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The local repressor AcrR plays a modulating role in the regulation of *acrAB* genes of *Escherichia coli* by global stress signals

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Summary

Genes *acrAB* encode a multidrug efflux pump in *Escherichia coli*. We have previously reported that transcription of *acrAB* is increased under general stress conditions (i.e. 4% ethanol, 0.5 M NaCl, and the stationary phase in Luria–Bertani medium). In this study, *lacZ* transcriptional fusions and an *in vitro* gel mobility shift assay have been utilized to study the mechanisms governing the regulation of *acrAB*. We found that a closely linked gene, *acrR*, encoded a repressor of *acrAB*. Nevertheless, the general stress conditions increased transcription of *acrAB* in the absence of functional AcrR, and such conditions surprisingly increased the transcription of *acrR* even more strongly than that of *acrAB*. These results suggest that the general-stress-induced transcription of *acrAB* is primarily mediated by global regulatory pathway(s), and that one major role of AcrR is to function as a specific secondary modulator to fine tune the level of *acrAB* transcription and to prevent the unwanted overexpression of *acrAB*. To our knowledge, this represents a novel mechanism of regulating gene expression in *E. coli*. Evidence also suggests that the up-regulation of *acrAB* expression under general stress conditions is not likely to be mediated by the known global regulators, such as MarA or SoxS, although elevated levels of these proteins were shown to increase the transcription of *acrAB*.

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Introduction

Mutations at the *acr* locus render *Escherichia coli* cells hypersusceptible to hydrophobic growth inhibitors such as basic dyes, detergents and many antibiotics (Nakamura, 1965). Recently, we and others have cloned the *acr* locus by functional complementation (Ma *et al*., 1993b; Xu *et al*., 1993). Sequence analysis has revealed three genes expressed on two divergent operons at this locus: *acrR*, *acrA* and *acrB* (formerly *acrE*) (Fig. 1). Gene *acrR* encodes a putative transcription regulator with a helix-turn-helix motif at its N-terminus (Pan and Spratt, 1994). Genes *acrA* and *acrB* are located on the same operon and encode membrane-associated polypeptides. Sequence comparison with other bacterial transport systems (Dinh *et al*., 1994) and time-course study of the accumulation of acriflavin (a hydrophobic dye) in *acrAB*$^+$ and *acrAB*$^-$ strains (Ma *et al*., 1993b) have strongly suggested that *acrAB* encodes a multidrug efflux pump in *E. coli*.

While AcrAB was originally recognized as a multidrug efflux pump, one of its physiological roles may be protection of *E. coli* from lipophilic inhibitors occurring in its natural habitat, such as bile salts and fatty acids (Ma *et al*., 1995). Since the transcription of *acrAB* is also up-regulated by general stress signals, including 4% ethanol, 0.5 M NaCl and the onset of stationary phase in Luria–Bertani (LB) medium (Ma *et al*., 1995) (throughout this paper, these conditions will be called 'general stress conditions'), it remains to be determined whether AcrAB also pumps out endogeneous metabolite(s). The MexAB system, a homologue of AcrAB found in *Pseudomonas aeruginosa*, has been suggested to export the endogenous siderophore pyoverdine into the medium (Poole *et al*., 1993a), and the same system also confers multidrug resistance when the cells are challenged with exogenous small molecules (Poole *et al*., 1993b).

Mutations at the *mar* locus affect drug susceptibility of *E. coli* (George and Levy, 1983). Genes *marR*, *marA* and *marB* were found to form an operon (Cohen *et al*., 1993a). Mutations in MarR repressor lead to activation of *marRAB* transcription and higher levels of intrinsic drug resistance in *E. coli*. Interruption of *marA* by Tn*5* insertion results in drug hypersusceptibility. Based on these and other results, Cohen *et al*. (1993a) have proposed that

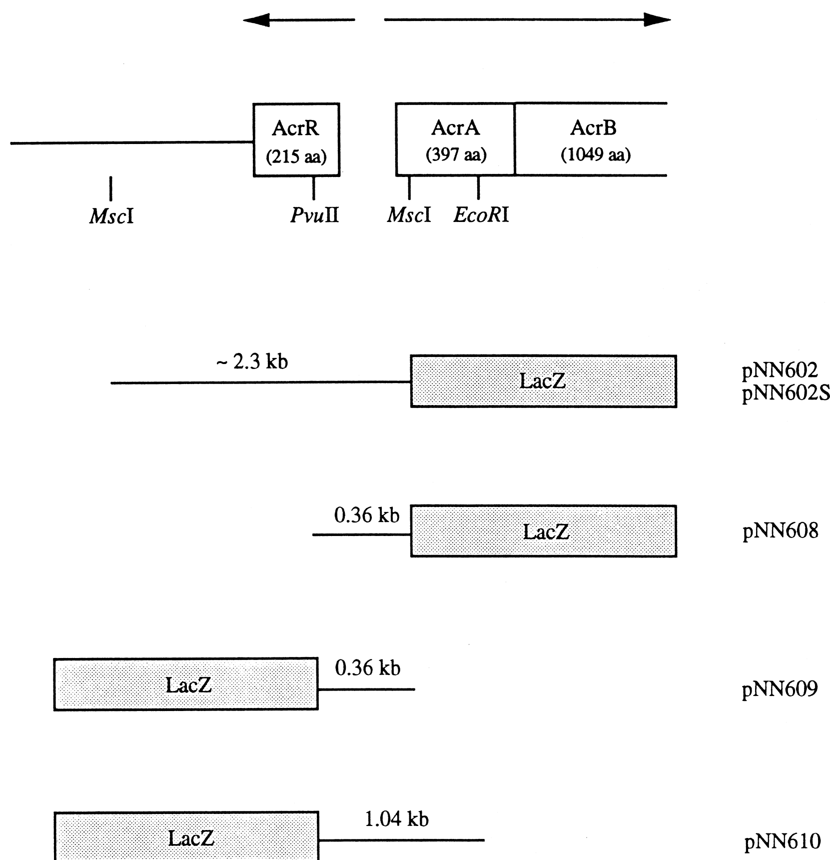

Fig. 1. Construction of single-copy reporter plasmids for the study of transcriptional regulation of *acrAB* and *acrR*. Plasmid pNN602S is the same as pNN602 except that it contains the streptomycin/spectinomycin-resistance gene cartridge of the omega fragment inserted at the *Bam*HI site of pNN602. Arrows at the top of the figure represent the direction of transcription.

MarA acts as a global transcription activator which is produced in increased amounts in response to antibiotic stress. Some of the defensive responses regulated by MarA include reduction of the synthesis of OmpF, the larger porin of *E. coli* (Cohen *et al.*, 1988), and an increase in drug efflux (McMurry *et al.*, 1994). We have previously reported that transcription of *acrAB* was increased in several *marR* mutants, a result consistent with the notion that the AcrAB pump contributes to the Mar-mediated antibiotic resistance in *E. coli* (Ma *et al.*, 1995).

Genes *soxRS* comprise another global regulatory system that triggers the expression of various genes in *E. coli* in response to superoxides (Greenberg *et al.*, 1990; Wu and Weiss, 1991). In this case, SoxS is the global transcription activator and its synthesis is controlled by SoxR (Nunoshiba *et al.*, 1992; Wu and Weiss, 1992). Both MarA and SoxS belong to the XylS/AraC family of transcription regulators, and they share 42% overall sequence identity (Cohen *et al.*, 1993a). At a functional level, a significant overlap has been observed between the genes activated by MarA and those activated by SoxS (Demple, 1991). Mutants constitutively expressing MarA are more resistant to oxidants, and those constitutively expressing SoxS display elevated resistance to drugs (Greenberg *et al.*, 1991; Miller *et al.*, 1994).

Recently, an *in vitro* study on proteins bound to the origin of replication in *E. coli* has led to the discovery of another homologue of MarA and SoxS, called Rob (right oriC binding) (Skarstad *et al.*, 1993). Rob is larger than either MarA or SoxS. However, the N-terminal 100 residues of Rob share about 50% sequence identity with MarA and SoxS.

In this study, we have attempted to elucidate some of the regulatory circuits controlling expression of *acrAB*. Gene *acrR* was shown to encode a repressor that regulated the transcription of *acrAB* operon. Unexpectedly, transcription of *acrR* was increased by the general stress conditions, which also increased the expression of *acrAB*. In addition, gel mobility shift assays demonstrated the formation of both AcrR-dependent and AcrR-independent protein–DNA complexes at the *acr* promoter region. These and other results suggest that general stress conditions play a major role in the regulation of *acrAB* transcription and that the local repressor AcrR probably plays a modulating, dampening role.

Results

Gene acrR *encodes a repressor of the* acrAB *operon*

Based on its sequence homology with other known DNA-binding proteins, we and others hypothesized that *acrR*, a gene located next to *acrAB* and transcribed in the opposite direction, may code for a protein that regulates the expression of *acrAB* (Ma *et al.*, 1995; Pan and Spratt, 1994). To verify this proposal, we have studied the effects of the insertional mutation or overexpression of *acrR* on the transcription of *acrAB*. Strain WZM124 was constructed by inserting the kanamycin-resistance gene cartridge from Tn*903* into the chromosomal *Bgl*II site of *acrR*. Since the *Bgl*II site is located at the 3′ end of the helix-turn-helix coding sequence of *acrR*, it is possible that the truncated AcrR could be expressed in WZM124 and retain the potential DNA-binding ability. Although we could not definitely exclude such a possibility, a gel mobility shift assay has failed to detect the formation of any truncated AcrR–DNA complex (see below). The DNA-binding ability of AcrR is greatly diminished, if not abolished, in WZM124. The expression of *acrAB* was then investigated using pNN608 as a reporter plasmid in WZM124. Plasmid pNN608 was chosen for this purpose because it lacks the majority of the AcrR-coding sequence and it contained a transcriptional fusion of the *acrA* promoter sequence to the *lacZ* gene (Fig. 1) (see also the *Experimental procedures*). Plasmid pNN608 retains a similar *lacZ* induction profile to plasmid pNN602, which contains a much larger DNA sequence upstream of the start of the *acrA* coding sequence (Ma *et al.*, 1995). Insertional inactivation of *acrR* enhanced the transcription of *acrAB* in WZM124 (Fig. 2, lanes 3 and 4). Enhanced expression was evident in the logarithmic phase and, particularly, at the onset of the stationary phase.

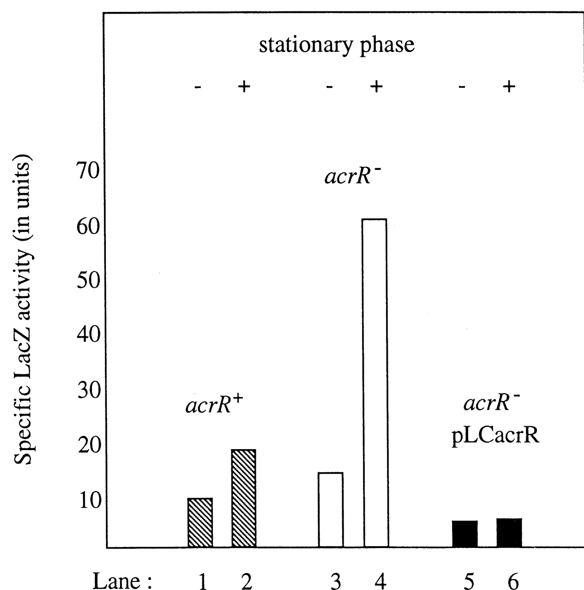

Fig. 2. Effects of mutation or overexpression of *acrR* on the growth phase-dependent induction of *acrAB*. Transcription of *acrAB* was assayed using plasmid pNN608 in either W4680 (the AcrR$^+$ wild-type strain) or WZM124 (the AcrR$^-$ strain derived from W4680). WZM124 contained the kanamycin-resistance gene cartridge inserted at codon 61 of *acrR*. Plasmid pLCacrR contained the *acrR* gene cloned into the multiple-copy vector pACYC177. For each pair of bars, the left one (i.e. lanes 1, 3 and 5) represents the specific LacZ activity of pNN608 in mid-log phase (A_{600} of 0.3), and the right one (i.e. lanes 2, 4 and 6) represents the specific LacZ activity of pNN608 at the onset of stationary phase (i.e. when the optical density at 600 nm had stopped increasing).

Overexpression of AcrR was achieved by transforming W4680 (the isogenic AcrR$^+$ parent of WZM124) with plasmid pLCacrR, which contained the *acrR* gene cloned into the multiple-copy vector pACYC177. Overexpression of AcrR repressed the transcription of *acrAB*. Furthermore, overexpression of AcrR was sufficient to abolish the growth-phase-dependent induction of *acrAB* (Fig. 2, lanes 5 and 6). These observations are consistent with AcrR functioning as a repressor for transcription of *acrAB*. This conclusion was further strengthened by gel mobility shift assays that gave direct evidence for the binding of AcrR to the promoter region of *acrAB* (see below).

It is noteworthy that not only could transcription of *acrAB* still be induced in WZM124 (*acrR*::Tn*903kan*^{r}) by the stationary phase but also the extent of induction (about fourfold) was higher than that in W4680 (*acrR*$^+$) (about twofold) (Fig. 2). Similarly, the *acrAB* promoter remained inducible by 4% ethanol and 0.5 M NaCl in WZM124 (data not shown). Therefore, these general stress signals can regulate the *acrAB* operon in the absence of functional AcrR.

General stress conditions, which induce acrAB *operon, unexpectedly increased transcription of* acrR

Transcription of *acrAB* increased in response to general stress conditions (Ma *et al.*, 1995). We, therefore, expected that the level of the transcription of *acrR*, coding for a repressor of this system, would decrease or at least remain unaltered under these conditions. Plasmid pNN609 was constructed as a transcriptional fusion in which the *acrR* promoter region was joined to *lacZ* (Fig. 1) (see also the *Experimental procedures*). Surprisingly, transcription of *acrR* was persistently increased by all these conditions, and the extent of increase was even higher than that seen for *acrA* (Fig. 3A): for example, 4% ethanol increased transcription of *acrR* about tenfold, whereas that of *acrA* increased less than fourfold. Repeating the experiments with pNN610, which contains a larger DNA sequence upstream of *acrR*, led to similar inductions (data not shown). While unexpected, this could explain

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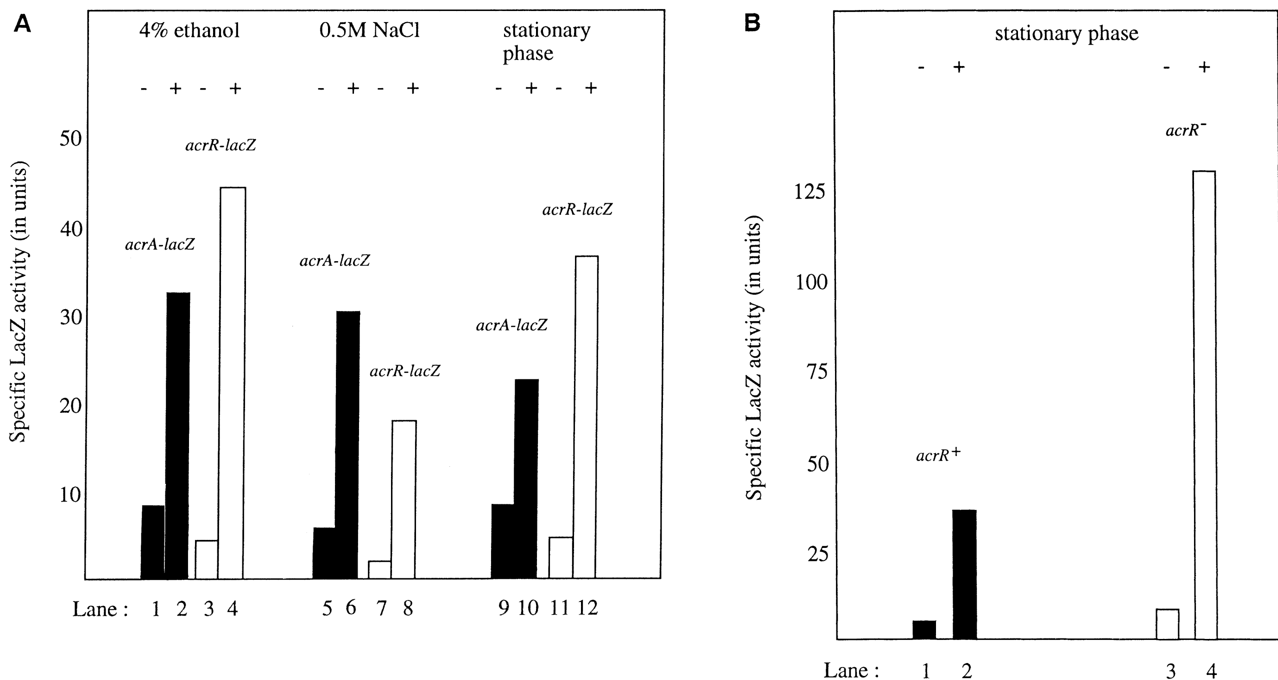

Fig. 3 A. Transcription of *acrAB* (filled-in bars; assayed using pNN602) and *acrR* (empty bars; assayed using pNN609) in the wild-type strain W4680 under general stress conditions. Cells were grown in LB medium except that those for NaCl shock experiments were grown in LB without NaCl. The pre-induction specific activity was assayed when the A_{600} had reached 0.3. Ethanol or NaCl was then added, and the post-induction LacZ specific activity was determined when the A_{600} reached 0.6. The post-induction activity for stationary phase was determined when the A_{600} had stopped increasing.
B. Effects of the *acrR* mutation on the growth-phase-dependent induction of *acrR*. Transcription of *acrR* was assayed by using pNN609 in W4680 (*acrR*$^{+}$) and in WZM124 (an isogenic *acrR*$^{-}$ derivative). The experimental conditions were similar to (A).

why the insertional inactivation of *acrR* led to higher induction of *acrAB* in the stationary phase, as described above (Fig. 2). These findings suggested that unidentified factor or factors are involved in the increased transcription of both *acrAB* and *acrR* operons under the general stress conditions.

Transcription of acrR *is autoregulated*

Many transcriptional regulators are subject to autoregulation. To explore this possibility, transcription of *acrR* in W4680 (*acrR*$^{+}$) and WZM124 (*acrR*$^{-}$) was compared in the logarithmic or stationary phase by using plasmid pNN609. As one can see from Fig. 3B, both the absolute amount and the extent of the induction level of *acrR* transcription were higher in WZM124 than in the AcrR^{+} parent strain W4680. Therefore, AcrR can repress its own synthesis.

Increased transcription of acrAB*, caused by the general stress conditions, is not mediated by MarA or SoxS*

acrAB transcription becomes elevated by the general

Table 1. MarA/SoxS is not involved in the general stress induction of *acrAB*.

Strain		Induction Ratio of LacZ[a] 4% ethanol	stationary phase	0.5 M NaCl
GC4468 (*mar*$^{+}$ *sox*$^{+}$)	pNN602S	3.8	2.5	ND
JHC1098 (Δ*mar* Δ*sox*)	pNN602S	4	2.1	ND
W4680 (*mar*$^{+}$)	pNN602	3.8	2.4	4.6
WZM1069 (*marR* (MarA^{const}))	pNN602	2.9	1.5	3.1
GC4468 (*sox*$^{+}$)	pNN602	3.5	3.3	ND
JTG1052 (*soxR* (SoxS^{const}))	pNN602	3.5	3.2	ND

ND, not determined.
a. The numbers show the ratio of LacZ specific activity (post-induction) to the initial, uninduced specific activity. Induction with 4% ethanol was carried out for 1 h, and that with 0.5 M NaCl for 2 h. The absolute levels of initial, pre-induction specific activity were around 4.5 and 8.5 U for GC4468 and W4680, respectively, except that in the 0.5 M NaCl experiment, W4680 showed a slightly lower activity (5.5 U) because LB without NaCl was used (see the *Experimental procedures*). The pre-induction specific activity was higher in MarA- and SoxS-constitutive strains (see Table 2). In all the experiments shown in this table and in Table 2, as well as in the Figures, experiments were repeated usually at least three times, and the average values are shown. The specific deviation of the data was usually within 5% of the average.

Table 2. Overexpression of MarA, SoxS or Rob induces AcrAB expression.

Strain	Genotype			LacZ specific activity[a]
	marRAB	*soxRS*	*rob*	
GC4468	+	+	+	4.6
JHC1069	Δ*marR*(*cfxB1*)	+	+	15.1
JHC1096	Δ*marRAB*	+	+	4.4
JTG1052	+	*soxR101*	+	8.6
DJ901	+	Δ*soxRS*	+	4.7
GC4068(pHCrob)	+	+	+++	8.5

a. pNN602 was present in all the strains, which were isogenic derivatives of GC4468. Plasmid pHCrob contains *rob* cloned into the multicopy plasmid pUC19. Transcription of *acrAB* was assayed by the specific LacZ activity encoded by pNN602 during the mid-log growth phase (A_{600} of 0.3) in LB medium. The unit of specific LacZ activity is defined in the *Experimental procedures*.

stress conditions (Ma *et al.*, 1995), but this response occurs in the absence of functional AcrR, as described above. Since it is known that *acrAB* expression is also induced by the 'global' regulators MarA (Ma *et al.*, 1995) and SoxS (see below), we examined the possibility that MarA or SoxS might be mediating the response to general stress conditions, by using strains that are totally lacking or constitutively producing these regulatory proteins (Table 1). We have previously shown that transcription of *acrAB* is up-regulated in response to 4% ethanol, 0.5 M NaCl and the onset of stationary-phase growth in LB medium (Ma *et al.*, 1995), and this observation was confirmed with strains GC4468 and W4680, as shown in Table 1. Notably, a double mutant with complete deletions of both *marRAB* and *soxRS* clusters (JHC1098) still allowed the full response to the global stress conditions tested (Table 1). In addition, in order to test for the effect of the constitutive production of MarA, WZM1069 was derived by transducing the Δ*marR* mutation from JHC1069 into W4680. Transcription of *acrAB* in WZM1069 was still inducible by the gobal stress conditions (Table 1), although the induction ratios were slightly lower, presumably because the pre-induction levels of LacZ were already quite high (see below). Induction ratios were essentially unaltered in JTG1052, which produces the SoxS regulatory protein constitutively (Table 1). These data suggest that the general stress response of *acrAB* in *E. coli* is unlikely to be mediated by *marRAB* or *soxRS*.

Transcription of acrAB *is increased in MarA- and SoxS-constitutive mutants, and in a strain overproducing Rob*

The transcription of *acrAB* is increased in *E. coli* strain JHC1069 (Ma *et al.*, 1995), a strain that produces the MarA regulator in a constitutive manner (Ariza *et al.*, 1994). Expression of *acrAB* was unaffected, however, by deletion of the entire *marRAB* operon in strain JHC1096 (Table 2). In light of the well-documented cross-talk in *E. coli* between multidrug resistance and resistance to oxidative stress, and of the striking homology among MarA, SoxS and Rob, we have tested whether the transcription of *acrAB* could also be increased in a SoxS-constitutive mutant or by overexpression of Rob. For this purpose, plasmid pNN602, which contains a transcriptional fusion of the *acrAB* promoter to *lacZ* reporter gene (Fig. 1) (Ma *et al.*, 1995), was transformed into strain JTG1052, a mutant producing the SoxS regulator constitutively. Measurement of LacZ activity has demonstrated that *acrAB* expression was elevated approximately twofold in the SoxS-constitutive background (Table 2). However, transcription of *acrAB* was unaffected in a *soxRS* deletion strain DJ901 (Table 2), and it was not decreased even in the *marRAB soxRS* double-deletion strain JHC1098 (not shown). To evaluate the effects of Rob on *acrAB* expression, the Rob protein was overexpressed by transforming the high-copy-number plasmid pHCrob (Table 3) into strain GC4468. Expression of *acrAB* was also increased approximately twofold in the presence of the plasmid containing *rob* (Table 2). The effect of deletion of *rob* on the transcription of *acrAB* was not determined.

In vitro *study of the interactions between the* acrR–acrAB *promoter region and cellular factors*

A gel mobility shift assay was utilized to study the protein–DNA interactions within the promoter region of *acrR–acrAB*. A 180 bp DNA fragment, which contained the sequence between the seventh codon of *acrR* and the seventh codon of *acrA*, was synthesized by the polymerase chain reaction (PCR) and was labelled with ^{32}P. When this fragment was mixed with a crude cell lysate from W4680 (*acrR*$^+$) in the presence of increasing amounts of poly(dI-dC), three shifted bands were visible (Fig. 4A, lanes 2–5). Control experiments using the cell lysates from WZM124 (*acrR*$^-$) and the AcrR-overexpressing strain (Fig. 4A, lanes 6 and 7, respectively) clearly identified band 2 as the AcrR-associated protein–DNA complex. Pre-incubation of the cell lysate with 10-fold molar excess of non-labelled *acr* promoter region (Fig. 4B, lanes 2–4), but not with 80-fold molar excess of pUC19 (Fig. 4B, lanes 5–7), was sufficient to eliminate band 2. Band 1 was not studied further since its intensity was relatively weak and seemed to decrease with increasing amounts of poly(dI-dC). However, the intensity of band 3 was much higher and not significantly affected by competing poly(dI-dC). Band 3 was still present in the binding mixture upon using a fivefold dilution of cell lysate (data not shown). Band 3 probably represents a protein–DNA complex because it was very sensitive to 100 μg ml^{-1} protease

Table 3. Strains and plasmids.

Strain/Plasmid	Description	Reference
Strain		
D1210	*lacI*^{q} derivative of HB101	Cook *et al.* (1992)
JC7623	*argE3 hisG4 leuB6* Δ(*gpt–proA*)*62 thr-1 thi-1 ara-14 galK2 lacY1 mtl-1 xyl-1 kdgK51 tsx33 recB21 recC22 sbcB15 supE44 rpsL31 rac*	Kushner *et al.* (1971)
W4680	F^{-} Δ*lacZ39 rpsL45 melB4*	Ma *et al.* (1995)
WZM120	Same as W4680; Δ*acrAB*::Tn*903kan*^{r}	This work
WZM1069	Same as W4680; *marR*(*cfxB1*) *zdd2207*::Tn*10kan*^{r}	This work
GC4468	F^{-} Δ*lacU169 rpsL*	Greenberg *et al.* (1991)
JHC1069	Same as GC4468; *marR*(*cfxB1*) *zdd2207*::Tn*10kan*^{r}	Greenberg *et al.* (1991)
JHC1096	Same as GC4468; *zee239*::Tn*9cm*^{r} *del1738* (Δ*marRAB*)	Greenberg *et al.* (1991)
JTG1052	Same as GC4468; *soxR101 zjc2206*::Tn*10kan*^{r}	Greenberg *et al.* (1991)
DJ901	Same as GC4468; Δ*(soxRS-zjc2204) zjc2207*::Tn*10kan*^{r}	Greenberg *et al.* (1991)
JHC1098	Same as DJ901; *zee239*::Tn*9cm*^{r} *del1738* (Δ*marRAB*)	Greenberg *et al.* (1991)
Plasmid		
pBR151B	pBR322 derivative containing *acrR* and *acrA*	Ma *et al.* (1993b)
pZM124	1.3 kb Tn*903kan*^{r} inserted at *Bgl*II site of *acrR* on pBR151EH	This work
pNN387	A single-copy vector with promoterless *lacZY* and Tn*9cm*^{r}	Elledge and Davis (1989)
pNN602	pNN387 containing 2.3 kb upstream of *Msc*I site of *acrA*	Ma *et al.* (1995)
pNN602S	1.8 kb *sm*^{r}/*spc*^{r} gene inserted at *Bam*HI site of pNN602	This work
pNN608	pNN387 with 0.36 kb DNA upstream of *Msc*I site of *acrA*	This work
pNN609	pNN387 with 0.36 kb DNA upstream of *Pvu*II site of *acrR*	This work
pNN610	pNN387 with 1.04 kb DNA upstream of *Pvu*II site of *acrR*	This work
pLCarcR	pACYC177 derivative containing *acrR*	This work
pUHE21.1	An IPTG-inducible overexpression vector	H. Bujard (personal communication)
pUHEacrR	pUHE21.2 derivative containing *acrR*	This work
pHCrob	pUC19 derivative containing *rob*	This work

K either in the presence or absence of 0.5% SDS (Fig. 4C, compare lanes 1, 3 and 4). Addition of 0.5% SDS alone to the binding mixture was enough to prevent its formation (Fig. 4C, lane 5). Surprisingly, the protein(s) involved in the formation of band 3 were resistant to heat treatment. Heating the cell lysate in a boiling water bath for 3 min before the binding reaction did not have any apparent effect on the formation of band 3, while the same treatment abolished the formation of AcrR-associated band 2 almost completely (Fig. 4C, lane 2). Although not titrated by an 80-fold molar excess of pUC19, the intensity of band 3 could be reduced by 80% (quantified on a Molecular Dynamics Model 400S Phosphorimager), but not eliminated, when a 40-fold molar excess of non-labelled *acr* promoter region was included in the binding mixture (Fig. 4B, lanes 2–7). Interestingly, the addition of a 40-fold molar excess of non-labelled *micF* promoter region (about 280 bp) or *oriC* DNA fragment (about 280 bp) reduced the intensity of band 3 by 80 or 60%, respectively (Fig. 4D). These data suggest that a protein factor in addition to AcrR binds to the promoter region of *acrR–acrAB*, and that this factor has some affinity also for the *micF* promoter region and *oriC*.

Discussion

Transcription of *acrAB* is up-regulated in mutants constitutively producing MarA or SoxS and in a strain that overexpresses Rob. The DNA-binding domains of MarA, SoxS and Rob share about 80% sequence identity, which suggests that these proteins have overlapping specificity. A large number of genes, including *micF*, encoding an antisense regulatory RNA for the synthesis of OmpF (Mizuno *et al.*, 1984; Hooper *et al.*, 1992), *sodA*, encoding a superoxide dismutase (Greenberg *et al.*, 1991), *fumC*, encoding a heat-stable fumarase (Liochev and Fridovich, 1992; Ariza *et al.*, 1994), and *inaA*, encoding a weak acid-inducible protein (Rosner and Slonczewski, 1994) have been shown to be activated by both MarA and SoxS. During the preparation of our manuscript, it has been reported that Rob can bind to several stress-inducible promoters (such as those of *micF* and *sodA*), and the overexpression of *rob* can induce the multiple antibiotic resistance in *E. coli* (Ariza *et al.*, 1995). The cross-induction of these genes is believed to provide the basis for the cross-resistance of *E. coli* to various stresses (Jenkins *et al.*, 1988; Demple, 1991). Our study has added *acrAB* to this growing list of MarA/SoxS/Rob-regulated stress-inducible genes. At present, however, we cannot exclude the possibility that one of these factors is acting indirectly through another factor, as SoxS has been reported to increase the expression of *marRAB* (Miller *et al.*, 1994).

Although MarA, SoxS and Rob could increase *acrAB* transcription when they were overexpressed, these gene

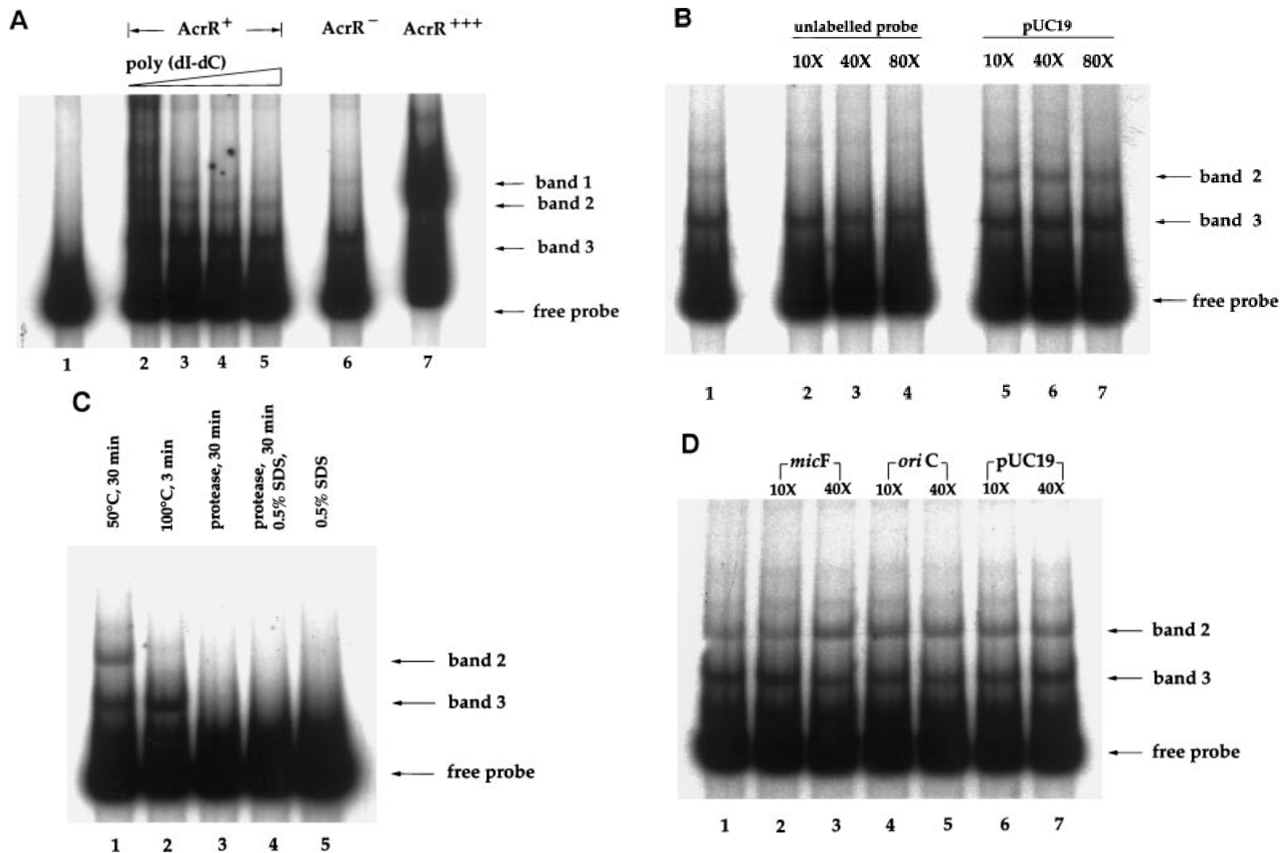

Fig. 4. Gel mobility shift assays of the promoter region of *acrR–acrAB*. A PCR-amplified 180 bp fragment, which contained the promoter region of *acrR–acrAB*, was labelled and used as the probe. Each binding mixture contained 0.85 ng labelled probe and 1 mg cellular proteins. Typically, a 1000× weight excess of poly(dI-dC) was also included unless otherwise indicated. Except in (A), cell lysate from the wild-type strain W4680 was used.

A. Effects of the AcrR dosage on the formation of various protein–DNA complexes in cell lysates. AcrR$^+$, W4680 (the wild-type strain); AcrR$^-$, WZM124 (the AcrR$^-$ mutant of W4680); AcrR^{+++}, IPTG-induced pUHEacrR-transformed W4680 (an AcrR-overexpressing strain). Lanes 2–5 contained increasing amounts (0, 500×, 1000× and 2000× weight excess) of poly(dI-dC) in the binding mixture. Bands 1, 2 and 3 represent the protein–DNA complexes. Lane 1 was the control lane and contained no cell lysate.

B. Effects of 10×, 40× or 80× molar excess unlabelled probe (lanes 2–4) or pUC19 (lanes 5–7) on the formation of band 2 and 3. Lane 1 was the control lane and contained no competitors.

C. Effects of various prior treatments of cell lysate on the formation of bands 2 and 3.

D. Effects of 10× or 40× molar excess *micF* promoter region (lanes 2–3), *oriC* DNA fragment (lanes 4–5), or pUC19 (lanes 6–7) on the formation of bands 2 and 3. Lane 1 was the control lane and contained no competitors.

products did not appear to be responsible for the *acrAB* induction by the general stress conditions such as 4% ethanol, 0.5 M NaCl or the onset of stationary-phase growth in LB medium (Table 1). This tentative conclusion is strengthened by three lines of evidence. First, induction by the general stress conditions was intact at least in a strain (JHC1098) that contained the deletions of both *marRAB* and soxRS genes (Table 1), a result that eliminates the possibility that one of the systems could substitute for the other. Second, the induction was not affected even when one of the regulators was present at a high, constitutive level (Table 1). Third, unlike the general stress conditions, the constitutive overexpression of MarA or SoxS did not increase the transcription of *acrR* at all (D. Ma, unpublished results), although it increased the transcription of *acrAB*, as shown in Table 2. We note that Mar/Sox-independent regulation of stress-inducible genes has been reported in the literature. Thus, both *mar*-dependent and *mar*-independent pathways are known to regulate salicylate-induced multidrug resistance in *E. coli* (Cohen *et al.*, 1993b). A *marRAB*/*soxRS*-independent pathway has also been proposed to regulate the induction of *inaA* by salicylate (Rosner and Slonczewski, 1994).

What might be the *marRAB*- and *soxRS*-independent regulatory pathway for induction of *acrAB* in response to the general stresses listed above? Our gel mobility shift assays indeed showed that some factor other than AcrR, which could correspond to the regulator responding to global stress conditions, can bind to the promoter region of *acrR–acrAB* to form band 3 (Fig. 4). The results dis-

cussed above suggest that such a regulator is unlikely to be MarA or SoxS. However, it could very well be other homologue(s) of MarA–SoxS–Rob. This hypothesis is consistent with the observations that the formation of band 3 can be specifically and competitively removed by the *micF* promoter region and the *oriC* DNA fragment (Fig. 4). To test this hypothesis, we are currently purifying the protein(s) involved in the formation of band 3.

Band 3 should represent a specific protein–DNA complex independent of AcrR. First, band 3 was sensitive to 100 μg ml^{-1} protease K or 0.5% SDS (Fig. 4C). Second, the formation of band 3 was resistant to a wide range of poly(dI-dC) concentrations (Fig. 4A) but could be competed away more efficiently by a specific competitor (i.e. unlabelled probe) than by a non-specific competitor (i.e. pUC19) (Fig. 4B). Third, band 3 was still formed when a cell lysate from an *acrR* transposon insertion mutant was tested (Fig. 4A). The reason why the specific competitor chased band 3 less efficiently than band 2 is not clear. However, this might be explained by differences in the abundance of the proteins involved in the formation of these bands or in the number of their binding sites on the chromosome. Since AcrR is probably a specific regulator for *acrAB*, it is likely to exist in lower amounts and have fewer target sites on the chromosome than the proteins involved in band 3, if these are global transcription regulators. Differences in abundance of these proteins would make band 3 more resistant to specific competition than band 2 when crude cell lysates were used.

The fact that *acrR* encodes a repressor (Fig. 2) but that the expression of *acrR* is induced when the *acrAB* transcription is increased under general stress conditions (Fig. 3A) was unexpected. Apparently, factor or factors other than AcrR are primarily responsible for the stress induction of *acrAB* (and probably *acrR* as well), and AcrR could, therefore, function as a secondary modulator to prevent the excessive expression of *acrAB*. Available data are consistent with the idea that overexpression of *acrAB* is deleterious to *E. coli*. First, it was difficult to clone the *acrAB* genes into a high-copy-number vector, because of the low efficiency of transformation (unpublished data). Similar observations were reported for the transcriptionally active form of *acrEF* (formerly *envCD*), a homologue of *acrAB* in *E. coli* (Klein *et al.*, 1991) and for *mtrCD*, a homologue of *acrAB* in *Neisseria gonorrhoeae* (Pan and Spratt, 1994). Second, the growth rate of WZM124, which lacks a functional AcrR and up-regulates *acrAB* slightly, was slower than that of the wild type after the mid-log phase in LB medium (D. Ma, unpublished data). Presumably, in addition to its response to various stresses, the level of the AcrAB efflux pump also has to be carefully controlled. While the response of *acrAB* to various general stresses can be made possible by exploiting the global transcription regulators, the fine-tuning of *acrAB* expression probably cannot be achieved solely by these global regulators. AcrR may, therefore, serve as a specific modulator to fine tune the production of AcrAB.

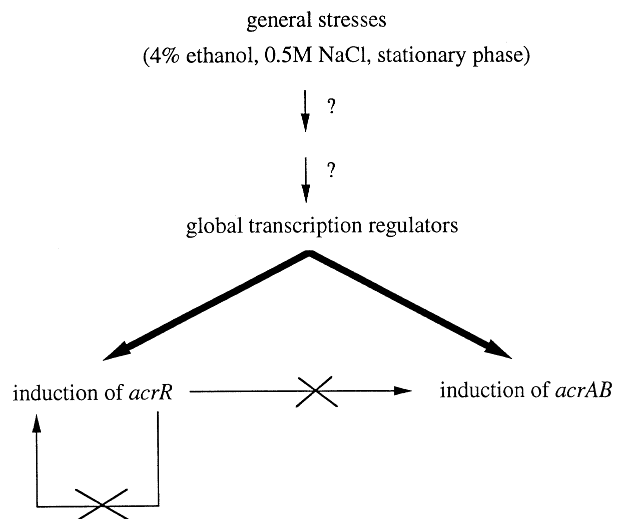

Fig. 5. The proposed mechanism for the transcriptional regulation of *acrAB* in *E. coli*. Based on the data presented, we assume that general stress conditions result in the generation of global transcription regulators, which are unlikely to be MarA, SoxS, or Rob, but could be homologues of them. Such regulators increase the transcription of both *acrAB* and *acrR*, and AcrR, whose production is self-repressed, will limit the extent of overexpression of *acrAB*.

As far as we are aware, the way in which AcrR regulates the transcription of *acrAB* reveals two novel features (Fig. 5). First, although many bacterial operons are under the control of both global (distal) and specific (local) regulators, none of those reported previously utilizes a global regulator as the primary activator (or repressor) and a specific regulator as the secondary modulator of transcription. In contrast, many examples exist for the 'opposite' mechanism, in which a specific regulator is utilized as the primary activator (or repressor) while a global regulator functions as the secondary modulator. Some known systems include the regulation of *nifDHK* operon (encoding a nitrogenase) by NifA (a specific activator) and IHF (a global modulator) in *Klebsiella pneumoniae* (Hoover *et al.*, 1990) and the regulation of *lacZYA* operon (encoding a lactose-utilizing system) by LacI (a specific repressor) and cAMP–CRP complex (a global modulator) in *E. coli*. Second, the prevention of overexpression (dampening) of *acrAB* appears to be achieved by directly increasing the amount of its repressor, AcrR. This is different from many other cases, in which the dampening of induced operons essentially relies on negative autoregulation of activators. For example, Nunoshiba *et al.* (1993) have shown that the autorepression by the SoxS global activator can limit the expression of *soxS* gene. Presumably, the MarR-mediated repression of *marRAB* operon could also dampen the expression of the MarA positive regula-

tor. Although *acrR* itself is autoregulated, the regulation of *acrAB* transcription seems to involve a mechanism that goes far beyond such locally limited feedback loops.

Several questions remain to be answered. First, what is the regulatory pathway for the induction of *acrAB* and *acrR* under general stress conditions (i.e. stationary phase in LB medium, 4% ethanol, and 0.5 M NaCl)? In principle, stress induction of *acrAB* can be achieved by a variety of regulatory proteins, each one specific for a particular stress, or by one or few regulatory proteins which sense common signals generated under various stresses. Interestingly, transcription of *acrAB* was not increased in the stationary phase during growth in several minimal media, and the addition of glucose in LB repressed the stationary-phase induction (D. Ma, unpublished data). When *E. coli* was grown in MOPS (3-(*N*-morpholino)propanesulphonic acid) minimal medium, the transcription of *acrAB* varied significantly with the different carbon sources used (D. Ma, unpublished data). These data are consistent with the possibility that some metabolite of *E. coli* serves as the signal in the regulation of *acrAB*. Second, does AcrR have other functions in addition to the prevention of overexpression of *acrAB*? Preliminary results showed that growth at 25°C induced *acrAB* expression, but that under these conditions the transcription of *acrR* was decreased (D. Ma, unpublished results). Therefore, in this case AcrR may indeed be functioning as a classical repressor. Third, three homologues of AcrAB have been identified on the *E. coli* chromosome so far (for review, see Ma *et al.*, 1994b). Like the *acrAB* mutants, *E. coli* with mutations in at least two of these homologues typically shows no apparent growth defects under laboratory conditions. Presumably, these AcrAB homologues have unrecognized yet essential functions under physiological conditions, which have not been duplicated in the laboratory. Preliminary analysis has indicated that at least some AcrAB homologues are subject to regulation by stress, and it seems likely that these proteins constitute a family of stress-inducible transmembrane transporters. It would be interesting to determine under what conditions these *acrAB* homologues are induced and how they respond to the global stress-induced regulators, including MarA, SoxS and (possibly) Rob.

Experimental procedures

Bacterial strains and growth conditions

The *E. coli* strains used in this study were all derivatives of K-12 and are listed in Table 3. All bacterial cells were grown at 37°C with shaking at 200 r.p.m. in LB medium (containing 10 g Bacto Tryptone, 5 g Bacto Yeast Extract, and 10 g NaCl per litre solution) except in the NaCl shock experiment, in which LB without NaCl was used. The antibiotics chloramphenicol (25 μg ml^{-1}), kanamycin (25 μg ml^{-1}), ampicillin (100 μg ml^{-1}), streptomycin (100 μg ml^{-1}) and spectinomycin (100 μg ml^{-1}) were used for the selection of plasmids.

Construction of plasmids

Plasmids used in this study are listed in Table 3. The construction of pBR151B has been described previously (Ma *et al.*, 1993b). For pZM124, pBR151B was partially digested by *Bgl*II and the 1.3 kb *Bam*HI fragment from pUC4K (Pharmacia Inc.) was inserted into the *Bgl*II site of the *acrR* gene. The 1.3 kb *Bam*HI fragment contains the kanamycin-resistance gene cartridge from Tn*903*. For pNN602, pNN608, pNN609 and pNN610, a two-step cloning strategy was adopted. The first step was the insertion of four restriction fragments from pBR151B (shown on Fig. 1) into the *Sna*BI site of pDC401 to make pDC602, pDC608, pDC609 and pDC610, respectively. Plasmid pDC401 is a multiple-copy-number reporter vector which contains the promoterless *lacZ* and the termination signals for both transcription and translation (Ma *et al.*, 1993a). The second step was to move the *acrAB* or *acrR* promoter regions from pDC plasmids to pNN387, a single-copy-number reporter vector which contains the promoterless *lacZY* genes (Elledge and Davis, 1989). Plasmids pDC602, pDC608, pDC609 and pDC610 were restricted with *Bam*HI, end-filled with Klenow fragment and restricted again with *Sst*I. The resulting DNA fragments, which contained the *acrAB* or *acrR* promoter regions and the 5′ portion of *lacZ*, were isolated by the gel electrophoresis. Plasmid pNN387 was restricted with *Hin*dIII, end-filled with Klenow fragment and restricted again with *Sst*I. The resulting 11 kb DNA fragment, which contained the 3′ portion of *lacZ* and the replicon, was isolated by gel electrophoresis. Plasmids pNN602, pNN608, pNN609 and pNN610 were then made by ligating the DNA fragments from the corresponding pDC plasmids into the 11 kb fragment from pNN387. Plasmid pNN602S was constructed by inserting the 1.8 kb *Bam*HI-restricted streptomycin/spectinomycin-resistance gene cartridge of the omega fragment (Prentki and Krisch, 1984) into the unique *Bam*HI site of pNN602. To construct pLCacrR, the 1.2 kb *Msc*I–*Pst*I fragment from pDC602 was ligated with *Nru*I–*Nsi*I-digested pACYC177. For the overexpression of AcrR, pDC602 was digested by *Nde*I, end-filled by Klenow and digested again by *Pst*I; and pUHE21.2 was digested by *Bam*HI, end-filled by Klenow and digested again by *Pst*I. Plasmid pUHE21.2 was made in the laboratory of Dr H. Bujard (University of Heidelberg, Germany) and has been described previously (Ma *et al.*, 1994a). The overexpressing plasmid pUHEacrR was then constructed by ligating the 0.9 kb *Nde*I/Klenow–*Pst*I fragment from pDC602 into the *Bam*HI/Klenow–*Pst*I-digested pUHE21.2. For the cloning of *rob* into pUC19, we amplified the *rob* gene from the genomic DNA of GC4468 by PCR. Primers used in PCR were: 5′-TCAGATGTCGACCGAACCAATCTCTT-3′ and 5′-GGGCGTATTCCTGAAGGCGA-3′. The 1.5 kb amplified DNA fragment was digested by *Sal*I and *Sst*I, purified from the agarose gel by the Geneclean Kit (Bio 101, Inc.) and ligated into *Sal*I–*Sst*I-digested pUC19 to make pHCrob. Plasmids from four independent clones were used to transform GC4468 for the LacZ assay.

It should be noted that all plasmids used for the measurement of transcriptional activity, including pNN602, pNN608,

pNN609 and pNN610, were true transcriptional fusions, in which the translation of the truncated AcrA or AcrR polypeptide is stopped by the presence of three stop codons in all possible reading frames (supplied by the plasmid pDC401, see Ma *et al*., 1993a) and the translation of LacZ is started with its own ribosome-binding sequence.

Mutagenesis of acrR *by homologous recombination*

Plasmid pZM124 was restricted by *Eco*RI which cuts within both pBR322 and *acrA*. The 6.5 kb *Eco*RI fragment, which contained *acrR*::Tn*903kan*^{r}, was separated by the gel electrophoresis, purified by the Geneclean Kit (Bio 101, Inc.) and used to transform the competent JC7623 cells, which were prepared with ice-cold $CaCl_2$ as described (Sambrook *et al*., 1989). Homologous recombinants were selected and purified on the LB plates containing 25 μg ml^{-1} kanamycin. The *acrR* mutation was transduced into W4680 by using the P1*clr100* phage according to the protocol described by Miller (1992). All the insertions and deletions were confirmed by the PCR amplication of the genomic DNA.

Beta-galactosidase assays

Beta-galactosidase (LacZ) activity was assayed essentially as described by Miller (1992) with the following modifications. Samples, which contained the equivalent amount of cells as contained in 1 ml of culture at an A_{600} of 0.3, were removed from 37°C and stored on ice before use. Cells were spun down and resuspended in 0.5 ml of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ and 50 mM β-mercaptoethanol). Toluene (20 μl) was then added into each resuspended sample, which was then vortexed for 10 s. Samples were left in the hood at room temperature for 45 min to allow the evaporation of toluene. For the LacZ assay, 100 μl of 4 mg ml^{-1} ONPG (*o*-nitrophenyl β-D-galactopyranoside) was added after the samples were preincubated at 28°C for 5 min. Typically, the reaction was allowed to proceed for 40 min and was stopped by the addition of 0.25 ml 1 M Na_2CO_3. After centrifugation, the absorptions of the supernatants at 420 nm and 550 nm were measured, and the LacZ specific activities were calculated as follows:

$$1000 \times (A_{420} - 1.75 \times A_{550})/(40\,\text{min} \times 1.0\,\text{ml} \times 0.3) = \text{units of } \beta\text{-galactosidase}$$

Preparations of DNA fragment and cell lysates for binding assays

DNA fragment used in the gel mobility shift assays was prepared by PCR. The template was plasmid pDC602 and two primers were 5′-TTGTTTGGTTTTTCGTGCCAT-3′ and 5′-AACCCTCTGTTTTTGTTCATA-3′. The resulting 182 bp fragment was isolated from the mermaid agarose gel (Bio 101, Inc.) and was then labelled by [γ-^{32}P]-ATP with T4 polynucleotide kinase and purified by Sephadex G-50 column in 50 mM Tris-HCl pH 7.5, 100 mM NaCl and 1 mM EDTA. The labelled fragment was stored at −20°C before use.

To make cell lysates, *E. coli* strains W4680 or WZM124 were grown as described above until they attained an A_{600} of 0.5. For the overexpression of AcrR, strain D1210 harbouring the plasmid pHCacrR was induced by 1 mM IPTG for 3 h before the A_{600} reached 0.5; the overproduction was confirmed by the SDS–PAGE denaturing gel. Cells were collected by first cooling in an ice-water bath for 15 min, followed by centrifugation for 10 min at 6000 × *g* at 4°C. Each culture was resuspended in 0.33% of its original volume in 40 mM Tris-HCl pH 7.5, 100 mM KCl, 2 mM DTT, 1 mM EDTA, 0.5 mM PMSF and 20% (v/v) glycerol. Cell lysates were obtained by three passage through a French pressure cell at 13 000 p.s.i. Large cell debris and unbroken cells were removed by centrifugation at 12 000 × *g* for 10 min at 4°C. Lysates were then divided into portions, frozen in liquid N_2 and stored at −70°C. Protein concentrations were determined using a Bradford assay.

Gel mobility shift assays

The binding reaction was done in 25 mM Tris-HCl pH 7.5, 50 mM KCl, 5 mM NaCl, 1 mM DTT, 1 mM EDTA and 10% (v/v) glycerol. Various amounts of poly(dI-dC) and cold competitors were first mixed with 1 μg cellular proteins at 22°C for 10 min. Labelled DNA fragment (about 0.85 ng or 7.2 fmole) was then added and incubation was continued for another 25 min. After this, the binding mixture was immediately loaded onto a 4% native polyacrylamide gel (acrylamide/bis: 29/1) and run at 8 V cm^{-1} for 4.5 h with the constant recirculation of the buffer. The gel was then dried under vacuum in a conventional gel drier (Heto Dry GD-1, Heto Lab Equipment) at 80°C for 40 min and subjected to the autoradiography. The buffer composition for the electrophoresis was 20 mM Tris-HCl pH 7.5, 3 mM sodium acetate and 1 mM EDTA. The gel was preelectrophoresed at 5 V cm^{-1} for 2 h before use.

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