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The FNR family of transcriptional regulators

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Abstract

Homologues of the transcriptional regulator FNR from *Escherichia coli* have been identified in a variety of taxonomically diverse bacterial species. Despite being structurally very similar, members of the FNR family have disparate regulatory roles. Those from *Shewanella putrefaciens, Pseudomonas aeruginosa, Pseudomonas stutzeri* and *Rhodopseudomonas palustris* are functionally similar to FNR in that they regulate anaerobic respiration or carbon metabolism. Four rhizobial proteins (from *Rhizobium meliloti, R. leguminosarum, B. japonicum* and *Azorhizobium caulinodans*) are involved in the regulation of nitrogen fixation; a fifth (from *Rhizobium* strain IC3342) has unknown function. Two proteins from mammalian pathogens (*Actinobacillus pleuropneumoniae* and *Bordetella pertussis*) may be involved in the regulation of toxin expression. The FNR protein of *Vibrio fischeri* regulates bioluminescence, and the function of the one known FNR homologue from a Gram-positive organism (*Lactobacillus casei*) remains to be elucidated. Some members of this family, like FNR itself, appear to function as sensors of oxygen availability, whereas others do not. The ability to sense and respond to oxygen limitation may be correlated with the presence of cysteine residues which, in the case of FNR, are thought to be involved in oxygen or redox sensing. The mechanism of DNA sequence recognition is probably conserved, or very similar, throughout this family. In a number of other Gram-negative species, there is good indirect evidence for the existence of FNR analogues; these include *Alcaligenes eutrophus, A. denitrificans, A. faecalis, Paracoccus denitrificans* and a number of *Pseudomonas* species.

Introduction

Facultatively anaerobic bacteria and many obligate aerobes are able to adapt rapidly to changes in the availability of oxygen in their environment. In facultative anaerobes such as *Escherichia coli,* short term adaptation involves regulation of the activities of particular enzymes, for example pyruvate dehydrogenase and pyruvate formate lyase, which play critical roles in aerobic and anaerobic carbon metabolism, respectively. Longer term adaptation involves regulating the expression of energy generating pathways such that the most efficient operates for a given growth condition. In *E. coli,* several regulatory systems have been characterised which are involved in the adaptive responses to aerobic and anaerobic growth (reviewed by Iuchi & Lin 1993). One mechanism involves the transcriptional activator and repressor FNR, the major role of which is to regulate the expression of genes involved in anaerobic respiration and pyruvate metabolism (Spiro & Guest 1991). In other enteric and non-enteric bacteria, adaptive responses to changes in oxygen availability have a wider significance. For example, both nitrogen fixation and anoxygenic photosynthesis are processes which are regulated by environmental oxygen. Disease causing organisms, whether plant or animal pathogens, often live in environments which are anoxic or microoxic. There can be little doubt that responses to anoxia are important in these organisms, though this is an area which has only recently begun to receive attention. Given the general importance of adaptive responses to oxygen limitation, it is not surprising that regulatory mechanisms have been sought and found in many bacterial species. Systems analogous to FNR from *E. coli* appear to be quite common and have been characterised in a taxonomically

Table 1. The FNR protein and its known relatives.

Protein	Organism	Regulates	Reference
FNR	*Escherichia coli*	anaerobic respiration	Spiro & Guest (1991)
EtrA	*Shewanella putrefaciens*	anaerobic respiration	Saffarini & Nealson (1993)
FNR	*Vibrio fischeri*	luminescence	U. Winkler, pers. comm.
HlyX	*Actinobacillus pleuropneumoniae*	haemolysin biosynthesis?	MacInnes et al. (1990)
BTR	*Bordetella pertussis*	haemolysin biosynthesis?	Bannan et al. (1993)
ANR	*Pseudomonas aeruginosa*	denitrification, arginine fermentation, cyanide synthesis	Zimmermann et al. (1991); Sawers (1991)
FnrA	*Pseudomonas stutzeri*	arginine fermentation	Cuypers & Zumft (1993)
FixK	*Rhizobium meliloti*	nitrogen fixation	Batut et al. (1989)
FnrN	*Rhizobium leguminosarum*	nitrogen fixation	Colonna-Romano et al. (1990)
FixK	*Bradyrhizobium japonicum*	nitrogen fixation, denitrification	Anthamatten et al. (1992)
FixK	*Azorhizobium caulinodans*	nitrogen fixation	Kaminski et al. (1991)
orf4 product	*Rhizobium* IC3342	not known	Upadhyaya et al. (1992)
AadR	*Rhodopseudomonas palustris*	aromatic acid metabolism	Dispensa et al. (1992)
FLP	*Lactobacillus casei*	not known	Irvine & Guest (1993)

diverse range of organisms, particularly in members of the Pseudomonadacae and Rhizobiaceae. The purpose of this article is to review current knowledge of FNR-like proteins and their regulatory roles in non-enteric bacteria.

Study of the FNR protein of *Escherichia coli* has led to a good understanding of its role in regulating anaerobic metabolism and the nature of its interactions with DNA and the transcriptional apparatus. However, the mechanism by which the FNR protein senses and responds to changes in oxygen availability remains rather poorly understood. Proteins related to FNR are involved in regulating rather disparate aspects of bacterial physiology (Table 1). In general, evidence for the presence of FNR-like proteins is of two sorts: homologues of the *E. coli fnr* gene have been isolated from a number of species, and characterised at the level of primary structure (Table 1). In other cases the evidence is at present only indirect, and involves the identification of DNA sequences potentially recognised by FNR (subsequently referred to as FNR boxes), observations on the behaviour of heterologous promoters that are consistent with the presence of an FNR homologue, or hybridisation to the *fnr* homologue of another organism (Table 2). This review shall describe FNR homologues in bacteria other than *E. coli*, focusing on the roles that these proteins play in regulating cellular physiology, and shall consider some of the conserved structural features in this family of regulatory proteins.

It is perhaps necessary to define what is meant by an FNR homologue, or FNR-like protein. This is in fact not straightforward because of the close similarity between FNR and the cyclic AMP receptor protein (CRP) and its relatives. If a protein is related to both FNR and CRP then on what basis should it be designated a CRP homologue, or an FNR homologue? There are a number of possible ways to resolve this question. A protein which is related to both FNR and CRP may be assigned to the FNR family on the basis of:

- an ability to complement an *fnr* mutation in *E. coli*, or its equivalent in another organism;
- a greater degree of sequence identity to FNR than to CRP;
- having the predicted DNA-binding specificity of FNR rather than that of CRP;
- failing to respond to cAMP, or lacking amino acids which are involved in liganding cAMP;
- responding to the same physiological signal as FNR, namely oxygen limitation or
- retaining some or all of the cysteine residues of FNR which are thought to be involved in its response to oxygen limitation, and which are absent from CRP.

A phylogenetic analysis of the entire FNR/CRP family suggests that distinctions drawn between FNR- and CRP- homologues using these criteria are, to a large extent, meaningful (Fig. 1). Most of the proteins described herein are clearly FNR rather than CRP homologues according to more than one of the crite-

Table 2. Species for which there is indirect evidence for the presence of an FNR homologue.

Organism	Evidence	Reference
Pseudomonas G-179	F	Ye et al. (1993)
Pseudomonas fluorescens	P	Zimmermann et al. (1991)
Pseudomonas putida	P	Zimmermann et al. (1991)
	H	Sawers (1991)
Pseudomonas syringae	P	Zimmermann et al. (1991)
Pseudomonas mendocina	P	Zimmermann et al. (1991)
Pseudomonas alcaligenes	H	Sawers (1991)
Rhodobacter sphaeroides	F	Neidle and Kaplan (1993)
Alcaligenes eutrophus	F	Zumft et al. (1993)
Alcaligenes faecalis	F	Nishiyama et al. (1993)
Alcaligenes denitrificans	F	Hoitink et al. (1990)
Paracoccus denitrificans	P	Spiro (1992)
	H	A. Hinsley and S. Spiro, unpublished
Vitreoscilla sp.	F	Khosla and Bailey (1989)

The evidence is of three sorts: F, the presence of FNR boxes in promoters which might reasonably be expected to be subject to regulation by an FNR homologue; H, positive hybridisation to the cloned *fnr* homologue of another organism; P, a known FNR sensitive promoter from another organism shows a pattern of expression (i.e. anaerobic activation) that is consistent with the presence of an FNR homologue.

ria mentioned above, and the phylogenetic analysis (with the possible exception of the *L. casei* FLP protein, see below). Each is predicted to have the same, or a very similar, DNA-binding specificity as that of FNR, as judged by the structures of their DNA-binding domains. Some however, are not themselves sensitive to changes in oxygen availability and for that reason may not, strictly, be analogous to FNR. So, all of the proteins discussed in this review are FNR homologues, and they are FNR analogues with respect to predicted DNA-binding specificity, though not necessarily with respect to signalling mechanisms. Proteins which are homologous to FNR but are clearly not analogous (ie CRP and its relatives) are not discussed, but are included in the phylogenetic analysis (Fig. 1).

Occurrence and regulatory roles of FNR-like proteins

For organisms where there is either direct or indirect evidence for the presence of an FNR homologue, the role (or potential role) of that protein is discussed.

Shewanella putrefaciens

Shewanella (formerly *Alteromonas*) *putrefaciens* is a Gram-negative bacterium, closely related to members of the family Vibrionaceae, which can couple anaerobic energy generation to the respiration of fumarate, nitrate, nitrite, Fe (III), Mn (IV), trimethylamine *N* -oxide (TMAO), dimethylsulphoxide (DMSO), thiosulphate and sulphite (Myers & Nealson 1990; Kita-Tsukamoto et al. 1993). Anaerobic growth on fumarate, nitrite, Fe (III), TMAO, DMSO, thiosulphate and sulphite is dependent on a gene designated *etrA* which encodes a protein very similar to FNR (Fig. 1; Saffarini & Nealson 1993). The EtrA protein is functionally as well as structurally similar to FNR, given that its major role appears to be in the regulation of anaerobic respiration. Anaerobic growth on nitrate and Mn (IV) is independent of *etrA* and hybridisation experiments have shown that *S. putrefaciens* may contain a second *etrA* -like gene (Saffarini & Nealson 1993). Thus, it is possible that this organism expresses two FNR homologues which have discrete regulatory functions; a similar situation occurs in *Pseudomonas stutzeri* and *Bradyrhizobium japonicum* (see below).

Vibrio fischeri

The expression of luciferase in *Vibrio fischeri* is regulated by a number of environmental factors including carbon source and oxygen tension. Microaerobic growth conditions are known to favour optimal expression of the luciferase genes (Nealson, 1977). Expression of the *V. fischeri lux* regulon has been studied in *E. coli* and found also to be maximal under microaerobic or anaerobic growth conditions. Expression is greatly reduced (microaerobically) or abolished (anaerobically) in an *fnr* mutant (Müller-Breitkreutz & Winkler 1993). The identification of an FNR box in the regulatory region of the *lux* regulon supports the notion that FNR is involved in the regulation of luminescence (Fig. 2; U. Winkler, personal communication). The *fnr* gene of *V. fischeri* has recently been sequenced and found to encode a protein very similar to FNR of *E. coli* (Fig. 1; U. Winkler, pers. comm.).

Actinobacillus pleuropneumoniae and Bordetella pertussis

Actinobacillus pleuropneumoniae is a Gram-negative bacterium belonging to the family Pasteurellaceae and

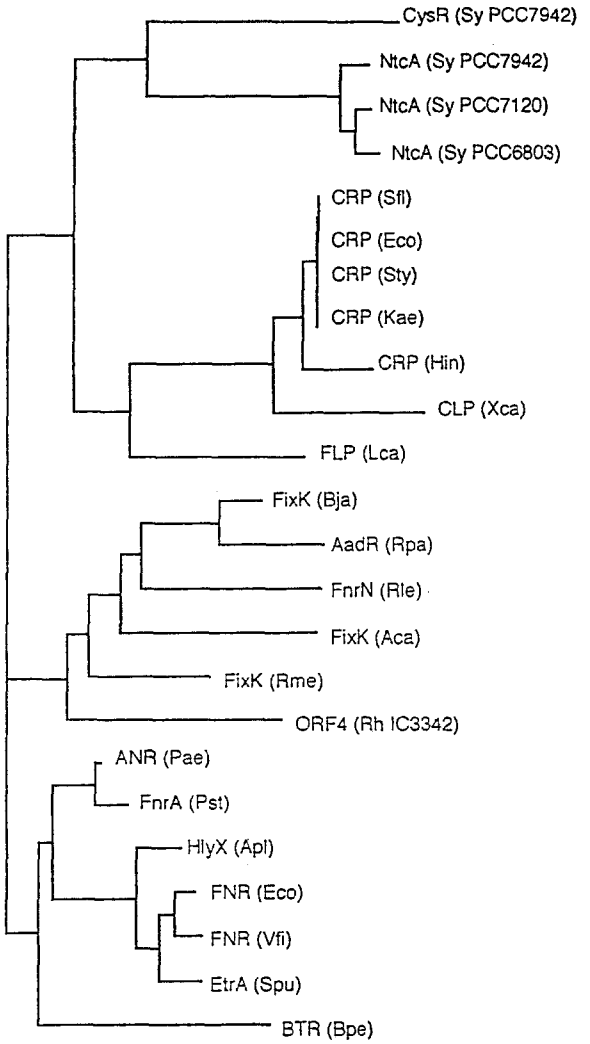

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Fig. 1. Phylogenetic relatedness of all known members of the CRP/FNR family of transcriptional regulators, derived from an alignment similar to that shown in Figure 3, but including 10 members of the cyclic AMP receptor (CRP) family of proteins: CRPs from *E. coli* (*Eco,* Accession Number P03020), *Salmonella typhimurium* (*Sty,* P06170), *Shigella flexneri* (*Sfl,* M13772), *Klebsiella aerogenes* (*Kae,* P29282) and *Haemophilus influenzae* (*Hin,* P29281); CLP, a CRP-like protein from *Xanthomonas campestris* (*Xca,* P22260), the nitrogen regulatory protein NtcA from *Synechococcus* (*Sy*) strains PCC 7942 (X60197) and PCC 6803 (X71607) and from *Anabaena* (*An*) strain PCC7120 (X71608) and the sulphur regulatory protein CysR from *Synechococcus* strain PCC 7942 (P27369). Other species abbreviations are: *Lca, Lactobacillus casei; Bja, Bradyrhizobium japonicum; Rpa, Rhodopseudomonas palustris; Rle, Rhizobium leguminosarum; Aca, Azorhizobium caulinodans; Rme, Rhizobium meliloti; Rh* IC3342, *Rhizobium* strain IC3342; *Pae, Pseudomonas aeruginosa; Pst, Pseudomonas stutzeri; Apl, Actinobacillus pleuropneumoniae; Vfi, Vibrio fischeri; Spu, Shewanella putrefaciens* and *Bpe, Bordetella pertussis.* The Figure shows an unrooted phylogenetic tree estimated by distance matrix and neighbour-joining methods using the programs PROTDIST, NEIGHBOUR and DRAWTREE of the PHYLIP package, version 5.3c (Felsenstein 1993). The programs were accessed through the HGMP Resource Centre, Clinical Research Centre, Harrow. Relative branch lengths are as determined by the neighbour-joining program.

is the causative agent of porcine pleuropneumonia. Isolates of *A. pleuropneumoniae* express a number of different haemolytic and cytotoxic activities. A DNA fragment that was isolated on the basis of its ability to confer haemolytic activity on *E. coli* was subsequently found to contain a gene (designated *hlyX*) homologous to *fnr* (MacInnes et al. 1990). This open reading frame was the sole requirement for haemolytic activity in *E. coli.* It was suggested that the *hlyX* gene encodes either a haemolysin, or a protein that activates expression of a haemolysin gene in *A. pleuropneumoniae* and of a normally silent haemolysin gene in laboratory strains of *E. coli.* The *hlyX* gene complements an *E. coli fnr* mutant with respect to anaerobic growth on non-fermentable carbon sources, and regulates FNR-sensitive promoters in response to anoxia (MacInnes et al. 1990; Green et al. 1992; Soltes & MacInnes 1994). Activation of haemolytic activity in *E. coli* by HlyX requires anaerobic growth conditions (Soltes & MacInnes 1994). There is however no haemolytic activity associated with expression of the *E. coli fnr* gene. The HlyX protein has been purified and shown not to have an intrinsic haemolytic activity (Green et al. 1992). The available evidence therefore supports the notion that HlyX activates the expression of a latent haemolysin gene in *E. coli*, and that the failure of FNR to activate the expression of that gene is a consequence, in part, of subtle differences between the properties of HlyX and FNR (Green et al. 1992).

A gene (designated *btr*) has been isolated from *Bordetella pertussis,* the causative agent of whooping cough, which also confers haemolytic activity on *E. coli* (Bannan et al. 1993). The *btr* gene is homologous to *fnr* and *hlyX,* and complements an *E. coli fnr* mutant. A *btr* mutant of *B. pertussis* has been constructed and has no detectable phenotype under either aerobic or microaerobic growth conditions (Bannan et al. 1993). It is possible that the HlyX and BTR proteins play roles in pathogenicity, in the oxygen depleted environment of host tissues (although *B. pertussis* is usually described as being an obligate aerobe); there is as yet no evidence to support this possibility.

It is of interest to examine the sequence alignment of FNR, HlyX and BTR (Fig. 3) in search of fea-

E. coli consensus			-a--TTGAT--a-ATCAAt---
Vfi		*luxR*	CCAATTTATTAGAATCAAATGT
Apl		*hlyX*	AAATTTGCTTGAAATCAAACTT
Pst		*nosR*	AGTCTTGATTGCAATCAAGGTA
Pst		*nirS*	AAGCTTGATTGCGATCAAGTCC
Pst		*nirS*	CTCTTTGATTGCCGTCAAGCGC
Pst	(JM300)	*nir*	AAGCTTGATTACGGTCAAGTCC
Pst	(JM300)	*nir*	ACTCTTGACTGCCATCAAGCGC
Pst		*nirM*	TGATTTGATTGCAATCAAGGAA
Pst		*orf8*	GGACTTGATCGCAATCAAGCTT
Pst		*norC*	TTTCTTGATTGCCATCAAGCTT
Pae		*arcDABC*	GCTATTGACGTGGATCAGCATT
Pae		*denAB*	AATCTTGATTCCGGTCAAGCAA
Pae		*azu*	GGGTTTGACCTGAATCAGTGGA
Ps	*G-179*	*nirU*	CGCCTTGATGAAAATCAAATTT
Ade		*azu*	GGGATTGATGTCCGTCAATAGC
Afa		*azu*	CTGTTTGATCCAGATCAAAGAG
Afa		*nir*	CACCTTGATCGCAGTCAAGGAA
Aeu		*nosZ*	CCTTTTGATTCGAGTCAAGTTC
Rme		*fixK*	CTTAGTGATCTAACCCAATTTC
Rme		*fixN*	GCACTTGATCTGGATCAAGGTG
Rme		*fixLJ*	TACATTGATCACGGTCAATACT
Rme		*fixGHJI*	AGACTTGACGCAGATCAAGGTG
Rle		*fnrN*	CGCTTTGATCTAGATCAAACAG
Rle		*fnrN*	GAAATTGATAATCCTCAAGCGG
Rle		*nifA*	AGACTTGATGAAACACAAAGAT
Bja		*fixK*	AGAATTGATCTGGGTCAACCGC
Aca		*nifA*	AAATTTGATCCAGATCAAAGCC
Rsp		*hemA*	GGATTTGATCCTTATCAAGGCC
Rpa		*aadR*	GACCTTGATCTGTGTCAAGCTG
Vi		*vhb*	AGTTTTGATGTGGATTAAGTTT
non-*E. coli* consensus			----TTGAT----ATCAAg---

Fig. 2. Compilation of potential binding sites for FNR homologues. Sequences were selected on the basis of their similarity to the core of the *E. coli* FNR binding site consensus (TTGAT—ATCAA), and if there is good reason to believe that the promoter concerned might be regulated by an FNR homologue. Sequences which match only half of the consensus are excluded from this analysis, since the physiological significance of such sites is not clear. The *E. coli* consensus is derived from a compilation of 14 known FNR binding sites. A consensus sequences derived here for non-enteric FNR boxes is based on the occurrence of a particular base in at least half of the sites. The core motif in each consensus is shown in capitals. Species abbreviations are as in Figure 1, and: *Pst, Pseudomonas stutzeri* (strain ZoBell); *Pst* (JM300), *P. stutzeri* (strain JM300); *Ps* G-179, *Pseudomonas* strain G-179; *Ade, Alcaligenes denitrificans; Afa, A. faecalis; Aeu, Alcaligenes eutrophus; Rsp, Rhodobacter sphaeroides; Vi, Vitreoscilla* sp. Details and references are in the text.

tures which might allow HlyX and BTR to activate the expression of an *E. coli* gene which is not activated by FNR. There are no obvious features of the DNA binding domains which might offer an explanation. However, Gln-84 of FNR is replaced by serine in both HlyX and BTR, but in no other member of this family (Fig. 3). This residue is in the so-called positive patch of FNR that is predicted to interact with RNA polymerase (Williams et al. 1991). It is possible that a serine residue at this position allows HlyX and BTR to activate transcription at a promoter at which FNR is unable so to do.

The Pseudomonadacae

Although often regarded as being obligate aerobes, a number of the Pseudomonads are able to adapt to microaerobic or anaerobic growth conditions. For example, *Pseudomonas aeruginosa* grows rapidly anaerobically using nitrate or nitrite as the terminal electron acceptor, and can ferment arginine, *via* the arginine deiminase pathway, as a means of anaerobic energy generation. Some FNR-dependent promoters from *E. coli* are active and anaerobically inducible in *P. aeruginosa*, and FNR boxes are associated with the *P. aeruginosa* genes encoding the arginine deiminase pathway enzymes (*arcDABC*) and azurin (*azu*), a redox protein associated with denitrification (Fig. 2; Lodge et al. 1990; Galimand et al. 1991; Hoitink et al. 1990). The promoter of the *P. aeruginosa* operon (*denAB*) which encodes cytochrome cd_1 nitrite reductase and its electron donor cytochrome c_{551} has been studied in some detail. The promoter, which has an FNR box in its vicinity, is activated some 20-fold by anaerobic growth (Fig. 2; Arai et al. 1991). The FNR homologue from *P. aeruginosa* (ANR) regulates arginine fermentation, nitrate respiration and cyanide synthesis, and *anr* (formerly *nirD*) mutants are defective in these processes. The ANR protein is oxygen sensitive, is structurally very similar to FNR, and the cloned *anr* gene complements an *E. coli fnr* mutant (Zimmermann et al. 1991; Sawers 1991; Galimand et al. 1991). Galimand et al. (1991) have drawn attention to a number of other FNR boxes upstream of *P. aeruginosa* genes, including one around the transcription start site of the *plcS* gene encoding phospholipase C. The position of this site suggests that ANR may act as a repressor, as does the *E. coli* FNR protein in some cases.

The characterisation of genes and proteins required for denitrification is well advanced in the case of *Pseudomonas stutzeri.* The expression of denitrification enzymes in this organism is clearly activated by anoxia at the level of biosynthesis (Körner & Zumft 1989). Sequences resembling FNR boxes are located in the promoter regions of the *nirS* and *nirM* genes encoding a cytochrome cd_1 nitrite reductase and cytochrome c_{551} respectively, the *norC* gene encoding a subunit of the nitric oxide reductase and a possible regulatory gene, *orf8* (Fig. 2; Jüngst et al. 1991; Cuypers & Zumft 1992). In two strains of *P. stutzeri* (JM300

and ZoBell) there are two FNR boxes in the upstream region of *nirS;* in strain JM300 the two sites are 55 base pairs further apart than in strain ZoBell (Fig. 2; Jüngst et al. 1991; Smith & Tiedje 1992).The *nosR* gene which encodes a protein required for the expression of an active nitrous oxide reductase also contains an FNR box in its promoter region (Fig. 2; Cuypers et al. 1992). It is perhaps surprising that FNR boxes are not apparent in the promoter region of the nitrous oxide reductase structural gene, although perfect half sites can be found (Viebrock & Zumft 1988). The denitrification genes are clearly regulated in a complex fashion in *P. stutzeri,* with both anoxia and nitrogen oxides having roles in that regulation. Maximal expression of the nitrate, NO and N_2O reductases requires anoxia and the presence of nitrate, whereas nitrite reductase is expressed at higher levels in anaerobic cultures grown with nitrite rather than nitrate. Anaerobic growth in the presence of N_2O as the sole N-oxide allows significant expression of nitrate and N_2O reductases but no expression of nitrite reductase (Körner & Zumft 1989; Körner 1993). The sequence of an *fnr* -like gene (designated *fnrA*) from *P. stutzeri* (strain ZoBell) has recently been determined (Cuypers & Zumft 1993). Apparently FnrA regulates arginine fermentation, but not denitrification in *P. stutzeri* , since an *fnrA* mutant is defective only in the former. An *E. coli* FNR-regulated promoter remains anaerobically inducible in the *fnrA* mutant of *P. stutzeri*, which raises the possibility that a second FNR homologue is involved in the regulation of denitrification genes (Cuypers & Zumft 1993).

Denitrifying bacteria can be distinguished according to the expression of a copper-containing or cytochrome cd_1-type nitrite reductase. Unlike *P. aeruginosa* and *P. stutzeri*, *Pseudomonas* G-179 expresses a copper-containing enzyme. Two FNR boxes have been indicated in the upstream region of the structural gene (*nirU*) for the copper-containing nitrite reductase of *Pseudomonas* G-179 (Fig. 2; Ye et al. 1993), although only one of them has the correct spacing between the two half-sites. This potential FNR box is more than 300 base pairs upstream of the start codon of *nirU,* and is located 5' to two potential σ^{54}-type promoters (Ye et al. 1993). Understanding of the significance, if any, of these sequences must await expression studies.

A *P. aeruginosa* ANR-dependent promoter has been introduced into a number of other *Pseudomonas* species. This promoter was active and anaerobically inducible in a range of species from the rRNA homology group I (*P. fluorescens, P. putida, P. syringae* and *P. mendocina*) but not in a representative of rRNA homology group II, *P. cepacia.* Thus, it seems likely that the group I species express FNR homologues (Zimmermann et al. 1991). There is also evidence from hybridisation studies for the existence of FNR homologues in *P. putida* and *P. alcaligenes* (Sawers 1991).

The Rhizobiaceae

Nitrogenases from all diazotrophs, whether free-living or symbiotic, are sensitive to oxygen and these organisms have evolved a variety of strategies to protect nitrogenase from oxygen damage. In many instances this involves regulating the expression of nitrogen fixation (*nif* and *fix*) genes according to the environmental concentration of oxygen. In the case of the free-living enteric diazotroph *Klebsiella pneumoniae*, the expression of *nif* genes is known to be independent of FNR (Hill, 1985). In symbiotic diazotrophs of the family Rhizobiaceae the expression of nitrogen fixation genes is regulated by complex cascade mechanisms which include FNR-like proteins. In *R. meliloti,* low oxygen tensions are sensed by the trans-membrane haemoprotein FixL, which autophosphorylates before phosphorylating the transcriptional regulator FixJ (Gilles-Gonzalez et al. 1991). Phosphorylated FixJ activates expression of the *fixK* gene, and of the *nifA* gene which is the major regulator of symbiotic nitrogen fixation. The FixK protein activates expression of the *fixN* gene which is required for symbiotic nitrogen fixation; thus *fixK* mutants are unable to fix nitrogen, but are unaffected in the expression of a respiratory nitrate reductase (Batut et al. 1989). The FixK protein also has a negative effect on expression of *nifA* and the *fixK* gene itself (Batut et al. 1989; Waelkens et al. 1992). Deletion analysis of the *fixK* promoter has identified an FNR box which is required for autoregulation (Waelkens et al. 1992). The *fixN* promoter also has an FNR box, and is active in anaerobic cultures of *fnr* $^+$ strains of *E. coli* (Fig. 2; Cherfils et al. 1989). However, the available evidence indicates that *R. meliloti* FixK is not a direct sensor of oxygen tension but is an intermediary component of a complex regulatory cascade. Moreover, it is not clear whether the activity of FixK is modulated in any way, or whether activation of *fixK* expression by the FixJ protein is the principal event in that branch of the regulatory cascade.

The FnrN protein of *R. leguminosarum* is functionally similar to FixK of *R. meliloti* in that it activates expression of *fixN*, and *fnrN* mutants have reduced nitrogenase activity (Colonna-Romano et al. 1990).

However, FnrN appears to be sensitive to oxygen, indeed when expressed in *E. coli,* FnrN behaves rather like the FNR protein itself (Schlüter et al. 1992). These differences in the activities of FixK and FnrN are correlated with differences in their primary structures (see below). In *Bradyrhizobium japonicum* the expression of the *fixK* gene is also activated by the FixLJ proteins in response to microoxic conditions (Anthamatten et al. 1992). The *B. japonicum* FixK protein is active in *E. coli* where it is apparently sensitive to oxygen (Anthamatten et al. 1992). Unlike *R. meliloti* and *R. leguminosarum, fixK* mutants of *B. japonicum* are unaffected in their ability to fix nitrogen. To account for this observation, it has been suggested that *B. japonicum* has two copies of the *fixK* gene, although the two are not sufficiently similar to be detectable by DNA hybridisation (Anthamatten et al. 1992). The two FixK proteins are hypothesised to be involved in the activation of expression of genes involved in nitrogen fixation and denitrification. However, the relative contribution of each protein remains to be elucidated, and the putative second copy of *fixK* has not yet been characterised.

A *fixK* gene which is subject to positive regulation by FixL and FixJ has also been characterised in *Azorhizobium caulinodans.* A *fixK* mutant of *A. caulinodans* does not fix nitrogen symbiotically and shows a greatly reduced nitrogenase activity (Kaminski et al. 1991). In contrast to the situation in *R. meliloti,* the *A. caulinodans* FixK protein acts as a positive regulator of microaerobic *nifA* expression (Kaminski et al. 1991). It is not known whether FixK regulates other *nif* and *fix* genes in this organism. There is no information to indicate whether FixK is an oxygen sensor in *A. caulinodans*, although the structure of the protein suggests that, as for *R. meliloti,* this may not be the case.

One further rhizobial FNR homologue has been described, for which a precise physiological function remains uncertain. The strain *Rhizobium* IC3342 induces a leaf curl syndrome in the pigeon pea *Cajanus cajun.* A cluster of genes that are required for leaf curling has been characterised, and localised to one of the Sym (symbiotic) plasmids of *Rhizobium* IC3342. One of the genes of this cluster (*orf4*) predicts a protein which is 29% identical to FNR (Fig. 3; Upadhyaya et al. 1992). Only a single transposon insertion has been isolated in this cluster and it is in another open reading frame which would appear not to be co-transcribed with *orf4*. Hence the precise role of the *orf4* -encoded protein of *Rhizobium* IC3342 is uncertain.

Homologues of the *E. coli* FNR protein are widespread, perhaps ubiquitous, amongst the Rhizobiaceae. In all cases where a function has been clearly ascribed, FNR-like proteins are involved in regulating microaerobic nitrogen fixation, as one component of a complex regulatory cascade, the precise roles of these proteins differing to some extent from one species to another. The expression of the Rhizobial FNR protein is microaerobically inducible in every case which has been studied, some of the proteins are themselves sensitive to oxygen.

Purple nonsulphur bacteria

The purple nonsulphur bacteria are metabolically highly versatile, and can generate energy from photosynthesis under anaerobic growth conditions. Some members of this group can also grow anaerobically in the dark, using fermentative or respiratory metabolism. Oxygen limitation is the primary signal which activates expression of the photosynthetic apparatus in the purple nonsulphur bacteria. The genes encoding components of the light harvesting and reaction centre complexes are induced by low oxygen tensions, as are the genes required for bacteriochlorophyll synthesis, though to a lesser extent (reviewed by Klug 1993). *Trans*-acting factors involved in this regulation are currently being characterised, and probably belong to the two-component family of regulatory proteins (Klug 1993). There is little evidence to implicate FNR-like proteins in the anaerobic induction of the expression of the photosynthetic apparatus. A possible FNR box has been pointed out in the *puc* operon (encoding components of a light-harvesting complex) of *Rhodobacter sphaeroides,* although it is not a very strong match to the consensus, and there is little evidence for it being functional (Lee & Kaplan 1992).

The *hemA* gene of *R. sphaeroides* encodes one of two isoenzymes of 5-aminolevulinic acid synthase and produces a transcript which is threefold more abundant under anaerobic growth conditions (Neidle & Kaplan 1993). The *hemA* promoter region includes a perfect FNR box which is centred on the transcription start site (Fig. 2; Neidle & Kaplan 1993). The FNR box is therefore in a position which is not indicative of anaerobic activation of transcription by an FNR-like protein and understanding of its role must await further study.

An FNR-like protein has been described in one other member of this group of organisms, and on taxonomic grounds it may be expected that others will follow. *Rhodopseudomonas palustris* is able to degrade benzoate under anaerobic growth conditions.

Strains mutant in a gene designated *aadR* are unable to grow photoheterotrophically on 4-hydroxybenzoate and grow very slowly on benzoate, but grow normally on malate and retain the ability to fix nitrogen. The product of the *aadR* gene is structurally related to FNR, and is proposed to be involved in regulating the biosynthesis of benzoate-CoA ligase and aromatic acid-CoA ligase II, which are required for benzoate and 4-hydroxybenzoate catabolism respectively (Dispensa et al. 1992). It is tempting to speculate that other members of the purple nonsulphur bacteria may have FNR homologues, which are involved not in the regulation of photosynthesis, but in other aspects of anaerobic metabolism such as nitrate respiration and degradation of aromatic compounds.

Other Gram-negative bacteria

In a number of other species of Gram-negative bacteria there is indirect evidence for the presence of FNR homologues, usually from the observation of FNR boxes in the upstream regions of genes involved in denitrification (Fig. 2). This is the case for the structural gene (*nosZ*) for nitrous oxide reductase in *Alcaligenes eutrophus,* which also has a σ^{54}-type promoter (Zumft et al. 1992). Denitrification is known to be dependent on σ^{54} in *A. eutrophus* (Römermann et al. 1989), so this may provide the first example of an FNR-like protein acting in concert with an alternative sigma factor. However, the relative orientations of the σ^{54} promoter and the FNR box upstream of *nosZ* argue against a simple model whereby the FNR homologue activates RNA polymerase containing the alternative σ factor. There are other examples of FNR boxes being associated with genes involved in denitrification, for example the gene encoding the copper-containing nitrite reductase of *Alcaligenes faecalis* (Nishiyama et al. 1993). In some species, the blue copper-containing azurins have roles in anaerobic nitrate respiration. FNR boxes are found in the promoter regions of the azurin genes from *Alcaligenes faecalis* S-6, *Alcaligenes denitrificans* as well as *Pseudomonas aeruginosa* (Fig 2; Hoitink et al. 1990). An FNR dependent promoter from *E. coli* is activated anaerobically in *Paracoccus denitrificans*, and the *fixK* gene of *Rhizobium meliloti* hybridises to genomic DNA from *P. denitrificans* (Spiro 1992; A. Hinsley & S. Spiro, unpublished observations). Thus there is good reason to suggest that an FNR homologue may regulate denitrification in *P. denitrificans,* and that FNR-like proteins may have a rather general role in the regulation of denitrification. Steinrücke and Ludwig (1993) have recently discussed aspects of oxygen-regulated gene expression in *P. denitrificans* and other species.

Vitreoscilla sp. is an obligate aerobe from the Beggiatoa family which synthesises a haemoglobin like molecule (product of the *vhb* gene) in response to oxygen-limited growth. When present on a plasmid in *E. coli,* the *vhb* promoter is induced when the dissolved oxygen tension (DOT) of a culture falls below 40% of air saturation, and is maximally active at a DOT of 2% or less (Khosla & Bailey 1989). The major *vhb* promoter (when expressed in *E. coli*) has an FNR box centred on position -41.5 with respect to the transcription start site (Fig. 2; Khosla & Bailey 1989).

Gram-positive bacteria

The recent discovery of an FNR homologue in *Lactobacillus casei* provides the first example of a member of this class of proteins in a Gram-positive organism (Bowie et al. 1991; Irvine & Guest 1993). FLP is more similar to CRP than to FNR on the basis of sequence similarity, and in a phylogenetic analysis groups closer to CRP than to FNR homologues (Irvine & Guest 1993; Fig. 1). The algorithm used by Bowie et al. (1991) predicts that the three-dimensional structure of FLP is more closely related to the known structure of CRP than is that of FNR. Nevertheless, FLP is regarded as an FNR homologue because of its likely DNA-binding specificity and its possession of two conserved cysteine residues (Irvine & Guest, 1993; see below). The gene encoding FLP was characterised as an open reading frame of unknown function linked to the *trpDCFBA* operon of *L. casei.* The physiological function of FLP remains to be elucidated.

Structural considerations

An alignment of the amino acid sequences of thirteen characterised members of the FNR group is shown in Fig. 3. It is clear from both the sequence information and the phylogenetic analysis (Fig. 1) that the six proteins from γ-proteobacteria (*E. coli, S. putrefaciens, V. fischeri, A. pleuropneumoniae, P. aeruginosa* and *P. stutzeri*) are the most closely related. The six proteins from α-proteobacteria (members of the Rhizobiaceae and *Rhodopseudomonas palustris*) are somewhat more divergent. On the basis of recent ribosomal RNA sequence data, it is likely that *B. pertussis* is a member of the ß-proteobacteria (Müller & Hilde-

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              1        10           20        30        40        50
              .        .            +  +     +.         .         .
FNR  (Eco)    MIPEKRIIRRIQSGG---CAIHCQDCSISQLCIPFTLNEHELDQLDNIIERKKPIQ
EtrA (Spu)    MTIEQNKNRRSAASG---CAIHCHDCSMGTLCMPFTLNANELDQLDDIIERKKPIQ
FNR  (Vfi)    MMSDNSANKRIQSGG---CAIHCQDCSISQLCIPFTLNDSELDQLDEIIERKKPIQ
HlyX (Apl)        MKIVSDAKHTGRTRCTIHCQNCSISQLCLPFTLSEHELTQLDNIIERKKPVQ
BTR  (Bpe)             MQRRVPLSPDAAHCSSCMLGHVCVPVACLPTKSRNWTNSSRRPCAWS
ANR  (Pae)            MAETIKVRALPQAHCKDCSLAPLCLPLSLTVEDMDSLDEIVKRGRPLK
FnrA (Pst)            MSESIKVRAQRQAHCKDCSLSGLCLPLSLNMQDMDALDDIVKRGRPLK
FixK (Rme)                   MYAAAQAKPQSIEVEHLGPAPMSGPR--------LVAT-YK
FnrN (Rle)          MDVARSEFFETGTPVACTSCQARHGVVCGALSKGQLRELNRHSLRRK-IE
FixK (Bja)          MKPSVVMIEPNGHF--CSDCAIRTSAVCSSLDAAELREFEHLGRRVH-FS
FixK (Aca)  MSIAASVIAHIAPVPAQAYAHPMPSNRWSEMARGVAADESARPAQVVAALGTPAV-FA
ORF4 (Rhi)                    MSLQLVTGHQSRVAAAVPFAAEKQDLSSLFDAQPVER-FT
AadR (Rpa)          MPHLAYPTTTCEGFRCETHCAVRGLAICGELGPADHEEFERLAQHVR-YG
FLP  (Lca)                               MAHSC-TAVVPLFKNLNDEARAAIDALTHERQV--

      60        70        80        90       100       110       120
       .         .         .** ****  .         .         .         . +
KGQTLFKAGDELKSLYAIRSGTIKSYTITEQGDEQITGFHLAGDLVGFDAIGS-GHHPSFAQALETSMVC
KGEQIFKSGDLLKSLFAIPSGTIKSYTITEQGDEQITGFHLAGDVIGFDGIHA-QSHQSFAQALETSMVC
KGQELFKAGDELKCLYAIRSGTIKSYTITEQGDEQITAFHLAGDLVGFDAITE-AQHPSFAQALETSMVC
KSQIIFQSGDELRSIYAIRSGTIKSYTISESGEEQITAFHLPGDLVGFDAIMN-MKHVGFAQALETSMIC
AAKRSTNSTIPW-SRLRVRFGSLKTQLEDSSGQLQITGFHLPGEIVGLVGMIE-SKHVSSAVALEDSEVC
KGEFLFRQGDPFGSVFAVRSGALKTFSITDAGEEQITGFHLPSELVGLSGMDT-ETYPVSAQALETTSVC
KGETLFRQGDTFSSVFAVRSGALRTFSVTDGGEEQITGFHLPSELVGLSGMDT-EMYPVTAQALETTSVC
PGREIYAQGDLNDKCYQVSTGAVRIYRLLSDGRRQVVSFHLPGEMFGFEA--G-SNHSFFAEAITETTLA
AGCEIIAQGSESSFYSNIMRGVMKLCKVMPDGRQQIVGLQFAPDFVGRPF--V-RESTLSAEAATDSEIC
SGETVFSEEDITTSFYNVLEGVMRLYKLLPDGRRQIVGFALPGDFLGMNL--S-GRHNFSADAIGAVTVC
RNSEIFGDDQVAENVYVVVSGVVRICKLMGDGRRQIEAFCLPGDAFGWET--G-ERYRFSAEAVSECRLV
PAAAVFWEGDQARHIFEVVEGTLRAVRILNDGRRVIIGFLRPGDLLGVSV--K-EHYLYTVEAITHVELR
PKEALFSEDEVADSVYSLIEGIARLYKLLPDGRRQIIGFALPGDFLGMAP--G-NRYSFSADSIGGVTVC
EKGTVLISPDTAAHLLVVAHGKLKTYQLATNGKEQLLRVDGVGDYEGEAGLLNIANPNVYTETLTAATVC

      130       140       150       160       170       180       190
       .         .         .         .         .         .         .
EIPFETLDDLSGKMPNLRQQMMRLMSGEIKGDQDMILLLSKKNAEERLAAFIYNLSRRFAQ-RGFSPREF
EIPFNILDELSGTMPKLRQQIMRLMSNEIMSDQEMILLLSKKNAEERLAAFISNLANRFGN-RGFSAKEF
EIPYEILDDLSGKMPKLRQQIMRLMSNEIKGDQEMILLLSKKNAEERLAAFLYNLSTRFHQ-RGFSPREF
EIPFDILDDLAGKMPKIRHQIMRLMSNEIKSDQEMILLLSKMSAEEKLAAFLHNLSQRYAA-PGFSAREF
VIRLPEIDRVSTQLPSLQQQFRRLMSREITRSHQMLATVGAMRSEQRLAAFLLNLSQATRR-SAIRRPNS
EIPFERLDELSEQLPQLRRQLMRLMSREIRDDQQMMLLLSKKTADERIATFLVNLSARFRA-RGFSAQQF
EIPFERLDELSVLLPQLRRQLMRIMSREIRDDQQMMLLLSKKTADERIATFLINLSARFSA-RGFSANQF
IFGRRNM-------QERSRELLALALTGMARAQQHLLVIGRQCAVERIAAFLVDLCERQGGGR-----QL
VFPRNLLDRMISETPELQRSLHDQALKELDAAREWMLTLGRRTAEEKVAS-LLHLIATHAEPQTATSTAF
QFAKAPFGRFIEERPQLLRRINELAIRELSQARDHMVLLGRRSADEKVAAFLLGWRERLLALKGASDT-V
RVKRSVLFARAGSDPELACALWALSFAELQRAQEHLLLLGRKTAQERVGSFLLDLARRSGTTNASHVTEV
RFSRRRFESESARAPHLREQLFSRLCDEMAAAQDQMVLLSRRSAEEKVAGFLLMMAR---GQSENRRPVI
KFFRGPFLRFIENRPQMLLRMNDFATRELSLAQDQMLLLGRRSAEEKVAAFLVGWRDRLARLEGVTKT-V
TISATDFQQLLLKQPQISLQLLTENARKMQALEKQAGYLGNDSINVRLTHYLLDLR------TAAGQDTV

       200       210        220       230       240       250
        .        =. =        .         .         .         .
RLTMTRGDIGNYLGLTVETISRLLGRFQKSGMLAV-KGKYITIENNDALAQLAGHTRNVA
RLTMTRGDIGNYLGLTVETISRLLGRFQKSGLIEV-KGKYIIIVDHHELNLLAGNARIAR
RLTMTRGDIGNYLGLTVETISRLLGRFQKTEMLTV-KGKYITINDHDALAELAGSAKEIK
RLTMTRGDIGNYLGLTIETISRLLGRFQKSGMITV-QGKYITINRMDELTV
CVRMSREEIGNYLGLTLETVSRLFSRFGREGLIRI-NQAEVRLIDLPGLKQLIGQESC
RLAMSRNEIGNYLGLAVETVSRVFTRFQQNGLISA-EGKEVHILDSIELCALAGGQLEG
RLPMSRNEIGNYLGLAVETVSRVFTRFQQNGLLEA-EGKEVRILDSIGLCALAGGAMDA
RLPMSRQDIADYLGLTIETVSRVVTKLKERSLIALRDARTIDIMKPEALRSLCN
DLPLSRAEIADFLGLTIETVSRQMTRLRKIGVIRIENFRHIIVPDMDELERMISA
PLPMSRQDIADYLGLTIETVSRTFTKLERHGAIAIIHG-GISLLDPARVEALAAA
TLAMSRQDIADFLGLTIETVSRTLTYLEEQGTISLPSSRRVLLRDRSALRRLDS
ELPMTRLDVADYLGMTIETVSRTITKLAGSGVIAIVGRHAIAILKMDALIALADGECDDGAQRSARYAKA
SLPMGRQDIADFLGLTIETVSRTFTKLEREKLIVIVPD-GVRVLDPKRFDALAAA
TVPMAWTQLADYLGTTPETVSRTLKRLAEEKLIE-RSGKQVRILNAEDMEDFAW
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Fig. 3. Alignment of the amino acid sequences of the FNR protein and its relatives. The predicted DNA-binding domain is underlined, and the following residues of the *E. coli* protein are highlighted: (+), cysteine residues which are essential for FNR activity; (*), residues which are implied to interact with RNA polymerase and (=), residues within the DNA recognition helix which are required for the sequence specific interaction with DNA. The numbering is that of the *E. coli* protein. Species abbreviations are as in Figure 1. The ORF4 protein is the predicted product of *orf4* of *Rhizobium* strain IC3342 (*Rhi*).

brandt 1993). The *L. casei* FLP protein groups with the CRP rather than the FNR family. In the present review, sequence similarities in particular regions of these proteins will be considered, focusing on those domains to which a function has either been ascribed, or suggested, in the case of the *E. coli* protein.

The cysteine cluster

The *E. coli* FNR protein has an *N*-terminal cluster of four cysteine residues, and a single internal cysteine. Site-directed mutagenesis has demonstrated that the internal cysteine, and three of the four cysteines in the *N*-terminal cluster are essential for the normal function of FNR (Spiro & Guest 1988; Sharrocks et al. 1990). There is evidence that FNR activity requires metal ions in vivo, and that the purified protein binds iron (Spiro et al. 1989; Trageser & Unden 1989; Green et al. 1991). There is an inverse relationship between the amount of iron bound by pure protein and the number of sulphydryl groups accessible to a modifying agent (Green et al. 1991). It has been suggested, therefore, that the essential cysteine residues might be involved in liganding an iron ion. More recently, it has been demonstrated that only the internal cysteine residue (Cys-122) is essential for iron binding (Green et al. 1993). Furthermore, it has been shown that FNR contains an intramolecular disulphide bridge, and that the relative amounts of oxidised and reduced forms differ during aerobic and anaerobic growth (Green et al. 1993). It seems that the disulphide may form between Cys-122 and any one of at least two cysteines in the *N*-terminal cluster. Additionally, FNR is subject to a post-translational modification by an unidentified substituent of molecular mass 71-85 Da; mutants with substitutions of Cys-122 do not show this modification. Of the five cysteine residues in FNR, Cys-122 appears to be the most important in that it is absolutely required for disulphide bond formation, iron binding and covalent modification (Green et al. 1993). It is of some interest to examine the corresponding regions in the FNR homologues from other organisms. It is apparent that a clear distinction should be drawn between those FNR homologues which contain cysteine residues in the *N*-terminal region, and those (the FixK proteins of *R. meliloti* and *A. caulinodans* and the *orf4* gene product of *Rhizobium* strain IC3342) which do not. Only FLP of *L. casei* does not fit easily into this classification, since it has only a single cysteine residue in the *N*-terminal region. The FixK proteins of *R. meliloti* and *A. caulinodans* do have internal cysteine residues, although only in the case of *A. caulinodans* is one of these near to a position corresponding to Cys-122 of the *E. coli* FNR protein. The absence of the *N*-terminal and conserved internal cysteine residues in the *R. meliloti* FixK protein may explain the fact that it does not behave as an oxygen sensor *in vivo*. The structures of the FixK protein of *A. caulinodans* and of the *orf4* product of *Rhizobium* IC3342 suggest that these too may not be oxygen sensors. It is something of a paradox that substitution of the cysteine residues of FNR renders the protein incompetent for transcriptional activation, whereas naturally occurring proteins which lack the cysteines are functional.

Of those proteins which have the conserved cysteine residues, ANR, HlyX, FnrN and FixK (*Bradyrhizobium japonicum*) are known to respond directly to oxygen in a manner that is, at least superficially, similar to the FNR protein itself (see above). Whilst there is conservation of the cysteine cluster, the number and spacing of cysteines is not completely conserved. The HlyX protein is identical to FNR in this respect, and ANR contains the three essential cysteines, with the same spacing. The FnrN (*R. leguminosarum*) and FixK (*B. japonicum*) proteins have the three essential cysteine residues, but with a greater spacing between those corresponding to Cys-23 and Cys-29. The AadR protein has a four-cysteine cluster, but with slightly different spacing to the other proteins. Of those FNR homologues which have an *N*-terminal cysteine cluster, AadR is the only one which does not have a Cys-X-X-Cys motif, the corresponding sequence being Cys-E-T-H-Cys in AadR. Interestingly, an FNR mutant which has a single amino acid insertion within the Cys-X-X-Cys motif is severely impaired for both activation and repression (Melville & Gunsalus 1990). However, it is difficult to extrapolate from FNR to AadR, since it is not known whether or not AadR is directly responsive to oxygen. Furthermore, until the precise role of the cysteine cluster in FNR has been elucidated, it is difficult to draw firm conclusions about the equivalent region in FNR homologues. It should not be assumed that the FNR proteins of non-enteric bacteria have exactly the same mode of action as the *E. coli* protein. An interesting possibility, which has yet to be tested, is that the redox potential of the metal centre in each protein depends upon the precise arrangement of the cysteine cluster. Some conclusions can nevertheless be drawn from these comparisons:

- FNR-like proteins which are known to behave as oxygen sensors retain three or four *N*-terminal cysteine residues and the internal cysteine residue, i.e.

– the converse is true, ie the one protein which is known not to be an oxygen sensor (FixK of *R. meliloti*) does not retain these cysteine residues.

A considerable amount remains to be learnt about the mechanism of anaerobic activation of FNR by anoxia in *E. coli*, and in non-enteric bacteria.

The positive control region

There is considerable interest in the mechanism of transcriptional activation by regulatory proteins such as FNR and CRP. A key question is the manner in which these proteins stimulate transcription, whether that be by making physical contacts with RNA polymerase, or by altering the structure of promoters so as to facilitate initiation. A common approach to this problem is to isolate 'positive control' mutations in the regulatory protein which do not affect its ability to bind to DNA, but abolish transcriptional activation. The location of such mutations identify regions of the protein which are potentially involved in interacting with RNA polymerase. In the sole study of positive control by FNR, it has been shown that mutations targeted to residues 81-87 of the protein do in some cases produce a positive control phenotype. In particular, substitutions of Ile-81, Thr-82, Gly-85, Asp-86, Glu-87 and Gln-88 produce proteins which are defective for transcriptional activation but not for DNA-binding (Williams et al. 1991). Substitutions of Thr-80, Glu-83 and Gln-84 had minimal effects on FNR activity. It was suggested that the former group of residues constitutes part of a positive control 'patch' on the protein which is a surface exposed loop capable of interacting with RNA polymerase. Since mutations were targeted to this part of the protein, it cannot be excluded that there may be other positive control 'patches'. Nevertheless, it is of some interest to examine regions of FNR homologues corresponding to this positive control 'patch' of the *E. coli* protein (Fig. 3). Substitutions of Gly-85 and Gln-88 of the *E. coli* protein produce proteins with a positive control phenotype. Both of these residues are completely conserved throughout this family of proteins, with the exception of the ORF4 protein which has Gln-88 substituted by valine. It is possible that the glycine residue has an important structural role in the predicted loop of the positive control 'patch'. The precise role of the glutamine is a matter for speculation, though it is possible that this residue is required for a specific interaction with RNA polymerase. The genetic evidence suggests that three other residues of FNR are involved in positive control: Ile-81, Thr-82, Asp-86 and Glu-87. There is a conservation of amino acids with non polar side chains at position 81. The residue at position 82 does not appear to be strongly conserved. However, the mutant form of FNR has a substitution with proline at this position, which may have a destabilising influence on the structure of the positive control 'patch'. It is at positions 86 and 87 that there is most divergence in this region of FNR homologues. FNR and its close relatives EtrA, ANR, FnrA and HlyX have acidic residues at these positions, whereas there is a preponderance of basic amino acids, particularly arginine, in the rhizobial proteins and AadR at the equivalent positions. This may point to a rather different mechanism of transcriptional activation in the case of the rhizobial proteins, or may indicate that this region of the protein does not in fact constitute a positive control 'patch' in the non-*E. coli* proteins. The fact that some of the rhizobial proteins are able to activate transcription in *E. coli* complicates interpretation of the sequence alignment in this region. As was the case for the redox domain, sequence comparisons must be interpreted with some caution. Further studies are required on the non-*E. coli* proteins and on FNR itself in order to achieve a complete description of the mechanism of positive control.

The DNA-binding domain

The DNA-binding domain of the *E. coli* FNR protein is well defined, both from genetic studies and on the basis of its similarity to the helix-turn-helix units of related DNA-binding proteins. Similarly, the sequence that FNR recognises (the FNR box) is well characterised and substantiated. FNR boxes can be found in the promoter regions of many non-enteric genes, and in most cases there is good reason to suppose that these are functional. However, only in the case of an FNR boxes in the *fixK* promoter of *R. meliloti* and the *arc* promoter of *P. aeruginosa* is there good evidence that the FNR homologue interacts with the FNR box (Haas et al. 1992; Waelkens et al. 1992; Galimand et al. 1991). Nevertheless, if the assumption is made that FNR boxes are functional (if they are found in the promoters of genes which might reasonably be expected to be under FNR control) then a consensus for non-*E. coli* FNR boxes can be derived which is remarkably similar to the *E. coli* consensus (Fig. 2). The tentative conclusion is that the FNR homologues recognise the same, or a very similar DNA sequence. This suggestion is supported by the results of experiments in which the regulation of promoters by heterologous

FNR-like proteins has been studied (see above). The DNA-binding domains are the most highly conserved regions of the FNR family of proteins as illustrated in Fig. 3. Site directed mutagenesis of the *E. coli* protein has implicated two residues of the DNA recognition helix in specific interactions with DNA, these are Glu-209 and Ser-212 (Spiro et al. 1990). Both residues are completely conserved throughout this family of proteins. It has been suggested that side chains of these residues make interactions with the T-A and G-C base pairs at positions 1 and 3 of the TTGAT motif of the FNR box. Model building studies have suggested that a hydrophobic residue at the position corresponding to Val-208 in FNR also contributes to specificity (Cherfils et al. 1989), although the phenotype of an FNR protein substituted at this position is not consistent with this suggestion. The conservation of sequences within the DNA-binding domains and FNR boxes from non-enteric bacteria lends support to the idea that there is a conservation of the mechanism of DNA sequence recognition. However, there may be subtle differences in the DNA-binding specificities in at least some members of the FNR family (Galimand et al. 1991; Sawers 1991; Green et al. 1992).

Conclusions

Proteins structurally homologous to FNR (and CRP) of *E. coli* are widespread amongst Gram-negative bacteria, and there is at least one example from a Gram-positive organism. There is every reason to suppose that more members of this family of regulatory proteins will be characterised as more species are examined. There is a striking degree of conservation of the DNA sequence which FNR-like proteins recognise. It is clear, on the other hand, that FNR homologues have been recruited to regulate a variety of functions in different organisms. In cases where FNR like proteins regulate similar functions, the regulatory system may show a markedly greater degree of conservation than the genes and proteins which are the target of regulation. A good example is found in the denitrifying bacteria, where there is evidence that FNR homologues regulate the expression of cytochrome *cd* $_1$-type (in *Pseudomonas aeruginosa, P. stutzeri* and *Paracoccus denitrificans*) and copper-containing (in *Pseudomonas* G-179 and *Alcaligenes faecalis*) nitrite reductases. The nature of the interaction of FNR homologues with environmental signals and with other regulatory proteins is somewhat variable. Further research will no doubt uncover new surprises from this fascinating group of proteins.

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References

Anthamatten D, Scherb B & Hennecke H (1992) Characterization of a *fixLJ* -regulated *Bradyrhizobium japonicum* gene sharing similarity with the *Escherichia coli fnr* and *Rhizobium meliloti fixK* Genes. J Bacteriol. 174: 2111-2120

Arai H, Igarashi Y & Kodama T (1991) Anaerobically induced expression of the nitrite reductase cytochrome *c* -551 operon from *Pseudomonas aeruginosa.* FEBS Lett 280, 351–353

Bannan JD, Moran MJ, MacInnes JI, Soltes GA & Friedman RL (1993) Cloning and characterization of *btr,* a *Bordetella pertussis* gene encoding a FNR-like transcriptional regulator. J. Bacteriol. 175: 7228–7235

Batut J, Daveran-Mingot M-L, David M, Jacobs J, Garnerone AM & Kahn D (1989) *fixK,* a gene homologous with *fnr* and *crp* from *Escherichia coli,* regulates nitrogen fixation genes both positively and negatively in *Rhizobium meliloti.* EMBO J 8: 1279–1286

Bowie JU, Lüthy R & Eisenberg D (1991) A method to identify protein sequences that fold into a known three-dimensional structure. Science 253:164–170

Cherfils J, Gibrat J-F, Levin J, Batut J & Kahn D (1989) Model-building of Fnr and FixK DNA-binding domains suggests a basis for specific DNA recognition. J. Mol. Recognition 2: 114–121

Colonna-Romano S, Arnold W, Schlüter A, Boistard P, Priefer UB (1990) An Fnr-like protein encoded in *Rhizobium leguminosarum* biovar *viciae* shows structural and functional homology to *Rhizobium meliloti* FixK. Mol. Gen. Genet. 223: 138–147

Cuypers H & Zumft WG (1992) Regulatory components of the denitrification gene cluster of *Pseudomonas stutzeri.* pp 188-197 in 'Pseudomonas molecular biology and biotechnology' (Eds Galli, E., Silver, S. and Witholt, B.) American Society for Microbiology, Washington, D.C.

Cuypers H & Zumft WG (1993) Anaerobic control of denitrification in *Pseudomonas stutzeri* escapes mutagenesis of an *fnr*-like gene. J. Bacteriol. 175: 7236–7246

Cuypers H, Viebrock-Sambale A & Zumft WG (1992) NosR, a membrane-bound regulatory component necessary for expression of nitrous oxide reductase in *Pseudomonas stutzeri.* J. Bacteriol. 174: 5332–5339

Dispensa M, Thomas C, Kim M-K, Perrotta JA, Gibson J & Harwood CS (1992) Anaerobic growth of *Rhodopseudomonas palustris* on 4-hydroxybenzoate is dependent on AadR, a member of the cyclic AMP receptor protein family of transcriptional regulators. J. Bacteriol. 174: 5803–5813

Felsenstein J (1993) PHYLIP (Phylogeny Inference Package) Version 3.5c. University of Washington, Seattle.

Galimand M, Gamper M, Zimmermann A & Haas D (1991) Positive FNR-like control of anaerobic arginine degradation and nitrate respiration in *Pseudomonas aeruginosa.* J. Bacteriol. 173: 1598–1606

Gilles-Gonzalez M, Ditta G & Helinski DR (1991) A haemoprotein with kinase activity encoded by the oxygen sensor of *Rhizobium meliloti.* Nature (London) 350: 170–172

Green J, Trageser M, Six S, Unden G & Guest JR (1991) Characterization of the FNR protein of *Escherichia coli,* an iron-binding transcriptional regulator. Proc. R. Soc. Lond. B244: 137–144

Green J, Sharrocks AD, MacInnes JI & Guest JR (1992) Purification of HlyX, a potential regulator of haemolysin synthesis, and properties of HlyX:FNR hybrids. Proc. R. Soc. Lond. B248: 79–84

Green J, Sharrocks AD, Green B, Geisow M & Guest JR (1993) Properties of FNR proteins substituted at each of the five cysteine residues. Mol. Microbiol. 8: 61–68

Haas D, Gamper M & Zimmermann A (1992) Anaerobic control in *Pseudomonas aeruginosa.* pp 177–187 in 'Pseudomonas molecular biology and biotechnology' (Eds Galli, E., Silver, S. and Witholt, B.) American Society for Microbiology, Washington, D.C.

Hill S (1985) Redox regulation of enteric *nif* expression is independent of the *fnr* gene product. FEMS Microbiol. Lett. 29: 5–9

Hoitink CWG, Woudt LP, Turenhout JCM, van de Kamp M & Canters GW (1990) Isolation and sequencing of the *Alcaligenes denitrificans* azurin-encoding gene: comparison with the genes encoding blue copper proteins from *Pseudomonas aeruginosa* and *Alcaligenes faecalis.* Gene 90: 15–20

Irvine AS & Guest JR (1993) *Lactobacillus casei* contains a member of the CRP-FNR family. Nucleic Acids Res. 21: 753

Iuchi S & Lin ECC (1993) Adaptation of *Escherichia coli* to redox environments by gene expression. Mol. Microbiol. 9, 9–15

Jüngst A, Wakabayashi S, Matsubara H & Zumft WG (1991) The *nirSTBM* region coding for cytochrome *cd* $_1$-dependent nitrite respiration of *Pseudomonas stutzeri* consists of a cluster of mono-, di-, and teraheme proteins. FEBS Lett. 279: 205–209

Kaminski PA, Mandon K, Arigoni F, Desnoues N & Elmerich C (1991) Regulation of nitrogen fixation in *Azorhizobium caulinodans*: identification of a *fixK* -like gene, a positive regulator of *nifA.* Mol. Microbiol. 5: 1983–1991

Khosla C & Bailey JE (1989) Characterization of the oxygen-dependent promoter of the *Vitreoscilla* hemoglobin gene in *Escherichia coli.* J. Bacteriol. 171: 5995–6004

Kita-Tsukamoto K, Oyaizu H, Nanba K & Simidu U (1993) Phylogenetic relationships of marine bacteria, mainly members of the family *Vibrionaceae,* determined on the basis of 16S rRNA sequences. Int. J. Syst. Bacteriol. 43: 8–19

Klug G (1993) Regulation of expression of photosynthesis genes in anoxygenic photosynthetic bacteria. Arch. Microbiol. 159: 397–404

Körner H (1993) Anaerobic expression of nitric oxide reductase from denitrifying *Pseudomonas stutzeri.* Arch. Microbiol. 159: 410–416

Körner H & Zumft WG (1989) Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of *Pseudomonas stutzeri.* Appl. Env. Microbiol. 55: 1670–1676

Lee JK & Kaplan S (1992) *cis* -acting regulatory elements involved in oxygen and light control of *puc* operon transcription in *Rhodobacter sphaeroides.* J. Bacteriol. 174: 1146–1157

Lodge J, Williams R, Bell A, Chan B & Busby S (1990) Comparison of promoter activities in *Escherichia coli* and *Pseudomonas aeruginosa* : use of a new broad-host-range promoter-probe plasmid. FEMS Microbiol. Lett. 67: 221–226

MacInnes JI, Kim JE, Lian C-J & Soltes GA (1990) *Actinobacillus pleuropneumoniae hlyX* gene homology with the *fnr* gene of *Escherichia coli.* J. Bacteriol. 172: 4587–4592

Melville S & Gunsalus RP (1990) Mutations in *fnr* that alter anaerobic regulation of electron transport-associated genes in *Escherichia coli.* J. Biol. Chem. 265: 18733–18736

Müller M & Hildebrandt A (1993) Nucleotide sequences of the 23S rRNA genes from *Bordetella pertussis, B. parapertussis, B. bronchiseptica* and *B. avium,* and their implications for phylogenetic analysis. Nucleic Acids Res. 21:3320.

Müller-Breitkreutz K & Winkler UK (1993) Anaerobic expression of the *Vibrio fischeri lux* regulon in *E. coli* is FNR–dependent. 7th International Symposium on Bioluminescence and Chemiluminescence. John Wiley & Sons, in press.

Myers CR & Nealson KH (1990) Respiration-linked proton translocation coupled to anaerobic reduction of manganese (IV) and iron (III) in *Shewanella putrefaciens* MR-1. J Bacteriol. 172: 6232–6238

Nealson KH (1977) Low oxygen is optimal for luciferase synthesis in some bacteria. Arch. Microbiol. 112: 9–16.

Neidle EL & Kaplan S (1993) Expression of the *Rhodobacter sphaeroides hemA* and *hemT* genes, encoding two 5-aminolevulinic acid synthase isoenzymes. J. Bacteriol. 175: 2292–2303

Nishiyama M, Suzuki J, Kukimoto M, Ohnuki T, Horinouchi S & Beppu T (1993) Cloning and characterization of a nitrite reductase gene from *Alcaligenes faecalis* and its expression in *Escherichia coli.* J. Gen. Microbiol. 139: 725–733

Römermann D, Warrelmann J, Bender RA & Friedrich B (1989) An *rpoN* -like gene of *Alcaligenes eutrophus* and *Pseudomonas faecalis* controls expression of diverse metabolic pathways, including hydrogen oxidation. J. Bacteriol. 171: 1093–1099

Saffarini DA & Nealson KH (1993) Sequence and genetic characterization of *etrA,* an *fnr* analog that regulates anaerobic respiration in *Shewanella putrefaciens* MR-1. J. Bacteriol. 175: 7938–7944

Sawers RG (1991) Identification and molecular characterization of a transcriptional regulator from *Pseudomonas aeruginosa* PAO1 exhibiting structural and functional similarity to the FNR protein of *Escherichia coli.* Mol. Microbiol. 5: 1469–1481

Schlüter A, Patschkowski T, Unden G & Priefer UB (1992) The *Rhizobium leguminosarum* FnrN protein is functionally similar to *Escherichia coli* Fnr and promotes heterologous oxygen-dependent activation of transcription. Mol. Microbiol. 6: 3395–3404

Sharrocks AD, Green J & Guest JR (1990) In vivo and in vitro mutants of FNR the anaerobic transcriptional regulator of *E. coli.* FEBS Lett. 270: 119–122

Smith GB & Tiedje JM (1992) Isolation and characterization of a nitrite reductase gene and its use as a probe for denitrifying bacteria. Appl. Env. Microbiol. 58: 376–384

Soltes GA & MacInnes JI (1994) Regulation of gene expression by the HlyX protein of *Actinobacillus pleuropneumoniae.* Microbiology In press

Spiro S (1992) An FNR-dependent promoter from *Escherichia coli* is active and anaerobically inducible in *Paracoccus denitrificans.* FEMS Microbiol. Lett. 98: 145–148

Spiro S & Guest JR (1988) Inactivation of the FNR protein of *Escherichia coli* by targeted mutagenesis in the *N* -terminal region. Mol. Microbiol. 2: 701–707

Spiro S & Guest JR (1991) Adaptive responses to oxygen limitation in *Escherichia coli.* TIBS 16: 310–314

Spiro S, Roberts RE & Guest JR (1989) FNR-dependent repression of the *ndh* gene of *Escherichia coli* and metal ion requirement for FNR-regulated gene expression. Mol. Microbiol. 3: 601–608

Spiro S, Gaston KL, Bell AI, Roberts RE, Busby SJW & Guest JR (1990) Interconversion of the DNA-binding specificities of two related transcription regulators, CRP and FNR. Mol. Microbiol. 4: 1831–1838

Steinrücke P & Ludwig B (1993) Genetics of *Paracoccus denitrificans*. FEMS Microbiol. Rev. 104:83–118.

Trageser M & Unden G (1989) Role of cysteine residues and of metal ions in the regulatory functioning of FNR, the transcriptional regulator of anaerobic respiration in *Escherichia coli*. Mol. Microbiol. 3: 593–599

Upadhyaya NM, Scott KF, Tucker WT, Watson JM & Dart PJ (1992) Isolation and characterisation of *Rhizobium* (IC3342) genes that determine leaf curl induction in pigeon pea. Mol Plant Microb. Interact. 5: 129–143

Viebrock A & Zumft WG (1988) Molecular cloning, heterologous expression, and primary structure of the structural gene for the copper enzyme nitrous oxide reductase from denitrifying *Pseudomonas stutzeri*. J. Bacteriol. 170: 4658–4668

Waelkens F, Foglia A, Morel J-B, Fourment J, Batut J & Boistard P (1992) Molecular genetic analysis of the *Rhizobium meliloti fixK* promoter: identification of sequences involved in positive and negative regulation. Mol. Microbiol. 6: 1447–1456

Williams R, Bell A, Sims G & Busby S (1991) The role of two surface exposed loops in transcription activation by the *Escherichia coli* CRP and FNR proteins. Nucleic Acids Res. 19: 6705–6712

Ye RW, Fries MR, Bezborodnikov SG, Averill BA & Tiedje JM (1993) Characterization of the structural gene encoding a copper-containing nitrite reductase and homology of this gene to other denitrifiers. Appl. Env. Microbiol. 59, 250–254

Zimmermann A, Reimmann C, Galimand M & Haas D (1991) Anaerobic growth and cyanide synthesis of *Pseudomonas aeruginosa* depend on *anr*, a regulatory gene homologous with *fnr* of *Escherichia coli*. Mol. Microbiol. 5: 1483–1490

Zumft WG, Dreusch A, Löchelt S, Cuypers H, Friedrich B & Schneider B (1992) Derived amino acid sequences of the *nosZ* gene (respiratory N_2O reductase) from *Alcaligenes eutrophus, Pseudomonas aeruginosa* and *Pseudomonas stutzeri*. Eur. J. Biochem. 208: 31–40