Alternating palindrome regulates the *E. coli* mazEF promoter

The regulation of the *Escherichia coli* mazEF promoter involves an unusual alternating palindrome

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Summary

The *Escherichia coli* mazEF system is a chromosomal “addiction module” that, under starvation conditions in which guanosine-3’,5’-bispyrophosphate (ppGpp) is produced, is responsible for programmed cell death. This module specifies for the toxic stable protein MazF and the labile antitoxic protein MazE. Upstream from the mazEF module are two promoters, P2 and P3 that are strongly negatively auto-regulated by MazE and MazF. We show that the expression of this module is positively regulated by the factor for inversion stimulation (FIS).

What seems to be responsible for the negative auto-regulation of mazEF is an unusual DNA structure, which we have called an “alternating palindrome.” The middle part, “a,” of this structure may complement either the downstream fragment, “b,” or the upstream fragment, “c”. When the MazE-MazF complex binds either of these arms of the “alternating palindrome”, strong negative auto-regulation results. We suggest that the combined presence of the two promoters, the “alternating palindrome” structure, and the FIS binding site all permit the expression of the mazEF module to be sensitively regulated under various growth conditions.
Introduction

In *Escherichia coli* (*E. coli*) programmed cell death is mediated through unique genetic elements called “addiction modules.” These consist of two genes, where the second gene specifies for a stable toxin, and the first gene specifies for a labile antitoxin. “Addiction modules” were first discovered in a number of extrachromosomal elements where they were found to be responsible for the post-segregational killing effect, that is, the death of cells from which these extrachromosomal elements have been removed. In other words, these cells are "addicted" to the continuous presence of a labile antitoxic element. Among the best studied addiction modules of this kind are *ccdAB* borne on factor F, *pemlK* borne on plasmid R100, and *phd-doc* borne on bacteriophage P1 [reviewed in 1; 2; 3; 4].

All known extrachromosomal addiction systems have been shown to be negatively auto-regulated at the level of transcription. For example, such modules as *ccdAB* of the F factor [5; 6; 7], *parD* of the plasmid R1 [8], *pemlK* of the plasmid R100 [9], or *phd-doc* of the plasmid P1 [10; 11]. Magnuson and Yarmolinsky [11] suggested that the auto-regulation of addiction modules might prevent fluctuations in the levels of the antidote and the toxin that would result in the activation of the toxin. During auto-regulation, both the toxin and the antidote bind to a palindrome sequence in their own promoter region thereby decreasing their own transcription. In a few cases, the binding of antitoxin by itself resulted in a low level of auto-regulation; the concomitant binding of the toxic element increased the level of binding [8; 10]. In more complicated cases, as has been found for the *pemlK* [9] and *phd-doc* [11] modules, the promoter region of addiction module contains two separate palindrome sequences. Based on the stoichiometry and dynamics of binding, Magnuson and Yarmolinsky [11] suggested a model in which the palindrome sequence binds the antidote dimer independently but cannot bind the toxin. When the toxin interacts with the antidote it increases the binding affinity of the antidote to the palindrome sequence, and thus increases half-life of the complex.
Pairs of genes homologous to some of the extra-chromosomal “addiction modules” have also been found on the *E. coli* chromosome [12; 13; 14; 15; 16]. As we have reported previously, the *E. coli mazEF* system is the first known regualtable prokaryotic chromosomal “addiction module” [12]. This system consists of the two genes, *mazE* and *mazF*, that are located in the *rel* operon downstream from the *relA* gene [16]. We found [12] that the *mazEF* gene pair has all the properties required for an addiction module. MazF is toxic and long-lived, while MazE is antitoxic and short-lived. MazE and MazF are co-expressed and they interact. In addition, the *mazEF* system has a unique property: its expression is regulated by guanosine-3',5'-bispyrophosphate (ppGpp), which is synthesized by the RelA protein under conditions of amino acid starvation [17]. Furthermore, overproduction of ppGpp induces *mazEF* mediated cell death [12; 18]. These properties suggest that the *mazEF* module may be responsible for programmed cell death under conditions of nutrient starvation [12].

Here we studied the regulation of the expression of the *mazEF* system. The promoter region of the chromosomally borne *mazEF* addiction module is partially homologous to that of the promoter of the *pemIK* plasmid borne addiction module [14]. The promoters of both modules contain similar palindrome sequences, though, like the promoter of the *phd-doc* module [11], the *pemIK* promoter includes two separated palindromes [9] and the *mazEF* promoter was found to include only one [14]. Since *pemIK* is auto-regulated, Masuda and colleagues [14] hypothesized that *mazEF* might also be auto-regulated.

Results of our previous in vitro work [12] revealed that the chromosomal “addiction module” *mazEF* can be expressed from two promoters, P2 and P3, which are located 13 bp apart. In *in vivo* studies, we found that, P2, the upstream promoter, was active in exponentially growing cells. Here we showed that the *in vivo* activity of the P3 promoter is only one tenth of that of the P2 promoter.

We found that the *mazEF* system is weakly auto-regulated by the antitoxic component MazE, and efficiently auto-regulated by the combined action of the
antitoxic component MazE and the toxic component MazF. In this respect, the chromosomal promoter of the \textit{mazEF} system is regulated as are most of the previously studied promoters of extra-chromosomal “addiction modules.” However, the \textit{mazEF} promoter has two unique properties: it has an unusual DNA structure that we call an “alternating palindrome,” and it carries a binding site for the Factor for Inversion Stimulation (FIS). In the following discussion, we shall consider the relevance to \textit{mazEF} regulation of these two sites.

**Experimental procedures**

**Media.** The media used were LB broth or LB agar (Bio101, Inc.) supplemented with the appropriate antibiotics at the following final concentrations: 100 \( \mu \text{g/ml} \) ampicillin, or 34 \( \mu \text{g/ml} \) chloramphenicol, or 50 \( \mu \text{g/ml} \) kanamycin, or 20 \( \mu \text{g/ml} \) tetracycline.

**Bacterial strains.** The bacterial strains used in this work are listed in Table 1. The bacterial strain MC4100 and its derivatives bearing either the \textit{mazEF} null allele or a \textit{fis} \textsuperscript{-} allele were constructed by P1 transduction using strains bearing corresponding mutations as we have described previously [12; 19].

**Plasmid construction**

**Construction of plasmids bearing a \textit{mazEF} promoter-\textit{lac}'Z gene fusion.** Using the EF-1 and EG-3 oligomer primers (Table 2), we synthesized a PCR fragment bearing the \textit{mazEF} promoter. This fragment contained the end of the \textit{relA} gene, the \textit{mazEF} promoter, and the first 17 codons of the \textit{mazE} gene. After cutting with \textit{Bam}HI, we cloned this fragment into the \textit{SmaI-Bam}HI sites of plasmid pSK10\textDelta6 that bears the \textit{lacZ} gene lacking both its promoter and its first eight codons, that is \textit{lac}'Z. We called this new plasmid pSK10\textDelta6-\textit{pef}. Clones of pSK10\textDelta6-\textit{pef} were selected on X-gal plates to which ampicillin had been added. Plasmids from the selected clones were purified and sequenced.
Construction of plasmids bearing *mazE* or *mazEF* under the control of *ptac*.

Using appropriate DNA primers (EE-1 and EF-2 for *mazE* gene, or EE-1 and FG-1 for *mazEF* genes, see Table 2), we synthesized PCR fragments bearing the ORF of the *mazE* or the *mazEF* genes. These PCR products were used for cloning the corresponding genes under the *tac* promoter present on the expression vector pKK223-3. We called the resulting plasmids pKK-*mazE* and pKK-*mazEF* (Table 1).

We also cloned these PCR fragments into the modified pSK10Δ6 compatible plasmid pLex1 that bears the IPTG inducible promoter *ptac* [20]. We could not use the plasmids of the pKK set for testing the influence of MazE and MazF proteins on their promoter because both pKK223-3 and pSK10Δ6 are derivatives of the plasmid pBR322 and they are not compatible. Using pLex1 as our parent plasmid, we constructed the plasmids pLex-*mazE* and pLex-*mazEF* by introducing the promoter *ptac*, the chloramphenicol resistance gene, the p15A replication origin, and either *mazE* or *mazEF* such that they were under the control of the *ptac* promoter. We used these plasmids to study the influence of the proteins MazE and MazE-MazF on the *mazEF* promoter when it was present on the pSK10Δ6-pef plasmid.

**Crude extracts of cellular proteins.** With the exception of the wild type N99 strain, we made crude cell extracts of all *E. coli* strains mentioned in Table 1. We grew all of the strains at 37°C to OD<sub>600</sub> 0.2 in LB medium supplemented with appropriate antibiotics. We transformed strain MC4100Δ*mazEF* with plasmids pKK-*mazE* or pKK-*mazEF*. We induced the expression of the genes cloned under the *ptac* promoter by adding 1mM IPTG, and then allowing growth to continue for one more hour. No plasmids were added to the control bacteria that were also grown to OD<sub>600</sub> 0.2. Cells were harvested, sonicated, and centrifuged, and the supernatants were dialyzed over night against 10mM HEPES, 50mM NaCl, 1mM dithiothreitol, and 50% glycerol at pH8.0. The amount of protein in the samples was estimated by use of the Bradford assay (BioRad, USA). Dialysed supernatants were stored at -80°C.
Proteins expressed either from the ptac promoter bearing plasmid pKK223 or from the chromosome of E.coli strain MC4100 were analyzed by electrophoresis on denaturing and native gels and by Western blot analysis using antibodies raised against MazE (Fig. 1A and 1C) and MazF (Fig. 1B). As a control, we tested the proteins expressed from the chromosome of MC4100ΔmazEF. As we found previously [12], we also found here that MazE and MazF interact directly. We observed no bands of the proteins from the cell extracts of the addiction module mazEF expressed from the cell chromosome, presumably because under such condition these proteins were expressed at very low physiological concentrations. When both MazE and MazF were present on native gels, we observed a complex between the toxin and its antidote (indicated by an arrow on the Fig. 1C). We used these crude protein extracts to study the influence of MazE and MazF on their own promoter.

**Preparation of DNA fragments.** DNA fragments for the gel mobility shift assays and DNase I footprint analysis were obtained by PCR with appropriate primers (Table 2) and purified with a gel extraction kit (Qiagen, USA). Short fragments (about 20-30bp) were obtained by slow annealing of complementary primers in the presence of 100mM NaCl and 1mM EDTA. The primers that were used are listed in Table 2.

The fragments obtained were end labelled by polynucleotide kinase (New England Biolabs, Inc.) with (γ-32P)-ATP (Amersham Pharmacia Biotech, USA) and purified on the Sephadex G-50 columns (Boehringer Mannheim, Germany).

**DNA sequencing.** DNA sequencing was done by the di-deoxy method [21] using the Thermo Sequenase Radiolabelled Terminator Cycle Sequencing kit (Amersham Pharmacia Biotech, USA).
Gel mobility shift assay and DNase I footprint analysis. Ten microgram samples of crude protein extracts were diluted with the binding buffer (0.1M Tris HCl, pH 7.4, 2mM EDTA, 1mM DTT, 5mM MgCl₂, 5% glycerol) to a final volume of 10µl. To inactivate the nucleases, the samples were then heated at 65°C for 3min. When the mixtures had cooled to room temperature, 2µg of poly(dIdC) (Boehringer Mannheim, Germany) and 2µl of labelled DNA fragments were added. The binding reactions were conducted at room temperature for 10min, after which they were loaded onto 6% native polyacrylamide gels (PAAG) and run in TAE buffer [22] at 200V.

DNase I footprinting analyses were done according to Giladi et al. [23].

β-galactosidase assays. β-Galactosidase assays were done according to Miller [24].

Gel filtration analysis. Crude cellular extract of E. coli MC4100ΔEF strain was loaded on Sephadex G-100 (Amersham Pharmacia Biotech) column (1.5x75cm, Bio-Rad, USA) equilibrated with binding buffer, and supplemented with 0.4 M NaCl to prevent unspecific adsorption. The column was initially calibrated with standard proteins having established molecular weights. Collected fractions were analyzed by the gel mobility shift assay.

RNA extraction and primer extension. RNA extraction was carried out using the RNeasy Mini kit (Qiagen, USA). Primer extension experiments were carried out with AMV reverse transcriptase (USB Corp., USA) according to Gafny and colleagues [25]. The oligonucleotide primer used for the pSK10Δ6-gef construct and its mutant derivatives was “-40 M13 forward” (Amersham Pharmacia Biotech, USA) from the lacZ gene to the transcription start sites of the mazEF promoter. The primer was end labelled as a DNA fragments (see above). Reaction products were resolved on a 6% sequencing gel. A DNA sequencing reaction was performed with the same primer and run on the gel parallel to the primer extension reaction. To quantify the
RNA levels, the gels were analysed and the bands were quantified using the Fujix BAS100 phosphoimager.

The mutagenesis of the promoter fragment. Point mutations were introduced into mazEF promoter region by PCR-based site-directed overlap extension mutagenesis [26] using appropriate primers (Table 2). All introduced mutational changes were verified by DNA sequence determination.

Results

The mazEF promoter is negatively auto-regulated. Like most addiction modules, sequence analysis of the promoter region of mazEF suggests that it is auto-regulated at the transcriptional level [15]. To test whether mazEF was indeed auto-regulated we chose lacZ as a reporter gene and fused it to the mazEF promoter region, where the beginning of mazE is fused to the eighth codon of lacZ. We introduced this gene fusion into plasmid pSK10Δ6-pef. We used pSK10Δ6-pef to transform MC4100ΔmazEF strain and then measured the cellular levels of β-galactosidase (Fig. 2A). Under optimal growth conditions at OD600 0.2-0.3, we found cellular levels of β-galactosidase around 7000-8000 Miller units. This high level of lacZ expression suggests that the mazEF promoter is very strong, similar to the promoters of other addiction modules that have been studied [5; 8; 9; 10].

Strain MC4100ΔmazEF, already harbouring pSK10Δ6-pef, was transformed with the compatible plasmids pLex-mazE or pLex-mazEF in which the mazE or mazEF genes were under the control of the IPTG inducible tac promoter. The levels of β-galactosidase activity were measured at mid-log phase. Inducing plasmid pLex-mazE to produce MazE led to moderate inhibition (about 40%) of mazEF promoter activity, as reflected by the reduction in β-galactosidase activity; however, inducing pLex-mazEF to produce both MazE and MazF led to a much higher level (up to 90%) of inhibition (Fig. 2A). Using Western blot analysis, we found increased cellular levels of protein MazE when plasmid pLex-mazE was induced, and similarly,
increased cellular levels of MazE and MazF when pLex-mazEF was induced (data not shown). Thus, the activity of mazEF promoter is about five times more inhibited by the combination of MazE and MazF then by MazE alone.

To verify that the regulation of the mazEF promoter took place at the transcriptional level, we performed a series of primer extension experiments using plasmid pSK10Δ6-pef as the template. Using RNA extracted from cells carrying this plasmid, we estimated the relative efficiency of transcription from the two promoters P2 and P3 (Fig. 2B). Transcription initiated from promoter P3 was about 10-fold weaker then that from P2, located 13bp upstream (Fig. 2B). This explains why we were unable to observe initiation from P3 by primer extension on RNA transcribed from a chromosome borne mazEF module, which is present in only one copy per cell [12].

Primer extension experiments under the same experimental conditions revealed that induction by IPTG led to repression of transcription from both P2 and P3 by MazE or by the MazE-MazF complex. Twenty minutes after induction, MazE repressed P2 expression by 53% compared to the activity of the unrepressed promoter; MazE-MazF complex repressed P2 by 92% (Fig. 2B and 2C). We believe that these two promoters are inhibited similarly; however, after repression, the levels of the P3 transcript may have been so low that we could not measure them (Fig. 2B). These results from our primer extension experiments (Figs. 1B and 1C) confirmed the data that we obtained in our assays for β-galactosidase activity (Fig. 2A). Thus, we concluded that the mazEF addiction module is auto-regulated at the transcriptional level.

**Over-expressing the MazE-MazF complex leads to a gel mobility shift of the mazEF promoter fragment.** To further investigate the mechanism of the action of MazE-MazF on the promoters, we studied how MazE and MazF bind to the promoter region of the mazEF module (see map in Fig. 3A). As the source of proteins for these assays we used crude cell extracts enriched for either MazE or MazE and
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MazF (Fig. 1). For our electrophoretic mobility shift assay we used the 74bp fragment of the mazEF promoter that extends from the multi-linker to the residue +2 of the P2 promoter (Fig. 3A). This DNA fragment was labeled and exposed to every one of the cell extracts that we had prepared (defined in the legend to Fig. 1). We found that the mazEF promoter was bound by the MazE-MazF complex (Fig. 4A, lane 4), confirming our hypothesis that mazEF is negatively auto-regulated (Fig. 2). The crude extract containing only MazE but lacking MazF also bound the promoter fragment (Fig. 4A, lane 3). Though MazE was present in approximately equal amounts when by itself or in the presence of MazF, here the shift was much weaker (Fig. 1A, compare lanes 3 and 4). Thus, MazE could bind to its own promoter, but, like the antidotes from most other addiction modules of plasmid origin, the binding affinity of MazE to its promoter was very low. The cooperative binding of the toxic protein, here MazF, greatly enhanced the binding of MazE (Fig. 4A, lane 4).

We also found that exposing the promoter fragment to the extract of MC4100ΔmazEF, that contained neither MazE nor MazF, resulted in an additional retarded band with a very low shift (Fig. 4A, lane 2). This same band was present when the promoter fragment was exposed to the extracts that either contain no MazF or contained it at very low level (Fig. 4A, lanes 1 and 3). This unexpected result suggested that in addition to the binding by its own proteins MazE and MazF, the promoter region of mazEF also bore the binding site of another as yet unidentified protein. The presence of MazF together with MazE and this unknown protein in a cell extract caused an over-shift of the promoter fragment (Fig. 4A, lane 4) (see below).

**Footprint analysis of the mazEF promoter revealed protection against DNase I by the MazE-MazF complex.** We used DNase I footprint analysis to define the binding sites in the mazEF promoter. The 180bp DNA fragment contained the whole region of the mazEF promoter from the *relA* stop codon to the start codon of *mazE*, and an additional 100bp of the *mazE* gene. Before digestion by DNase I, we
exposed each strand of this fragment to crude cell extracts: (i) *E. coli* strain MC4100Δ*mazEF* (Fig. 5, lanes 4); (ii) MC4100Δ*mazEF* with MazE over-expressed from a plasmid (Fig. 5, lanes 3); (iii) MC4100Δ*mazEF* with the MazE-MazF complex over-expressed from a plasmid (Fig. 5, lanes 2). As seen in the schematic diagram (Fig. 5, bottom), the protein complex MazE-MazF protects the large area including (-10) and (-35) elements of both promoters (P2 and P3). Unfortunately, we were not able to show DNA protection by MazE alone, probably because of the low affinity of MazE for the promoter region (Fig. 5, lanes 3).

**Further characterising the mazEF promoter region by dividing it into two overlapping fragments.** To define the binding sites of the mazEF promoter more precisely, we divided it into two overlapping fragments: the upstream fragment (-72 to -19) and the downstream fragment (-38 to +2) (Fig. 3B). These two overlapping fragments shared a common element (-38 to -19). When we exposed this common element to each of our cell extracts, we observed no shift in its electrophoretic mobility (data not shown), suggesting that the sequence that it bears is not long enough to permit binding. In contrast, using this same set of cell extracts to expose each of the overlapping fragments, the upstream fragment and the downstream fragment, caused each of them to be shifted very differently (Fig. 4B and 4C). The upstream element was shifted identically by every extract used; it was not at all affected by the presence of MazE or of MazE and MazF (Fig. 4B). In fact, the upstream element appeared to have bound only the unidentified protein with which some of the molecules of the whole 74bp promoter fragment were observed to bind (Fig. 4A). In contrast, the retardation of the downstream fragment revealed a strong dependence on the presence of MazE and MazF (Fig. 4C). As shown above (Fig. 4A), high concentrations of the MazE-MazF complex caused an overshift of this DNA probe (Fig. 4C, lane 4) compared to the retardation level obtained with the cell extract containing only MazE (Fig. 4C, lane 3). When the fragment of the whole promoter region was exposed to a cellular extract containing high levels of MazE and
MazF, we observed both a supershift and an additional band above it. However, this highest band was missing from the gel when this same cellular extract was used to retard the downstream fragment (compare Fig. 4A and 4C, lanes 4). We propose that this overshifted band may have been formed by the mazEF promoter fragment binding to all three proteins: MazE, MazF, and the unidentified protein factor. The mobility pattern of the downstream DNA fragment exposed to the cell extract of E. coli strain from which the mazEF genes had been deleted and (Fig. 4C, lane 2) looked like that of the probe alone (Fig. 4C, lane C), that is, there was no binding.

It did not seem to matter if the downstream fragment were exposed to MazE and MazF expressed from the E. coli chromosome (Fig. 4C, lane 1) or to MazE alone expressed from a plasmid (Fig. 4C, lane 3). We propose that because the promoter region binding affinity of the MazE-MazF complex is much higher than that of MazE alone, high concentrations of MazE result in retardation of the mazEF promoter DNA fragment to the same extent as do low amounts of the MazE-MazF complex (Fig. 4D). High concentrations of the MazE-MazF complex caused an overshift of the whole promoter fragment, though this was not observed in the presence of low concentrations of these proteins (Fig. 4D). These dynamics probably indicate that there are several available binding sites in the promoter region.

**FIS (Factor for Inversion Stimulation) is the cellular protein responsible for the gel mobility shift of the upstream mazEF promoter fragment.** We showed above that an unidentified E. coli protein bound the upstream mazEF promoter fragment (-72 to -19) (Fig. 4B). To determine the size of this protein, we loaded the crude extract of E. coli MC4100ΔmazEF on a Sephadex G-100 column. The fractions collected were subjected to a gel mobility shift assay with the upstream fragment of the mazEF promoter (data not shown). In these fractions, the factor that bound the promoter corresponded to a protein with a molecular weight of 20-25kDa. To identify this promoter-binding protein, we used our broad collection of E. coli mutants. We
prepared crude cell extracts (see Experimental Procedures) of the mutants defective in the following DNA-binding factors: the Factor for Inversion Stimulation (FIS), Integration Host Factor (IHF), rpoS (stationary phase sigma factor), gyrase, and histone-like proteins H-NS and HU. Gel mobility shift assays on the upstream DNA fragment of the mazEF promoter exposed to each of these extracts (Fig. 6A) revealed retardation in every case except when FIS was absent (Fig. 6A, lane 2). The addition to that same DNA fragment of increasing amounts of the pure protein Factor for Inversion Stimulation (FIS) (kindly provided by G. Mushkelishvili) (Fig. 6B, lanes 9-11) resulted in retardation at the same level as that observed by the unidentified protein factor from the crude extract of E. coli MC4100ΔmazEF (Fig. 6B, lane 8). FIS, a protein of molecular weight 12kDa that in vivo forms homodimers of molecular weight 25kDa [27] corresponded perfectly to the protein obtained in our gel filtration experiment (see above). Thus, we concluded that the unidentified protein was in fact FIS.

That the effect of the addition of FIS on the mobility of the upstream fragment represented a specific DNA binding event was further supported by the results of two additional sets of experiments: (i) The character of the gel shift was not changed (data not shown) when we cut the upstream mazEF promoter fragment with the enzyme SalI whose restriction site is in the multi-linker (see map in Fig. 3B); (ii) On the other hand, when we used the enzyme HaeIII, whose restriction site is within the promoter region, neither of the two resulting fragments were retarded by the addition of FIS (data not shown).

We determined the extent of the influence of the FIS on mazEF promoter expression by primer extension experiments. Wild type E. coli strain MC4100 and its fis- mutant derivative were transformed with the plasmid pSK10Δ6-pef bearing the lac'Z gene under the control of the mazEF promoter. We measured the levels of transcription in these transformants at the entry to logarithmic growth (Fig. 6C and 6E). The level of RNA transcription from this plasmid in the wild strain was about 1.6-fold higher then that in the mutant strain, indicating that FIS activates the mazEF
promoter as it acts on most other promoters that it regulates [28]. A mutation in the putative FIS-binding site of the mazEF promoter, in which the T residue at position (-40) is replaced by a G residue, caused the mazEF promoter to be insensitive to activation by FIS (Fig. 6D and 6F).

**Analysis of the auto-regulation region of the mazEF promoter.** Since we were able to show that the downstream fragment of the mazEF promoter was responsible for mazEF auto-regulation (Fig. 4C), the next step was to analyze its sequence (Fig. 7A). The auto-regulation regions of all known addiction modules contain palindrome structures, and mazEF was no exception: the palindrome sequence “a-b,” analogous to the palindrome of pemIK, had already been predicted by Masuda and colleagues [14].

Sequence analysis of the area protected by the MazE-MazF complex revealed an unusually complicated structure (Fig. 7): (i) fragment “a” of the palindrome could be complemented not only by fragment “b”, but also by fragment “c”. We called this “c-a-b” component an “alternating palindrome”, (ii) the centre of an additional palindrome, “d-e,” is located 4bp upstream from the centre of the “c-a” palindrome (Fig. 7).

We present a possible alignment of the palindrome fragments (Fig. 7B) and a possible base pairing among them (Fig. 7C). The “d” and “e” parts of the palindrome show a perfect complementation (Fig. 7B and C). The common element (-38 - -19) of the overlapping upstream and downstream fragments, tested before, contained almost the whole “d-e” palindrome, except for its last nucleotide T (-18). In the gel shift mobility assay the cell extracts contain either MazE alone or MazE-MazF complex did not show retardation with this DNA fragment (data not shown).

Within the alternative palindrome the homology was high, especially between parts “c” and “b.” We hypothesized that at any given moment the “alternating palindrome” might exist in one of two possible configurations: “c-a” or “a-b” (Fig. 7C). To test our model, we performed gel mobility shift experiments using the two
overlapping parts of the double palindrome, “a-b” (-20 to +6) and “a-c” (-34 to -7) and of their common fragment “a” (-23 to -7) alone. The MazE-MazF protein complex bound each of the two palindrome sequences (“a-b” or “a-c”), while none of the cell extracts caused a shift of the middle fragment “a” alone (Fig. 8A).

To verify our model further, we constructed a variation of the downstream promoter fragment “c-m-b” (-38 to +6), in which we replaced the middle part “a” with an unrelated multi-linker sequence that we called “m.” After exposing this “c-m-b” fragment to our standard set of crude cellular extracts, we ran the mixtures in a gel mobility shift assay. As we observed for fragment “a” alone, none of the cell extracts led to retardation of the “c-m-b” fragment (Fig. 8A). In these experiments, we observed no binding of MazE by itself to any other fragment than to the complete “c-a-b” fragment (Fig. 8A).

We asked: what was the factor that was important for binding? Was it simply the sequence of the double palindrome or was the secondary structure of the promoter region required? To distinguish between these two possibilities we introduced several mutations into the sequence of the mazEF promoter region (indicated by the black points in Fig. 7C and specified in Table 2). Every mutation we introduced destroyed a hydrogen bond in the proposed structure, and in the case of mutations (-25; -26; -27) three such bonds were destroyed simultaneously. Each mutated fragment was exposed to cell extracts enriched for the MazE-MazF complex and was run on a gel mobility shift assay. It appears that introducing these mutations caused no changes in the mobility of the promoter fragment (data not shown).

Discussion

In previous studies we have shown that the chromosomal genes mazE and mazF, located in the E. coli rel operon, have all the properties required to be an addiction module [12]. Along with properties shared with other known addiction systems, the mazEF addiction module has two additional properties: (a) it is directed from two
promoters, P2 and P3, located thirteen nucleotides apart; and (b) under conditions of amino acid starvation, mazEF expression is regulated by the cellular level of ppGpp, the product of the RelA protein. Here we have shown the following: (i) the P2 promoter is about 10-fold stronger than is the P3 promoter; (ii) expression from both P2 and P3 is repressed by MazE and is highly repressed by the MazE-MazF complex; (iii) MazE and MazF could bind to an “alternating palindrome” that we found in the promoter region (-34 to +6). This alternating palindrome, which in fact is the operator of mazEF, could exist in one of two alternative states: its middle part “a” complemented with either of the outer parts “b” or “c” (Fig. 7); (iv) expression from mazEF promoters is activated by FIS.

When β-galactosidase was expressed under the control of the mazEF promoters, we observed as much as 7000-8000 Miller units at mid-logarithmic growth (Fig. 2A). This high level of synthesis indicated that the mazEF promoter was very strong, as are the promoters of most addiction modules [5; 8; 9; 10]. Moreover, the presence in trans of the gene products of the mazEF module led to negative auto-regulation, and about ten-fold repression of transcription (Fig. 2).

In further experiments, we obtained cell extracts containing the MazE and MazF proteins, either transcribed from the chromosome or over-expressed from a plasmid under the control of the tac promoter. When MazE and MazF are present together in a cell extract they form a complex. Here, and in other studies, we observed the formation of such a complex in native gel electrophoresis with (35S)-methionine labelled proteins [12], with antibodies against MazE (Fig. 1C), and also in an E. coli two hybrid system (Marianovsky and Glaser, in preparation).

By gel mobility shift assays we clearly showed that the retardation of the promoter fragment depended on exposure to either the MazE or to MazE-MazF complex. We were surprised to find that this DNA fragment could also be retarded by an initially unidentified protein present in the crude cellular extract of a strain from which mazEF had been deleted (Fig. 4A). On the basis of our results reported above, we have concluded that this regulation protein is FIS. FIS is one of the
nucleoid-associated proteins that regulate various processes, including transcription, recombination, and replication [28; 29]. Here we found that FIS increased the activity of the mazEF promoter by 1.6-fold (Fig. 6C and 6E). However, it is possible that under certain specific stressful conditions the effect of FIS on mazEF could be more profound. In this regard, we suggest that FIS may affect the role of mazEF in programmed cell death. Under various physiological conditions, the cellular levels of FIS vary over a large interval (up to 100-fold) and they depend on both the growth phase and on nutritional conditions [29; 30]. In rich medium, the concentrations of FIS are very high in the early exponential phase, but sharply decrease towards stationary phase. FIS is known to act as a homodimer [27]; the molecular weight that we calculated for the initially unidentified protein from the results of our gel filtration experiments corresponded to the molecular weight of FIS as a homodimer. It has been shown that by binding to the DNA region upstream from promoters, this homodimer causes the DNA to bend, thus increasing the binding efficiency of the RNA polymerase [31]. Thus, positive regulation of the mazEF promoter by FIS must be maximal under conditions of rapid growth on rich media.

We found a high level of conformity between the sequence of the upstream fragment of the mazEF promoter and the consensus sequence of the known FIS-binding sites [28]. When we introduced a point mutation in the FIS binding site of the mazEF promoter, the influence of FIS on the promoter was abolished (Fig. 6D and 6F), further confirming that FIS participates in mazEF gene regulation. We were able to ascertain the precise location of the FIS binding site in the mazEF promoter (underlined in the schematic diagram in Fig. 3C). FIS regulation of the promoter of this addiction module seems to be a unique feature of the mazEF module.

While FIS caused positive regulation of the mazEF promoter, auto-regulation of mazEF promoter was strongly negative as it is for most known addiction modules. We found that the auto-regulation site of mazEF was longer and more complicated than would have been predicted by the results of previous studies [14]. In our DNA footprint experiments, we found that an area more extended than the “a-b”
palindromic was protected against DNase I digestion by the MazE-MazF protein complex (Fig. 5). In addition to this “a-b” palindrome, which is similar to the palindrome in the pemIJK promoter [14], we discovered an unusual structure that we have called an “alternating palindrome.” Thus, our results suggest that the middle fragment “a” may complement not only the downstream fragment “b”, but also fragment “c” located upstream from “a” (Fig. 7C). Comparing fragments “b” and “c” revealed that they were highly similar (Fig. 7B). The “alternating binding model” that we have proposed here is supported by the results of our gel mobility shift experiments: the MazE-MazF complex could bind both alternate structures, “c-a” and “a-b,” but not the central “a” fragment by itself. Moreover, the MazE-MazF complex could not bind the “c-m-b” fragment in which the “a” fragment was replaced by an unrelated sequence (Fig. 8A). Based on mutational analyses, we propose that the mazEF promoter can exist in two possible alternate states (Fig. 8). The MazE-MazF complex can bind either of these structures, resulting in strong negative auto-regulation (see below).

The role of the additional “d-e” palindrome is not yet clear. The regulation areas of the promoters of many known addiction modules contain a palindrome sequence [9; 10; 11; 32]. Moreover, some addiction modules, like phd-doc and pemIJK, also contain two palindromes [9; 10; 11; 14]. It has been suggested that in the regulation of the phd-doc addiction module, the toxic and the anti-toxic proteins bind to the two palindromes cooperatively. This binding process is accompanied by an increased affinity of the protein for the DNA, and hence an increasing stability of the DNA-protein complex [11].

The numerous mutations that we introduced into the “alternating palindrome” did not at all affect the binding efficiency of the Maze-MazF complex, suggesting that the secondary structures of the regulating region is more important than its DNA sequence per se. The “alternating palindrome” that we have described seems to be a unique feature of the mazEF promoter. In this structure, it is as though the two palindromes often found in the promoters of other addiction modules [9; 10; 11; 14].
Alternating palindrome regulates the *E. coli* *mazEF* promoter

have been collapsed, thus minimizing the space required for the regulating elements without losing efficiency.

We suggest that the combined presence of two promoters, a complicated palindrome structure, and the FIS binding site permits regulation of expression that is simultaneously safe and dynamic, enabling quick responses to changes in physiological conditions. The duplication of the structural elements (promoters or binding sites for auto-regulation) assures that *mazEF* regulation will be adequate even in the case that one of these elements may be destroyed. The action of two promoters, P₂ and P₃, is additive; during exponential growth they are repressed by auto-regulation to 10-12% of their full capacity. On the one hand, cellular levels of the positive regulator FIS are high in cells growing in rich medium; on the other hand, cellular levels of the negative regulator ppGpp increase under conditions of starvation. Thus the intracellular concentrations of *mazEF* products strongly depend on the growth conditions.

The regulation of the systems responsible for programmed cell death must be very carefully controlled; otherwise, fluctuations in the concentrations of the system’s products may result in death. The duplication of the regulatory elements in the operator of the *mazEF* operon provides such safety to the “addiction system”. We propose that limited space on the *E.coli* chromosome is the reason for the metamorphosis of two palindromes for autoregulation into the alternating structure. Thus, the *mazEF* promoter is elegantly engineered to respond to any possible changes in the nutritional environment of the bacterium.

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Figure legends

Fig. 1. Western blots of cell extracts of *E. coli* strains MC4100 or MC4100Δ*mazEF*. *E. coli* wild type strain MC4100 or strain MC4100Δ*mazEF* were transformed with various plasmids, and crude cell extracts were obtained as described in Experimental Procedures. These cell extracts were analyzed by electrophoresis on denaturing gels (A, B) or native gel (C), and subjected to Western blot analysis with antibodies against MazE (A, C) or against MazF (B). Lanes 1: MC4100 without any plasmids; lanes 2: MC4100Δ*mazEF* without any plasmids; lanes 3: MC4100Δ*mazEF* transformed by pKK-*mazE*; lanes 4: MC4100Δ*mazEF* transformed by pKK-*mazEF*. The MazE-MazF complex on the native gel (C) is indicated by arrow.

Fig. 2. The effects of MazE or MazEF on β-galactosidase transcription directed by a *mazEF-lacZ* gene fusion. *E. coli* strain MC4100Δ*mazEF*, harboring the pSK10Δ6-pef plasmid bearing a gene fusion of *lacZ* under the control of the *mazEF* promoter, was transformed with pLex-*mazE* or with pLex-*mazEF* (see Table 1). At OD$_{600}$ 0.2, the synthesis of MazE or MazE-MazF proteins was induced by the addition of 1mM IPTG. (A) At various times, β-galactosidase activity was measured according to Miller [24]. (B) RNA was extracted and primer extension was carried out as described in Experimental Procedures. The arrows indicate the positions of P$_2$ and P$_3$ promoters. The lanes, marked A, C, G, and T, indicate the sequence of promoter in plasmid pSK10Δ6-pef. (C) Graphical presentation of the signals of the P$_2$ promoter from the experiment shown above (B). (For (A) and (C): ● - *mazEF* promoter alone; ▲ - *mazEF* promoter in presence of MazE protein; ■ - *mazEF* promoter in presence of the MazE and MazF proteins).
Fig. 3. Sequence of the **mazEF** promoter region.  (A) The nucleotide sequence of the **mazEF** promoter. The arrows indicate the transcriptional starting sites P2 and P3, the start codon of **mazE**, the stop codon of **relA**, and the multi-linker of the PCR primer. The transcriptional starting point of the P2 promoter has been designated “+1.”  (B) To further our analysis, we divided the promoter fragment into two overlapping fragments that we call the upstream and the downstream fragments.  (C) Sites of the regulatory elements on the **mazEF** promoter.

Fig. 4. The effects of MazE and of MazF on the gel mobility of the **mazEF** promoter.  The two overlapping DNA fragments of the **mazEF** promoter were exposed to the crude cell extracts described in the legend to Fig. 1. Bound DNA was run on a 6% polyacrylamide gel after exposure to the following protein extracts (A-C): Lanes 1: *E. coli* MC4100 wild type; lanes 2: MC4100Δ*mazEF*; lanes 3: MC4100Δ*mazEF* transformed with the pKK-**mazE**; lanes 4: MC4100Δ*mazEF* transformed with pKK-**mazEF**; lanes C: the DNA promoter fragment without any cell extracts. The DNA fragments used were: (A) the whole 74bp **mazEF** promoter region (from the multi-linker to the NdeI restriction site (see the map in Fig.3A)); (B) the upstream fragment of the **mazEF** promoter (see Fig. 3B); (C) the downstream fragment of the **mazEF** promoter (see Fig. 3B above).  (D) The whole 74bp **mazEF** promoter fragment was exposed to the following increasing amounts of protein extract from MC4100Δ*mazEF* transformed with pKK-**mazEF**: lane 1: 0.2µg; lane 2: 0.5µg; lane 3: 1.0µg; lane 4: 2.0µg; lane 5: 5.0µg; lane 6: 10.0µg; lane 7: 20.0µg; lane C: DNA probe without protein.

Fig. 5. Footprint analysis of the **mazEF** promoter protected against DNAse I digestion by either MazE or by the MazE-MazF complex. (A) The lower strand of the **mazEF** promoter (as shown in the schematic diagram at the bottom of the figure). The promoter fragments were protected against DNAse I by *E. coli* MC4100Δ*mazEF* cell extracts containing the following: Lane 1: no extract added;
lanes 2: MazE-MazF complex; lanes 3: MazE; lanes 4: cell extract containing neither MazE nor MazF. Far left lanes: sequence of the fragments. The following amounts of protein extracts were used (in the direction of arrows): 0.1µg, 1.0µg, 10.0µg, 25.0µg, 100.0µg. (B) as in (A) but for the upper strand of the mazEF promoter as seen in the schematic diagram.

Fig. 6 The FIS protein binds the mazEF promoter as shown by gel mobility shifts and primer extension assays. (A) Gel shift mobility assay of the upstream fragment of mazEF promoter exposed to the extracts of E. coli strains mutant for various DNA binding proteins. (B) As in (A) but with increasing concentrations of purified FIS protein. Lanes C: DNA probe without protein extract; lane 1: MC4100ΔmazEF; lane 2: N99fis+; lane 3: N99hu−; lane 4: N99hif ; lane 5: N99rpoS−; lane 6: N99gyrase−; lane 7: N99hns−; lane 8: cell extract of MC4100ΔmazEF; lane 9: 100nM of pure FIS protein; lane 10: 1µM of pure FIS; lane 11: 10µM of the pure FIS. The DNA fragment used was the same as that described in the legend to Fig. 4B. (C) Primer extension signals of the P2 and P3 promoters with E. coli MC4100 wild type and its mutant fis−. (D) As in (C), but with DNA from a mutated allele of the mazEF promoter in which there is a point mutation in bp −40. (E) Graphic representation of the signals from the P2 promoter in MC4100 (▲■) and in MC4100fis− (●). (F) As in (E) but with DNA as in (D) above. The RNA samples were obtained and primer extension was carried out at various times after the cultures reached OD600 0.2 as described in Experimental Procedures.

Fig. 7 Palindromes in the downstream fragment of the mazEF promoter. (A) DNA sequence of the fragment and the localisation of the palindromes. (B) Alignment of the palindromes. (C) Putative secondary DNA structures that can be generated by the sequences shown in (A) and (B) above (see text). The black points indicate the mutations introduced into the sequences.
Fig. 8  A schematic presentation of the possible states of the “alternating palindrome” during auto-regulation.  (A) Table showing the binding pattern of MazE and the MazE-MazF complex to various fragments of the “alternating palindrome” region (see text).  (B) The MazE-MazF complex binding to the “a-b” side of the palindrome.  (C) The MazE-MazF complex binding to the “c-a” side of the palindrome.
Alternating palindrome regulates the \textit{E. coli} \textit{mazEF} promoter

Table 1. Bacterial strains and plasmids

| Strain/Plasmid | Relevant genotype/Construction | Source/Reference |
|----------------|--------------------------------|-----------------|
| **E. coli strain** |                                |                 |
| MC4100         | \textit{araD139 (argF-lac)205 flbB5301 ptsF25 rpsL150 deoC1 relA1} | 33              |
| MC4100Δ\textit{mazEF} | a Δ\textit{mazEF} derivative of MC4100, kan\textsuperscript{R} | 12          |
| MC4100\textit{fis}\textsuperscript{−} N99 | a \textit{fis}\textsuperscript{−} derivative of MC4100, kan\textsuperscript{R} | this work |
| MG474          | A \textit{hu}\textsuperscript{−} derivative of N99, kan\textsuperscript{R} | NIH collection |
| MG475          | A \textit{ihf}\textsuperscript{−} derivative of N99, tet\textsuperscript{R} | laboratory collection |
| MG487          | A \textit{kutF rpoS}\textsuperscript{−} derivative of N99, kan\textsuperscript{R} | laboratory collection |
| MG521          | A \textit{hns}\textsuperscript{−} derivative of N99, tet\textsuperscript{R} | 19          |
| MG524          | A \textit{fis}\textsuperscript{−} derivative of N99, kan\textsuperscript{R} | 19          |
| MG533          | A \textit{gyrase}\textsuperscript{−} derivative of N99, tet\textsuperscript{R}, temperature sensitive | laboratory collection |
| **Plasmid**    |                                |                 |
| pKK223-3       | expression vector with strong \textit{tac} promoter, Amp\textsuperscript{R} | Amersham Pharmacia Biotech |
| pKK-\textit{mazE} | a pKK223-3 derivative carrying the \textit{mazE} gene | this work |
| pKK-\textit{mazEF} | a pKK223-3 derivative carrying the \textit{mazE} and \textit{mazF} genes | this work |
| MC1403         | a \textit{lacYZ} gene fusion vector | 34          |
| pSK10Δ6       | a MC1403 derivative lacking \textit{lacY} through partial cleavage by \textit{Aval} | this work |
| pSK10Δ6-\textit{pef} | a pSK10Δ6 derivative carrying the gene \textit{lacZ} fusion of the \textit{mazEF} promoter from the end of \textit{relA} to the 17\textsuperscript{th} codon of \textit{mazE} | this work |
| pLex1         | a pLex1 derivative carrying the \textit{mazE} gene under the \textit{tac} promoter, cam\textsuperscript{R} | 20          |
| pLex-\textit{mazE} | a pLex1 derivative carrying the \textit{mazE} gene under the \textit{tac} promoter, cam\textsuperscript{R} | this work |
| pLex-\textit{mazEF} | a pLex1 derivative carrying the \textit{mazE} and \textit{mazF} genes under the \textit{tac} promoter, cam\textsuperscript{R} | this work |
### Table 2. Oligonucleotides used in this study

| Name  | Sequence (5’-3’) | Orientation and Localization | Purpose |
|-------|-----------------|-------------------------------|---------|
| EE-1  | GCGCCGAGATCTGAAGGATATACATATGAGATCCACAGTACGCTCGTAAG | + (29 to 50) | cl’ |
| TFPE  | GACGCGCTCGACAAACGCGCTACATATGAGATCCACAGTACGCTCGTAAG | - (17 to 2) | cl’ |
| FG-1  | GGAGCGCTCGGCTACCAATCGACGGGAGTTAGGCGCAA | - (594 to 613) | cl’ |
| EF-1  | CCGGAATTCGTCGACGGGAGTTAGGCGCAA | + (29 to 50) | cl’ |
| EF-2  | GCGAGCGCTCCTTTACCAGACTTCTTATCT | - (260 to 278) | cl’ |
| EG-3  | ATCATCAATATTCCAGATTGA | - (+128 to 118) | cl’ |
| EF-4  | GCTCGTATCTCAACATATGAGATCCACAGTACGCTCGTAAG | + (-38 to -19) | cl’ and gms’’’ |
| EF-5  | TCTACATTGTAGATACGAC | - (-19 to -38) | cl’ and gms’’’ |
| EF-6  | GTATCTCAATGTAGATATGATATCGCAA | + (-33 to -7) | gms’’’ |
| EF-7  | GTATATATCAATCTACATTGTAGATCCACAGTACGCTCGTAAG | - (-7 to -33) | gms’’’ |
| EF-8  | GATTGATATATACTGTATCTACATATGAGATCCACAGTACGCTCGTAAG | + (-20 to +7) | gms’’’ |
| EF-9  | CATATGAGATACGACTTCTATATCAATCTTATCTAATCTTCGCTCTGCTCTCATTGTAGAAGTCGTGTTACGAGC | - (+7 to -20) | gms’’’ |
| EF-10 | GATTGATATATACTGTATCTACATATGAGATCCACAGTACGCTCGTAAG | - (-23 to -7) | gms’’’ |
| EF-11 | AGTATATCAATCTACATTGTAGAAGTCGTGTTACGAGC | - (-7 to -23) | gms’’’ |
| EF-12 | GCTCGTATCTGCTACATATGAGATCCACAGTACGCTCGTAAG | - (-39 to -20) | mut.’’’’ (-32) |
| EF-13 | TCTACATTGTAGATACGAC | - (-20 to -39) | mut.’’’’ (-32) |
| EF-14 | GCTCGTATCTGCTACATATGAGATCCACAGTACGCTCGTAAG | + (-38 to +6) | mut.s’’’’ (-32) and (-10) |
| EF-15 | ATATGAGATACGACTTCTATATCAATCTTATCTAATCTTCGCTCTGCTCTCATTGTAGAAGTCGTGTTACGAGC | - (+6 to -38) | mut.s’’’’ (-32) and (-10) |
| EF-16 | GCTCGTATCTGCTACATATGAGATCCACAGTACGCTCGTAAG | + (-38 to -14) | mut.s’’’’ (-32) and (-18) |
| EF-17 | ATCAGTCTCATTGTAGAAGTCGTGTTACGAGC | - (-14 to -38) | mut.s’’’’ (-32) and (-18) |
| EF-18 | CAATGAGATACGACTTCTATATCATTGTAGAAGTCGTGTTACGAGC | + (-27 to -8) | mut.’’’’ (-18) |
| EF-19 | GTATATACATCTACATTGAGATCCACAGTACGCTCGTAAG | - (-8 to -27) | mut.’’’’ (-18) |
| EF-20 | TTTGCTCGATCTCCTGTAATGAGGATTGATATGAGATCCACAGTACGCTCGTAAG | + (-41 to -11) | mut.s’’’’ (-25, -26, -27) |
| EF-21 | TATATACATCTACAGCGATCATGATACGAGC | - (-11 to -41) | mut.s’’’’ (-25, -26, -27) |
Alternating palindrome regulates the *E. coli* mazEF promoter

|    | Sequence          | Direction (bp range) | Mutation |
|----|-------------------|----------------------|----------|
| EF-22 | GCCGAAATGTCTCGAT | + (-49 to -30) | mut. *** (-40) |
| EF-23 | GATACGAGCACATTTGGC | - (-30 to -49) | mut. *** (-40) |

* cloning
** gel mobility shift
*** introduce mutation; the bold letters show the mutated nucleotides
### Figure 2

#### Panel A

![Graph with time vs. β-galactosidase activity](image)

- **β-galactosidase activity (Miller units)**
- **Time (min):** 0, 20, 40, 60

#### Panel B

| Sequence of mazEF promoter | mazEF promoter alone | mazEF promoter +MazE | mazEF promoter + (MazE-MazF) complex |
|---------------------------|----------------------|----------------------|-------------------------------------|
| A C G T                    | 0' 5' 10' 20'        | 0' 5' 10' 20'        | 0' 5' 10' 20'                       |

#### Panel C

![Graph with time vs. relative units](image)

- **Relative units:**
- **Time (min):** 0, 10, 20

---

**B**

- **Alert:**
  - **P2**
  - **P3**
Figure 3

A

multilinker → C terminus of relA ORF

\[ \text{Sal I} \]

\[ \text{Hae III} \]

CCGGAATTTCGAGCAGCGGAGTTAGGCCGAAATTTGCTCGATCTACAATGTAGATATATATATCTGTATCTACATATGATAGCGGTGTTGAGGAAGGGTATG
-35 -10

\[ \text{NdI} \]

B

multilinker → C terminus of relA ORF

CCGGAATTTCGAGCAGCGGAGTTAGGCCGAAATTTGCTCGATCTACAATGTAGATATATATATCTGTATCTACATATGATAGCGGTGTTGAGGAAGGGTATG
-72 -60 -40 -20

\[ \text{NdI} \]

upstream fragment

GCTCGATCTACAATGTAGATATATATATCTGTATCTACATATGATAGCGGTGTTGAGGAAGGGTATG
-38 -20

-10

C

multilinker → C terminus of relA ORF

CCGGAATTTCGAGCAGCGGAGTTAGGCCGAAATTTGCTCGATCTACAATGTAGATATATATATCTGTATCTACATATGATAGCGGTGTTGAGGAAGGGTATG
-60 -40 -20

\[ \text{NdI} \]

downstream fragment

\[ \text{FIS binding site} \]

d e c a b

\[ +1 \]

\[ +20 \]
mazE ORF

P_2

P_3
Figure 4

A

MazE+MazF+
+unidentified protein →
MazE+MazF →
MazE →
unidentified protein →
probe →

B

C

D

1 2 3 4  C
1 2 3 4
C 1 2 3 4
1 2 3 4  C
1 2 3 4 5 6 7  C
Alternating palindrome’s fragments and their binding by MazE and MazF containing extracts.

| The fragment length and position | Binding by MazE | Binding by MazE-MazF |
|---------------------------------|----------------|---------------------|
| c a b                           | +              | +                   |
| c a                             | -              | +                   |
| a b                             | -              | +                   |
| a                               | -              | -                   |
| c m b                           | -              | -                   |
The regulation of the Escherichia coli mazEF promoter involves an unusual
alternating palindrome
Irina Marianovsky, Einat Aizenman, Hanna Engelberg-Kulka and Gad Glaser

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