Molecular Microbiology (1994) 11(5), 943–954

Repression of *tonB* transcription during anaerobic growth requires Fur binding at the promoter and a second factor binding upstream

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Summary

Although iron is an essential nutrient, its toxicity at high levels necessitates regulated transport. In Gram-negative bacteria a central target for regulation is the TonB protein, an energy transducer that couples the cytoplasmic membrane proton motive force to active transport of (Fe^{III})–siderophore complexes across the outer membrane. We have previously demonstrated the threefold repression of *tonB* transcription by excess iron in the presence of Fur repressor protein under aerobic conditions. In this report, we examine *tonB* regulation under anaerobic conditions where the solubility of iron is not a limiting factor and, presumably, siderophore-mediated transport is not required. Under these conditions, *tonB* transcription is repressed at least 10-fold by excess iron in the presence of Fur, but can be fully derepressed in the absence of Fur. Based on several lines of evidence, this anaerobic repression is not due to increased negative supercoiling as previously postulated. Our results rule out both supercoiling-mediated decreased promoter function and increased Fur binding as mediators of anaerobic repression. Under iron-limiting anaerobic conditions *tonB* expression is as high or higher than under iron-limiting aerobic conditions, suggesting that promoter function has not decreased anaerobically. Furthermore, under anaerobic conditions in *tonB*$^+$ strains, *tonB* promoter function is insensitive to the gyrase inhibitor novobiocin and to changes in medium osmolarity and temperature, three conditions known to change levels of supercoiling. We also rule out effects of mutations in *arcA* or *fnr* as mediators of anaerobic repression. Results from *in vivo* dimethyl sulphate protection footprinting indicate that Fur binds to an operator site between the −10 and −35 regions of the promoter, but not to a less homologous operator site centered at +26. The binding is, if anything, weaker under anaerobic conditions, indicating that anaerobic repression is not mediated through Fur. Additional changes in the *in vivo* footprint upstream from the promoter implicate a second factor in *tonB* anaerobic repression. Together, these results suggest that the mechanism responsible for this regulation (and, by analogy, that of other anaerobically repressed, iron-regulated genes such as *cir*, *exbB*, and *fhuA*) is a novel one.

Received 14 July, 1993; revised 5 November, 1993; accepted 16 November, 1993. *For correspondence. Tel. (509) 3355614; Fax (509) 3353517.

Introduction

In *Escherichia coli*, iron is required for deoxyribonucleotide synthesis, oxidative phosphorylation and other important biological processes. While naturally abundant, iron is not readily available to microbes in aerobic aqueous environments at neutral pH, where it exists as insoluble ferric hydroxide complexes. *E. coli* solubilizes and scavenges ferric iron (Fe^{III}) by means of excreted siderophores, molecules with extremely high affinities for Fe^{III}. Soluble (Fe^{III})–siderophore complexes are actively transported across the outer and cytoplasmic membranes into the cell (for a review see Braun, 1985). An Fe^{II} uptake system has also been characterized recently (Kammler *et al.*, 1993). While iron is an essential nutrient, it is also potentially toxic as it promotes the formation of reactive hydroxyl radicals (Imlay *et al.*, 1988).

To accommodate the need for iron while preventing a toxic accumulation, *E. coli* regulates the transcription of virtually all genes involved in iron acquisition via the Fur repressor protein (Imlay *et al.*, 1988). For example, genes required for biosynthesis of the siderophore enterochelin — also known as enterobactin — (Fleming *et al.*, 1983), the high affinity outer membrane receptors for active transport of iron–siderophore complexes across the outer membrane (Hantke, 1981; Bindereif and Neilands, 1985), and the binding protein-dependent cytoplasmic membrane iron transport proteins are all negatively regulated at the level of transcription by the Fur repressor protein (Brickman *et al.*, 1990). Fur is a 16.8 kDa protein that, as a complex with cytoplasmic

ferrous iron (Fe^{II}), binds promoter regions of iron-regulated genes to prevent transcription (Bagg and Neilands, 1987a). Fur has a significantly lower affinity for Fe^{III} (Bagg and Neilands, 1987b). Unlike many repressor proteins, Fur seems to lack a helix-turn-helix motif in its deduced amino acid sequence (Schäffer *et al.*, 1985). It is rich in cysteine and histidine residues, which appear to play a role in the binding of iron. Mn^{II}, which can also bind Fur, has been substituted as a co-repressor in *in vitro* experiments performed so far owing to the difficulties in maintaining an environment compatible with Fe^{II} binding (DeLorenzo *et al.*, 1987; 1988; Griggs and Konisky, 1989; Brickman *et al.*, 1990). However, the use of Mn^{II} as co-repressor *in vitro* does not always reflect results observed *in vivo* (Privalle and Fridovich, 1993).

A central player in Fe^{III} transport, and an obvious target for regulation, is the TonB protein (Postle, 1990b; 1993). TonB is anchored in the cytoplasmic membrane by its uncleaved amino terminus and extends across the periplasmic space to interact directly with outer membrane receptors (Skare *et al.*, 1993), coupling cytoplasmic proton motive force to active transport across the outer — and essentially unenergized — membrane (Postle and Skare, 1988; Hannavy, *et al.*, 1990; Roof *et al.*, 1991; Bradbeer, 1993).

Since *E. coli* is a facultative anaerobe and since iron can clearly exist in two redox states, which are dependent upon pH and oxygen availability, *tonB* regulation should be characterized under both aerobic and anaerobic conditions. Expression of *tonB* under aerobic conditions is regulated threefold by iron availability and by Fur protein, but not, as previously postulated (Dorman *et al.*, 1988), by growth phase (Postle, 1990a). Under anaerobic conditions, Fe^{II} predominates and can be transported by a TonB-independent mechanism (Hantke, 1987; Kammler *et al.*, 1993). Previous studies of *tonB* transcription have been performed in *tonB* strains and consequently detected only the anaerobic repression of *tonB* (Hantke, 1981; Dorman *et al.*, 1988) or no regulation at all (Fleming *et al.*, 1983). Increased negative supercoiling was proposed to mediate anaerobic repression of *tonB*, again using *tonB* strains (Dorman *et al.*, 1988). We wanted to re-examine and quantify anaerobic regulation of *tonB* in $tonB^+$ strains and also determine to which sites Fur binds *in vivo* with Fe^{II} as corepressor.

The nucleotide sequence of *tonB* contains two potential Fur-binding sites in the vicinity of the *tonB* promoter (Postle and Good, 1983; Postle, 1990a). In this study, we show that Fur binds to a site between the −10 and −35 regions of the *tonB* promoter and not to the site centred at +26. Furthermore, *tonB* is more repressed under anaerobic conditions compared to aerobic conditions. The anaerobic repression is not due to decreased promoter function owing to changes in supercoiling or increased Fur binding at the operator, but rather appears to involve an upstream sequence and an additional, uncharacterized factor.

Results

Transcription of tonB *is more repressed under anaerobic conditions*

The *tonB–lacZ* transcriptional fusions from an earlier study (Postle, 1990a, Fig. 1) were used to investigate the anaerobic regulation of *tonB* in $tonB^+$ strains. In contrast to the iron-repressed levels of *tonB–lacZ* fusions under aerobic conditions (271 β-galactosidase units for KP1027 and 1350 β-galactosidase units for KP1030), assays on anaerobically grown cultures resulted in significantly lower iron-repressed levels for KP1027 (49 β-galactosidase units) and KP1030 (320 β-galactosidase units) (Table 1). Thus, rather than the two- to threefold iron-dependent repression observed aerobically, *tonB* expression is repressed more than 10-fold anaerobically. Because the absence of Fur resulted in constitutive expression, anaerobic repression was Fur dependent.

TonB expression is not post-transcriptionally regulated by anaerobiosis

The unique orientation of *tonB* relative to the opposing gene *P14* and the fact that they share a factor-independent transcription terminator suggested that *tonB* could be regulated by antisense mRNA originating in *P14*

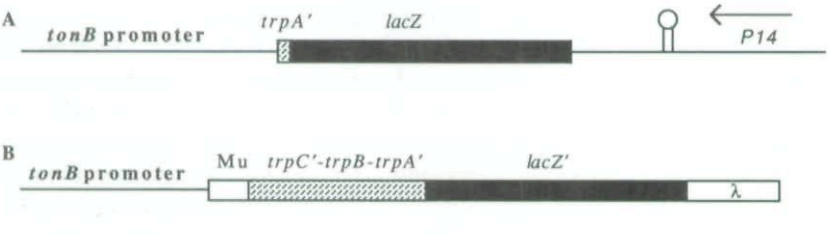


Fig. 1. Chromosomally encoded *tonB–lacZ* fusions (not to scale). Positions of the *tonB* promoters relative to the *lacZ* genes and intervening DNA sequences are shown.
A. The *tonB–trpA'–lacZ* fusion in strain KP1029.
B. The *tonB–Mu–trpC'–trpB–trpA'–lacZ* fusion in strain KP1027. *Trp* DNA is represented by stippled boxes, and *lacZ* DNA is represented by filled boxes. Details of the construction were previously decribed by Postle (1990a).

Table 1. Units of β-galactosidase for *E. coli* strains containing *tonB–lacZ* transcriptional fusions.[a]

	KP1027				KP1030			
	Aerobic		Anaerobic		Aerobic		Anaerobic	
Conditions	+iron[b]	+dipyridyl[c]	+iron	+dipyridyl	+iron	+dipyridyl	+iron	+dipyridyl
Wild type	271 ± 58	499 ± 109	49 ± 15	464 ± 63	1350 ± 423	4769 ± 1081	320 ± 119	7070 ± 1872
fur	541 ± 20	490 ± 45	482 ± 102	438 ± 67	3758 ± 413	5221 ± 1063	6437 ± 883	6730 ± 1890
arcA							345 ± 16	6360 ± 279
fnr							354 ± 57[d]	7065 ± 299[d]
pMH15(*fur*$^+$)	134 ± 40	378 ± 30						
Growth at 30°C			44 ± 8	413 ± 28				
Low osmolarity					689 ± 52[e]			
High osmolarity (NaCl)					674 ± 81			
High osmolarity (sucrose)					762 ± 88			

a. Beta-galactosidase units were measured as described in the *Experimental procedures.*
b. Cultures grown in minimal M9 media supplemented with 90 μM $FeCl_3$.
c. Cultures grown in minimal M9 media supplemented with 200 μM dipyridyl.
d. The *tonB–trpA'–lacZ* fusion was transduced in to strain JRG1728 (strain KP1049).
e. Media used for osmolarity experiments is described in the *Experimental procedures.*

(Postle and Good, 1985). Based on immunoblot analysis, there appeared to be no post-transcriptional regulation by iron limitation or anaerobicity, since changes in TonB protein levels in either KP1030, (Fig. 2) KP1006, or W3110 (data not shown) corresponded to changes in *tonB* transcription from the *tonB* promoter.

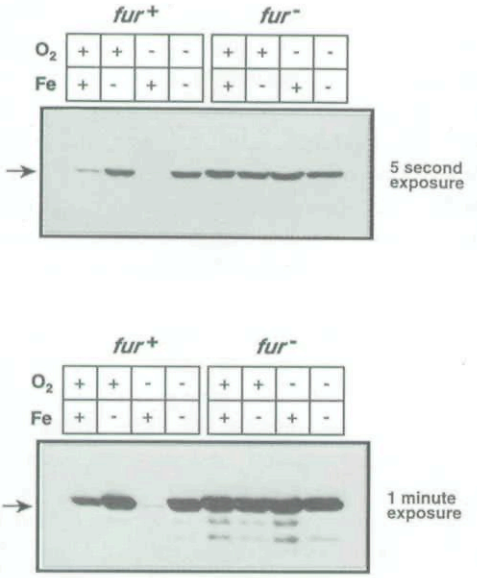
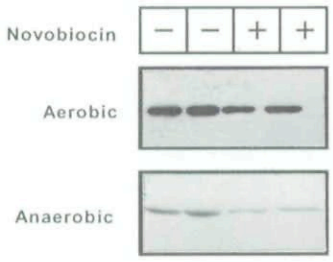


Fig. 2. Levels of TonB protein reflect levels of *tonB* transcription under aerobic and anaerobic conditions. Samples of KP1030 grown under the conditions used for β-galactosidase assays were examined by immunoblot analysis. Identical amounts of whole-cell lysates (0.5 OD ml equivalents) were loaded in each lane, electrophoresed on SDS–polyacrylamide gels, electroblotted, and probed with TonB-specific monoclonal antibody. Exposures of 5 sec and 1 min are shown.

Increased repression of tonB *under anaerobic conditions is not due to changes in DNA supercoiling or increased levels of Fur, ArcA, or Fnr*

It has been suggested that repression of *tonB* under anaerobic conditions is due to increased negative supercoiling (Dorman *et al.*, 1988). In that study, addition of the gyrase inhibitor novobiocin was also observed to increase aerobic expression of *tonB* by approximately 2.5-fold. To re-examine effects of supercoiling changes on *tonB* expression, levels of TonB protein were measured in MC4100/pSN5 grown aerobically or anaerobically with or without the DNA gyrase inhibitor novobiocin (Fig. 3). Plasmid pSN5 carries a synthetic *gyrA–lacZ* fusion known to respond to changes in DNA supercoiling (R. Krah and R. Menzel, personal communication) in a manner similar to that postulated for *tonB*. In the presence of oxygen, *gyrA–lacZ* fusion expression was slightly induced by novobiocin. As expected, growth under anaerobic conditions repressed *gyrA–lacZ* expression, while addition of novobiocin resulted in induction. Immunoblots of TonB protein from the same cultures indicated that, if anything, there was a slight decrease in TonB expression in the presence of novobiocin suggesting increases in DNA supercoiling were not responsible for anaerobic repression. While this decrease could reflect an effect of novobiocin on protein synthesis sufficient to mask its effects on TonB expression, Coomassie brilliant blue-stained immunoblots did not reveal significant differences in protein levels (data not shown). To further test this idea, levels of β-galactosidase were measured in *tonB*$^+$ strain KP1030 grown semi-aerobically in the presence of a range of concentrations of the DNA gyrase inhibitor novobiocin (Fig. 4). Increased repression was observed as the cultures became progressively more anaerobic.

A



B

	- Novobiocin		+ Novobiocin	
Aerobic	12789	12710	14098	13293
Anaerobic	7874	8267	10697	12965

Fig. 3. Inhibition of DNA gyrase does not induce TonB expression. Overnight cultures of MC4100 carrying pSN5 were subcultured (1:100) into fresh minimal M9 medium supplemented with 90 μM $FeCl_3$ and grown aerobically or anaerobically as described in the *Experimental procedures*. Novobiocin at 200 μg ml^{-1}, or water, was added at OD_{600} = 0.3. Incubation was continued until OD_{600} reached 0.5, at which time immunoblot analysis and β-galactosidase assays were performed on each culture.
A. Immunoblot analysis with anti-TonB mAb. To ensure detection of TonB protein in the same gel exposure, 0.125 OD ml equivalents and 0.4 OD ml equivalents were loaded for aerobic and anaerobic samples respectively.
B. Beta-galactosidase values for cultures immunoblotted in (A).

Levels of β-galactosidase from cultures containing novobiocin (at 10, 20, or 40 μg ml^{-1}) remained the same as the control culture lacking novobiocin, suggesting that the *tonB* promoter is insensitive to changes in DNA supercoiling.

It has been shown that genes whose expression is affected by supercoiling are similarly affected by environmental stimuli that are known to alter degrees of DNA supercoiling (O'Byrne *et al.*, 1992). For example, growth at high osmolarity increases negative supercoiling, which should mimic the anaerobic situation, whereas growth at 30°C results in decreased negative supercoiling. If *tonB* transcription were supercoiling dependent, we would expect increased repression compared to cultures grown at low osmolarity under aerobic conditions in the first instance, and derepression of anaerobically repressed cultures in the second. Although the basic osmolarity medium resulted in overall decreased *tonB* expression compared to M9 medium, variations in osmolarity had no effect. Furthermore, growth of KP1027 at 30°C had no effect (Table 1). We have previously observed the insensitivity of *tonB* expression to medium osmolarity under aerobic conditions (Larsen *et al.*, 1993).

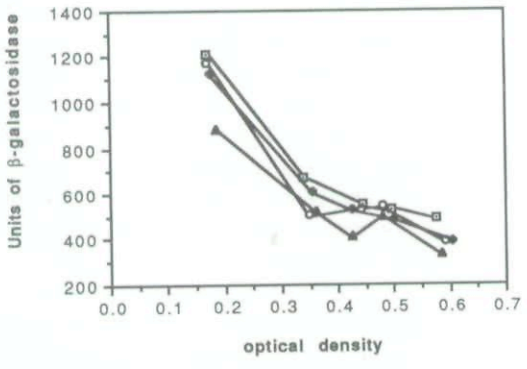


Fig. 4. Expression of *tonB* is insensitive to changes in supercoiling. Strain KP1030 was grown semi-aerobically in M9 medium without (□) or with the DNA gyrase inhibitor novobiocin at 10 mg ml^{-1} (◆), 20 mg ml^{-1} (○), or 40 mg ml^{-1} (▲). Beta-galactosidase activity was assayed at the indicated OD_{600} values.

To determine if anaerobic repression of *tonB* under anaerobic conditions might be due to increased levels of Fur in the cell, strain KP1027 carrying *fur* on a multicopy plasmid (pMH15) was assayed under aerobic conditions. The presence of *fur* on a multicopy plasmid resulted in a modest decrease in β-galactosidase activity for a culture of KP1027(+pMH15) compared to a culture of KP1027 (−pMH15), suggesting that, although not measured directly, Fur was being expressed at higher levels (Table 1). The fact that the aerobic repression ratios remained the same whether or not Fur was overexpressed suggested that increased levels of Fur were probably not the mechanism of anaerobic repression.

Transcriptional regulation of the *E. coli sodA* gene is simultaneously dependent upon *fur*, *arcA* and *fnr* (Hassan and Sun, 1992; Compan and Touati, 1993). The *arcA* and *fnr* genes encode pleiotropic regulators that affect, both negatively and positively, the expression of many genes in cultures grown aerobically as opposed to anaerobically (Iuchi and Linn, 1988; Spiro and Guest, 1990). To investigate the possibility that either of these genes might be responsible for anaerobic repression of *tonB*, β-galactosidase levels from the chromosomally encoded *tonB–trpA′–lacZ* transcriptional fusion in KP1030 were examined in strains KP1049 (*fnr*) and KP1051(*arcA*). The results show that neither the presence of an *arcA* nor an *fnr* mutation affected the regulation of *tonB* transcription under anaerobic conditions (Table 1).

Fur binds tonB *DNA at a site between the −10 and −35 regions of the promoter under aerobic and anaerobic conditions*

The existence of two DNA sequences with similarity to the consensus Fur-binding sites (FBS) proximal to the *tonB* promoter suggested that Fur bound to one or both of these sites *in vivo* (Fig. 5). Since anaerobic repression is Fur dependent, we wanted to determine where Fur binds and whether the extent of Fur binding could account for anaerobic repression. Dimethyl sulphate (DMS) methylation protection experiments were performed on the *tonB*$^+$ plasmid pKP167 in KP1052 under several sets of conditions: with or without plasmid-encoded Fur (pMH15), aerobic or anaerobic growth, and in the presence of excess iron or the iron chelator dipyridyl. Levels of TonB protein were determined by immunoblot analysis of cultures grown under identical conditions used for *in vivo* footprinting and were identical to the transcriptional regulation results (data not shown). The pattern of guanosine residues protected from methylation indicated that Fur bound between the −10 and −35 regions of the *tonB* promoter under both aerobic and anaerobic conditions, but only when iron was present in excess (Fig. 6, A and B). Fur did not bind to the potential FBS which was centrally located at position +26. Protection of residues −14, −21, −25 and −31 on the coding strand was slightly decreased under anaerobic compared to aerobic conditions (Fig. 6A). However, the degree of protection at positions −13 and −20 (Fig. 6B) on the non-coding strand was similar under both aerobic and anaerobic conditions (approximately four- to sixfold by densitometer scan, data not shown). The fact that only a subset of guanosine residues are differentially protected in the presence and absence of O_2 suggests that while tighter binding is not the cause of increased anaerobic repression, Fur may undergo a conformational change.

Upstream of the Fur-binding site, protection of residues at positions −85 and −86 compared to the *in vitro* control reaction, which indicates all possible DMS reactive residues, was observed under all conditions (except anaerobically when iron was present in excess). The protection of these residues was independent of Fur binding, suggesting that a second factor bound to this region (Fig. 6C).

Discussion

The phenomenon of anaerobic repression has been previously observed in the iron-regulated genes *cir*, *fhuA*, *exbB* and *tonB* (Hantke, 1981; Braun, 1985). The fact that these genes participate in ferric-siderophore transport suggests that other genes involved in this process may also exhibit anaerobic repression. In this paper we showed, using two *tonB–lacZ* transcriptional fusions in *tonB*$^+$ backgrounds and immunoblot analysis that *tonB* transcription was repressed more than 10-fold anaerobically, compared to threefold aerobically. The *tonB* promoter is, if anything, more active under anaerobic than aerobic conditions when iron is limiting. Like repression of *tonB* under aerobic conditions (Postle, 1990a), Fur was necessary for the increased repression of *tonB* expression under anaerobic conditions, but, as will be discussed later, it was by itself not sufficient. These results are consistent with the central role that TonB protein plays in ferric, but not ferrous, iron acquisition (Hantke, 1987; Kammler *et al.*, 1993). Under anaerobic conditions, ferrous iron is soluble and can be transported in a TonB-independent fashion. Thus, under anaerobic conditions, unless *E. coli* finds itself in an iron-limited environment, it does not expend energy on TonB synthesis.

Results from our laboratory suggested that expression of *tonB* could be post-transcriptionally regulated from expression of an antisense messenger RNA expressed by the opposing *P14* gene (Postle and Good, 1985). In this study, however, immunoblot analysis indicated that steady-state levels of TonB protein corresponded with the degree of *tonB–lacZ* transcription under all conditions examined, suggesting that TonB was not post-transcriptionally regulated by iron or oxygen availability.

It has been reported previously, using *tonB* strains, that anaerobic repression of *tonB* is due to the increased

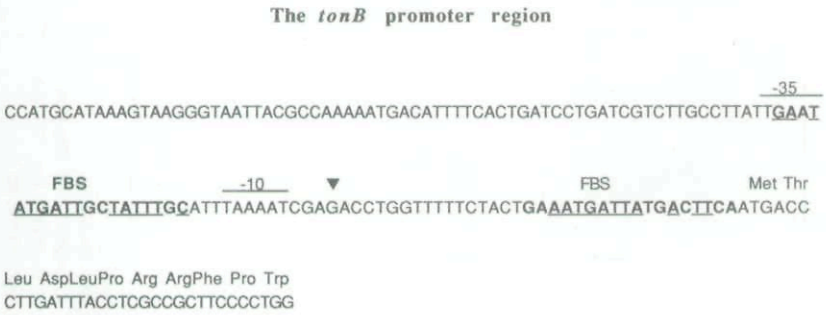


Fig. 5. The DNA sequence of the *tonB* promoter region. The −10 and −35 regions are overlined. The start of transcription (Postle and Good, 1983) is indicated by the arrowhead. Sequences similar to consensus Fur-binding sites (FBS) are indicated in bold and identical bases are underlined. The initiating methionine (Postle and Skare, 1988) and subsequent amino acids are indicated by three letter symbols above their respective codons.

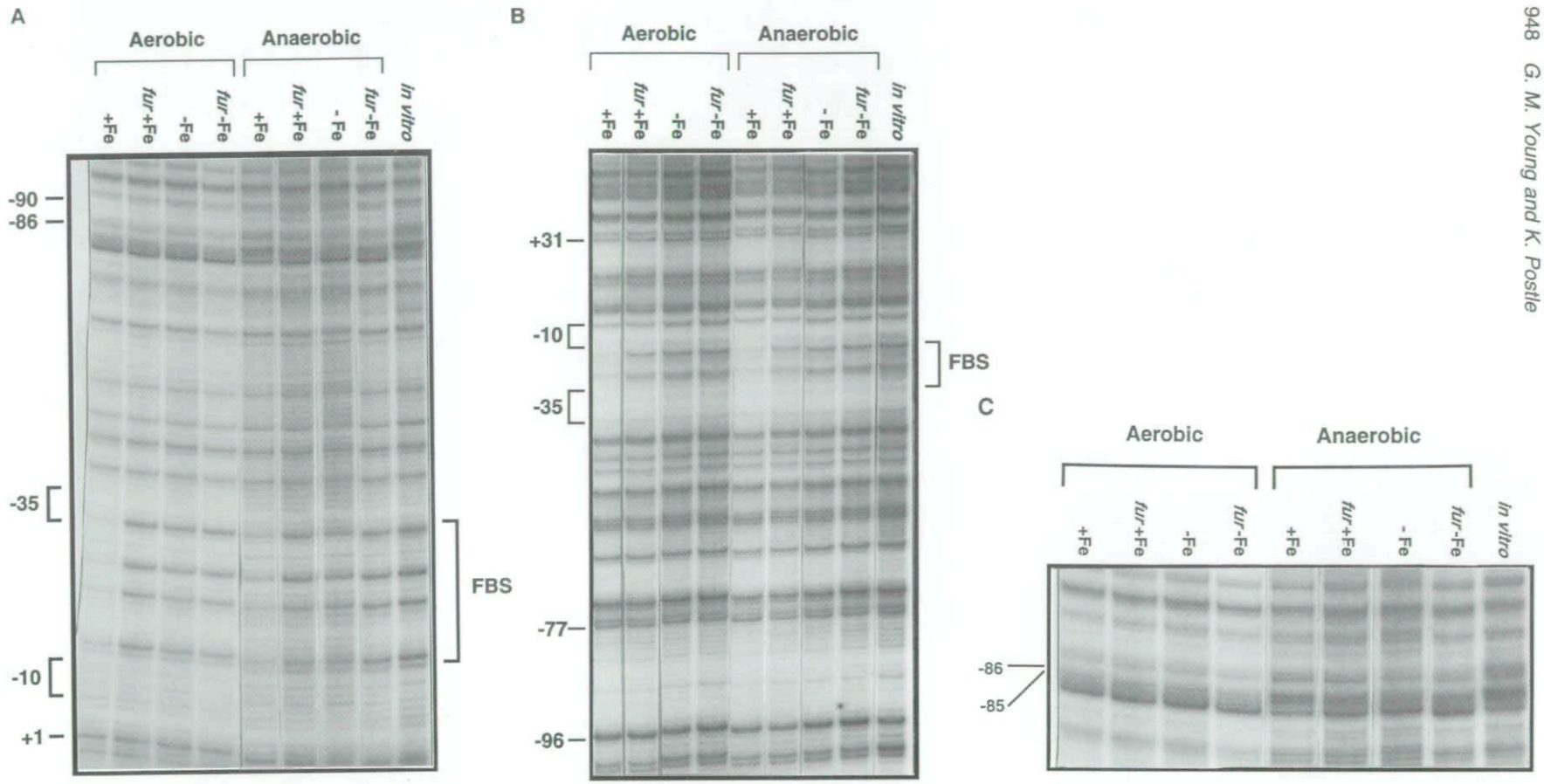


Fig. 6. *In vivo* DMS protection footprinting of the *tonB* promoter region in KP1052 carrying pKP167 and pMH15 grown under aerobic and anaerobic conditions.
A. Coding strand.
B. Non-coding strand.
C. Enlargement of the area of protected nucleic acid residues on the coding strand upstream of the *tonB* promoter. The numbers along the left margin indicate the location of nucleic acid residues relative to the start of transcription. The Fur-binding site (FBS) is indicated by a bracket. For *in vitro* lanes, purified plasmid was treated with DMS to serve as a control for all possible modified residues.

negative supercoiling that occurs both under anaerobic conditions and as cells move into stationary phase (Dorman *et al.*, 1988). However, it was subsequently shown that the postulated growth-phase regulation of *tonB* occurs only in *tonB* strains and not in *tonB*$^+$ strains, occurs only in media with intermediate levels of iron, and is due to iron starvation-induced derepression as the *tonB* cultures approached stationary phase (Postle, 1990a). These results suggest that *tonB* expression is not sensitive to the decreased negative supercoiling that accompanies growth at stationary phase in aerobically grown cultures.

We wanted to investigate more thoroughly the hypothesis that supercoiling plays a role in anaerobic repression of *tonB* using *tonB*$^+$ strains. Changes in DNA supercoiling require the activity of DNA gyrase (Menzel and Gellert, 1983). In direct tests of supercoiling changes, the *gyrA* promoter was used as a control for efficacy of the gyrase inhibitor, novobiocin, since it responds to novobiocin in the same way that *tonB* was reported to respond. Inhibition of DNA gyrase with novobiocin and growth under anaerobic conditions changed expression from a synthetic *gyrA* promoter as predicted: anaerobic growth inhibited expression from *gyrA* and novobiocin induced expression. In contrast, immunoblot analysis of the same cultures indicated that chromosomally encoded TonB protein levels were, if anything, decreased rather than increased by novobiocin addition. Furthermore, anaerobic expression from a *tonB–lacZ* fusion was unaffected by growth in the presence of novobiocin.

Anaerobiosis and other environmental factors such as high osmolarity and low temperature are known to influence the supercoiling of *E. coli* DNA, which both positively and negatively affects the expression of many genes (Ni Bhriain *et al.*, 1989; Ueshima *et al.*, 1989; Göransson, *et al.*, 1990; O'Byrne *et al.*, 1992). An additional set of experiments was based on the observation that when gene expression is sensitive to levels of DNA supercoiling, changes in these environmental factors should cause corresponding changes in gene expression (O'Byrne *et al.*, 1992). Consistent with our inability to detect sensitivity of *tonB* expression to gyrase inhibitor, our results showed that *tonB* regulation was unaffected by changes either in osmolarity or temperature. Thus the *tonB* promoter appears insensitive to changes in supercoiling.

Changes in supercoiling could potentially affect Fur binding. We have examined the *tonB* promoter region *in vivo* using DMS methylation protection footprinting to determine the site and extent of Fur binding under aerobic and anaerobic conditions. There are several advantages to *in vivo* footprinting. First, the DNA-binding activity of Fur requires the redox-sensitive ferrous form of iron (Fe^{II}) (Bagg and Neilands, 1987b) which is maintained *in vivo*. *In vitro*, under aerobic conditions, iron is normally present in the ferric form (Fe^{III}), therefore, conventional footprinting protocols require iron to be replaced by the alternate divalent metal ion, manganese (Mn^{II}) (DeLorenzo *et al.*, 1988). Second, changes in the redox state of the cell under aerobic and anaerobic conditions may affect the ratio of Fe^{II}/Fe^{III} which is difficult, if not impossible, to mimic *in vitro*. Third, Fur binds Fe^{II} in a pH-dependent fashion (Saito *et al.*, 1991b). Finally, by performing these experiments *in vivo* we have been able to examine regions upstream of the *tonB* promoter for the binding of other factors that may also contribute to the regulation of *tonB* transcription. This study represents the first *in vivo* characterization of Fur binding to an iron-regulated gene.

In vivo DMS protection footprinting indicated that Fur protein bound a single site between the −10 and −35 region of the *tonB* promoter under both aerobic and anaerobic conditions when iron is present in excess. The binding site (Fig. 5) has considerable homology to other Fur-binding sites determined by *in vitro* footprinting and sequence comparisons amongst other iron-regulated promoters (DeLorenzo *et al.*, 1988; Griggs and Konisky, 1989). The putative Fur-binding site centred at +26 was not detectably protected from DMS modification. Presumably this reflects the fact that it shows homology to the consensus Fur-binding site.

The degree of DMS protection can be used to detect relative changes in the extent of Fur binding to the operator (Sasse-Dwight and Gralla, 1988). The degree of Fur protection of guanosine residues at −13 and −20 on the non-coding strand was essentially identical under aerobic and anaerobic conditions. Furthermore, there was consistently less protection of positions −14, −21, −25, and −31 on the coding strand under anaerobic compared to aerobic conditions. Because these footprints were performed *in vivo*, they should accurately reflect the biologically relevant degree of Fur binding to its operator. It is clear that increased negative supercoiling resulting from an aerobic to anaerobic shift does not enhance Fur binding. These results also rule out models invoking increased binding owing to greater concentrations of active Fur repressor, such as the possibility that anaerobic conditions increase the Fe^{II}/Fe^{III} ratio.

Assuming the DNA is a B-form helix, the fact that the degree of DMS protection changes for only a subset of the residues in the Fur-binding site suggests that Fur undergoes a conformational change under anaerobic conditions that affects it positioning within the major groove (Fig. 7). These results are consistent with *in vitro* studies of Fur demonstrating that binding of metal ion induces a conformational change in the DNA-binding domain of the Fur protein (Coy and Neilands, 1991). Furthermore, the Fur protein contains 12 histidine residues, which is unusual for a protein of 16.8 kDa, and nuclear magnetic resonance

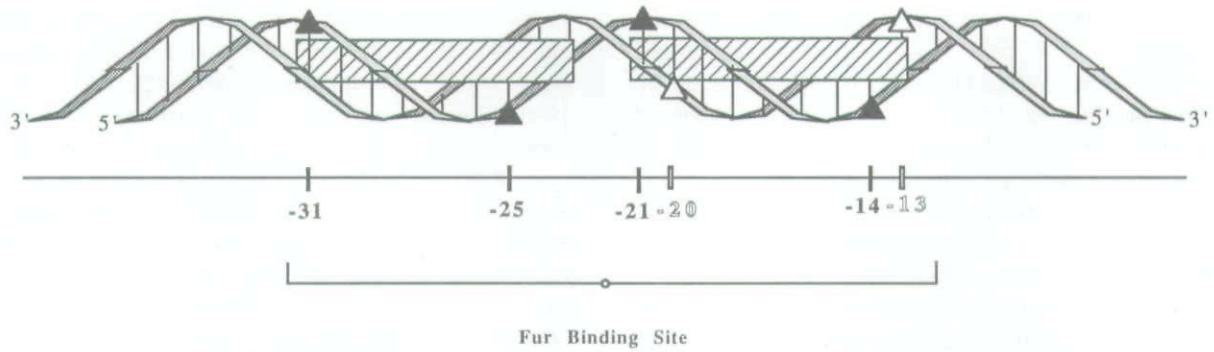


Fig. 7. A summary of the *in vivo* DMS footprinting results. The DNA is shown as a B-form helix. Guanosine residues that show similar protection under aerobic and anaerobic conditions are indicated by open triangles. Guanosine residues that are less protected under anaerobic conditions compared to aerobic conditions are indicated by filled triangles. Positions of each residue indicated is relative to the start of transcription. The location of the Fur-binding site is indicated by a bracket, with the circle indicating the centre of the site. Hatched boxes are included to represent a possible location of potential Fur-recognition helices (Saito *et al.*, 1991b) within the major groove of the DNA helix.

(NMR) studies have shown that all have ionization values of pH 6–8 suggesting that Fur is sensitive to the redox state of it surroundings (Saito *et al.*, 1991b). Alternatively, a localized change in the DNA topology or binding of a second factor could affect the protection pattern of sequences within the Fur-binding site, which, if relevant to *tonB* regulation, is not mediated through gyrase-dependent DNA supercoiling.

In summary, if anaerobic repression of *tonB* were due to increased negative supercoiling, we would expect the repression to be manifested by either decreased activity of the promoter or increased binding of Fur protein to the operator. The fact that under anaerobic conditions, maximal derepressed *tonB* promoter function remains as high or higher than it does under aerobic conditions suggests that there has been no decrease in promoter activity. Direct tests on the effects of novobiocin, or changes in osmolarity and temperature confirm the insensitivity of the *tonB* promoter to changes in supercoiling. The fact that the degree of Fur binding under anaerobic conditions was equal to or less than that seen under aerobic conditions rules out the possibility that supercoiling causes tighter Fur binding. Also ruled out is the idea that anaerobic repression resulted from an increase in active Fur levels (with correspondingly tighter binding) owing to increased Fe^{II} levels under anaerobic conditions. These results confirm and extend results with plasmid-encoded Fur, which does not mimic the anaerobic repression result. Thus we must look elsewhere for the mechanism of anaerobic repression.

The expression of other genes affected by anaerobicity has been described in *E. coli*. Regulation of many of these genes is mediated by the global regulators ArcA and Fnr (Iuchi and Linn, 1988; Spiro and Guest, 1990). Our results show that anaerobic repression of *tonB* was unaffected in strains carrying mutations in *arcA* and *fnr*.

In vivo footprinting results revealed that sequences upstream of the *tonB* promoter are conditionally protected. Guanosine residues at positions −85 and −86 were protected in contrast to the *in vitro* control reaction, except when iron is provided in excess under anaerobic conditions. We have found similar sequences (not always on the coding strand) in similar regions of *exbB* (Eick-Helmerich and Braun, 1989), *fepA* (Pettis *et al.*, 1988), and *cir* (Griggs *et al.*, 1987), genes which are also anaerobically repressed, suggesting that this region may be generally important in the regulation of all iron-dependent anaerobically repressed genes. The protection of residues −85 and −86 of *tonB* did not require Fur protein suggesting that another factor is responsible. The fact that not all Fur-regulated genes exhibit anaerobic repression (Niehaus *et al.*, 1991) also predicts the existence of another factor. There is no direct evidence for the nature of this upstream binding factor; the fact that it binds to sequences upstream of the promoter under conditions when the *tonB* gene is actively transcribed suggests that it may be a positive regulator. Clearly a more complete characterization of sequences upstream of *tonB* is required.

Experimental procedures

Bacterial strains and plasmids

The bacterial strains used in this study were all derivatives of *E. coli* K-12. The details of strains and plasmids are given in Table 2.

Growth media and conditions

Bacterial strains were grown in liquid Luria–Bertani (LB) medium and on LB plates for general maintenance and genetic manipulations. M9 minimal medium consisting of M9 salts (Shedlovsky and Brenner, 1963) with 0.2% glucose,

Table 2. *E. coli* strains, bacteriophages, and plasmids.

Strain/Phage /Plasmid	Relevant properties	Source/ Reference
E. coli strain		
AB4020	*fur*::Tn*5*	J. B. Neilands
ECL618	*arcA2 zjj*::Tn*10*	Iuchi *et al.* (1989)
JRG1728	Δ(*tyrA–fnr–rac–trg*)	Spiro *et al.* (1989)
KP1006	*polA* Δ(*argF–lac*)*U169*	Postle (1990a)
MC4100	*araD139 rpsL150 deoC1 ptsF25 rbsR flbB5301* Δ(*argF–lac*)*U169 relA thi rpsL200* Δ*srllac srl*::dTn*10*	Casadaban (1976)
W3110	F⁻ I⁻ IN(*rrnD–rrnE*)1	B. Bachmann
KP1027	*tonB*:: Mu *d*(lac)	Postle (1990a)
KP1028	KP1027 *fur*::Tn*5*	Postle (1990a)
KP1029	KP1006 *tonB–trpA'–lacZ*	Postle (1990a)
KP1030	KP1029·λgt11(*tonB*)	Postle (1990a)
KP1031	KP1030 *fur*::Tn*5*	Postle (1990a)
KP1049	JRG1728 *tonB–trpA'–lacZ*	This study
KP1051	KP1030 *arcA2 zjj*::Tn*10*	This study
KP1052	W3110 *fur*::Tn*5*	This study
Phages		
λgt11(*tonB*)	*tonB*⁺	Postle (1990a)
Plasmid		
pKP167	*tonB*⁺ derivative of pBR322	Postle (1990a)
pMH15	*fur*⁺ derivative of pACYC184	Hantke (1984)
pSN5	*gyrA–lacZ* derivative of pMB1109	Regis Krah

4 μg ml^{-1} thiamine, 1 mM magnesium sulphate, and 0.2% casamino acids (technical grade, Difco Laboratories) was used for all other experiments. To vary the iron availability, M9 minimal medium was supplemented with either 90 μM ferric chloride, or the iron chelators dipyridyl (200 μM), as previously described, (Postle, 1990a), or diethylenetriaminepentaacetic acid (100 μM) (DTPA; Niederhoffer and Fee, 1990) . Although only results with dipyridyl are reported, they were identical to those obtained using DTPA. The following concentrations of antibiotics were used where appropriate: ampicillin, 100 μg ml^{-1}; chloramphenicol, 34 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; and tetracycline, 20 μg ml^{-1}.

Aerobic cultures were grown in 10 ml of media in 300 ml sidearm baffle-bottom flasks with constant, vigorous aeration (290 r.p.m.). Anaerobic cultures were grown in 25 ml screw-cap test tubes filled completely with media and capped. Each screw-cap tube included a mini-magnetic stir bar and was incubated on top of a magnetic stir plate to allow for slow circulation of media throughout the culture. Confirmation of anaerobic conditions was based on the reduction of the redox indicator resazurine and induction of a *frdA–lacZ* fusion (Jones and Gunsalus, 1987).

Genetic manipulations

Generalized transduction of *E. coli* strains was carried out using bacteriophage P1*vir*, as described previously by Miller (1972). The *fur*::Tn*5* from strain AB4020 was introduced into strain W3110 by P1*vir* transduction, selecting for kanamycin resistance and screening for the hypersecretion of enterochelin as described previously by Arnow (1937) in order to create strain KP1052. The *arcA2* mutation from strain ECL618 was introduced into strain KP1029 by transduction of a linked Tn*10* insertion and screening for sensitivity to the dye toluidine blue (S. Iuchi, personal communication). The cotransduction frequency of *arcA2* with Tn*10* was 30 per cent. This strain was designated KP1051. To examine the effect of *fnr* on the regulation of *tonB*, the *tonB–trpA'–lacZ* fusion from strain KP1029 was transduced into strain JRG1728 (*fnr*⁻) by selecting for simultaneous resistance to bacteriophage φ80 and colicin B and screening for the *lacZ*⁺ phenotype on LB plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XG, Sigma Chemical) at 40 μg ml^{-1}. This strain was designated KP1049.

Transformation of bacterial strains with plasmid DNA was performed by the TSS procedure (Chung *et al.*, 1989). Routine plasmid purification was performed by the CTAB (cetyl trimethyl ammonium bromide) procedure (De Sal *et al.*, 1988).

Beta-galactosidase assays

Cultures were grown to an optical density at 600 nm $(OD)_{600}$ of 0.5–0.6 and, after chilling on ice, β-galactosidase activity was determined and expressed as Miller units (Miller, 1972). Strains in Table 1 were assayed at least three different times, with triplicate assays each time. For Fig. 3, triplicate assays were performed on each individual culture. For Fig. 4, duplicate assays were performed at each-time point on each culture.

Effects of gyrase inhibitor, osmolarity, and temperature on TonB expression

For experiments presented in Fig. 3, fresh subcultures from overnight samples were grown to $OD_{600} = 0.3$ in M9 minimal medium, at which point novobiocin at 200 μg ml^{-1} was added. Cultures were incubated until OD_{600} reached 0.5 then chilled on ice (Menzel and Gellert, 1983). Samples were divided and, after chilling on ice, assayed for β-galactosidase, or immediately precipitated with trichloroacetic acid and processed for polyacrylamide gel electrophoresis.

For the experiments presented in Fig. 4, fresh, aerobically grown saturated cultures were inoculated (1:100) into 25 ml screw-cap culture tubes containing 25 ml of M9 minimal medium and novobiocin at 0, 10, 20, or 40 μg ml^{-1}. Samples were removed at various time-points as the cultures became progressively more anaerobic (Dorman *et al.*, 1988), and were assayed for β-galactosidase activity.

To examine whether changes in osmolarity affect *tonB* expression, KP1030 cultures were grown in a low-osmolarity minimal salts medium (10 mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 0.8 mM $MgCl_2$, 2 μg ml^{-1} vitamin B_1, 0.2% casamino acids, 0.2% glucose) or in high-osmolarity medium (addition of either 0.3 M NaCl or 0.3 M sucrose to low-osmolarity medium) under aerobic or anaerobic conditions. The effect of temperature on *tonB* expression was determined by assaying KP1027 cultures grown anaerobically at 30°C and 37°C (Dorman *et al.*, 1990) in M9 minimal medium.

In vivo *DMS protection footprinting*

The procedures used for *in vivo* footprinting were as

described by Sasse-Dwight and Gralla (1990), except for the following modifications. Cultures were grown aerobically (10 ml) and anaerobically (25 ml) to $OD_{600} = 0.5–0.7$ and treated with 10 μM DMS with continued incubation for 4 min. Bacteria were poured into pre-chilled tubes and pelleted by centrifugation at 7000 × *g* for 7 min at 4°C. DMS-modified plasmid DNA was purified by a modified CTAB procedure (Del Sal, 1988). Protein was removed from plasmid DNA by extensive phenol:chloroform extractions followed by precipitation with 0.3 M sodium acetate pH 6.5, and 1.5 volumes of isopropanol. The final pellet was washed with 70% ethanol and air dried. Plasmid DNA was cleaved with piperidine and analysed by the PCR-modified primer extension analysis procedure previously described by Sasse-Dwight and Gralla (1991). The oligonucleotide 5′-CGG CTG CGC AGG CGC AGG-3′, which corresponds to the complementary sequence of the published *tonB* sequence (Postle and Good, 1983) from position 451 to 468 was used to examine the template strand. Examination of the non-template strand (5′–3′) was carried out using the oligonucleotide primer 5′- GAG GCT ATC AGC TTT GTC-3′, which corresponds to position 72–89 of the published *tonB* sequence. The *in vitro* controls, and treatment of purified plasmid with DMS, serve as indicators of all possible modified residues. All experiments were performed in duplicate and repeated at least three times. Densitometric analysis was performed on an Imagemaster Scanning System (Pharmacia).

Immunobot analysis

Identical amounts of cells were harvested by precipitation in equal volumes of 10% trichloro-acetic acid, washed in 10 mM Tris-HCl, pH 8.0, and prepared for SDS gel electrophoresis (Skare *et al.*, 1993). After electrotransfer to Immobilon P membranes (Millipore), the immunoblots were developed using an Enhanced Chemiluminscence (ECL) Western Blotting Kit, Amersham. The primary antibody used was a monoclonal antibody specific for TonB protein designated 34-1C11-186 (Skare *et al.*, 1993). Levels of TonB were quantified by comparison to a set of samples from serial dilutions of cultures grown under conditions where *tonB* expression is constitutive (data not shown).

Acknowledgements

We thank John Guest, Klaus Hantke, Shiro Iuchi, Regis Krah, and Rolf Menzel for strains and plasmids, Louis Mallavia for the use of Imagemaster Scanning System, Ray Larsen for critical reading of the manuscript, and Kevin Bertrand, Nancy Federspiel, Rob Gunsalus, and Scott Minnich for helpful discussions. This work was supported by a grant to K. P. from NIH (GM42146), and in the early stages by NSF (DCB-8903334).

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