Regulation of gene expression by the HlyX protein of *Actinobacillus pleuropneumoniae*

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The *hlyX* gene of the swine pathogen *Actinobacillus pleuropneumoniae* and the *fnr* gene of *Escherichia coli* encode very similar proteins. The *hlyX* gene is able to complement Δfnr mutations and will permit the growth of *E. coli fnr* strains in nitrate minimal salts medium under anoxic conditions. In addition, the *hlyX* gene product appears to induce the expression of a latent haemolytic activity as evidenced by the presence of a strong zone of haemolysis around *E. coli* (*hlyX*⁺) colonies grown on bovine or ovine blood. In this study, the ability of the *hlyX* gene product to induce haemolytic activity and regulate expression of *frdA* and its own gene was examined in the presence of various carbon sources and in the presence and absence of iron; *fnr* was included for comparison. The HlyX protein was able to induce the synthesis of the latent *E. coli* haemolytic activity only under anoxic conditions. Haemolytic activity was highest during the late exponential phase and then levelled off in the stationary phase. The *hlyX* gene product was able to activate the expression of a $\Phi(frdA'-lacZ)$ in *E. coli* JRG1787 (Δfnr); however, the level of expression depended on carbon source, growth phase and copy number. Like *fnr*, the *hlyX* gene product appeared to affect its own synthesis but the nature and extent of regulation depended not only on the presence of oxygen but also on growth conditions.

Keywords: *Actinobacillus pleuropneumoniae*, HlyX protein, FNR, BTR, haemolytic activity

INTRODUCTION

The Gram-negative bacterium *Actinobacillus pleuropneumoniae* is an important pathogen of swine throughout the world (Nicolet, 1992). It can cause an acute, and often fatal, pleuropneumonia in pigs of all ages. Capsule, outer membrane proteins, lipopolysaccharide and three related RTX toxins have been implicated in the pathogenesis of this organism (Frey *et al.*, 1993a, b; Inzana, 1991). The RTX toxins, now designated ApxI, ApxII and ApxIII, can damage neutrophils and macrophages (Frey *et al.*, 1993b). ApxI and, to a lesser degree, ApxII, can also lyse red blood cells from several different species (Frey & Nicolet, 1988; Rosendal *et al.*, 1988). In addition to these toxins, *A. pleuropneumoniae* carries a gene which can confer a strong haemolytic phenotype on a variety of *Escherichia coli* K12 strains (Frey *et al.*, 1989; Lian *et al.*, 1989). This gene, *hlyX* (*cfp*), does not appear to encode a haemolysin

per se but, rather, it seems to induce a latent haemolysin in *E. coli*.

Consistent with this notion, the deduced amino acid sequence of the HlyX protein does not share any homology with that of any known haemolysin or cytotoxin (MacInnes *et al.*, 1990). It does, however, have a high degree of similarity with the *E. coli* protein FNR (Shaw & Guest, 1982). FNR belongs to a growing family of transcriptional regulators with a helix-turn-helix DNA-binding domain. This family includes the CRP (cAMP receptor protein) of *E. coli*, FnrN and FnrK from *Rhizobium leguminosarum* and *Rhizobium meliloti*, respectively, and ANR from *Pseudomonas aeruginosa* (Batut *et al.*, 1989; Colonna-Romano *et al.*, 1990; Galimand *et al.*, 1991; Sawers, 1991; Spiro & Guest, 1990). FNR homologues often function as oxygen sensors, and they can regulate a variety of genes involved in such diverse processes as anaerobic carbon metabolism and respiration, nitrogen fixation or luciferase expression (reviewed by Spiro, 1994).

The FNR protein has been shown to bind to specific sequences in the promoters of its target genes (Green *et*

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Abbreviations: CIP, calf intestinal phosphatase; MU, Miller units.

al., 1991; Sharrocks *et al.*, 1991). Under anoxic conditions, FNR activates the transcription of at least 15 genes that code for enzymes which are required for anaerobic electron transport to alternative terminal electron acceptors such as nitrate or fumarate (Spiro & Guest, 1990, 1991). FNR-activated targets include the *narGHJI* operon, which encodes nitrate reductase, the *frdABCD* operon, which encodes fumarate reductase, *pfl*, which encodes pyruvate formate-lyase, and the *dmsABC* operon, which encodes DMSO reductase (reviewed by Spiro & Guest, 1990). It is also able to repress the synthesis of cytochrome *o* oxidase (*cyoABCD*), NADH dehydrogenase II (*ndh*) and manganese-containing superoxide dismutase (*sodA*) (Cotter *et al.*, 1990; Hassan & Sun, 1992; Spiro *et al.*, 1989). Although there has been some debate, FNR is also thought to negatively regulate its own synthesis under anoxic conditions (Jones & Gunsalus, 1985; Pascal *et al.*, 1986; Spiro & Guest, 1987, 1990). Regulation of gene expression by FNR requires four iron-binding cysteine residues, and it has been speculated that FNR changes from an active to inactive conformation as the iron molecule is oxidized from ferrous to ferric iron under aerobic conditions (Green *et al.*, 1991).

The *hlyX* gene shares considerable sequence homology with *fnr*, including regions thought to be important in regulation. HlyX has also been shown to activate the expression of FNR targets such as the *narABCD* operon and *FF-melR-lacZ*, which contains a semi-synthetic promoter with a perfect FNR recognition sequence (Green *et al.*, 1992; MacInnes *et al.*, 1990). Despite sequence similarities, there do appear to be both quantitative and qualitative differences in the way these proteins regulate gene expression (Green *et al.*, 1992). Therefore, the purpose of these experiments was to look, in detail, at the ability of HlyX to regulate the well-studied FNR target, *frdA'-lacZ*, and the latent *E. coli* haemolysin. We also wanted to determine if HlyX expression was autoregulated and to confirm that iron was required for its activity.

METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study and their sources are listed in Table 1. For routine culture, cells were grown in Luria-Bertani (LB) medium at 37 °C or, for λ CI857 lysogens, at 30 °C in the presence of the appropriate antibiotic. For growth and promoter fusion studies, cultures were grown in minimal salts medium (Lambden & Guest, 1976) supplemented with 0.6% (w/v) fumarate or 0.4% (w/v) nitrate and 0.2% (w/v) glucose or 0.2% (v/v) glycerol. A 1% (v/v) inoculum of cells grown in minimal medium was used to inoculate cultures. When anoxic conditions were required, media were bubbled with N₂ for 1 h immediately before use. Anoxic cultures were grown in 10 ml aliquots in sealed N₂-purged 12 ml serum vials. Aerobic cultures were grown in 10 ml aliquots in 125 ml Erlenmeyer flasks on a rotating shaker at 125 r.p.m. When required, free Fe³⁺ and Fe²⁺ were removed by the addition of 10–50 μ M 1,10-phenanthroline or 2% (w/v) Chelex-100 beads. Iron-depleted media were prepared using acid-treated glassware and Milli-Q purified water.

Calculation of doubling times. When grown under anoxic

conditions, cultures were sampled approximately every 3 h under a continuous flow of N₂; aerobic cultures were sampled every 30 min. The doubling times of the cultures were calculated from the linear portion of the logarithmic plots of growth curves according to Koch (1981). The data shown are means $\pm$ SE; they are representative of three experiments, each with three replicates.

β -Galactosidase and haemolytic activity assays. The β -galactosidase assays were performed essentially as described by Miller (1972). Expression of the *frdA'-lacZ* promoter fusion was measured after 5 h of aerobic growth and 18–22 h of anoxic growth. The data shown are means $\pm$ SE; they are representative of three experiments, each with three replicates. Haemolytic activity assays were done using a modification of the method of Rosendal *et al.* (1988). Since previous experiments had shown that the haemolytic activity remains within the cell, it was only necessary to measure haemolytic activity in the cell pellet. All measurements were done on four replicates at each time point. The data shown are means $\pm$ SE; they are representative of three independent experiments.

Construction of *hlyX* recombinant plasmids. Unless otherwise indicated, chemicals were purchased from Fisher Scientific and enzymes were purchased from Boehringer Mannheim. Recombinant plasmids were constructed using standard procedures (Sambrook *et al.*, 1989). The 6 kb *Hind*III fragment of pA44 (Lian *et al.*, 1989), which carries the *hlyX* gene and flanking sequences, was gel purified and ligated to *Hind*III-digested, CIP-treated pBR322 DNA to create a tetracycline resistant *hlyX* recombinant, pGSG2. A *hlyX* promoter *lacZ* fusion was created using the plasmid pCB267, which carries a multiple cloning site followed by strong translation termination sequences in all three reading frames (Schneider & Beck, 1986). These sequences are upstream of a promoterless *lacZ* cistron

Table 1. *E. coli* strains, bacteriophage and plasmids

	Genotype	Source or reference
Strains		
JRG1728	MC1000 Δ (<i>tyrR-fnr-rac-try</i>)	Spiro & Guest (1988)
JRG1787	MC1000 Δ (<i>tyrR-fnr-rac-try</i>) λ Φ (<i>frdAp'-lacZ</i>)	Spiro & Guest (1987)
Phage		
λ RZ5	λ ' <i>bla</i> ' ⁺ ' <i>lacZ</i> ' ⁺ ' <i>lacY</i> ' ⁺	R. Zagursky, unpublished
λ GSG10	λ ' <i>bla</i> ' ⁺ Φ (<i>hlyXp'-lacZ</i>) ⁺ <i>lacY</i> ' ⁺	This work
Plasmids		
pGEM4	<i>bla</i> ' ⁺	Promega Corp., Madison, Wisconsin
pBR322	<i>bla</i> ' ⁺ <i>tet</i> ' ⁺	Bolivar <i>et al.</i> (1977)
pA44	<i>bla</i> ' ⁺ <i>hlyX</i> ' ⁺	Lian <i>et al.</i> (1989)
pT51	<i>bla</i> ' ⁺ <i>hlyX</i> ' ⁺	Lian <i>et al.</i> (1989)
pGS24	<i>bla</i> ' ⁺ <i>fnr</i> ' ⁺	Shaw & Guest (1982)
pCB267	<i>bla</i> ' ⁺ ' <i>lacZ</i> ' ⁺ ' <i>pboA</i> ' ⁺	Schneider & Beck (1986)
pGSG5	<i>bla</i> ' ⁺ Φ (<i>hlyXp'-lacZ</i>) ⁺	This work
pGSG3	<i>bla</i> ' ⁺ Φ (<i>hlyXp'-lacZ</i>) ⁺	This work
pGSG2	<i>tet</i> ' ⁺ <i>hlyX</i> ' ⁺	This work

with an intact Shine–Dalgarno sequence. A 300 bp *SspI* fragment from pT51 was gel purified and ligated to *HindIII*-digested pCB267 which had been made blunt-ended using mung bean nuclease. The 300 bp fragment carries 99 bp of sequence upstream from the putative transcription start site of the *hlyX* gene and encodes the first 42 amino acids of the HlyX protein (MacInnes *et al.*, 1990). One recombinant, which had a single copy of the 300 bp insert in the correct orientation, was selected and designated pGSG5. A plasmid construct with $\Phi(hlyXp-lacZ)$ and *bla* transcribed in opposite directions was required for construction of an *E. coli* λ RZ5 ($\Phi(hlyXp-lacZ)$) lysogen. Plasmid pGSG5 DNA was digested with *PstI* and the 4 kb fragment, which carries the *hlyXp-lacZ* promoter fusion, was purified. The 4 kb fragment was ligated to *PstI*-digested, CIP-treated pGEM4 (Promega). One recombinant with the *hlyXp-lacZ* fusion in the correct orientation was identified and designated pGSG3.

Construction of λ GSG10, containing $\Phi(hlyXp-lacZ)$.

Recombinant phage containing a *hlyXp-lacZ* fusion were created by a double crossover event between λ RZ5 containing partial *bla* and *lacZ* genes and pGSG3, which contains $\Phi(hlyXp-lacZ)$ and an intact *bla* gene. A high-titre lysate of λ RZ5 was used to infect *E. coli* JRG1728(pGSG3). Following infection, a lysate containing both wild-type (Lac^- Ap^s) and recombinant λ RZ5 (Lac^+ Ap^r) was recovered and used to infect *E. coli* JRG1728 under conditions that favoured lysogeny (Casadaban, 1976). Putative $\Phi(hlyXp-lacZ)$ lysogens were screened in the presence of 75 μ g ampicillin ml^{-1} and 50 μ g 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside ml^{-1} . Six blue, Ap^r colonies were obtained and shown to contain prophage by cross-streaking with wild-type phage. Bacteriophage were induced from one of the lysogens, *E. coli* JRG1728(λ GSG10), and characterized further by restriction mapping and Southern blotting. The recombinant phage λ GSG10 was again introduced into the chromosome of *E. coli* JRG1728 by lysogeny. One lysogen, shown to contain a single prophage by Southern blotting, was selected for the autoregulation studies.

RESULTS

Regulation of haemolytic activity by the HlyX protein

Haemolytic activity in *E. coli* JRG1728 (Δfnr) in the presence of plasmids pT51 (*hlyX*), pGS24 (*fnr*) or pBR322 was measured quantitatively. In glycerol-nitrate minimal salts medium, haemolytic activity was detected only in the presence of the HlyX protein, and only under anoxic conditions (Fig. 1). The activity peaked in the late exponential phase and dropped slightly during the stationary phase of growth. No haemolytic activity could be detected in *E. coli* JRG1728 (Δfnr) with plasmids pGS24 (*fnr*) or pBR322 grown under either aerobic or anoxic conditions.

Doubling times of *E. coli* JRG1728 in minimal salts medium

The doubling time of *E. coli* JRG1728 (Δfnr) in the presence of plasmids pT51 (*hlyX*), pGS24 (*fnr*) or pBR322 was measured in minimal salts medium supplemented with fumarate or nitrate as the terminal electron acceptor and glycerol or glucose as the carbon source (Table 2). The doubling times were shortest with oxygen as the terminal electron acceptor regardless of the carbon source

or the presence of HlyX or FNR. As shown previously, either HlyX or FNR was required for growth of JRG1728 in glycerol-nitrate minimal salts medium under anoxic conditions. By contrast, with this same medium containing fumarate, neither HlyX nor FNR was needed; however, FNR did enhance growth. When glucose rather than glycerol was present as the carbon source, neither HlyX nor FNR had a significant effect on growth.

Regulation of *frdA'-lacZ* expression by HlyX and FNR

Expression of *frdA'-lacZ* was measured under aerobic and anoxic conditions in glycerol-fumarate minimal salts medium using *E. coli* JRG1787 ($\Phi(frdA'-lacZ)$ Δfnr) in the presence and absence of HlyX; FNR was included for comparison (Table 3). Under anoxic conditions, the HlyX

Fig. 1. Growth and haemolysin titres of *E. coli* JRG1728 (Δfnr) with plasmid pT51 (*hlyX*⁺). Cells were grown under anoxic conditions in glycerol-nitrate minimal salts medium at 30 °C. No haemolytic activity was detected under aerobic conditions or in *E. coli* JRG1728 with plasmids pBR322 or pGS24 (*fnr*⁺). —, Haemolytic units; ·····, OD₆₅₀.

Table 2. Doubling times of *E. coli* JRG1728 Δfnr with pT51, pGS24 or pBR322 grown at 30 °C under aerobic and anoxic conditions in minimal salts medium with glycerol and fumarate, glucose and fumarate and glycerol and nitrate

Medium	Plasmid	Doubling time (h)	
		Aerobic	Anoxic
Glycerol + fumarate	pT51 (<i>hlyX</i> ⁺)	2.0 ± 0.1	10.6 ± 0.2
	pGS24 (<i>fnr</i> ⁺)	2.1 ± 0.1	7.8 ± 0.2
	pBR322	2.2 ± 0.1	10.0 ± 0.4
Glucose + fumarate	pT51 (<i>hlyX</i> ⁺)	1.6 ± 0.1	4.7 ± 0.6
	pGS24 (<i>fnr</i> ⁺)	1.6 ± 0.1	3.2 ± 0.4
	pBR322	1.9 ± 0.1	5.0 ± 0.4
Glycerol + nitrate	pT51 (<i>hlyX</i> ⁺)	2.3 ± 0.1	8.7 ± 0.3
	pGS24 (<i>fnr</i> ⁺)	2.4 ± 0.1	6.8 ± 0.3
	pBR322	2.5 ± 0.1	21 ± 1.0

Table 3. Expression of *frd'-lacZ* in *E. coli* JRG1787 ($\Phi(\text{frd}'\text{-lacZ}) \Delta\text{fnr}$) with plasmids pT51 (*hlyX*⁺), pGSG2 (*hlyX*⁺), pGS24 (*fnr*⁺) or pBR322

Cells were grown at 30 °C in minimal salts medium with glycerol and fumarate.

Plasmid	<i>frd'-lacZ</i> expression (MU)	
	Anoxic	Aerobic
pT51 (pGEM4 derivative)	810 ± 31	230 ± 5
pGSG2 (pBR322 derivative)	590 ± 32	180 ± 3
pGS24 (pBR322 derivative)	1700 ± 54	280 ± 5
pBR322	250 ± 14	95 ± 1

protein activated *frdA'-lacZ* expression two- to threefold as compared to the vector control when expressed from pGSG2. Somewhat higher levels of expression were obtained when the HlyX protein was expressed from plasmid pT51. FNR activated *frdA'-lacZ* expression about sixfold. Under aerobic conditions, the expression of *frdA'-lacZ* in the presence of the HlyX or FNR proteins was comparable to expression under anoxic conditions with the vector alone.

The effects of different carbon sources on the regulation of the *frdA'-lacZ* expression by the HlyX and FNR proteins

were also examined at various times (Fig. 2). Under anoxic conditions in fumarate minimal salts medium with glucose, *frdA'-lacZ* expression was maximal at 4 h at 7 times and 13 times the vector control levels for the HlyX and FNR proteins, respectively. The level of *frdA'-lacZ* expression was slightly lower at 8 and 18 h. In medium containing glycerol, *frdA'-lacZ* expression increased over time. In glycerol medium, the maximal level of *frdA'-lacZ* expression was about 1.4-fold higher than that detected in glucose medium.

Regulation of expression of a chromosomal *hlyX* promoter fusion in JRG1728 ($\lambda\text{GSG10 } \Phi(\text{hlyXp-lacZ})$)

In order to study the regulation of expression of a single copy of the *hlyX* gene, the *hlyXp-lacZ* fusion from pGSG3 was transferred to λRZ5 by homologous recombination to produce $\lambda\text{GSG10 } (\Phi(\text{hlyXp-lacZ}))$, which was then introduced into the chromosome of *E. coli* JRG1728 ($\Delta\text{fnr } \Delta\text{lac}$). The expression of the *hlyXp-lacZ* fusion in cells with pGSG2 (*hlyX*) or pBR322 was examined (Table 4). The expression of the *hlyXp-lacZ* promoter fusion could not be detected using the Miller (1972) assay in cells grown to late exponential or stationary phase in LB medium or in glycerol-fumarate minimal salts medium. In early exponential phase in both media, expression could be detected in cells containing pBR322 but not in cells with pGSG2 (*hlyX*⁺). The level of expression varied with the growth conditions, however. In minimal medium, no

Fig. 2. Growth of *E. coli* JRG1787 ($\Phi(\text{frdA}'\text{-lacZ}) \Delta\text{fnr}$) (a, c) (—■—, HlyX; ---▲---, FNR; ···●···, vector) and expression of *frdA'-lacZ* (b, d) (▨, 4 h; ▩, 8 h; ▤, 18 h) with plasmids pT51 (*hlyX*⁺), pGS24 (*fnr*⁺) and pBR322. Cells were grown from 4 to 18 h at 30 °C under anoxic conditions in minimal salts medium with fumarate and 0.2% glucose (a, b) or 0.2% glycerol (c, d) as the carbon source.

Table 4. Expression of a chromosomal *hlyX* promoter fusion, JRG1728(λ GSG10 Φ (*hlyXp-lacZ*) Δ *fnr*)

Medium	Plasmid	<i>hlyXp-lacZ</i> expression (MU)	
		Aerobic	Anoxic
Minimal salts with glycerol and fumarate	pBR322 (vector)	22 $\pm$ 1	< 1.0
	pGSG2 (<i>hlyX</i>)	< 1.0	< 1.0
Luria-Bertani	pBR322 (vector)	43 $\pm$ 1	101 $\pm$ 1
	pGSG2 (<i>hlyX</i>)	< 1.0	< 1.0

Table 5. Expression of *frdA-lacZ* in *E. coli* JRG1787(Φ (*frdA-lacZ*) Δ *fnr*) grown under anoxic conditions with plasmids pT51, pGS24 or pBR322 in the presence of various concentrations of the iron chelator 1,10-phenanthroline

1,10-Phenanthroline concentration	<i>frdA-lacZ</i> expression (MU)		
	pT51 (<i>hlyX</i> ⁺)	pGS24 (<i>fnr</i> ⁺)	pBR322
0 μ M	980	1580	160
10 μ M	130	970	60
20 μ M	110	130	60
50 μ M	100	140	60
50 μ M + 50 μ M iron	610	1400	170

expression could be measured when cells were grown under anoxic conditions; low levels were detected under aerobic conditions. By contrast, in LB medium, expression of the *hlyXp-lacZ* promoter fusion was greatest when cells were grown under anoxic conditions.

Regulation of *frdA-lacZ* expression in the presence of iron chelators

E. coli JRG1787 (Φ (*frdA-lacZ*) Δ *fnr*) was used to study the effects of iron limitation on the regulation of the *frdA-lacZ* expression by HlyX; FNR was included as a control (Table 5). The iron chelator 1,10-phenanthroline reduced cell growth and expression of *frdA-lacZ* in a concentration-dependent manner. Growth was reduced gradually with increasing 1,10-phenanthroline concentrations under aerobic conditions whereas concentrations of 20 μ M phenanthroline or greater abolished growth abruptly under anoxic conditions. Addition of excess iron completely abrogated the effect of 20 mM 1,10-phenanthroline. This result suggests that the effect of 1,10-phenanthroline under aerobic conditions is due to generalized iron limitation whereas the effect under anoxic conditions is due to inactivation of the FNR and HlyX proteins and/or direct or indirect reduction in the activity of iron-containing anaerobic respiratory enzymes.

The nontoxic chelator Chelex-100 reduced *frdA-lacZ*

expression and cell growth in a similar manner (data not shown). However, there was only a moderate reduction in growth and expression of *frdA-lacZ* as compared to 1,10-phenanthroline-treated cultures. Again, the inhibition of growth and *frdA-lacZ* expression could be completely reversed by the addition of excess iron.

DISCUSSION

Regulation of expression of haemolytic activity by HlyX

HlyX, but not FNR, was able to activate haemolytic activity in *E. coli*, and the activity could only be detected in cells grown under anoxic conditions (Fig. 1). The haemolytic activity peaked in the late exponential phase and dropped slightly during the stationary phase, suggesting that expression ceases or is reduced after the exponential phase. The same pattern of expression was also seen with *frdA-lacZ*. To date, it has not been possible to characterize the HlyX-induced haemolytic activity. Although the activity is fairly stable and easily followed through various purification steps, the protein is present in vanishingly small quantities.

Regulation of gene expression under different growth conditions

Under anoxic conditions, HlyX protein activated *frdA-lacZ* in *E. coli* JRG1787 approximately half as well as FNR (Table 3). The lower levels of gene activation may be a function of concentration or the ability of HlyX to interact with *E. coli* promoters or polymerase. In cells lacking both the HlyX and FNR proteins, *frdA-lacZ* expression was activated threefold under anoxic conditions as compared to aerobic conditions. This result suggests that some other redox-sensitive mechanism also affects *frdA-lacZ* expression.

Carbon source and, to some extent, growth phase affected HlyX- and FNR-dependent regulation of the *frdA-lacZ* gene (Fig. 2). In cultures containing glucose, the expression of *frdA-lacZ* was relatively constant during the exponential growth phase (1–8 h). With glycerol as a carbon source, *frdA-lacZ* expression increased steadily as the cells grew exponentially (5–20 h). Although the levels of *frdA-lacZ* expression were lower at early time points, maximal expression was always detected in glycerol rather than glucose medium. The effector of this carbon-source-dependent regulation may be the CRP protein, since the putative FNR target sequences in the *frdA* promoter have approximately the same level of homology with the CRP consensus sequence as with the FNR consensus sequence (Spiro & Guest, 1990). Alternatively, the effect might be indirectly mediated by cAMP (Uden & Duschene, 1987). The nature of the growth-phase-dependent effector is unclear but it may be a regulatory protein responsive to fumarate concentration. Another possible explanation is that the Miller (1972) assay, which uses A_{600} to estimate total cellular protein, may have over-estimated *frdA-lacZ* expression because of alterations in the cell surface (e.g. capsule) of glycerol- versus glucose-grown cells.

Autoregulation of *hlyX* gene expression

The promoter of the FNR gene contains an FNR consensus sequence, and Spiro & Guest (1987) reported that under anoxic conditions, expression of an *fnr-lacZ* promoter fusion was reduced in *fnr*⁺ strains, but not in Δ *fnr* strains. In similar experiments, Pascal *et al.* (1986) found that the expression of *fnr* was negatively regulated by its own product but expression was independent of anaerobiosis. Others have reported that *fnr*-specific mRNA levels and the abundance of the FNR protein are not significantly affected by changes in oxygen tension (Unden & Duschene, 1987; Eiglmeier *et al.*, 1989).

The promoter region of the *hlyX* gene contains a region of dyad symmetry immediately downstream of a putative -10 region, which is almost identical to the FNR consensus sequence (MacInnes *et al.*, 1990). In the absence of the HlyX protein, the *hlyXp-lacZ* promoter fusion was expressed in both LB and minimal salts medium; however, the pattern of expression was reversed. In LB medium, *hlyXp-lacZ* expression was highest when cells were grown under anoxic conditions, whereas in minimal medium, *hlyXp-lacZ* expression could only be detected in aerobic cultures. Taken together, these data suggest that the *hlyX* gene product is able to repress its own synthesis under both aerobic and anaerobic conditions; however, the basal level of *hlyX* transcription is a function of not only oxygen levels, but also growth conditions.

Role of iron in HlyX-regulated gene expression

Consistent with previous studies on FNR, the activation of the *frdA'-lacZ* operon promoter by the HlyX was iron dependent (Table 5; Green *et al.*, 1991; Trageser & Unden, 1989). Since the chelator 1,10-phenanthroline is known to have toxic and DNA-damaging properties in bacterial cells (Sigman *et al.*, 1979) these experiments were repeated with the Chelex-100, an iron chelator, attached to latex beads, which does not enter the bacterial cell. The effects of iron chelation with Chelex-100 were consistent with those obtained using 1,10-phenanthroline, but were much less pronounced.

The identity of the latent haemolytic activity induced in *E. coli* is not yet known. Frey *et al.* (1989) suggested that the *hlyX* gene, which they call the *cfp* (CAMP factor protein) gene, encodes a CAMP cohaemolysin. They reported that this cohaemolysin is antigenically related to protein B of *Streptococcus agalactiae*, and it is able to produce a distinct CAMP reaction in the presence of both phospholipase C and phospholipase D. Although *hlyX* (*cfp*) appears to induce (rather than to actually encode) a haemolysin, the *E. coli* gene activated by HlyX could encode a protein which is related to protein B or other CAMP factors. It is likely that there is also a similar HlyX-regulated gene in *A. pleuropneumoniae*. Convalescent serum from *A. pleuropneumoniae*-infected animals can neutralize the haemolytic activity induced by HlyX. Furthermore, Frey *et al.* (1989) found that antiserum produced against *E. coli* containing the *cfp* gene was able to neutralize the CAMP reaction of *A. pleuropneumoniae* strains.

Recently, a similar *fnr* homologue has been cloned from *Bordetella pertussis*, *btr* (*Bordetella* transcriptional regulator), and from *Haemophilus ducreyi* (Bannan *et al.*, 1993; P. A. Totten, personal communication). These genes are also able to induce haemolytic activity in *E. coli*, and *btr* has been shown to complement *E. coli fnr* mutants (Bannan *et al.*, 1993). It might be noted that although *B. pertussis*, *A. pleuropneumoniae* and probably *H. ducreyi* all have one or more related haemolysins (belonging to the RTX family) there is no evidence that *fnr*-like genes are involved in the regulation of their expression (Ehrmann *et al.*, 1991; Frey *et al.*, 1993a). The role of these 'haemolysin-inducing' FNR homologues in virulence remains to be established.

ACKNOWLEDGEMENTS

We thank J. R. Guest, R. P. Gunsalus and A. M. Kropinski for their generous gifts of *E. coli* strains, plasmids and phage, and P. H. S. Ling for technical assistance. We are also grateful to P. A. Totten for sharing unpublished results. Financial support for this work was provided by a grant from the Natural Sciences and Engineering Research Council of Canada.

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Received 18 August 1993; revised 11 October 1993; accepted 20 October 1993.