The molecular basis for the differential regulation of the *hlyE*-encoded haemolysin of *Escherichia coli* by FNR and HlyX lies in the improved Activating Region 1 contact of HlyX

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The regulator of fumarate and nitrate reduction (FNR) protein of *Escherichia coli* is an oxygen-responsive transcription regulator that acts mainly to activate the transcription of genes associated with anaerobic energy generation during periods of oxygen starvation. The *hlyX* gene of the swine pathogen *Actinobacillus pleuropneumoniae* encodes an FNR homologue, HlyX, which can complement the anaerobic respiratory deficiencies of an *fnr* mutant. However, FNR and HlyX have distinct but overlapping regulons because during anaerobic incubation, *hlyX*-expressing *E. coli* K-12 strains produce an otherwise latent haemolysin. The gene encoding the 'latent' haemolysin has been designated *hlyE* and analysis of the promoter region by DNase I footprinting reveals the presence of an FNR- (HlyX-) binding site. Anaerobic expression of an *hlyE::lacZ* reporter was 6.5-fold higher in *hlyX* compared to *fnr*-expressing cells. Both FNR and HlyX recruited RNA polymerase to the *hlyE* promoter but formed different ternary complexes. One major transcript (*tsp1*) initiating at 78.5 bp downstream of the FNR-binding site and four minor transcripts initiating at 73.5 (*tsp2*), 71.5 (*tsp3*), 63.5 (*tsp4*) and 62.5 (*tsp5*) bp from the FNR site were detected. From the position of the FNR box relative to the transcript starts, *hlyE* is expressed from a Class I FNR-regulated promoter. Substitution of selected FNR amino acids with the residues found in the equivalent positions in HlyX indicated that Activating Region 1 (AR1) of FNR forms a surface encompassing β_9 to β_{11} and that the AR1 contact at Class I promoters is different to that at Class II promoters, although the same surface is involved. The FNR variant, FNR-A225T, combined the properties of FNR (good activation from Class II promoters) and HlyX (good activation of Class I promoters) and conferred the haemolytic phenotype.

Keywords: FNR, HlyX, HlyE, Class I promoter, haemolysin

INTRODUCTION

The regulator of fumarate and nitrate reduction (FNR) protein of *Escherichia coli* is an oxygen-responsive member of the cAMP receptor protein (CRP) family of transcription regulators. All the members of this family are structurally related to CRP and, with the exception of the *Bacillus subtilis* FNR (Ramos *et al.*, 1995), have a C-terminal helix–turn–helix DNA-binding domain and an N-terminal sensory domain (Guest *et al.*, 1996). The

hlyX gene of the pig pathogen *Actinobacillus pleuropneumoniae* encodes an FNR protein, known as HlyX, that is 73% identical to *E. coli* FNR at the amino acid level. Both FNR and HlyX sense oxygen starvation through the anaerobic acquisition of an oxygen-labile [4Fe–4S] cluster (Lazazzera *et al.*, 1996; Green *et al.*, 1996a, b; Green & Baldwin, 1997). Incorporation of the iron–sulphur cluster promotes dimerization of FNR and enhances binding to the FNR (HlyX) DNA target, TTGAT---ATCAA (Lazazzera *et al.*, 1996; Green & Baldwin, 1997).

Unsurprisingly, HlyX is able to complement the anaerobic growth deficiencies of an *fnr* mutant. However,

Abbreviations: AR1, Activating Region 1; CRP, cAMP receptor protein; FNR, regulator of fumarate and nitrate reduction.

HlyX also anaerobically activates the expression of a number of genes in *E. coli* K-12 strains, including a haemolysin, that FNR apparently does not (MacInnes *et al.*, 1990; Green *et al.*, 1992; Green & Baldwin, 1997). Thus FNR and HlyX have distinct but overlapping modulons. The construction of FNR-HlyX hybrid proteins led to the proposal that the endowment of the haemolytic phenotype required unique features of both the N- and C-terminal regions of HlyX (Green *et al.*, 1992). Recently, the N-terminal component has been shown to reside in the β_9 - β_{10} loop (Activating Region 1, AR1) of HlyX and is important in transcription activation from Class I promoters. Thus it was predicted that the promoter region of the 'latent haemolysin' gene would contain an FNR box located at -61 or beyond (Green & Baldwin, 1997).

Recently, an *E. coli* gene, designated *hlyE*, has been identified. It is located at 26.4 min and encodes a protein (34 kDa) which, when overproduced, confers a haemolytic phenotype upon *E. coli* (GenBank accession no. U57430). The molecular mass of the product is the same as the previously described cryptic contact-dependent haemolysin ClyA (cytolysin A), which is secreted by some strains of *E. coli* K-12, including *hns* mutants and strains overexpressing *clyA* and *slyA* (Oscarsson *et al.*, 1996). Although the *clyA* sequence is unavailable, it is likely that *clyA* is identical to *hlyE*. The *slyA* gene is thought to encode a regulator of cytolysin A expression (Oscarsson *et al.*, 1996). SlyA is homologous to several known regulatory proteins but the nature of the SlyA stimulus and response is unknown.

In this study *hlyE* expression is shown to be driven from a Class I FNR-dependent promoter that is differentially regulated by FNR and HlyX. Single substitutions of amino acids in the β_9 - β_{10} region of FNR with those present in HlyX generally resulted in improved activation from the *hlyE* promoter and two model Class I promoters, but this was not sufficient in itself to confer a haemolytic phenotype. A substitution in β_{11} resulted in an FNR which combined the properties of FNR (good Class II activation) and HlyX (good Class I activation) and allowed the detection of the haemolytic phenotype.

METHODS

Plasmids and bacterial strains. Plasmids used to test the effects of the anaerobic expression of *fnr*, *hlyX* and the *fnr* and *hlyX* mutants were the *ptac85* derivatives: pGS330, FNR (Green *et al.*, 1991); pGS415, HlyX (Green *et al.*, 1992); pGS1003, HlyX-A187P (Green & Baldwin, 1997); pGS1053, FNR-R179Q; pGS1054, FNR-F181Y; pGS1055, FNR-Q183A; pGS1056, FNR-R184P; pGS1057, FNR-P188A; pGS1086, FNR-A225T; pGS1085, FNR-Cdel17, which lacks 17 C-terminal amino acids; pGS1094, FNR-Cdel10, which lacks 10 C-terminal amino acids. Western blotting with anti-FNR serum indicated that expression of all the FNR derivatives was similar to that observed with FNR. However, the products of pGS1085, FNR-Cdel17 and pGS1094, FNR-Cdel10 were insoluble.

The source of the *hlyE* promoter DNA used in the footprinting analyses was pGS1051 constructed by ligating a PCR-gener-

ated *hlyE* promoter fragment (-97 to +61) into *EcoRI*-*Bam*HI-digested pUC118.

The ability of FNR, HlyX and altered FNR and HlyX proteins to activate transcription from *hlyE* and model Class I and Class II promoters was determined using: pGS1065, a pRW50 derivative, constructed by ligating the *EcoRI*-*Bam*HI *hlyE* promoter-containing fragment from pGS1051 into pRW50; and the semi-synthetic *lacZ* reporter fusions, *FFpmelR* (Class II, FNR site at -41.5), *FF+20pmelR* (Class I, FNR site at -61.5) and *FF-71.5pmelR* (Class I, FNR site at -71.5 plus an improved -35 element) (Wing *et al.*, 1995) in JRG1728 [$\Delta(lacIPOZYA)$ $\chi 74$ *galU galK rpsL* $\Delta(ara-leu)$ $\Delta(tyrR-fnr-rac-trg)17$ *zdd-230::Tn9*] containing pGS330 to generate JRG3653, 3402, 3403 and 3404, pGS415 to generate JRG3654, 3405, 3206 and 3407, pGS1003 to generate JRG3703, 3452, 3453 and 3454, pGS1053 to generate JRG3704, 3657, 3655 and 3656, pGS1054 to generate JRG3705, 3660, 3658 and 3659, pGS1055 to generate JRG3706, 3663, 3661 and 3662, pGS1056 to generate JRG3739, 3735, 3737 and 3736, pGS1057 to generate JRG3707, 3666, 3664 and 3665, pGS1086 to generate JRG3749, 3745, 3747 and 3746 or pGS1094 to generate JRG3761, 3762, 3763, and 3764. JRG3702 is JRG1728(pGS1065). The authenticity of pGS1065 was confirmed by automated DNA sequencing. Cultures were grown aerobically in L broth with vigorous shaking or anaerobically in L broth supplemented with 0.4% glucose to an OD₆₀₀ of 0.3-0.5 (Pye Unicam UV4) in sealed bottles at 37 °C. β -Galactosidase activity was determined according to Miller (1972).

DNase I footprinting. DNase I footprinting was carried out as described previously (Green *et al.*, 1991) except that the reactions were performed anaerobically and contained (in a total volume of 10 μ l): *hlyE* promoter DNA (approximately 10 ng), end-labelled at the *Bam*HI site (top strand) of the *Pvu*I-*Bam*HI fragment of pGS1051 (275 bp); HlyX or FNR (0-100 nM) or RNA polymerase (0.2-1 U); 2 μ l 5 $\times$ binding buffer (5 $\times$ binding buffer is 0.1 M Tris/HCl, pH 8.0; 0.05 M MgCl₂; 50 mM dithiothreitol; 25%, v/v, glycerol). The mixtures were incubated for 2 min at 25 °C followed by digestion with DNase I (1 μ l of 1 U ml⁻¹ for 15-60 s at 25 °C). Reactions were stopped by addition of 200 μ l 0.3 M sodium acetate containing 10 mM EDTA followed by phenol/chloroform extraction. The DNA was ethanol-precipitated and resuspended in 10 μ l loading buffer (40%, v/v, formamide; 5 M urea; 5 mM NaOH; 1 mM EDTA; 0.03% bromophenol blue; 0.03% xylene cyanol) for electrophoretic fractionation on polyacrylamide-urea gels and autoradiographic analysis. A Maxam & Gilbert G track of the same fragment was used to provide a calibration (Maxam & Gilbert, 1980).

Transcript mapping. The transcription start point of the *hlyE* promoter was determined by primer extension. Total RNA was prepared from JRG2269 (*fnr hlyX*⁺), JRG3350 (*fnr*⁺) after anaerobic growth in the presence of 30 mg IPTG l⁻¹, or from MC4100, JRG1728 (*fnr*), RH90 (*rpoS*), SJP3 (*ihfA*), RJ1802 (*fis*) or ECL585 (*arcA*) after anaerobic growth in the absence of IPTG, as described by Aiba *et al.* (1981). For primer extension, the method of Gerischer & Durre (1992) was used with 10 pmol primer S420 (CAGTCATAATCATTCGCCTC, *hlyE* co-ordinates 154-135), approximately 100 μ g RNA and 50 U AMV reverse transcriptase (Nbl). After ethanol precipitation, the DNA was fractionated on calibrated urea-polyacrylamide gels and analysed by autoradiography.

Other methods. FNR and HlyX were purified from the respective GST-fusion proteins and their [4Fe-4S] clusters reconstituted as described previously (Green *et al.*, 1996a, b; Green & Baldwin, 1997). The altered FNR proteins used in

this study were generated using the Altered Sites II System (Promega) with the appropriate mutagenic oligonucleotides and presence of the desired substitutions was confirmed by automated DNA sequencing.

In vitro transcription was as described previously (Green *et al.*, 1996c) except *ndh* DNA was replaced by *hlyE* (*Pvu*I–*Bam*HI fragment from pGS1051) and Nbp was replaced by FNR or HlyX. Gels were calibrated with a Maxam & Gilbert G track.

The ability of FNR, HlyX and the altered HlyX proteins to confer a haemolytic phenotype was assessed after anaerobic incubation in the absence or presence of 30 mg IPTG l⁻¹ for 24 h at 37 °C on blood agar (7% w/v, horse blood) of *E. coli* JRG1728 expressing *fnr*, *hlyX* or the mutant *fnr* or *hlyX* genes from the *tac* promoter of p_{tac}85 (Green *et al.*, 1992).

Western blotting with anti-FNR serum was as described by Spiro & Guest (1987).

RESULTS

The *hlyE* promoter contains an FNR site and is differentially regulated by FNR and HlyX

Analysis of the DNA upstream of the *hlyE* coding region revealed the presence of an FNR-binding site which matches the consensus in 9 out of 10 bases (Fig. 1). The FNR site was shown to be functional by estimating the anaerobic induction of β -galactosidase activity from a *hlyE*::*lacZ* reporter plasmid (pGS1065) in the *fnr* strain,

Fig. 1. Nucleotide sequence of the *hlyE* promoter region. The following features are indicated: the FNR-binding site (boxed); three -10 promoter elements (single and double underlined, consensus bases in bold); five transcript start points (arrowed, the major *tsp*, *tsp1*, is indicated by +1 and a large arrow); the ribosome-binding site (rbs) and two possible initiating methionine (met) residues.

JRG1728 and the same strain carrying plasmids expressing *fnr* or *hlyX* (Table 1). In rich medium, expression of *hlyE* was anaerobically induced 32-fold in cultures expressing HlyX, but only ninefold by those expressing FNR, relative to aerobic expression in JRG3702. However, in the absence of either regulator (JRG3702), after growth on rich or minimal medium, a 5–7-fold anaerobic induction of *hlyE* expression was still observed. Therefore, using the anaerobic expression of *hlyE* in JRG3702 (Δ *fnr*) as a baseline, the FNR-dependent anaerobic induction of *hlyE* was only 1.5-fold compared to a HlyX-mediated induction of 5.6-fold. Growth in the presence of 30 mg IPTG l⁻¹ increases the cellular content of the regulators (FNR and HlyX) and resulted in a small increase in FNR-dependent *hlyE* expression (265 Miller units without IPTG compared to 390 Miller units in the presence of IPTG), but the addition of IPTG increased HlyX-dependent expression to 2540 Miller units from 950 Miller units observed in the absence of IPTG, equivalent to an overall HlyX-mediated 15-fold induction relative to JRG3702 (Table 1). Thus, it is clear that HlyX is a more efficient anaerobic activator of *hlyE* transcription than FNR.

The *lacZ* fusion data were consistent with the production of haemolysin by the three strains. After anaerobic growth on blood agar without added IPTG, only the *hlyX*-expressing strain, JRG2269, displayed the haemolytic phenotype, whereas in the presence of IPTG, low levels of haemolysin were produced by the *fnr*-expressing strain, JRG3350. Irrespective of IPTG addition, JRG1728 (Δ *fnr*) did not produce detectable levels of haemolysin.

Analyses of the FNR–*hlyE* and HlyX–*hlyE* DNase I footprints revealed similar protection patterns. Both regulators occupied the region between -97 and -61 (Fig. 2, lanes 2 and 3). A hypersensitive site at position -71 indicates that both FNR and HlyX distort the DNA in a similar manner. In the absence of FNR or HlyX, addition of 1 U RNA polymerase caused the protection of a region between -83 and $+5$ (Fig. 2, lane 4). Hypersensitive bases at -41 and -42 were present, indicative of DNA distortion in the *hlyE*–RNA polymerase binary complex. However, both FNR and HlyX were able to recruit RNA polymerase to the *hlyE*

Table 1. Anaerobic activation of *hlyE* expression by FNR and HlyX

FNR- and HlyX-driven *hlyE* promoter activities were estimated by measuring β -galactosidase activity after growth of JRG3702 (Δ *fnr* *hlyE*::*lacZ*) and with transformants containing a second plasmid expressing either FNR (pGS330) or HlyX (pGS415) in the absence or presence of 30 mg IPTG l⁻¹. The values quoted are the mean of duplicate assays from three independent cultures.

Strain	β -Galactosidase activity (Miller units)		
	Aerobic	Anaerobic	Anaerobic + IPTG
JRG3702 (Δ <i>fnr</i>)	30	170	120
JRG3653 (<i>fnr</i>)	37	265	390
JRG3654 (<i>hlyX</i>)	50	950	2540

Fig. 2. DNase I footprints of the *hlyE* promoter region. Lanes: 1, 8 and 10, no protein; 2, FNR (100 nM); 3, HlyX (100 nM); 4, RNA polymerase (1 U, 100 nM); 5, RNA polymerase (1 U) plus FNR (100 nM); 6, RNA polymerase (1 U) plus HlyX (100 nM); 7 and 17, calibrating Maxam & Gilbert G tracks; 9, FNR (15 nM); 11, HlyX (15 nM); 12, FNR (15 nM) plus RNA polymerase (0.1 U, 10 nM); 13, FNR (30 nM) plus RNA polymerase (0.1 U); 14, HlyX (15 nM) plus RNA polymerase (0.1 U); 15, RNA polymerase (1 U); 16, RNA polymerase (0.1 U). The regions of FNR or HlyX (open box), RNA polymerase (dashed line), HlyX (FNR) plus RNA polymerase (solid line) protection and some hypersensitive positions (arrows) are indicated.

promoter because at concentrations insufficient to produce protection when present individually, FNR or HlyX (15 nM) in combination with RNA polymerase (0.1 U), generated a region of protection from -97 to at least +5 corresponding to *hlyE*-RNA polymerase-FNR (-HlyX) ternary complexes (Fig. 2, lanes 12-14). Although the protection of the FNR site was not as strong as that observed when higher concentrations of regulator were used (Fig. 2, lanes 5 and 6), these observations indicate that RNA polymerase and FNR (and HlyX)

may bind co-operatively. The regions of protection for the FNR- and HlyX-containing ternary complexes were not identical. The HlyX-containing ternary complex protected the *hlyE* promoter from -97 to +17 compared to -97 to +5 for the equivalent FNR-containing complex. Increasing the concentration of FNR did allow the formation of a complex similar to that formed with HlyX (Fig. 2, lanes 5, 6 and 13). These observations indicate that HlyX is probably better able to direct RNA polymerase to the optimal position for transcription to

Fig. 3. (a) Location of transcript start sites for the *hlyE* promoter driven by FNR or HlyX. The 5' ends of the transcripts were determined by primer extension analysis and comparing the resulting molecules (lane 1, FNR-expressing cultures; lanes 4 and 6, HlyX-expressing cultures, lane 6 is a shorter exposure time of the sample in lane 4 to illustrate that tsp1 is the major transcript start) with calibrating Maxam & Gilbert G tracks (lane 2, *yfiD* DNA; lanes 3 and 5, *ndh* DNA). The transcript start points (tsp1–5) are indicated. (b) Location of *hlyE* transcript starts in a variety of genetic backgrounds. The 5' ends of the transcripts were estimated by primer extension, all strains (except lanes 1 and 4) expressed *fnr* from the chromosome and the cultures were grown anaerobically for 24 h. Lanes: 1, multicopy *fnr*; 2, wild-type; 3, *rpoS*; 4, *fnr*; 5, *ihfA*; 6, *fis*; 7, *arcA*; 8, calibrating Maxam & Gilbert G track of *yfiD* DNA (Green & Baldwin, 1997).

occur. This re-alignment of polymerase is accompanied by the reduction/loss of hypersensitivity at positions -41 and -42. However, transcription *in vitro* with σ^{70} RNA polymerase indicated that, even in the presence of FNR or HlyX, the *hlyE* transcript was not detectable (not shown), indicating that the ternary complexes formed are not transcriptionally competent.

The *hlyE* promoter is a Class I FNR-dependent promoter

Further analysis of the *hlyE* promoter region revealed the presence of a ribosome-binding site (7 out of 9 consensus match, TAAAGAGGC) but several possible -10 elements (Fig. 1). Transcript mapping by primer extension with RNA isolated from the *hlyX*-expressing strain JRG2269 indicated that the *hlyE* transcript is initiated from three positions: the major transcript start places the FNR site at -78.5 (tsp1); the minor transcripts place the FNR site at -73.5 (tsp2) and -71.5 (tsp3), respectively (Fig. 3a). The major transcript

represented 80% of the total *hlyE* message in this strain. The same three transcript start points were observed in an *fnr*-expressing strain although the relative proportions of the transcripts differed (Fig. 3a).

Overexposure of the autoradiographs allowed the detection of two additional minor ($\leq 1\%$ of the total transcripts) transcript starts, tsp4 and tsp5, which placed the FNR box at -63.5 and -62.5, respectively. Therefore, as the position of the FNR-binding site at the *hlyE* promoter is always beyond -41, *hlyE* is a Class I promoter (Ebright, 1993).

Expression of *hlyE* resembled that observed for a wild-type (*fnr*-expressing) strain in *rpoS*, *ihfA*, *fis* and *arcA* strains as judged by transcript mapping (Fig. 3b). However, in the *fnr* strain, JRG1728, the amount of *hlyE* transcript (tsp1) produced was reduced by 12.5-fold, relative to the wild-type, but was still detectable. This apparent FNR-independent *hlyE* transcription may account for the FNR-independent anaerobic induction observed in the *hlyE::lacZ* reporter experiments (see

Table 2. Anaerobic activation of the *FF* series and *hlyE* promoters by FNR and HlyX variants

The effect of various amino acid substitutions on anaerobic activation of FNR-dependent promoters. Promoter activity was estimated by measuring β -galactosidase activity after anaerobic growth of JRG1728 carrying the indicated pRW50 derivatives which encode the *lac* operon under the control of the following FNR-dependent promoters: *FFpmelR*, FNR site at -41.5 ; *FF+20pmelR*, FNR site at -61.5 ; *FF-71.5pmelR*, FNR site at -71.5 and an improved -35 element; *hlyE*. FNR, HlyX and the derivatives were introduced on a second plasmid encoding FNR (pGS330), HlyX (pGS415), FNR-R179Q (pGS1053), FNR-F181Y (pGS1054), FNR-Q183A (pGS1055), FNR-R184P (pGS1056), FNR-P188A (pGS1057), FNR-A225T (pGS1086) or HlyX-A187P (pGS1003). The values quoted are the mean from duplicate assays of three independent cultures and the figures in parentheses represent the activity of the indicated FNR derivative relative to FNR.

FNR protein	β -Galactosidase activity (Miller units)			
	<i>FFpmelR</i>	<i>FF+20pmelR</i>	<i>FF-71.5pmelR</i>	<i>hlyE</i>
FNR	3800 (1.00)	130 (1.00)	1600 (1.00)	275 (1.00)
FNR-R179Q	3800 (1.00)	260 (2.00)	1820 (1.13)	285 (1.04)
FNR-F181Y	3850 (1.01)	290 (2.23)	2310 (1.44)	360 (1.31)
FNR-Q183A	4135 (1.09)	270 (2.07)	2295 (1.43)	275 (1.00)
FNR-R184P	1030 (0.27)	150 (1.15)	1070 (0.66)	185 (0.67)
FNR-P188A	4025 (1.06)	305 (2.34)	2680 (1.68)	360 (1.31)
FNR-A225T	5380 (1.42)	570 (4.36)	3290 (2.05)	585 (2.13)
HlyX-A187P	800 (0.21)	200 (1.53)	1550 (0.96)	510 (1.85)
HlyX	1920 (0.51)	770 (5.92)	3330 (2.08)	950 (3.45)

above). Quantitative densitometry indicated that anaerobic cultures expressing HlyX produced at least 20 times more *hlyE* transcripts than those expressing FNR in multicopy or from the chromosome.

The region encompassing β_9 and β_{10} of FNR contributes to FNR activation of Class I promoters

The FNR and HlyX proteins bind to the same site at the Class I *hlyE* promoter and initiate transcription from the same positions. However, only HlyX efficiently induces haemolysin production, indicating that the quality of the HlyX AR1 contacts with RNA polymerase are likely to be superior to those of FNR at the *hlyE* and other Class I promoters (Green & Baldwin, 1997). The AR1 of HlyX has been partially defined by the substitution A187P and lies in the loop between β_9 and β_{10} (Green & Baldwin, 1997). This region of FNR was analysed using site-directed mutagenesis to substitute singly those amino acids unique to FNR with those present in HlyX (Fig. 4) and then testing the ability of the resulting proteins to activate transcription from the *hlyE* promoter and the model, semi-synthetic Class I (*FF+20pmelR* and *FF-71.5pmelR*) and Class II (*FFpmelR*) promoters.

Most of the substitutions made in the β_9 - β_{10} region had some effect on transcription activation from the Class I model promoters but little effect on the Class II *FFpmelR* promoter (Table 2). Such FNR variants displayed enhanced activation from Class I promoters compared with FNR. The FNR variant FNR-P188A was the most like HlyX in its regulation of Class I promoters but

although it was some 2.3-fold better than FNR in activating transcription from *FF+20pmelR* it was still poorer than HlyX (sixfold better than FNR). Studies with the *hlyE::lacZ* reporter plasmid indicated that the replacement of FNR residues F181 or P188 with the amino acids found in the equivalent positions in HlyX increased *hlyE* expression to 1.3 times that observed with FNR (Table 2), indicating that this region of FNR does contribute toward transcription activation at the *hlyE* promoter. Interestingly, substitution of R184 of FNR by P (found in the equivalent position in HlyX) resulted in an FNR protein, FNR-R184P, which was defective in activation from the Class II promoter *FFpmelR* but was much less affected in the ability to activate transcription from Class I promoters. This contrasts with the transcriptional activities of the other FNR variants in the β_9 - β_{10} region which displayed increased activity at Class I promoters and unaltered activity at the Class II promoter, suggesting that the AR1 contacts at Class I and Class II promoters are not identical.

None of the substitutions in the β_9 - β_{10} region of FNR was sufficient to confer a haemolytic phenotype upon *E. coli* JRG1728.

The β_9 - β_{10} region of HlyX has been shown to be involved in transcription activation from Class I promoters and the substitution A187P reduced activation from two model Class I promoters (Table 2, Green & Baldwin, 1997). Accordingly, for bacteria expressing HlyX-A187P, anaerobic expression of *hlyE* was reduced to only 54% of that observed with HlyX (Table 2).

The C-terminal element required for the expression of HlyX is defined by the substitution A255T

The failure to generate an FNR protein with the capacity to confer a haemolytic phenotype on *E. coli* by making substitutions in the β_9 – β_{10} region was not surprising. Even though these variants increased *hlyE* expression up to 1.3-fold relative to FNR, observation of the haemolytic phenotype is known to require elements of both the N- and C-terminal regions of HlyX (Green *et al.*, 1992) and the β_9 – β_{10} region represents only the N-terminal element. Thus the contribution of the β_{11} – β_{12} region of FNR/HlyX was investigated. Position 225 (A in FNR, T in HlyX) was targeted because it is the only non-conservative substitution in β_{11} (Fig. 4). Substitution of A225 of FNR with T had dramatic effects on transcription activation from model promoters (Table 2). The altered FNR (FNR-A225T) combined the transcriptional activation properties of FNR at Class II promoters and HlyX at Class I promoters. Also, it allowed increased *hlyE* expression and conferred a haemolytic phenotype upon JRG1728 (*fnr*). Thus, it is likely that A225 is part of AR1 of FNR and represents the C-terminal element required for the anaerobic induction of haemolytic activity in *E. coli* K-12 strains (Green *et al.*, 1992), the N-terminal element being previously defined as part of the β_9 – β_{10} loop (Green & Baldwin, 1997).

The C-terminal regions of FNR and HlyX are rather different as FNR has an extra 9 aa. Investigation of the contribution of this region was attempted by generating two FNR proteins in which 17 and 10 C-terminal amino acids were absent. Unfortunately, both altered FNR proteins were insoluble and their transcription activation properties were therefore difficult to study. Neither derivative could anaerobically activate expression from *hlyE* or any of the model promoters at 37 °C, even in the presence of IPTG. Consequently they did not confer a haemolytic phenotype at this temperature. However, if FNR-Cdel10-expressing cultures were stored at 4 °C for 14 d after anaerobic incubation for 24 h at 37 °C on blood agar, the haemolytic phenotype was detected. An FNR-expressing control culture was haemolysin-negative.

DISCUSSION

Following analysis of the relative efficiencies of FNR- and HlyX-mediated activation of model Class I and Class II promoters it was predicted that the *hlyE* gene would possess an FNR site positioned at or beyond –61.5, i.e. *hlyE* would be expressed from an FNR-dependent Class I promoter (Green & Baldwin, 1997). The present work shows that this is the case with the major transcript initiating 78.5 bp downstream of the FNR site. Thus, the molecular basis of the ability of HlyX but not FNR to confer a haemolytic phenotype upon *E. coli* probably lies in the relative efficiencies of FNR- and HlyX-mediated activation from Class I promoters. Studies with model promoters indicated that FNR is unable to activate transcription when positioned

beyond –61.5 unless an improved –35 element is present in the promoter (Wing *et al.*, 1995). The –35 element of *hlyE* is very poor (GCTAAC, having only one base in common with the consensus) and thus it is not surprising that FNR is a weak activator of *hlyE* expression. HlyX-mediated activation of Class I promoters is better than that of FNR because HlyX makes an enhanced AR1 contact with RNA polymerase (Green & Baldwin, 1997). The AR1 of HlyX was characterized by the mutation A187P which lies in the loop between β_9 and β_{10} . This region is equivalent to the AR1 of CRP and may be extended at least as far as the α_D – β_9 loop of HlyX (Green & Baldwin, 1997). The role of this β_9 – β_{10} region of the CRP/FNR family of transcription regulators in activation from Class I promoters was confirmed by making individual replacements of the amino acids found in FNR with those of HlyX. As a result, in most cases FNR became more HlyX-like in its ability to activate expression of model Class I promoters and *hlyE* itself. The exception was FNR-R184P which displayed much reduced activity at Class II promoters but was less compromised at Class I and *hlyE* promoters. The AR1 contact between regulator RNA polymerase is a feature of both Class I and Class II promoters (Savery *et al.*, 1996). For CRP at Class II promoters the role of the AR1 contact is that of anti-inhibition (Niu *et al.*, 1996), whereas at Class I promoters AR1 makes an activating contact with the α subunit of RNA polymerase (Ebright, 1993). The results obtained with FNR-R184P suggest that the AR1 contact at Class I promoters (FNR contacts the downstream α subunit of RNA polymerase) may not be identical to that at Class II promoters (FNR contacts the upstream α subunit) and that R184 may be more involved in making an anti-inhibition (Class II) rather than an activating contact (Class I). It has been suggested that the AR1 interaction between CRP and RNA polymerase is different at Class I and Class II promoters (Zhou *et al.*, 1994). An inferior anti-inhibition contact provides a plausible explanation for the relatively poor performance of HlyX at Class II promoters (Green & Baldwin, 1997).

None of the replacements in the β_9 – β_{10} region enabled FNR to induce a haemolytic phenotype in *E. coli*, nor did they increase *hlyE* expression to the levels observed with HlyX. This indicated that there were other regions of the protein involved in activating transcription from Class I promoters. Replacement of A225 in β_{11} of FNR with the equivalent T of HlyX indicated that AR1 may extend into β_{11} . This substitution allowed the altered FNR to confer the haemolytic phenotype. Even more striking were the effects on transcription activation from a set of model promoters. The A225T substitution generated an FNR which combined the transcription activation properties of FNR and HlyX. Thus, the AR1 of FNR/HlyX extends at least from β_9 – β_{10} through to β_{11} . These results indicate that FNR has a much larger AR1 surface for contacting the α subunit of RNA polymerase compared to CRP (Niu *et al.*, 1994; Zhou *et al.*, 1994). Another obvious region in which FNR and HlyX differ is at their C termini where FNR has a 9 aa

Fig. 4. Amino acid sequence of the β₉–β₁₀ and β₁₁–β₁₂ regions of FNR and HlyX. The amino acids selected for replacement are arrowed, the deleted C-terminal region of FNR is underlined and the numbering is for FNR.

extension. Two FNR proteins (FNR-Cdel17 and FNR-Cdel10) which lack 17 and 10 C-terminal amino acids, respectively, were insoluble at 37 °C and were thus unsuitable for rigorous characterization, but it seems clear that the C-terminal region of FNR inhibits *hlyE* expression at lower temperatures and is required for solubility and correct folding at 37 °C, even though it is absent from HlyX. It is possible that the C-terminal extension present in FNR acts to mask AR1, preventing efficient AR1:α contacts but, because of the low solubility of the C-terminally deleted FNRs, it has not been possible to investigate this further.

Reports in the literature indicate that *clyA* (*hlyE*) can be expressed in strains of *E. coli* which lack the histone-like protein HNS (Oscarsson *et al.*, 1996). The transcript mapping presented here shows that the other histone-like proteins, integration host factor (IHF) and factor for inversion stimulation (Fis) do not affect *hlyE* expression under anaerobic conditions. The *slyA* gene product is also thought to regulate *clyA* (*hlyE*) expression but its mechanism of action and binding site are unknown (Oscarsson *et al.*, 1996). It is possible that FNR (HlyX) and SlyA act in concert to maximize *hlyE* expression and that HlyX–SlyA contacts may be important and more productive than the equivalent FNR–SlyA contacts. There is, however, more to the endowment of a haemolytic phenotype than the production of the haemolysin polypeptide and other gene products may be required to allow efficient export of the haemolysin. SlyA, and indeed FNR and HlyX, may have a role in the regulation of these processes.

In conclusion, analysis of FNR–*hlyE* and HlyX–*hlyE* interactions indicates that *hlyE* is a Class I FNR-activated promoter but, even though both regulators are able to recruit RNA polymerase, only HlyX positions RNA polymerase optimally to allow transcription. This suggests that the AR1 of HlyX, with a crucial contribution from T224, is superior to that of FNR. An obvious implication of these results is that a single substitution in FNR (or other transcription regulators) may be sufficient to permit the expression of otherwise silent virulence genes. It will be interesting to investigate AR1 of FNR proteins isolated from pathogenic strains of *E. coli* to establish if they possess AR1 surfaces that resemble that of FNR from *E. coli* K-12 or that of HlyX.

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