Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 10449–10452, September 1998
Biochemistry

Altering the anaerobic transcription factor FNR confers a hemolytic phenotype on *Escherichia coli* K12

EDWARD T. RALPH, JOHN R. GUEST, AND JEFFREY GREEN*

The Krebs Institute, Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield, S10 2TN, United Kingdom

Communicated by Sankar Adhya, National Cancer Institute, Bethesda, MD, July 8, 1998 (received for review March 28, 1998)

ABSTRACT **The recent outbreaks of *Escherichia coli* 0157-associated food poisoning have focused attention on the virulence determinants of *E. coli*. Here, it is reported that single base substitutions in the *fnr* gene encoding the oxygen-responsive transcription regulator FNR (fumarate and nitrate reduction regulator) are sufficient to confer a hemolytic phenotype on *E. coli* K12, the widely used laboratory strain. The mechanism involves enhancing the expression of a normally dormant hemolysin gene (*hlyE*) located in the *E. coli* chromosome. The mutations direct single amino acid substitutions in the activating regions (AR1 and AR3) of FNR that contact RNA polymerase. It is concluded that altering a resident transcription regulator, or acquisition of a competent heterologous regulator, could generate a pool of hemolytic, and therefore more virulent, strains of *E. coli* in nature.**

The FNR protein of the facultative anaerobe *Escherichia coli* is a well characterized global transcription regulator that is homologous to CRP (1). Under anaerobic conditions, FNR monomers acquire a $[4Fe\text{-}4S]^{2+}$ cluster that enhances both dimerization and binding to the FNR sites (TTGAT—ATCAA consensus) of relevant promoters, leading to the activation or repression of target genes (2, 3). This provides a reversible switch because, under aerobic conditions, the $[4Fe\text{-}4S]^{2+}$ clusters are destroyed, monomers are formed, and the regulator no longer binds to FNR sites (3–5). The main role of FNR in *E. coli* is to control the expression of at least 29 transcriptional units, mainly associated with anaerobic energy generation (1). However, it has been observed that, when the *fnr* homologue (*hlyX*) of a pig pathogen (*Actinobacillus pleuropneumoniae*) is expressed in *E. coli* K12, it confers a hemolytic phenotype (6, 7). The heterologous regulator (HlyX) activates a previously undetected *E. coli* gene (*hlyE*) encoding a novel hemolysin (8). The activation of a dormant hemolysin gene by a heterologous regulator emphasizes the need for caution when cloning foreign genes in *E. coli* K12. The *hlyE* promoter binds FNR at a site that is centered at −78.5 relative to the transcriptional start site (8), which is much further upstream than normally is found in natural FNR-activated promoters. Indeed, FNR-activated promoters are typically class II promoters (1, 9), where the FNR-sites are centered at approximately −41 (Fig. 1*a*), and are quite distinct from class I promoters, where the regulator binding sites are centered at or upstream of −61 (see Fig. 1*b*) (10).

It has been established that members of the CRP-FNR family use different sets of contacts with RNA polymerase to activate transcription from each of the two classes of promoter (9–11). At class I promoters, the C-terminal domain of the α-subunit (α_C) of RNA polymerase interacts with an activating region (AR1, ♦ in Fig. 1*b*) in the downstream subunit of the regulator (9, 11). Mutational analysis has located AR1 on the face of FNR that extends from α_A to the β_{11}-β_{12} loop via the β_3-β_4 loop (73-loop) in the predicted structure (see Fig. 2) (11, 12). At class II promoters, α_C interacts with AR1 in the upstream subunit of the regulator, and there is an additional contact between the σ^{70} subunit and another activating region (AR3, ⋆ in Fig. 1*a*) in the downstream subunit (9). In FNR, the AR3 binding face was defined by the isolation of mutants with a G85A (85-loop, β_4-β_5) replacement or other substitutions, K60E (β_2), A61T (β_2), or F112L (β_7), which impair transcription activation at class II promoters (see Fig. 2) (13). It would appear that FNR is adapted specifically to activate class II promoters and that HlyX is better adapted to activate class I promoters (12). As a result, HlyX can activate transcription from the class I *hlyE* promoter of *E. coli* K12 and thus confer a hemolytic phenotype. This in turn raises the question of how readily the activation specificity of FNR could be changed to that of HlyX. Here, it is reported that single base substitutions in *fnr* are sufficient to confer a hemolytic phenotype on *E. coli* K12.

MATERIALS AND METHODS

Error-Prone PCR Mutagenesis. The template for error-prone PCR was pGS24, a derivative of pBR322 containing the *fnr* gene in a *Hin*dIII–*Bam*HI fragment. Random mutations in the *fnr* gene were introduced by using *Taq* DNA polymerase and the following synthetic primers, as described (13): 5′-GCTTATCATCGATAAGCTTCGTGAATATTTTGCCGG (*fnr* co-ordinates 1–23) and 5′-CGTAGAGGATCCAGGCTGTACGC (1625–1641), where the unique *Hin*dIII and *Bam*HI targets are underlined. The PCR products were digested with *Hin*dIII and *Bam*HI before ligating between the corresponding sites in pBR322. Plasmids were isolated by standard methods, and mutational alterations in the *fnr* gene were defined by Applied Biosystems cycle sequencing with the aid of two primers: 5′-AAACATATGGTCCCGGAAAAGCG (520–536) and 5′-GGAAACCTCGATGGTAGCTGAAATCCCGTTCG (866–898).

Assay of hlyE Expression and Phenotypic Tests. The library of mutagenized *fnr* genes in pBR322 was used to transform JRG1728 [Δ(*lacIPOZYA*)*X74 galU galK rpsL* Δ(*ara-leu*) Δ(*tyrR-fnr-rac-trg*)*17 zdd-230*::Tn*9*] and JRG3702, a derivative of JRG1728 containing a compatible *hlyE-lacZ* reporter plasmid, pGS1065 (8). Ampicillin-resistant transformants then were screened for the hemolytic phenotype on blood agar (5% vol/vol horse blood) containing ampicillin (200 μg/ml) and for enhanced *hlyE-lacZ* expression on L-agar containing: X-Gal (5-bromo-4-chloro-3-indoyl β-D-galactoside; 40 μg/ml), ampicillin (200 μg/ml), and tetracycline (35 μg/ml). The plates were incubated under anaerobic conditions at 37°C. The levels of *hlyE-lacZ* expression were determined by measuring β-galactosidase activity (15) with cultures that had been grown anaerobically for 16 h at 37°C in sealed bottles containing L broth supplemented with glucose (0.4% wt/vol), ampicillin

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

*To whom reprint requests should be addressed. e-mail: jeff.green@sheffield.ac.uk.

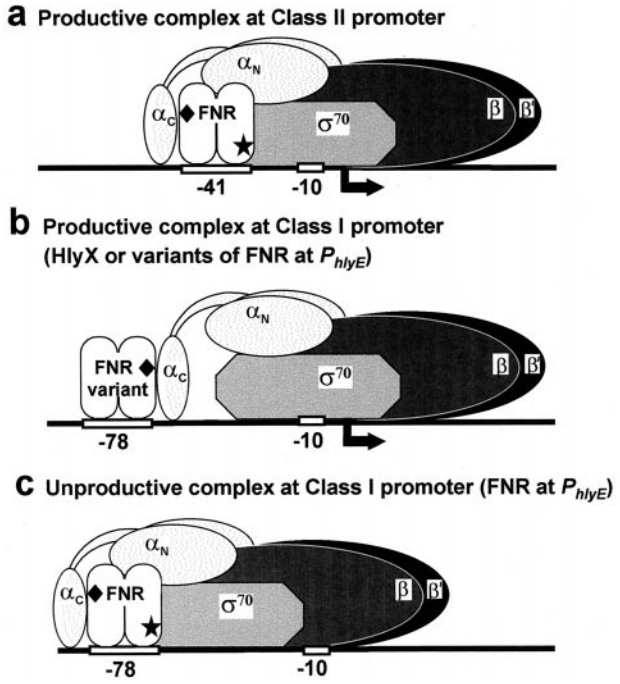

FIG. 1. Diagrammatic representations of the ternary complexes formed at the two classes of FNR-regulated promoter: (*a*) wild-type FNR at a class II promoter; (*b*) correct positioning of RNA polymerase by HlyX and FNR variants at a class I promoter (P_{hlyE}); and (*c*) incorrect positioning of RNA polymerase by wild-type FNR at a class I promoter (P_{hlyE}). The FNR variant proteins favor the formation of productive ternary complexes at the *hlyE* promoter by either enhancing interaction between AR1 (◆) and α_C or by weakening the interaction between AR3 (⋆) and σ^{70}, thus positioning RNA polymerase correctly with respect to the −10 element.

(200 μg/ml), and tetracycline (35 μg/ml). The *hlyX:fnr* hybrid (pGS412 and pGS413) and the corresponding *fnr* (pGS408) and *hlyX* (pGS409) plasmids have been described (7). Two class I promoters (FF+20p*melR*, which has an FNR-site centered at −61.5, and FF-71.5p*melR*, which has an FNR-site at −71.5 and an improved −35 element) and a class II promoter (FFp*melR*, equivalent to FF+20p*melR* but with the FNR-site at −41.5) fused to *lac* in pRW50 (16) were used to determine the effects of selected amino acid substitutions on the function of FNR AR1 and AR3. Complementation of the *fnr* lesion of JRG1728 by FNR variants was tested by the restoration of anaerobic growth on leucine supplemented glycerol-nitrate agar (17). Western blotting with polyclonal anti-FNR serum has been described (18).

RESULTS AND DISCUSSION

In preliminary studies, hybrid *fnr:hlyX* and *hlyX:fnr* genes expressing hybrid proteins containing the N-terminal region of FNR fused at β_{10} (Fig. 2) to the C-terminal region of HlyX, or *vice versa*, were constructed (7). These indicated that both the N- and C- terminal regions of HlyX contribute to its ability to confer a hemolytic phenotype (Table 1). Furthermore, both hybrids increased expression of a *hlyE-lacZ* transcriptional fusion, but neither was as efficient as HlyX itself (Table 1). Of interest, the degree of *hlyE-lacZ* expression observed with FNR was disproportionately higher than would be expected from the absence of hemolysis. This implies that a threshold level of HlyE synthesis has to be exceeded before the hemolysin is exported. Nevertheless, these observations suggested that relatively minor changes to FNR might create FNR variants that resemble HlyX in being able to confer a hemolytic phenotype.

The acquisition of a HlyX-like activity by FNR was explored by using error-prone PCR mutagenesis to generate a library of randomly mutated *fnr* genes in pBR322. Transformants of two *E. coli* strains, JRG1728 (Δ*fnr* Δ*lac*) and a derivative, JRG3702 (containing a compatible *hlyE-lacZ* reporter plasmid, pGS1065), were screened accordingly for an anaerobic hemolytic reaction on blood agar plates or for an elevated *hlyE-lacZ* expression on X-Gal plates (respectively). From ≈10,000 independent transformants, 13 were found to contain plasmids that both enhance *hlyE-lacZ* expression and confer a hemolytic phenotype. Anaerobic growth tests on glycerol-nitrate medium further showed that the corresponding FNR variants retain the ability to complement the *fnr* lesion of JRG1728. The variant proteins were produced in normal amounts, as judged by Western blotting, and as expected, the hemolytic phenotype was anaerobically inducible. Nucleotide sequence analysis revealed that plasmids encoding two distinct classes of FNR variant had been recovered: type A variants were substituted in AR1 whereas type B variants had single amino acid substitutions in AR3 (Table 1; Fig. 2).

The type A (AR1) substitutions affected residues contributing to the AR1 surface (11, 12): Q41R (α_A); F92L (β_5); A95P (β_6); and R197H (α_E) (see Fig. 2). In only one type A variant (A95P) was the amino acid present in FNR (A95) directly replaced by the residue occupying the corresponding position in HlyX (P94). The type A substitutions are presumed to improve the contact between FNR and RNA polymerase at the class I *hlyE* promoter (Fig. 1*b*). Accordingly, they enhanced expression from the model class I promoters, FF+20p*melR* and FF-71.5p*melR*, but had little effect on expression from the model class II promoter FFp*melR* (Table 1). The increased efficiency of the AR1 contact is more evident at FF+20p*melR*, which lacks the improved −35 element of FF-71.5p*melR* and therefore more closely resembles the *hlyE* promoter (8). Independent tests with the archetypal AR1 variant of FNR that is inactivated by an S73F substitution (13) confirmed that AR1 is essential for *hlyE* expression because *hlyE-lacZ* expression fell to its lowest level and no hemolytic phenotype was observed with this variant (Table 1).

The type B (AR3) substitutions affected residues contributing to the AR3 surface: I81T (β_4); G85D (β_4-β_5 loop); D86Q (β_4-β_5 loop); and F112S (β_7) (see Fig. 2). Each variant increased *hlyE-lacZ* expression and conferred a hemolytic phenotype (Table 1). Transcription from the model class II promoter FFp*melR* was impaired with all of the type B variants, indicating that they have defects in their AR3 contacts (Table 1). However, this loss was accompanied by a corresponding increase in transcription from the model class I promoters FF+20p*melR* and FF-71.5p*melR* (Table 1). Likewise, the archetypal G85A variant of FNR, which first defined the importance of AR3 at class II promoters (13) and was later shown to produce a 1.7-fold increase in transcription from a model FNR-dependent class I promoter (11), here was found to effect a 2.1-fold improvement in *hlyE-lacZ* expression and to confer a hemolytic phenotype (Table 1).

The increase in *hlyE* expression conferred by both types of FNR variant was generally small (as little as 1.6-fold) but sufficient to confer a hemolytic phenotype. This suggests that HlyE production has to reach a threshold before hemolysis is observed and that this can be attained by relatively small increases in *hlyE* expression, which then have profound effects on the hemolytic phenotype.

It would thus appear that the ability to endow *E. coli* K12 with a hemolytic phenotype can arise from two types of *fnr* mutation, *viz*. those that improve AR1 activity (type A) and those that compromise AR3 activity so that AR1 assumes a more prominent role in the formation of the transcription complex (type B). Consistent with this view, it has been shown that FNR and HlyX can each recruit RNA polymerase to the *hlyE* promoter (8), but it would appear that HlyX better directs

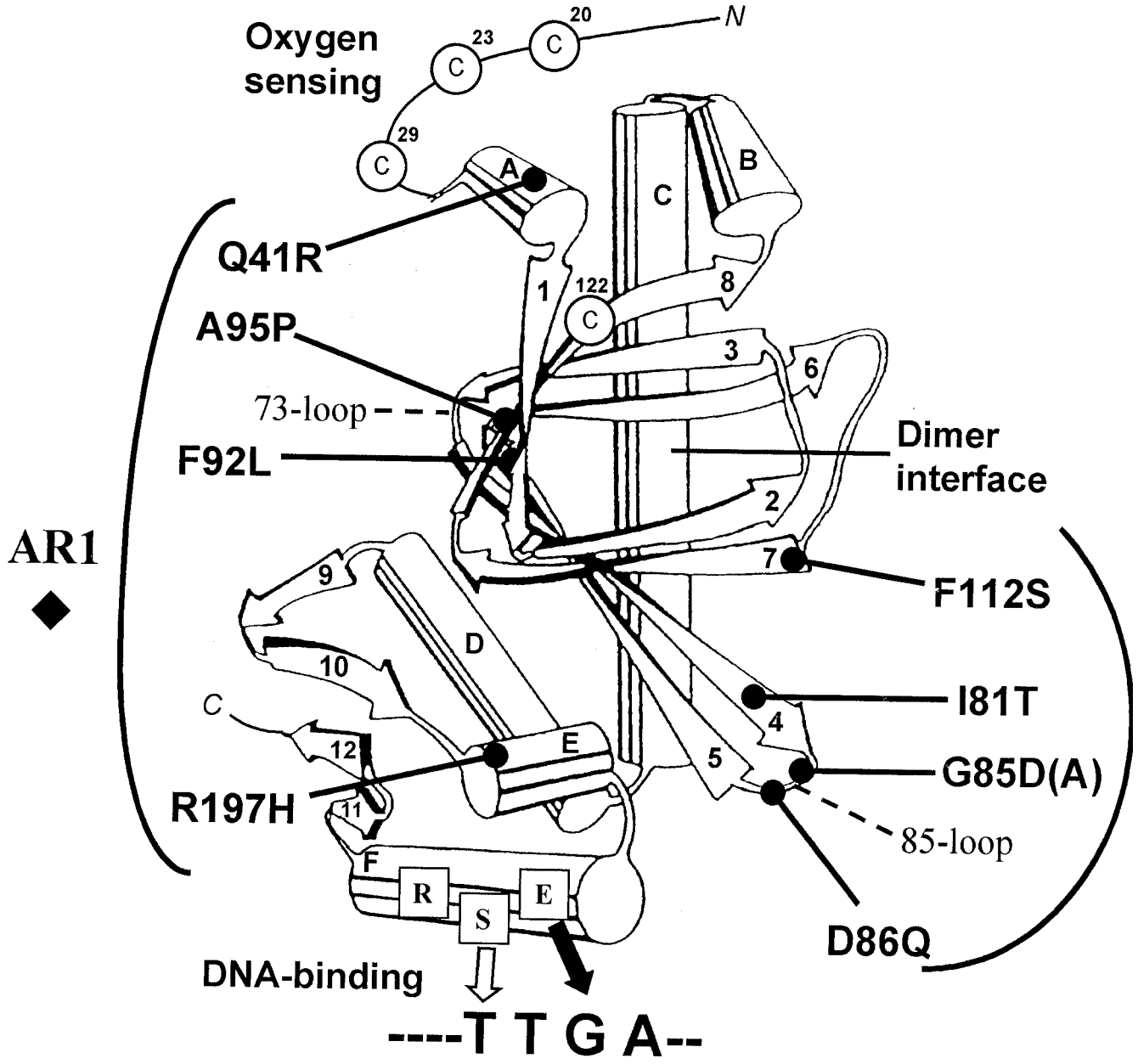

FIG. 2. Predicted structure of an FNR monomer based on that of CRP (14) showing the positions of amino acid substitutions that confer a hemolytic phenotype on *E. coli* K12. Also shown are: the helix–turn–helix motif (α_E-α_F) in the DNA-binding domain; key amino acid to base pair (protein–DNA) interactions; the essential cysteine ligands of the [4Fe-4S] cluster; and previously identified activating regions, the 73-loop in AR1 (13) and the 85-loop in AR3 (13).

the polymerase to an optimal position for transcription activation, as shown in Fig. 1*b*, whereas FNR tends to direct the polymerase to a region further upstream, as illustrated in Fig. 1*c*. This presumably reflects differences between the two regulators in the relative binding affinities of AR1 and AR3 for RNA polymerase, such that with HlyX the AR1-α_C, interaction is dominant, whereas with FNR the AR3-σ^{70} interaction is dominant (Fig. 1). So, by strengthening the AR1 contact or weakening the AR3 contact, in the respective type A and type B variants of FNR, the polymerase can be repositioned to allow an enhanced expression of the *hlyE* gene and the appearance of the hemolytic phenotype (Fig. 1). This model assumes that the AR1 substitutions identified here promote the formation of the transcriptionally competent complex (Fig. 1*b*) via improved activating contacts (AR1-α contact) rather than forming the unproductive complex (Fig. 1*c*) that incorporates an anti-inhibition FNR-α contact. There is good evidence to indicate that these two types of regulator-RNA polymerase contacts are different, although they involve the same face of the protein (8, 19).

Oxygen availability is known to influence the virulence of some bacterial pathogens. Furthermore, the internalization of enteric bacteria normally occurs in an anaerobic environment. So, the anaerobic activation of FNR potentially could participate in establishing a host–pathogen relationship. It will be of interest to determine whether any clinical isolates of *E. coli* possess variant FNR proteins having altered AR1 or AR3 regions or, alternatively, whether any have acquired regulators of the HlyX type. Such changes could contribute to the development of pathogenic traits. The capacity of a pathogen to infect a host normally depends on a suite of virulence factors, so the production of one such factor (e.g., HlyE) on its own is unlikely to turn a commensal into a pathogen. Nevertheless, it is clear that single base substitutions in a global

Table 1. Properties of two types of FNR variant

Class	Modification or replacement	Codon changes	Hemolytic phenotype	Anaerobic β-galactosidase activities with different *promoter-lacZ* reporters			
				hlyE-	FFp*melR*-	FF+20p*melR*-	FF-71.5p*melR*-
Parental and hybrid	FNR		−	100	100	100	100
	FNR-HlyX		+++	265	nd	nd	nd
	HlyX-FNR		++++	215	nd	nd	nd
	HlyX		+++++	455	nd	nd	nd
Type A (AR1)	Q41R + F92L (1)	CAG-CGG + TTC-CTC	++++	305	91	507	118
	A95P (1)	GCA-CCA	++	160	95	131	120
	R197H (3)	CGT-CAT	++++	210	100	128	110
	S73F*	TCC-TTC	−	43	34	8	5
Type B (AR3)	I81T (2)	ATC-ACC	++	205	33	259	188
	G85D (3)	GAT-GAC	++++	210	10	443	230
	D86Q (1)	GAC-AAC	++	240	24	252	189
	F112S (2)	TTC-TCC	++	240	36	237	163
	G85A*	GGC-GCC	++++	205	17	255	193

The hemolytic phenotypes were scored after 16 h of anaerobic growth on blood agar using a scale of − to +++++, where − denotes no detectable hemolysis and +++++ represents the level observed with HlyX-containing cultures. The values for anaerobic *hlyE-lacZ* (*hlyE*), FFp*melR-lacZ* (FFp*melR*), FF+20p*melR-lacZ* (FF+20p*melR*), and FF-71.5p*melR-lacZ* (FF-71.5p*mel*R) expression were derived from the β-galactosidase specific activities of different strains expressed as percentages of the values (610 Miller units for *hlyE*, 7,890 for FFp*melR*, 470 for FF+20p*melR*, and 3,680 for FF-71.5p*melR*) observed with cultures containing a wild-type *fnr* plasmid (pGS24): three independent anaerobic cultures of each strain were grown in sealed bottles for 16 h at 37°C and assayed in duplicate. Figures in parentheses denote the numbers of independent isolates of a given type that were recovered.

*Archetypal FNR variants (13) that define AR1 (S73F) and AR3 (G85A).

†nd, not determined.

transcription regulator gene can have a profound effect on phenotype of an organism. Furthermore, it seems likely that a group of virulence genes will share a common promoter architecture that has evolved to mediate their coexpression. If so, mutations in the corresponding transcription factor could either potentiate or lessen the production of a pathologically significant group of toxins. Deletion of the *hlyE* gene of *E. coli* K12 also might create a "safer" laboratory strain.

We thank Dr. A. J. G. Moir for DNA sequencing, Prof. S. J. W. Busby for some of the plasmids used in this study, and the Biotechnology and Biological Sciences Research Council and The Royal Society for financial support. We also thank Gary Coleman and Syakira Mohammed Hussein for contributions made during undergraduate research projects.

1. Guest, J. R., Green, J., Irvine, A. S. & Spiro, S (1996) in *Regulation of Gene Expression in Escherichia coli*, eds. Lin, E. C. C. & Lynch, A. S. (Landes, Austin, TX), pp. 317–342.
2. Green, J., Bennett, B., Jordan, P., Ralph, E. T., Thomson, A. J. & Guest, J. R. (1996) *Biochem. J.* **316,** 887–892.
3. Lazazzera, B. A., Beinert, H., Khoroshilova, N., Kennedy, M. C. & Kiley, P. J. (1996) *J. Biol. Chem.* **271,** 2762–2768.
4. Khoroshilova, N., Popescu, C., Munck, E., Beinert, H. & Kiley, P. J. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 6087–6092.
5. Jordan, P., Thomson, A. J., Ralph, E. T., Guest, J. R. & Green, J. (1997) *FEBS Lett.* **416,** 349–352.
6. MacInnes, J. I., Kim, J. E., Lian, C. J. & Soltes, G. A. (1990) *J. Bacteriol.* **172,** 4587–4592.
7. Green, J., Sharrocks, A. D., MacInnes, J. I. & Guest, J. R. (1992) *Proc. R. Soc. London Ser. B* **248,** 79–84.
8. Green, J. & Baldwin, M. L. (1997) *Microbiology* **143,** 3785–3793.
9. Busby, S. J. W. & Ebright, R. H. (1997) *Mol. Microbiol.* **23,** 853–859.
10. Ebright, R. H. (1993) *Mol. Microbiol.* **8,** 797–802.
11. Williams, S. M., Savery, N. J., Busby, S. J. W. & Wing, H. (1997) *Nucleic Acids Res.* **25,** 4028–4034.
12. Green, J. & Baldwin, M. L. (1997) *Mol. Microbiol.* **24,** 593–605.
13. Bell, A. & Busby, S. J. W. (1994) *Mol. Microbiol.* **11,** 383–390.
14. Weber, I. & Steitz, T. (1987) *J. Mol. Biol.* **198,** 311–326.
15. Miller, J. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab. Press, Plainview, NY).
16. Wing, H. J., Williams, S. M. & Busby, S. J. W. (1995) *J. Bacteriol.* **177,** 6704–6710.
17. Lambden, P. R. & Guest, J. R. (1976) *J. Gen. Microbiol.* **97,** 145–160.
18. Spiro, S. & Guest, J. R. (1987) *J. Gen. Microbiol.* **133,** 3279–3288.
19. Zhou, Y., Merkel, T. J. & Ebright, R. H. (1994) *J. Mol. Biol.* **243,** 603–610.