanine present) and are reproducible (Fig. 3). However, when grown anaerobically, *tppB* expression depends to a considerable extent on the osmolarity of the growth medium. At low osmolarity, expression is only 15% of that observed in the presence of 0.15 M or 0.3 M NaCl. Thus, both anaerobiosis and osmolarity play important roles in regulation of the 'anaerobic' *tppB* gene.

Our data imply a relationship between promoters such as *ompC, proU* and *tppB*, all of which are regulated by more than one stimulus. Other promoters also fall into this category (see below; unpublished results). Furthermore, data presented and discussed below suggest that these genes are regulated by a common mechanism involving changes in DNA supercoiling. We suggest that this class of genes be designated 'stress-regulated', rather than being referred to by the restrictive classifications of osmotically or anaerobically regulated genes.

Effects of gyrase inhibitors

Expression of the *tppB, ompC* and *proU* genes is stimulated both by osmolarity and by anaerobiosis. As both

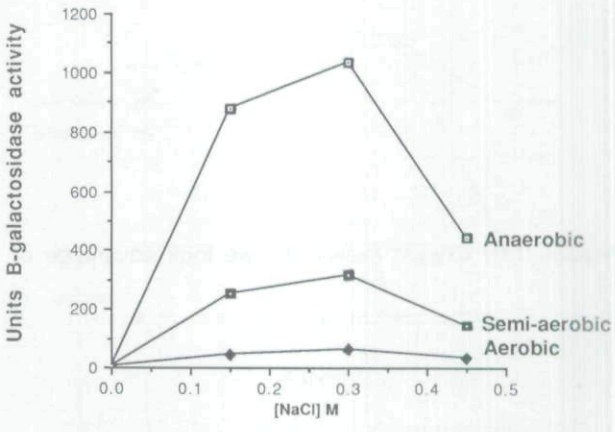

Fig. 2. The effect of osmolarity and anaerobiosis on *proU* expression. Cells of CH1301 (*proU-lacZ*) were grown at 37°C in minimal glucose medium containing the indicated amounts of added NaCl. β-galactosidase activity was assayed in exponentially growing cultures grown aerobically with extremely vigorous agitation (◆), semi-aerobically in unshaken test tubes (■), or anaerobically (□).

Table 2. Effect of novobiocin on expression of a *tppB-lacZ* fusion.

Medium	MMA	MMA+NaCl	MMA +Novobiocin	MMA+NaCl +Novobiocin
Units β-galactosidase	148	1182	149	281

Strain CH776 (*tppB-lacZ*) was grown anaerobically in MMA ± 0.3 M NaCl. Where indicated, novobiocin was added at 25 μg ml^{-1}. β-galactosidase activity was assayed for cells in mid-exponential growth.

these stresses cause similar increases in DNA supercoiling, these results are consistent with the idea that changes in DNA supercoiling play an important role in their regulation. We have shown elsewhere that the *proU* and *ompC* promoters are highly supercoiling-sensitive. For example, their expression is affected by inhibitors of DNA gyrase (Higgins *et al.* 1988; Graeme-Cook *et al.*, 1989). The data in Table 2 show that, as predicted, the osmotic induction of *tppB* expression is also strongly reduced in the presence of novobiocin. This provides further evidence for a regulatory link between these 'stress-regulated' genes and demonstrates that changes in DNA supercoiling can have major effects on these 'stress-regulated' promoters.

The pleiotropic oxrC *(oxygen regulation) mutation affects DNA supercoiling*

The *oxrC* gene was originally identified as being essential for the anaerobic induction of *tppB* expression. In addition, *oxrC* mutations are highly pleiotropic and inhibit the anaerobic induction of a number of other anaerobically regulated functions, including the formate dehydrogenase component of formate hydrogen lyase, threonine dehydratase and two of the three hydrogenase isoenzymes (Jamieson and Higgins, 1986). Surprisingly, *oxrC* mutations were found to be allelic with *pgi*, the structural gene for the glycolytic enzyme phosphoglucose isomerase (PGI), and the loss of PGI activity was found to be the primary defect in the reduced expression of anaerobic genes. As *oxrC* mutations could be phenotypically suppressed by the addition of fructose to the growth medium it was apparent that it was not loss of the PGI protein *per se* which prevented the anaerobic induction of gene expression, but simply the reduced flux of carbon through glycolysis.

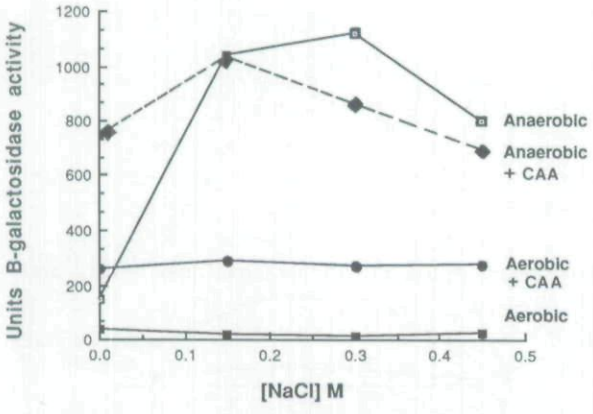

Fig. 3. The effect of osmolarity on expression of *tppB*. Strain CH776 (*tppB-lacZ*) was grown at 30°C in minimal glucose medium containing, where appropriate, 1 mg ml^{-1} casamino acids (CAA; containing leucine and alanine, inducers of *tppB* expression) and the indicated concentrations of NaCl (■), aerobic growth without CAA; (●), aerobic growth with CAA; (□), anaerobic growth without CAA; (◆), anaerobic growth with CAA.

Recently, Balke and Gralla (1987) found that carbon limitation could affect the supercoiling of plasmid DNA. This suggested to us that *oxrC* (*pgi*) mutants might mimic 'carbon-starvation', resulting in an alteration in DNA supercoiling and, consequently, altered levels of expression of certain anaerobic genes. If this were the case, the regulatory role of *oxrC* would not be expected to be specific to anaerobically induced genes but might also affect other supercoiling-dependent promoters. Table 3 shows that this is the case and that *oxrC* strains show reduced osmotic induction of *proU* expression. This provides further evidence for a regulatory overlap between the osmotic and anaerobic responses and the concept of 'stress-regulated' promoters.

The observed effects of *oxrC* mutations on gene expression are consistent with an indirect effect via changes in DNA topology. We therefore examined the topoisomer distribution of plasmid DNA isolated from congenic *oxrC*$^+$ and *oxrC*$^-$ strains (Fig. 4). As expected (Higgins *et al.*, 1988), growth at high osmolarity increased

Table 3. Expression of *proU* in an *oxrC* mutant.

Strain	NB	NBS	NBSF	MFS	MGS
CH946 (*proU-lacZ*)	4	544	643	1010	1422
CH1723 (*proU-lacZ oxrC*::Tn*5*)	4	243	228	1388	ND

Cells were grown anaerobically in nutrient broth (NB) or minimal medium (M) containing, where indicated: fructose (0.4%; F), glucose (0.4% G), NaCl (0.3 M; S). ND: not determined (*oxrC* mutants do not grow on MGS). The phenotypes of *oxrC* mutations are normally suppressed by fructose (Jamieson and Higgins, 1986). Surprisingly, fructose added to NB at high osmolarity (NBSF) failed to suppress the effect of *oxrC* mutations on *proU* expression. This observation could be explained if high osmolarity inhibits fructose uptake. The experiment was therefore repeated in minimal medium with fructose as sole carbon source (MFS). Under these conditions the effect of *oxrC* on *proU* expression was suppressed.

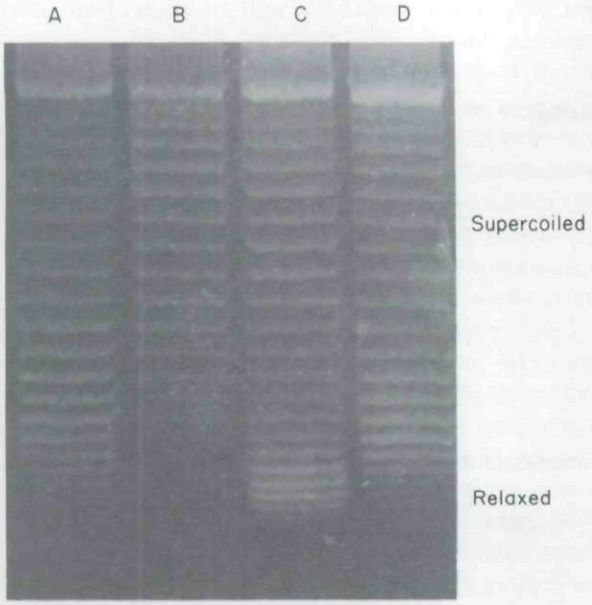

Fig. 4. The effect of an *oxrC* mutation on DNA supercoiling. Strains CH44 (*oxrC*$^+$) and CH881 (*oxrC*$^-$), each harbouring plasmid pACYC184, were grown in NB-tetracycline in the presence or absence of 0.3 M NaCl. After overnight growth (10-ml cultures in universal bottles with gentle shaking) plasmid DNA was extracted and topoisomers separated by electrophoresis as described in the *Experimental procedures*. Lane A, CH44 NB; lane B, CH44 NB + NCl; lane C, CH881 NB; lane D, CH881 NB + NaCl.

the negative supercoiling of isolated plasmid DNA. Whether the cells were grown at low- or at high osmolarity, the *oxrC* lesion resulted in a relaxation of the DNA. This is consistent with the observation that the *oxrC* mutation reduces *proU* expression. Although these measurements were on plasmid DNA, the fact that *oxrC* mutations affect expression from several supercoiling-sensitive chromosomal promoters implies a similar change in chromosomal DNA topology. Thus, the effects of *oxrC* mutations on gene expression appear to be purely indirect, causing a change in DNA supercoiling which has a 'knock-on' effect on gene expression. *oxrC* should no longer be considered as an anaerobic regulatory locus.

Increased osmolarity can overcome the requirement for activator proteins

Changes in DNA supercoiling appear to provide the primary means of regulating expression of 'stress-regulated' promoters, such as *proU*; no evidence for the involvement of a specific regulatory protein has been obtained (Higgins *et al.*, 1988). In contrast, the *ompC* and *tppB* promoters require the positive regulatory proteins OmpR and EnvZ for normal expression (Hall and Silhavy, 1981b; Gibson *et al.*, 1987). How can the requirement for these regulatory proteins be reconciled with a role for changes in DNA supercoiling in determining levels of expression?

OmpR and EnvZ are examples of a large family of two-component regulatory systems which regulate transcription (or other processes) in response to environmental stimuli (Ronson *et al.*, 1987). EnvZ is believed to be a membrane-associated protein which acts as a sensor and modifies OmpR (Slauch *et al.*, 1988). OmpR then interacts with the *ompC* and *ompF* promoter DNA to activate transcription (Norioka *et al.*, 1986; Maeda and Mizuno, 1988). However, the mechanisms by which EnvZ senses osmolarity and by which OmpR activates transcription remain obscure.

We examined the effects of anaerobiosis and osmolarity on *tppB* and *ompC* expression in *ompR* mutants. Two different *ompR* insertion mutants were introduced into *tppB* strains, one of which is towards the *C*-terminal end of OmpR and leaves a partially functional protein while the other insertion completely inactivates OmpR (Gibson *et al.*, 1987). Figure 5 shows that, for *tppB*, the requirement for *ompR* can be overcome at elevated osmolarities. Indeed, expression of *tppB* in an *ompR* mutant at high osmolarity is greater than that seen for the OmpR$^+$ strain at 'normal' osmolarities. Thus, OmpR is not essential for high-level expression of *tppB*. Similar experiments with the *E. coli ompC-lacZ* and *ompF-lacZ* fusions show that anaerobic induction of *ompC* expression still occurs in an *ompR* mutant, although at a reduced level (Table 1). It has been shown elsewhere that osmotic regulation of porin synthesis can also occur in OmpR and/or EnvZ mutants (Ramakrishnan *et al.*, 1985; Forst *et al.*, 1988). However, in contrast to *tppB*, the OmpR requirement for porin expression cannot be fully overcome by varying the growth conditions, at least within the range examined.

These results lead to two important conclusions. First, while OmpR is clearly required for optimum expression of *tppB* and *ompC*, anaerobic and osmotic regulation can still occur in the absence of this positive regulator. Thus, the OmpR-EnvZ proteins are not the sole mediators of their osmotic regulation. Second, it is possible to achieve high-level expression in the complete absence of OmpR and EnvZ, at least for the *tppB* promoter, simply by altering the osmolarity of the growth medium. These results, and those elsewhere (Graeme-Cook *et al.*, 1989), suggest that osmotic regulation of the porins is mediated by two distinct mechanisms: one involving the OmpR and EnvZ proteins, and the other involving changes in DNA supercoiling. The two regulatory systems interact to determine the efficiency of productive initiation of transcription by RNA polymerase. The involvement of specific control systems, superimposed on stress-induced supercoiling changes, can add an extra specificity to the responses of 'stress-regulated' genes.

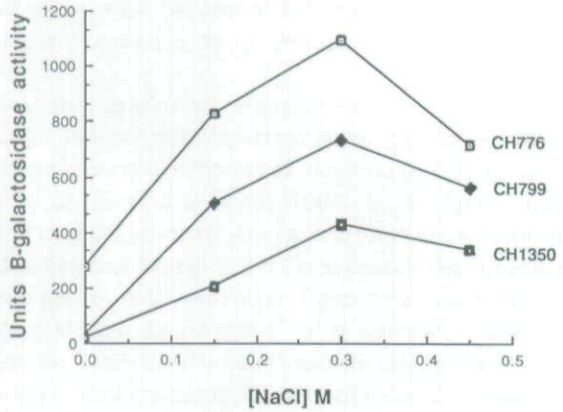

Fig. 5. Expression of *tppB* in *ompR* mutants. Cells were grown at 30°C, anaerobically in minimal glucose medium with NaCl added as indicated. β-galactosidase activity was assayed during exponential growth. Strains used were CH776 (*tppB-lacZ*); CH799 (*tppB-lacZ ompR*::Tn*5*); and CH1350 (*tppB-lacZ ompR*::Tn*10*).

A general class of 'stress-regulated' genes

We suggest that genes such as *tppB*, *proU* and *ompC* are members of a family of 'stress-regulated' genes linked by a common regulatory mechanism involving environmentally induced changes in DNA supercoiling. How many other known genes fall into this category?

Osmoregulated genes. Several genes have been identified whose expression is altered by medium osmolarity (Higgins *et al.*, 1987; Guitierez *et al.*, 1987). Besides *proU* and the porins, the only well-characterized genes are the *bet* genes encoding the glycine-betaine biosynthetic enzymes. The *bet* genes are sensitive to stimuli other than osmolarity, including anaerobiosis and temperature (Eshoo, 1988). *bet* expression is also altered by mutations which affect DNA supercoiling (unpublished data). Thus, the *bet* genes are also 'stress-regulated'. The little that is known about other osmotically induced genes suggests that they also respond to stimuli in a manner similar to *proU* and hence are also probably 'stress-regulated' (Higgins *et al.*, 1987). It is important, however, to emphasize that although most genes designated as osmotically regulated may well be examples of supercoiling-dependent, 'stress-regulated' genes, this is not always the case. For example, expression of the *kdp* operon (encoding a K^+ transport system) is affected by changes in medium osmolarity but is actually regulated by a rather specific mechanism which responds to cell turgor (Laimins *et al.*, 1981; Sutherland *et al.*, 1986).

Anaerobic regulation. There are two distinct classes of anaerobically regulated genes: those which require the positive activator protein FNR, and those which are FNR-independent (Jamieson and Higgins, 1984; 1986). This distinction is consistent with the roles of the gene products. In general, FNR-dependent genes encode specific respiratory proteins which are required only when oxygen is absent and are induced specifically during anaerobic growth. In contrast, many FNR-independent genes serve more general metabolic functions which may play roles in adapting the cell to stress conditions other than simply anaerobiosis. Many, if not all of these FNR-independent anaerobic genes should perhaps be designated 'stress-regulated'. Furthermore, there is increasing evidence of a role for DNA supercoiling in the regulation of many FNR-independent anaerobic genes besides those such as *tppB* discussed above. For example: (i) DNA supercoiling plays a central role in the anaerobic repression of *tonB* expression (Dorman *et al.*, 1988); (ii) certain mutations which map near the genes encoding DNA gyrase result in a failure to grow anaerobically (Yamamoto and Droffner, 1985; K. Drlica, personal communication); (iii) the *oxrC* mutation which appears to affect DNA supercoiling (see above) affects many FNR-independent promoters (Jamieson and Higgins, 1986); and (iv) expression of many FNR-independent anaerobic genes is affected by gyrase inhibitors (Kranz and Haselkorn, 1986; Novak and Maier, 1987; Axley and Stadtman, 1988; Dimri and Das, 1988; Dixon *et al.*, 1988).

In contrast, osmolarity or mutations which alter supercoiling have little effect on the activities of FNR-dependent enzymes including peptidase T, nitrate reductase and fumarate reductase (unpublished results). In addition, anaerobic changes in DNA supercoiling appear to be insufficient to activate FNR binding and promoter function (N. Ni Bhriain, C. F. Higgins, S. Jayaraman and S. Busby, unpublished results). Thus, although DNA supercoiling is likely to contribute to FNR function at some level (as it does for all DNA-protein interactions), environmentally induced changes in DNA supercoiling do not appear to contribute significantly to the regulation of FNR-dependent promoters.

Other environmental stresses. Our considerations have been limited to osmotic and anaerobic stress. However, available evidence suggests that this overlap in stress responses is not confined to osmolarity and anaerobiosis. Other stimuli such as pH, temperature, and nutritional status also influence expression of 'stress-regulated' genes. For example, porin expression is affected by pH, temperature and other undefined aspects of medium composition (Kawaji *et al.*, 1979; van Alphen and Lugtenberg, 1977; Graeme-Cook *et al.*, 1989; Heyde and Portalier, 1987; Lundrigan and Earhart, 1984; Scott and Harwood, 1980; Barron *et al.*, 1986). Several anaerobically regulated genes are also pH-sensitive (Aliabadi *et al.*, 1988), as is the osmotically regulated *proU* gene (unpublished results). The osmotically regulated betaine

biosynthetic genes are also regulated by anaerobiosis and temperature (Eshoo, 1988). Indeed, there is much published data showing an overlap between responses to different stress conditions (Aliabadi *et al.*, 1988; Christman *et al.*, 1985; Groat *et al.*, 1986; Wanner and McSharry, 1982; Spector *et al.*, 1986; Schultz *et al.*, 1988). Finally, parameters such as temperature (Goldstein and Drlica, 1984), growth phase (Dorman *et al.*, 1988), and nutritional status (Balke and Gralla, 1987) can affect supercoiling.

Conclusions

In this paper we define a class of 'stress-regulated' genes that are regulated in response to a number of apparently independent environmental stimuli. These genes are related by their function, (i.e. adaptation of cellular physiology to adverse growth conditions) as well as by their regulatory mechanism which involves environmentally induced changes in DNA supercoiling. Many genes previously considered to be regulated by a single parameter are in fact 'stress-regulated' genes. Indeed, for many genes specific designations such as 'osmotically regulated' or 'anaerobically regulated' are really historical accidents and do not necessarily reflect their primary mode of control.

Environmentally induced changes in DNA supercoiling provide a simple means of achieving such a regulatory overlap. By altering DNA supercoiling, any environmental stimulus can affect the expression of the same, large set of supercoiling-sensitive genes. For many of the 'stress-regulated' promoters there is now strong evidence of a central role for topological changes in the control of their expression (Dorman *et al.*, 1988; Higgins *et al.*, 1988; 1989). We suggest that such topological changes underlie the regulation, by different stresses, of this entire class of genes. Thus, environmentally induced changes in DNA supercoiling provide a global communications network, coordinating the cell's response to different stresses. Superimposed upon this underlying process are more specific regulatory processes (e.g. OmpR).

It must be stressed that not all genes induced in response to a specific stress are members of this control network. For any given stress there are at least two classes of responsive gene. One class is induced only by that stress, is required by the cell only under those conditions, and specific regulatory mechanisms exist to achieve this (e.g. FNR for anaerobic nitrate reductase; KdpDE for osmoregulation of *kdp*). The second class, the supercoiling-sensitive, 'stress-regulated' genes, function more generally in the adaptation of the stressed cell and their expression must be fine-tuned in response to many stimuli. The distinction between the specific and 'stress-regulated' genes may explain the finding that positive regulators associated with many 'stress-regulated' genes (e.g. OmpR) appear to function by a mechanism entirely different from that of classical regulatory proteins such as CRP and FNR.

Finally, it is worth emphasizing that, in their natural state, it is unlikely that *E. coli* or *S. typhimurium* will encounter just a single cause of stress. They will, instead, be subject to a continual fluctuation of many parameters. Adjustment in the expression of 'stress-regulated' genes must be in a coordinated fashion. While there is still much to understand, the overlapping response to supercoiling changes provides a relatively simple mechanism by which this can readily be achieved, maximizing the cell's ability to grow under a broad range of stressful conditions.

Experimental procedures

Bacterial strains and growth conditions

The strains used in this study are listed in Table 4. Bacteria were grown at 37°C unless otherwise indicated, in LB medium (Miller, 1972), Nutrient broth (NB; Difco) or MMA minimal medium (Miller, 1972) containing 0.4% glucose (or fructose for certain experiments) as carbon source. In certain experiments (indicated in the text), 0.1% w/v casmaino acids was added to the minimal medium. Where appropriate, NaCl was added to increase medium osmolarity at the concentrations indicated. Kanamycin, ampicillin, tetracycline and novobiocin were used at concentrations of 40 μg ml^{-1}, 50 μg ml^{-1}, 12.5 μg ml^{-1} and 25 μg ml^{-1}, respectively. Chloramphenicol was used at 25 μg ml^{-1} in solid media and at 12.5 μg ml^{-1} in liquid media. MacConkey-lactose medium was from Difco. Anaerobic incubations were carried out in BBL gas jars using GasPacks (Oxoid). Aerobic growth was achieved by vigorous shaking during incubation using flasks of at least ten times the volume of the culture, and harvesting cells at $OD_{600} < 0.3$.

Genetic manipulations

Transductions were carried out using a high-transducing derivative of phage P22-*int4* as described (Roth, 1970). Transductants were purified on Green plates (Roth, 1970) before being used. Strain CH1723 was constructed by transducing the *oxrC*::Tn*5* lesion from CH881 into CH946, selecting for kanamycin resistance. The correct location of the element in the transductant was confirmed phenotypically and by marker rescue. Plasmid pACYC184 (Chang and Cohen, 1978) was transduced into strains CH44 and CH881, selecting for tetracycline resistance.

DNA manipulations

Plasmid DNA was isolated using the alkaline lysis method (Ish-Horowitz and Burke, 1981). Plasmid topoisomers were separated by electrophoresis in 1.0% agarose gels containing 25 μg ml^{-1} chloroquine at 2.5 V cm^{-1} for 22 h using a TBE (90 mM Tris (pH 8.3), 90 mM borate, 10 mM EDTA) buffer system. Following electrophoresis, the chloroquine was removed by rinsing for 4–12 h in tap water and the DNA visualized by staining with ethidium bromide (5 μg ml^{-1}).

Table 4. Bacterial strains.

Strain	Genotype	Source/construction
Escherichia coli		
MC4100	*araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 deoC1 ptsF25 rbsR flbB5301*	Casadaban (1976)
MH225	MC4100Φ(*ompC-lacZ*)10-25	Hall and Silhavy (1981a,b)
MH513	MC4100Φ(*ompF-lacZ*)16-13	Hall and Silhavy (1981a,b)
CH1145	MH225 *ompR*::Tn*10*	Graeme-Cook *et al.* (1989)
CH1147	MH513 *ompR*::Tn*10*	Graeme-Cook *et al.* (1989)
Salmonella typhimurium		
LT2	Wild type	B. N. Ames
CH44	Δ*oppBC250*	Higgins *et al.* (1983)
CH881	CH44 *oxrC*::Tn*5*	Jamieson and Higgins (1986)
CH776	CH44 *tppB84*::Mud1-8	Jamieson and Higgins (1986)
CH799	CH776 *ompR1001*::Tn*5*	Gibson *et al.* (1987)
CH1350	CH776 *ompR1009*::Tn*10Δ16Δ17*	Gibson *et al.* (1987)
CH1301	*proU*::MudJ	Sutherland *et al.* (1986)
CH946	*proU*::Mud1-8	Cairney *et al.* (1985)
CH1723	CH946 *oxrC*::Tn*5*	This study

Preparation of cell extracts and polyacrylamide gel electrophoresis

Total cell proteins were prepared by harvesting 1 ml of exponentially growing cells, and resuspending the cell pellet in 400 μl of Laemmli sample buffer (Laemmli, 1970). The samples were boiled for 5 min and debris removed by centrifugation in a microcentrifuge prior to loading of 10 μl samples onto a gel. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was on 12.5% slab gels (30:0.8 acrylamide:bisacrylamide ratio) as described (Laemmli, 1970; Ames, 1974).

β-galactosidase assays

β-galactosidase activity was measured and quantified as described by Miller (1972) using the chloroform-sodium dodecylsulphate permeabilization procedure. Each determination was carried out at least in triplicate. The deviation between samples was less than ±10%.

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References

Aliabadi, Z., Park, Y.K., Slonczewski, J.L., and Foster, J.W. (1988) Novel regulatory loci controlling oxygen- and pH-regulated gene expression in *Salmonella typhimurium*. *J Bacteriol* **170:** 842–851.

Ames, G.F.-L. (1974) Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. *J Biol Chem* **249:** 634–644.

van Alphen, W., and Lugtenberg, B. (1977) Influence of osmolarity and the growth medium on the outer membrane protein pattern of *Escherichia coli*. *J Bacteriol* **131:** 623–630.

Axley, M.J., and Stadtman, T.C. (1988) Anaerobic induction of *Escherichia coli* formate dehydrogenase (hydrogenase-linked) is enhanced by gyrase inactivation. *Proc Natl Acad Sci USA* **85:** 1023–1027.

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

Barron, A., May, G., Bremer, E., and Villarejo, M. (1986) Regulation of envelope composition during adaptation to osmotic stress in *Escherichia coli*. *J Bacteriol* **167:** 433–438.

Borowiec, J.A., and Gralla, J.D. (1987) All three elements of the *lac* p^S promoter mediate its transcriptional response to DNA supercoiling. *J Mol Biol* **195:** 89–97.

Cairney, J., Booth, I.R., and Higgins, C.F. (1985) Osmoregulation of gene expression in *Salmonella typhimurium*: *proU* encodes an osmotically induced betaine transport system. *J Bacteriol* **164:** 1224–1232.

Casadaban, M.J. (1976) Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. *J Mol Biol* **104:** 541–555.

Chang, A.C.Y., and Cohen, S.N. (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J Bacteriol* **134:** 1141–1156.

Christman, M.F., Morgan, R.W., Jacobson, F.S., and Ames, B.N. (1985) Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. *Cell* **41:** 753–762.

Csonka, L.N. (1982) A third L-proline permease in *Salmonella typhimurium* which functions in media of elevated osmotic strength. *J Bacteriol* **151:** 1433–1443.

Dimri, G.P., and Das, H.K. (1988) Transcriptional regulation of nitrogen fixation genes by DNA supercoiling. *Mol Gen Genet* **212:** 360–363.

Dixon, R.A., Henderson, N.C., and Austin, S. (1988) DNA supercoiling and aerobic regulation of transcription from the *Klebsiella pneumoniae nifLA* promoter. *Nucl Acids Res* **16:** 9933–9946.

Dorman, C.J., Barr, G.C., Ni Bhriain, N., and Higgins, C.F. (1988) DNA supercoiling and the anaerobic and growth phase regulation of *tonB* gene expression. *J Bacteriol* **170:** 2816–2826.

Drlica, K. (1984) Biology of bacterial deoxyribonucleic acid topoisomerases. *Microbiol Rev* **48:** 273–289.

Drlica, K. (1987) The nucleoid. In Escherichia coli *and* Salmonella typhimurium. Neidhardt, F.C. (ed.). Washington, D.C.: ASM Press, pp. 91–103.

Eshoo, M.W. (1988) *lac* fusion analysis of the *bet* genes of *Escherichia coli*: regulation by osmolarity, temperature, oxygen, choline and glycine betaine. *J Bacteriol* **170:** 5208–5215.

Forst, S., Delgado, J., Ramakrishnan, G., and Inouye, M. (1988) Regulation of *ompC* and *ompF* expression in the absence of *envZ*. *J Bacteriol* **170:** 5080–5085.

Gibson, M.M., Price, M., and Higgins, C.F. (1984) Genetic characterization and molecular cloning of the tripeptide permease (*tpp*) genes of *Salmonella typhimurium*. *J Bacteriol* **160:** 122–130.

Gibson, M.M., Ellis, E.M., Graeme-Cook, K.A., and Higgins, C.F. (1987) OmpR and EnvZ are pleiotropic regulatory proteins: positive regulation of the tripeptide permease (*tppB*) of *Salmonella typhimurium*. *Mol Gen Genet* **207:** 120–129.

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

Gottesman, S. (1984) Bacterial regulation: global regulatory networks. *Annu Rev Genet* **18:** 415–441.

Graeme-Cook, K.A., May, G., Bremer, E., and Higgins, C.F. (1989) Osmotic regulation of porin expression: a role for DNA supercoiling. *Mol Microbiol*, in press.

Groat, R.G., Schultz, J.E., Zychlinsky, E., Bockman, A., and Matin, A. (1986) Starvation proteins in *E. coli*:: kinetics of synthesis and role in starvation survival. *J Bacteriol* **168:** 486–493.

Guitierrez, C., Barondess, J., Manoil, C., and Beckwith, J. (1987) The use of transposon Tn*phoA* to detect genes for cell envelope proteins subject to a common regulatory stimulus. *J Mol Biol* **195:** 289–297.

Hall, M.N., and Silhavy, T.J. (1981a) Genetic analysis of the major outer membrane proteins of *Escherichia coli*. *Annu Rev Genet* **15:** 91–142.

Hall, M.N., and Silhavy, T.J. (1981b) Genetic analysis of the *ompB* locus in *Escherichia coli* K-12. *J Mol Biol* **151:** 1–15.

Heyde, M., and Portalier, R. (1987) Regulation of major outer membrane porin proteins of *Escherichia coli* K-12 by pH. *Mol Gen Genet* **208:** 511–517.

Higgins, C.F., Hardie, M.M., Jamieson, D.J., and Powell, L.M. (1983) Genetic map of the *opp* (oligopeptide permease) locus of *Salmonella typhimurium*. *J Bacteriol* **153:** 830–836.

Higgins, C.F., Cairney, J., Stirling, D.A., Sutherland, L., and Booth, I.R. (1987) Osmotic regulation of gene expression: ionic strength as an intracellular signal? *Trends Biochem Sci* **12:** 339–344.

Higgins, C.F., Dorman, C.J., Stirling, D.A., Waddell, L., Booth, I.R., May, G., and Bremer, E. (1988) A physiological role for DNA supercoiling in the osmotic control of gene expression in *S. typhimurium* and *E. coli*. *Cell* **52:** 569–584.

Higgins, C.F., Dorman, C.J., and Ni Bhriain, N. (1989) Environmental influences on DNA supercoiling: a novel mechanism for the regulation of gene expression. In *The Bacterial Chromosome*. Riley, M., and Drlica, K., (eds). Chichester: John Wiley and Sons, in press.

Ish-Horowitz, D., and Burke J.F. (1981) Rapid and efficient cosmid vector cloning. *Nucl Acids Res* **9:** 2989–2994.

Jamieson, D.J., and Higgins, C.F. (1984) Anaerobic and leucine-dependent expression of a peptide transport gene in *Salmonella typhimurium*. *J Bacteriol* **160:** 131–136.

Jamieson, D.J., and Higgins, C.F. (1986) Two genetically distinct pathways for transcriptional regulation of anaerobic gene expression in *Salmonella typhimurium*. *J Bacteriol* **168:** 389–397.

Jovanovich, S.B., and Lebowitz, J. (1987) Estimation of the effect of coumermycin A_1 on *Salmonella typhimurium* promoters by using random operon fusions. *J Bacteriol* **169:** 4431–4435.

Kawaji, H., Mizuno, T., and Mizushima, S. (1979) Influence of molecular size and osmolarity of sugars and dextrans on the synthesis of outer membrane proteins O-8 and O-9 of *Escherichia coli* K-12. *J Bacteriol* **140:** 843–847.

Kranz, R.G., and Haselkorn, R. (1986) Anaerobic regulation of nitrogen-fixation genes in *Rhodopseudomonas capsulata*. *Proc Natl Acad Sci USA* **83:** 6805–6809.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

Laimins, L.A., Rhoads, D.B., and Epstein, W. (1981) Osmotic control of *kdp* operon expression in *Escherichia coli*. *Proc Natl Acad Sci USA* **78:** 464–468.

Lambden, P.R., and Guest, J.R. (1976) Mutants of *Escherichia coli* K-12 unable to use fumarate as an anaerobic electron acceptor. *J Gen Microbiol* **97:** 145–160.

Lundrigan, M.D., and Earhart, C.F. (1984) Gene *envY* of *Escherichia coli* K-12 affects thermoregulation of major porin expression. *J Bacteriol* **157:** 262–268.

Maeda, S., and Mizuno, T. (1988) Activation of the *ompC* gene by the OmpR protein in *Escherichia coli*. *J Biol Chem* **263:** 14629–14633.

May, G., Faatz, E., Villarejo, M., and Bremer, E. (1986) Binding protein dependent transport of glycine betaine and its osmotic regulation in *Escherichia coli* K-12. *Mol Gen Genet* **205:** 225–233.

Menzel, R., and Gellert, M. (1987) Fusions of the *Escherichia coli gyrA* and *gyrB* control regions to the galactokinase gene are inducible by coumermycin treatment. *J Bacteriol* **169:** 1272–1278.

Miller, J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Mizuno, T. (1987) Static bend of DNA helix at the activator recognition site of the *ompF* promoter in *E. coli*. *Gene* **54:** 57–64.

Newman, B.M., and Cole, J.A. (1978) The chromosomal location and pleiotropic effects of mutation of the *nirA*$^+$ gene of *Escherichia coli* K-12: the essential role of *nirA*$^+$ in nitrite reduction and in other anaerobic redox reactions. *J Gen Microbiol* **106:** 1–12.

Norioka, S., Ramakrishnan, G., Ikenaka, K., and Inouye, M. (1986) Interaction of a transcriptional activator, OmpR, with reciprocally osmoreguated genes, *ompF* and *ompC* of *Escherichia coli*. *J Biol Chem* **261:** 17113–17119.

Novak, P.D., and Maier, R.J. (1987) Inhibition of hydrogenase synthesis by DNA gyrase inhibitors in *Bradyrhizobium japonicum*. *J Bacteriol* **169:** 2708–2712.

Ramakrishnan, G., Ikenaka, K., and Inouye, M. (1985) Uncoupling of osmoregulation of the *Escherichia coli* K-12 *ompF* gene from *ompB*-dependent transcription. *J Bacteriol* **163:** 82–87.

Richardson, S.M.H., Higgins, C.F., and Lilley, D.M.J. (1984) The genetic control of DNA supercoiling in *Salmonella typhimurium*. *EMBO J* **3:** 1745–1752.

Ronson, C.W., Nixon, B.T., and Ausubel, F.M. (1987) Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. *Cell* **49:** 579–581.

Roth, J.R. (1970) Genetic techniques in studies of bacterial metabolism. *Meth Enzymol* **17A:** 3–35.

Sanderson, K.E., and Roth, J.R. (1988) Linkage map of *Salmonella typhimurium*, edition VII. *Microbiol Rev* **52:** 485–532.

Sanzey, B. (1979) Modulation of gene expression by drugs affecting deoxyribonucleic acid gyrase. *J Bacteriol* **138:** 40–47.

Schultz, J.E., Latter, G.I., and Matin, A. (1988) Differential regulation by cyclic AMP of starvation protein synthesis in *Escherichia coli*. *J Bacteriol* **170:** 3903–3909.

Scott, N.W., and Harwood, C.R. (1980) Studies on the influence of the cyclic AMP system on major outer membrane proteins of *Escherichia coli* K-12. *FEMS Microbial Letts* **9:** 95–98.

Slauch, J.M., Garretts, S., Jackson, D.E., and Silhavy, T.S. (1988) EnvZ functions through OmpR to control porin gene expression in *Escherichia coli*. *J Bacteriol* **170:** 439–441.

Smith, M.W., and Neidhardt, F.C. (1983a) Proteins induced by anaerobiosis in *Escherichia coli*. *J Bacteriol* **154:** 336–343.

Smith, M.W., and Neidhardt, F.C. (1983b) Proteins induced by aerobiosis in *Escherichia coli*. *J Bacteriol* **154:** 344–350.

Spector, M.P., Aliabadi, Z., Gonzalez, T., and Foster, J.W. (1986) Global control in *Salmonella typhimurium*: two-dimensional electrophoretic analysis of starvation-, anaerobiosis-, and heat shock-inducible proteins. *J Bacteriol* **168:** 420–424.

Spiro, S., and Guest, J.R. (1987) Activation of the *lac* operon of *Escherichia coli* by a mutant FNR protein. *Mol Microbiol* **1:** 53–58.

Sutherland, L., Cairney, J., Elmore, M.J., Booth, I.R., and Higgins, C.F. (1986) Osmotic regulation of transcription: induction of the *proU* betaine transport gene is dependent on the accumulation of intracellular potassium. *J Bacteriol* **168:** 805–814.

Tsui, P., Helu, V., and Freundlich, M. (1988) Altered osmoregulation of *ompF* in integration host factor mutants of *Escherichia coli*. *J Bacteriol* **170:** 4950–4953.

Wanner, B.L., and McSharry, R. (1982) Phosphate-controlled gene expression in *Escherichia coli* K12 using Mu*d1*-directed *lacZ* fusions. *J Mol Biol* **158:** 347–363.

Wood, D.C., and Lebowitz, J. (1984) Effect of supercoiling on the abortive initiation kinetics of the RNA-1 promoter of ColE1 plasmid DNA. *J Biol Chem* **259:** 11184–11187.

Yamamoto, N., and Droffner, M.L. (1985) Mechanisms determining aerobic and anaerobic growth in the facultative anaerobe *Salmonella typhimurium*. *Proc Natl Acad Sci USA* **82:** 2077–2081.