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Actinobacillus pleuropneumoniae hlyX Gene Homology with the *fnr* Gene of *Escherichia coli*

JANET I. MACINNES,* JOHN E. KIM, CHANG-JOO LIAN,† AND GLENN A. SOLTES

Department of Veterinary Microbiology and Immunology, University of Guelph, Guelph, Ontario, Canada N1G 2W1

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The *hlyX* gene from *Actinobacillus pleuropneumoniae*, which confers a hemolytic phenotype on *Escherichia coli*, was sequenced, and its role in regulation of gene expression was investigated. No similarity was found between the *hlyX* sequence and sequences of known hemolysin or cytotoxin genes. However, the *hlyX* sequence was very similar to that of the *fnr* gene of *Escherichia coli* which encodes the global regulatory protein, FNR. Comparison of the deduced amino acid sequence of the *hlyX* gene product (HlyX) with that of FNR revealed a high degree of well-aligned sequence correlation throughout the polypeptide chain. For example, 23 of 24 amino acids in the DNA-binding region of FNR are identical in the corresponding region of HlyX. Four cysteine residues in the amino-terminal region are also conserved. The promoter region of *hlyX* is very similar to that of *fnr*. It has a putative −10 sequence which closely resembles the *E. coli* −10 consensus sequence. This sequence is overlapped by a potential operator which is very similar to the FNR-binding-site consensus sequence. Functional homology between HlyX and FNR was also demonstrated. Plasmids carrying *hlyX* complemented the nutritional lesion of an *fnr* deletion strain of *E. coli*. These data suggest that HlyX may regulate, rather than mediate, hemolytic activity in *E. coli*, but the possibility that HlyX is both a regulator of gene expression and a hemolysin cannot be excluded.

The gram-negative bacterium *Actinobacillus* (*Haemophilus*) *pleuropneumoniae* can cause an acute and frequently fatal pleuropneumonia in swine (23, 30, 37). The disease is marked by extensive hemorrhagic lesions and fibrin exudation in the lungs (2, 30). Animals which survive the initial infection become chronic carriers with persistent lung lesions (30).

An intact capsule (15), lipopolysaccharide (9, 45), and one or more toxins (19, 28, 34) are thought to play a role in the virulence of *A. pleuropneumoniae*. To date, several different hemolytic and cytotoxic activities have been reported for *A. pleuropneumoniae* serotypes 1 to 12 (6, 10–13a, 16, 17, 19, 25, 34). The best-characterized *A. pleuropneumoniae* toxin is a 105-kilodalton (kDa) protein from serotype 1 strains which has both cytotoxic and hemolytic activities (4–6, 10, 11, 34). The 105-kDa toxin (HlyIA) has been purified (5, 10) from serotype 1 strains, and recently, the gene (*appA*) encoding this protein has been cloned and sequenced from a serotype 5 strain (4). On the basis of the nucleotide sequence and physiochemical data, the 105-kDa toxin of *A. pleuropneumoniae* appears to be related to the *Escherichia coli* alpha-hemolysin family of toxins (4, 6, 10, 13a).

A second hemolysin gene has been cloned from *A. pleuropneumoniae* by two groups, but the protein product has not yet been purified (13, 21). Frey et al. (13) have described the molecular cloning and characterization of a hemolysin gene, *cfp*, that confers a positive CAMP reaction. The *cfp*-encoded protein, CFP (CAMP factor protein), is similar in size and function to protein B from *Streptococcus agalactiae*; however, CFP requires different cofactors. In a parallel study with another serotype 1 strain (CM5), we isolated a recombinant plasmid, A44, with a similar restriction map which also conferred hemolytic activity on *E. coli* (21). This activity could not be neutralized with antisera raised against *S. agalactiae*, hemolytic *E. coli*, or purified streptolysin O, but it could be neutralized with antiserum prepared against A44 and with sera from some convalescent pigs.

In this paper, we report the complete nucleotide sequence of the A44-encoded hemolysin. The region of plasmid A44 which conferred the hemolytic phenotype was initially localized by deletion analysis of the cloned insert, and the nucleotide sequence of the corresponding region was determined. It contained a gene that is very similar to *fnr*, which encodes the global regulatory protein (FNR) of *E. coli* (39). We propose to designate this *A. pleuropneumoniae* "hemolysin" gene *hlyX* to indicate that it may regulate, rather than mediate, hemolytic activity and to reflect the fact that it may be a member of the family of DNA-binding proteins which have a helix-turn-helix motif.

Under anaerobic conditions, FNR activates the expression of a variety of genes for anaerobic respiratory activities, e.g., nitrate and fumarate reductases and pyruvate formate-lyase (14, 22). It also represses the synthesis of NADH hydrogenase II as well as its own synthesis (32, 43). Since *A. pleuropneumoniae* is also a facultative anaerobe (18), the *hlyX*-encoded protein (HlyX) could regulate the expression of similar genes. If so, HlyX may play a role in the persistence of the organism late in acute infection or in chronic infection. The HlyX protein could also play a role in the regulation of genes encoding virulence factors.

MATERIALS AND METHODS

Bacterial strains and plasmids. A gene library of a highly virulent serotype 1 strain of *A. pleuropneumoniae*, CM5, was prepared by cloning size-selected products of a partial *Sau*3A digest in pBR322 (21). A strongly hemolytic recombinant plasmid, A44, was selected, and plasmids pG1 and pG2 were constructed by subcloning a 6.1-kilobase *Hin*dIII fragment in pGEM4 (Promega Biotec, Madison, Wis.). Plasmid T51, which contains an insert of 1,530 base pairs (bp), is a 3′-deletion derivative of pG2 (21). *E. coli* JRG1728 (Δ*fnr*)

* Corresponding author.
† Present address: Urology Division, Department of Surgery, College of Medicine, University of Cincinnati, Cincinnati, OH 45267-0589.

and pGS24, a pBR322 derivative carrying the *fnr* gene, have been described previously (38, 41). These were the generous gifts of J. R. Guest (University of Sheffield, U.K.).

Generation and sequencing of deletion clones. Deletion clones were prepared from pG1 and pG2 by using the Erase-a-Base system (Promega Biotec), as described earlier (21). The limits of the deletions were determined, and the region which conferred hemolytic activity was localized to a segment of approximately 850 bp. Twelve overlapping deletion clones spanning this region were sequenced by using the dideoxynucleotide plasmid-sequencing kit, GEMSEQ K/RT (Promega Biotec), according to the instructions of the manufacturer. Overlapping sequence was obtained from both strands and compiled by using a program in the public domain written by William Schwindinger, Department of Biochemistry, Albert Einstein College of Medicine, Bronx, N.Y. The GenBank data library was searched by using the IFIND program available from the Bionet Resource (Intelligenetics Inc., Mountain View, Calif.). Substitutions of the following groups of amino acids were considered to be conservative: ILVM, RKH, ASTPG, YFW, and NQDE.

Complementation experiments. Glycerol nitrate medium was prepared as described elsewhere (20). Anaerobic cultures were grown at 37°C in 5-ml tubes overlaid with sterile mineral oil. The optical density was measured at 650 nm. The points shown are the averages of duplicate measurements from three independent experiments with a variance of no more than 10%.

RESULTS

The recombinant plasmid, A44, from an *A. pleuropneumoniae* serotype 1 gene library was initially selected because it conferred a hemolytic phenotype on *E. coli*. This property was localized to a segment of approximately 850 bp by deletion analysis. From the size of the region of DNA which encoded the hemolytic activity and the physiochemical properties of that hemolytic activity, it was apparent that the A44 plasmid did not encode the major 105-kDa hemolysin of *A. pleuropneumoniae* nor did it encode a hemolysin resembling other known hemolysins or toxins (3). To gain some insight into the nature of the A44-encoded hemolysin, the region conferring the hemolytic activity was sequenced.

Nucleotide sequence of the *hlyX* gene. The complete nucleotide sequence of a 1,080-bp region was determined (Fig. 1). It has a long open reading frame of 720 bases corresponding to the *hlyX* gene. The open reading frame, which encodes a protein with a predicted molecular mass of 27.1 kDa, is preceded by a putative ribosome-binding site situated 5 bp upstream (positions 175 to 181) of the translation initiation codon, AUG. Downstream, the open reading frame is followed by a region of strong dyad symmetry and a poly(T) tract which resembles a rho-independent terminator (positions 962 to 997). A putative −10 sequence with good homology to the *E. coli* consensus sequence (26) is present upstream of the coding region (positions 135 to 140). A sequence with relatively weak homology to the −35 consensus sequence (26) is located 17 bp upstream from the putative −10 sequence (positions 112 to 117). Consistent with restriction maps published previously, there are unique *Dra*I (positions 137 to 142), *Eco*RI (positions 751 to 756), and *Eco*RV (positions 778 to 783) sites (13, 21). In addition to the 720-bp open reading frame, there are two shorter open reading frames of 195 bp each in this region, but they are not preceded by translation initiation- or promoterlike elements.

A search of GenBank revealed that the *hlyX* gene is very similar to the *fnr* gene of *E. coli* but not to any other gene. The data base was also searched for similarity between the HlyX protein and other proteins, including protein B of *S. agalactiae* (36). Again, the only significant similarity was with FNR. This result was surprising in view of the fact that *fnr* does not confer a hemolytic phenotype on *E. coli*.

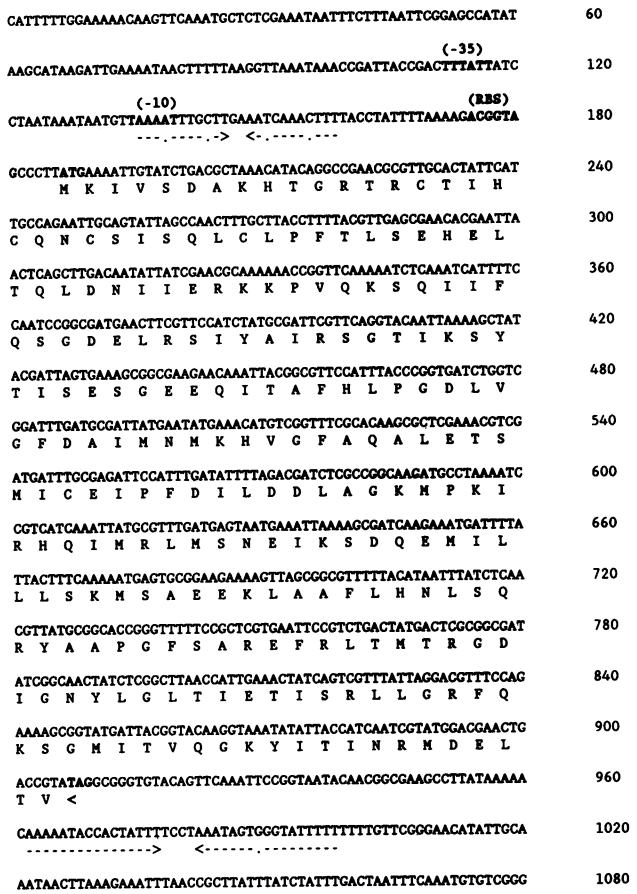

FIG. 1. Nucleotide sequence of the 1.1-kilobase DNA fragment containing the *hlyX* gene. The deduced amino acid sequence of the *hlyX* gene product is indicated. Putative ribosome-binding site (RBS), −10 and −35 sequences, and initiation and stop codons are shown in boldface type. Two regions of dyad symmetry corresponding to the putative FNR (HlyX)-binding site and the rho-independent terminator are underlined. The nucleotide sequence data reported in this paper have been submitted to GenBank and assigned the accession number M34443.

A comparison of the nucleotide sequence of *hlyX* with that of *fnr* revealed that 65% of 720 equivalent bases in the *hlyX*-coding region are identical, despite the difference in the G+C contents of the two genes. The moles percent G+C content of *hlyX* is 41%, which is almost identical to the moles percent G+C reported for *A. pleuropneumoniae* (18) and suggests that *hlyX* is an endogenous gene. The moles percent G+C of *fnr* (50%) is also within the range (48 to 52%) of the moles percent G+C content reported for *E. coli* (31).

The codon usage of the *hlyX* gene is nonrandom, and clear preferences for AT-rich codons are apparent when the gene is compared with the *E. coli fnr* gene (Table 1). In *hlyX*, glycine, isoleucine, and leucine are preferentially coded for by CAA, ATT, and TTA, respectively, whereas CAG, ATC, and CTG are used more frequently in *fnr*. Since this is, to our knowledge, the first endogenous *Actinobacillus* gene which

TABLE 1. Codon usage in *fnr* and *hlyX*

Amino acid	Codon	No. of times used in:		Codon	No. of times used in:	
		hlyX	*fnr*		*hlyX*	*fnr*
Ala	GCT	2	4	GCC	1	6
	GCA	2	3	GCG	8	3
Arg	CGT	11	9	CGC	2	5
	CGA	1	1	CGG	0	2
	AGA	0	0	AGG	0	0
Asn	AAT	6	4	AAC	1	5
Asp	GAT	7	8	GAC	4	5
Cys	TGT	0	2	TGC	5	3
Gln	CAA	10	4	CAG	3	11
Glu	GAA	15	10	GAG	1	5
Gly	GGT	6	10	GGC	7	11
	GGA	2	1	GGG	0	0
His	CAT	6	6	CAC	1	1
Ile	ATT	19	5	ATC	7	17
	ATA	0	1			
Leu	TTA	12	1	TTG	2	2
	CTT	4	6	CTC	3	2
	CTA	0	0	CTG	3	16
Lys	AAA	12	9	AAG	2	5
Met	ATG	12	9			
Phe	TTT	5	3	TTC	5	8
Pro	CCT	2	2	CCC	1	0
	CCA	1	0	CCG	2	5
Ser	TCT	3	1	TCC	3	4
	TCA	2	0	TCG	1	3
	AGT	5	1	AGC	6	7
Thr	ACT	5	3	ACC	3	4
	ACA	2	1	ACG	6	6
Trp	TGG	0	0			
Tyr	TAT	5	3	TAC	0	2
Val	GTT	1	1	GTC	2	1
	GTA	3	2	GTG	0	1

has been sequenced, it is not possible to determine whether the codon usage in *hlyX* is typical.

Similarity between the deduced amino acid sequences of *hlyX* and *fnr*. The deduced protein sequences of *fnr* and *hlyX* are very similar (Fig. 2 and 3A). Overall, 71% of 239 equivalent residues are identical, and an additional 15% are conservatively substituted. This similarity is most marked in the central 218 amino acids of FNR and is especially high in the DNA-binding region of FNR (positions 194 to 217 of HlyX) where 23 of 24 amino acids are identical with a single conservative (isoleucine to valine) substitution (Fig. 3B). This region of the HlyX protein is also similar to the binding regions of CRP, FixK, and other DNA-binding proteins with a helix-turn-helix motif (Fig. 3B).

Four cysteine residues (positions 14, 18, 21, and 27) are present in the amino-terminal region of both HlyX and FNR. In FNR, the N-terminal region and the cysteine residue at position 20 are required for regulation of gene expression by FNR (42). A fifth cysteine residue at position 120 is also conserved. As with FNR, none of the amino acid residues corresponding to those associated with cyclic AMP binding in CRP are conserved (40). Except for very brief stretches at the extreme ends of the proteins, there were no differences in the hydrophobicity profiles of HlyX and FNR.

Comparison of *hlyX* and *fnr* promoter regions. In addition to sharing a high degree of sequence similarity with *fnr* in the coding region of the gene, *hlyX* appears to have comparable regulatory features in the promoter region (Fig. 4). The

```
          .   >     .20       .        .40       .
-MKIVSDAKHTGRTRCTIHCQNCSISQLCLPFTLSEHELTQLDNIIERKKPVQKS  HlyX
 .     ..   . :.:::::::::::::.:::: :::: ::::::::::::.::.
MIPEKRIIRRIQSGGCAIHCQDCSISQLCIPFTLNEHELDQLDNIIERKKPIQKG  FNR

      .60       .        .80       .        .100
QIIFQSGDELRSIYAIRSGTIKSYTISESGEEQITAFHLPGDLVGFDAIMNMKHV  HlyX
: .: .::::.:.:::::::::::::.: :.:::::.:::.:::::::::  .:
QTLFKAGDELKSLYAIRSGTIKSYTITEQGDEQITGFHLAGDLVGFDAIGSGHHP  FNR

 .        .120      .        .140      .        .160
GFAQALETSMICEIPFDILDDLAGKMPKIRHQIMRLMSNEIKSDQEMILLLSKMS  HlyX
.::::::::::.:::::. ::::.:::: .:.:.::::: :::.::.:::::::
SFAQALETSMVCEIPFETLDDLSGKMPNLRQQMMRLMSGEIKGDQDMILLLSKKN  FNR

      .        .180      .        .200      .
AEEKLAAFLHNLSQRYAAPGFSAREFRLTMTRGDIGNYLGLTIETISRLLGRFQK  HlyX
:::.::::. ::: :.:  :::.:::::::::::::::::::.::::::::::::
AEERLAAFIYNLSRRFAQRGFSPREFRLTMTRGDIGNYLGLTVETISRLLGRFQK  FNR

 .220      <
SGMITVQGKYITINRMDELTV
:::..: ::::::.  : :.
SGMLAVKGKYITIENNDALAQLAGHTRNVA
```

FIG. 2. Similarity between the deduced amino acid sequences of *hlyX* and *fnr*. Identical amino acids are indicated by a colon, and positions where there are conservative changes are marked by a period. The positions of the amino acids in HlyX (discounting the initial formyl methionine) are indicated above the sequence. The limits of the conserved central core (amino acids 14 to 231) are indicated by arrowheads. The FNR sequence is based on the original report by Shaw and Guest (39) later emended (Guest, personal communication) to replace serine with cysteine at position 29 in FNR.

putative −10 sequence of *hlyX*, TAAAAT, closely resembles the canonical −10 sequence of *E. coli*, TATAAT (26), and is present at positions identical to the −10 element of *fnr*. Seventeen base pairs upstream of the putative −10 element is a sequence, TTTATT, which has comparatively poor homology with the −35 region consensus sequence, TTGACA (26). A similar sequence, TTACTT, with poorly conserved homology is present in *fnr*.

The promoter region of *hlyX* also contains sequences which are very similar (15 of 18 positions) to the FNR-binding-site consensus sequence (positions 133 to 161; Fig. 1 and 4). This sequence includes a perfect FNR half site, ATCAA (positions 150 to 154). A second region with some

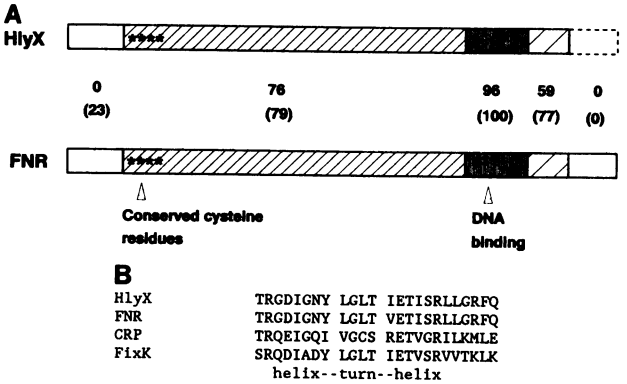

FIG. 3. Percent similarity between FNR and HlyX (A) and comparison of the putative DNA-binding region of HlyX with the corresponding regions of FNR, CRP, and FixK (B). The percentages of identical residues and conservative substitutions (in parentheses) are indicated. FNR and CRP (CAP) sequences are from Shaw and Guest (39), and the FixK sequence is from Batut et al. (1).

```
        (-35)
TTACCGACTTTATTATCCTAATAAATAAT      hlyX
     *****  **   *    ***  **
GCTTAGACTTACTTGCTCCCTAAAAAGAT      fnr

  (-10)
GTTAaaatttgcttgaaatcaaactTTTA      hlyX
******* ***    * *****
GTTaaaa-ttgacaaatatcaattaCGGC      fnr

g---aaa-ttgat----atcaa-ttt--c      FNR consensus (8)
    aa-tgtga------tcaca-tt         CRP consensus (7)

              RBS
CCTATTTTAAAAGACGGTAGCCCTTATG       hlyX
              *      * ****
------------TTGAGCAGACCTATG        fnr
```

FIG. 4. Comparison of the nucleotide sequences in the promoter regions of *hlyX* and *fnr* (39). The FNR-binding consensus sequence of Eiglmeier et al. (8) and the CRP consensus sequence of Ebright et al. (7) are also shown. RBS, Ribosome-binding site.

homology to the FNR-binding consensus sequence (9 of 18 bases) is present at positions 63 to 91 (Fig. 1). The sequence from positions 137 to 158 is also very similar (11 of 14 bases) to the CRP-binding-site consensus sequence; however, the guanosine and cytosine residues demonstrated to be necessary for CRP binding (7) are not conserved in *hlyX* (Fig. 4).

Complementation of an *fnr* mutant with *hlyX*. On nonfermentable carbon sources under anaerobic conditions, wild-type *E. coli* strains are able to use a variety of terminal electron acceptors, such as nitrate, nitrite, or fumarate (14, 22). This mode of energy generation requires the synthesis of the corresponding terminal reductase (e.g., nitrate reductase). Since expression of the genes encoding these proteins requires FNR, *E. coli* strains, such as JRG1728, that cannot make functional FNR are unable to grow under anaerobic conditions in minimal medium. In glycerol-nitrate minimal medium, *E. coli* JRG1728 was able to grow when either pGS24 carrying *fnr* or plasmid T51 carrying *hlyX* was present (Fig. 5). Neither vector, pBR322 (data not shown) or pGEM4, was able to complement the *fnr* mutation. Another plasmid carrying the *hlyX* gene, pG2, was able to induce the expression of a *frd-lacZ* fusion and a *melR-lacZ* fusion preceded by a synthetic FNR site in *E. coli* under anaerobic conditions. The levels of induction were 100 and 25% of those induced by pGS24, respectively (J. R. Guest, personal communication).

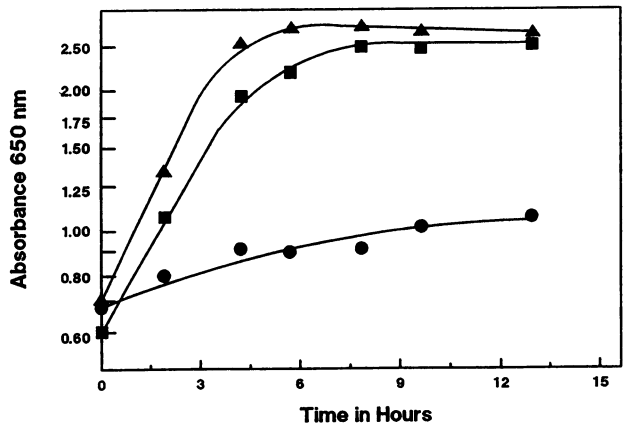

FIG. 5. Growth in glycerol-nitrate medium. *E. coli* JRG1728 (*fnr*) containing the vector pGEM4 (●) or plasmids bearing the *fnr* (pGS24) (▲) or *hlyX* (plasmid T51) (■) gene were grown anaerobically in glycerol-nitrate medium at 37°C.

DISCUSSION

Hemolytic activity associated with *hlyX*. Isolates of all serotypes of *A. pleuropneumoniae* carry one or more hemolytic activities (6, 10–13a, 16, 19, 25, 29, 34). In some cases, the hemolytic activity appears to be mediated by the same molecule that carries cytotoxic activity (6, 16, 34). To date, the genes associated with two distinct hemolysins have been cloned from *A. pleuropneumoniae* (4, 13, 13a, 21). The *appA* (*hlyIA*) gene (4, 13a) encodes the major 105-kDa hemolysin which is similar to *E. coli* alpha-hemolysin. The *hlyX* (*cfp*) gene encodes a smaller protein which is immunologically and physicochemically unrelated to the major hemolysin (13, 21).

Despite the fact that plasmids carrying *hlyX* conferred a hemolytic phenotype on a variety of *E. coli* strains, the sequence of the *hlyX* gene product does not share any homology with that of any known hemolysins or cytotoxins, including protein B from *S. agalactiae* (36). This finding is consistent with the idea that although HlyX could have activity similar to that of protein B, it is a different protein. Alternatively, HlyX could activate the expression of a silent gene in *E. coli* which encodes a product which is similar to protein B. In view of the strong homology with FNR, it seems more likely that HlyX is a regulator of hemolytic activity rather than a hemolysin per se. To distinguish between these possibilities, it will be necessary to purify and sequence the protein which confers the hemolytic activity.

HlyX homology with FNR, CRP, and FixK. The fact that plasmids carrying the *hlyX* gene could complement an *fnr* mutation suggests that the observed similarities between *fnr* and *hlyX* are not merely coincidental. However, that *hlyX* can confer a hemolytic phenotype on *E. coli* and *fnr* cannot suggests that their targets overlap but are not identical.

A comparison of the deduced amino acid sequences of *hlyX* revealed a region virtually identical to the DNA-binding region of FNR with only a single conservative amino acid substitution (Fig. 3B). This single amino acid change may allow HlyX to bind to a slightly different set of genes. These sequences are similar to the DNA-binding region of CRP (40) and to the comparable region of the *fixK* gene from *Rhizobium meliloti* (1). The genes regulated by FixK and CRP are very different from those regulated by FNR, and the signals for activation are also different.

Unlike CRP or FixK, both FNR and HlyX activate gene expression under anaerobic conditions and both FNR and HlyX contain a cysteine-rich amino-terminal region. This region is essential for FNR-regulated gene expression (42, 44). Experiments are in progress to determine whether the conserved cysteine residues play a comparable role in HlyX. These data suggest that FNR, HlyX, FixK, and CRP may have a common evolutionary origin, with the central core being required for DNA binding and altered specificity of activation being associated with the divergent termini of the molecules.

Comparison of the *hlyX* and *fnr* regulatory regions. The upstream region of the *hlyX* gene has sequences which are virtually identical to those of regulatory elements present in *fnr*. The putative −10 sequence of *hlyX* is preceded 2 bp upstream by a G residue. The presence of this residue has been shown to contribute greatly to promoter strength, especially in cases in which the −35 sequences are not well conserved (8, 33). The −10 sequence of *hlyX* overlaps a

region which is very similar (15 of 18 positions) to the FNR-binding consensus sequence. The level of similarity is comparable to that of FNR-regulated genes such as *frdA*, *dmsA*, *narG*, and *nirB* (8). A second, less-well-conserved, FNR-binding sequence is present upstream of the −10 element. Its significance is not known, but a similar sequence is also present in *fnr*.

Possible role of HlyX in virulence. In view of the fact that *A. pleuropneumoniae* is a facultative anaerobe which can reduce nitrates (18), it is tempting to speculate that *A. pleuropneumoniae* has a hierarchical system, similar to that found in *E. coli*, for the regulation of gene expression in the presence of reduced O_2 levels. Such a system could play an important role in gene expression when *A. pleuropneumoniae* is in O_2-poor sites such as fibrinous lesions in the lungs or in the tonsular crypts. HlyX might also play a role in pathogenesis in a way which is analagous to, for example, the *toxR* locus in *Vibrio cholerae* (27) or to the *bvg* locus in *Bordetella pertussis* which is required for the expression of several virulence factors, including a hemolysin (35). It is possible that *hlyX* is, in a more general sense, related to regulators of stress-regulated genes which affect expression by mediating changes in DNA supercoiling (29). Experiments are currently under way to expand our studies in *E. coli* and to initiate studies on the regulation of gene expression by HlyX in *A. pleuropneumoniae*.

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