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Anaerobic transcription activation in *Bacillus subtilis*: identification of distinct FNR-dependent and -independent regulatory mechanisms

Hugo Cruz Ramos, Laurent Boursier, Ivan Moszer, Frank Kunst[1], Antoine Danchin[2] and Philippe Glaser[2]

Unité de Régulation de l'Expression Génétique and [1]Unité de Biochimie Microbienne, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris Cedex 15, France

[2]Corresponding authors

H.Cruz Ramos and L.Boursier contributed equally to this work

***Bacillus subtilis* is able to grow anaerobically using alternative electron acceptors, including nitrate or fumarate. We characterized an operon encoding the dissimilatory nitrate reductase subunits homologous to the *Escherichia coli narGHJI* operon and the *narK* gene encoding a protein with nitrite extrusion activity. Downstream from *narK* and co-transcribed with it a gene (*fnr*) encoding a protein homologous to *E.coli* FNR was found. Disruption of *fnr* abolished both nitrate and fumarate utilization as electron acceptors and anaerobic induction of *narK*. Four putative FNR binding sites were found in *B.subtilis* sequences. The consensus sequence, centred at position –41.5, is identical to the consensus for the DNA site for *E.coli* CAP. Bs-FNR contained a four cysteine residue cluster at its C-terminal end. This is in contrast to Ec-FNR, where a similar cluster is present at the N-terminal end. It is possible that oxygen modulates the activity of both activators by a similar mechanism involving iron. Unlike in *E.coli*, where *fnr* expression is weakly repressed by anaerobiosis, *fnr* gene expression in *B.subtilis* is strongly activated by anaerobiosis. We have identified in the *narK–fnr* intergenic region a promoter activated by anaerobiosis independently of FNR. Thus induction of genes involved in anaerobic respiration requires in *B.subtilis* at least two levels of regulation: activation of *fnr* transcription and activation of FNR to induce transcription of FNR-dependent promoters.**

Keywords: anaerobic respiration/*Bacillus subtilis*/evolution/FNR/nitrate reductase

Introduction

The re-oxidation of excess reducing equivalent is indispensable in the metabolism of living cells. This reaction is important for both anabolism and catabolism. Most of the reduced molecules are produced by glycolysis and by the Krebs cycle (10 NADH and 2 FADH equivalents for the complete consumption of one molecule of glucose). Both soluble and membrane-bound enzymes are involved in re-oxidation. The membrane-bound enzymes couple the reaction to an electron transport chain generating a transmembrane proton gradient. This gradient is used as an energy supply for various processes, such as transport of molecules through the cytoplasmic membrane and ATP synthesis by the membrane bound F_0F_1 ATPase.

In the absence of an external terminal electron acceptor NADH is re-oxidized by endogenous electron acceptors produced in a redox-balanced dismutation of the substrate. This process, known as fermentation, does not lead to production of a transmembrane proton gradient. The energetic yield of catabolism is greatly increased when electrons are transferred through the respiratory chain to an external electron acceptor. Unlike some *Bacillus* species, including *Bacillus macerans*, *Bacillus subtilis* is considered to be unable to ferment (Priest, 1993). *Bacillus subtilis* is, however, able to grow in the absence of oxygen if an alternative electron acceptor (e.g. nitrate or fumarate) is added to the medium (Priest, 1993; Schirawski and Unden, 1995). *Bacillus subtilis* requires an external electron acceptor to be able to re-oxidize NADH, but ATP synthesis by soluble enzyme systems is sufficient to sustain growth, although with a lower yield. Indeed, *B.subtilis* mutants deficient in F_0F_1 ATPase grow on fermentable carbon sources, for example glucose (Santana *et al.*, 1994).

The genetics and the biochemistry of respiration with alternative electron acceptors has been studied for more than 25 years in *Escherichia coli*. During nitrate respiration nitrate is reduced to nitrite in the cytoplasm by a membrane-bound nitrate reductase. This reaction couples the transfer of electrons from menaquinol to nitrate to the translocation of protons across the membrane. The three subunits of the nitrate reductase complex and a fourth polypeptide required for assembly of the complex (NarJ) are encoded by the *narGHJI* operon (Blasco *et al.*, 1989). Upstream of this operon and in the same orientation the *narK* gene constitutes a second transcription unit (Noji *et al.*, 1989). The *narK* gene product is an integral membrane protein involved in excretion of the nitrite produced by the dissimilatory reduction of nitrate, intracellular accumulation of nitrite being toxic to the cell (DeMoss and Hsu, 1991; Rowe *et al.*, 1994). In *E.coli* a second nitrate reductase, highly similar to that encoded by *narGHJI*, is also present. It is encoded by the *narZYWV* operon, but unlike *narGHJI*, the *narZYWV* operon is expressed constitutively and weakly (Blasco *et al.*, 1990).

In *E.coli* regulation of the expression of genes involved in anaerobic respiration is complex. Expression of these genes is activated in the absence of oxygen, mainly mediated by the activator protein FNR (Spiro and Guest, 1990; Iuchi and Lin, 1993). FNR is encoded by the *fnr* gene, 123 kb from *narK* on the *E.coli* genome physical map (Médigue *et al.*, 1993). It is a pleiotropic regulator similar in amino acid sequence to catabolite activator protein (CAP). During a shift from aerobiosis to anaerobiosis *E.coli fnr* mutant strains fail to induce synthesis of

22 proteins normally induced in the wild-type, as assessed by two-dimensional gel electrophoresis (Sawers *et al.*, 1988). A few genes are repressed in anaerobiosis by FNR; *fnr* itself and the *ndh* gene encoding the membrane-associated flavoenzyme NADH dehydrogenase II (Spiro and Guest, 1990). There is a second network of oxygen-regulated genes in *E.coli* under the control of the two-component sensor–regulator system ArcB–ArcA, which represses a wide variety of aerobic enzymes under anaerobic conditions (Spiro and Guest, 1991; Iuchi and Lin, 1993).

Gene functions involved in reduction of the various alternative electron acceptors are also induced by their specific substrates. The expression of a number of reductases for less efficient electron acceptors, such as fumarate, are repressed by nitrate, such that the system ressembles that for aerobiosis. Nitrate-dependent regulation, including activation of the *narGHJI* operon and of the *narK* gene, and repression of genes involved in the reduction of other electron acceptors is mediated by two interacting two-component systems: NarX and NarQ are two homologous sensor proteins; NarL and NarP the corresponding response regulators (Stewart and Rabin, 1995).

Bacillus subtilis synthesizes two different nitrate reductases. An assimilatory nitrate reductase, encoded by the recently characterized *nasB* operon, is responsible for the utilization of nitrate as nitrogen source (Nakano *et al.*, 1995). This soluble enzyme reduces nitrate to nitrite, which is subsequently reduced by the nitrite reductase also encoded by the *nasB* operon. In anaerobiosis *B.subtilis* synthesizes a dissimilatory nitrate reductase. Nitrite, the product of the reduction of nitrate, is excreted into the external medium (Schirawski and Unden, 1995).

In this report we have characterized the chromosomal region homologous to the *E.coli narK–narGHJI* region. We have identified a regulatory gene between *narK* and *narG* encoding a protein homologous to *E.coli* FNR. Expression of the *narK* and *fnr* genes was analysed to identify the molecular details of the anaerobic induction of genes involved in reduction of alternative electron acceptors in *B.subtilis*. Comparison of anaerobic respiration and its regulation in *B.subtilis* and *E.coli* raises the question of the evolution of this part of energetic metabolism in these two distantly related bacteria.

Results

Cloning and sequencing of the narK and fnr genes

The *gerB–sacXY* (314–333° on the genetic map) region of the *B.subtilis* chromosome has been completely cloned using several cloning procedures (P.Glaser, unpublished). In this work plasmid pDIA5348 was obtained by the plasmid rescue method (Glaser *et al.*, 1993). Its chromosomal insert is located between the *thrZ* and *spo0F* genes at 328° on the *B.subtilis* genetic map (see Figure 1; Anagnostopoulos *et al.*, 1993). The nucleotide sequence of the pDIA5348 insert was completely determined on both strands after a single shotgun cloning experiment. Thirteen putative coding sequences (CDS) were predicted in the six frames using previously described criteria (Glaser *et al.*, 1993). Comparison of the deduced amino acid sequences of the products of these CDS with known protein sequences revealed a gene cluster whose products are similar to proteins involved in *E.coli* anaerobic respiration; NarK, FNR, the three subunits of the nitrate reductase (encoded by *narG*, *narH* and *narI*) and NarJ (Figure 1). Investigations of the nitrate reductase operon *narGHJI* will be published elsewhere.

The DNA sequence of the *narK* and *fnr* genes and the deduced protein sequences are presented in Figure 2. The start codon of both genes was designated according to protein sequence alignments (see below) and positions of potential ribosome binding sites (RBS). Upstream of the *narK* gene is a palindromic sequence followed by seven T residues. This is a likely transcription terminator for the preceding *argS* gene. A second palindromic sequence preceded by seven A and followed by seven T residues is found between *fnr* and the convergent gene *ywiC* and may be the transcription terminator for these two genes.

The narK gene product

The deduced *narK* gene product is a 395 residue hydrophobic protein similar to *E.coli* NarK (29% identity and a sum probability in the BLAST similarity search of $P(n)$ = 2.8e–35; Figure 3). The *B.subtilis* protein is notably shorter than its *E.coli* counterpart (395 residues versus 463), however, both proteins have similar hydropathy profiles and the conservation extends over the entire length of the proteins.

Escherichia coli NarK is involved in the excretion of nitrite during nitrate respiration (DeMoss and Hsu, 1991; Rowe *et al.*, 1994). We tested the *B.subtilis* protein for a similar activity by two approaches. The *B.subtilis narK* gene was first inactivated by the insertion of a spectinomycin cassette by homologous recombination between the chromosome and pDIA5349 (Figure 1). In the resulting strain the *fnr* gene was still expressed under the control of its own promoter (see below) and nitrate reductase was anaerobically induced at the wild-type level, as evidenced by measurements of nitrate reductase activity (Table I). In anaerobiosis when nitrate was the terminal electron acceptor the doubling time of the mutant was 1.7-fold higher than that in the wild-type; this was not the case when fumarate was the terminal electron acceptor (Table I). We compared the nitrite content of the growth medium during growth of the mutant with that for the wild-type strain (Table I). The *B.subtilis narK* strain, like the *E.coli narK* strain, but unlike the wild-type, does not excrete nitrite when grown with nitrate as the terminal electron acceptor.

We then tested whether the *B.subtilis narK* gene was able to complement the defect of an *E.coli narK* strain. The *narK–lacZ* gene fusion in pDIA5351 (Figure 1) was expressed in *E.coli* from the *narK* promoter, as indicated by β-galactosidase activity assays (see below and Table III). The *narK* gene on pDIA5352 (Figure 1) was thus expected to be expressed. pDIA5352 and the parental vector pJM783, a ColE1 derivative vector for *lacZ* fusion construction (Perego, 1993), were introduced into the *E.coli narK* mutant RK5266 (Stewart and MacGregor, 1982). pDIA5352, but not pJM783, complemented the *narK* defect; nitrite was detected in the medium of RKS5266 (pDIA5352) but not in the control (data not shown).

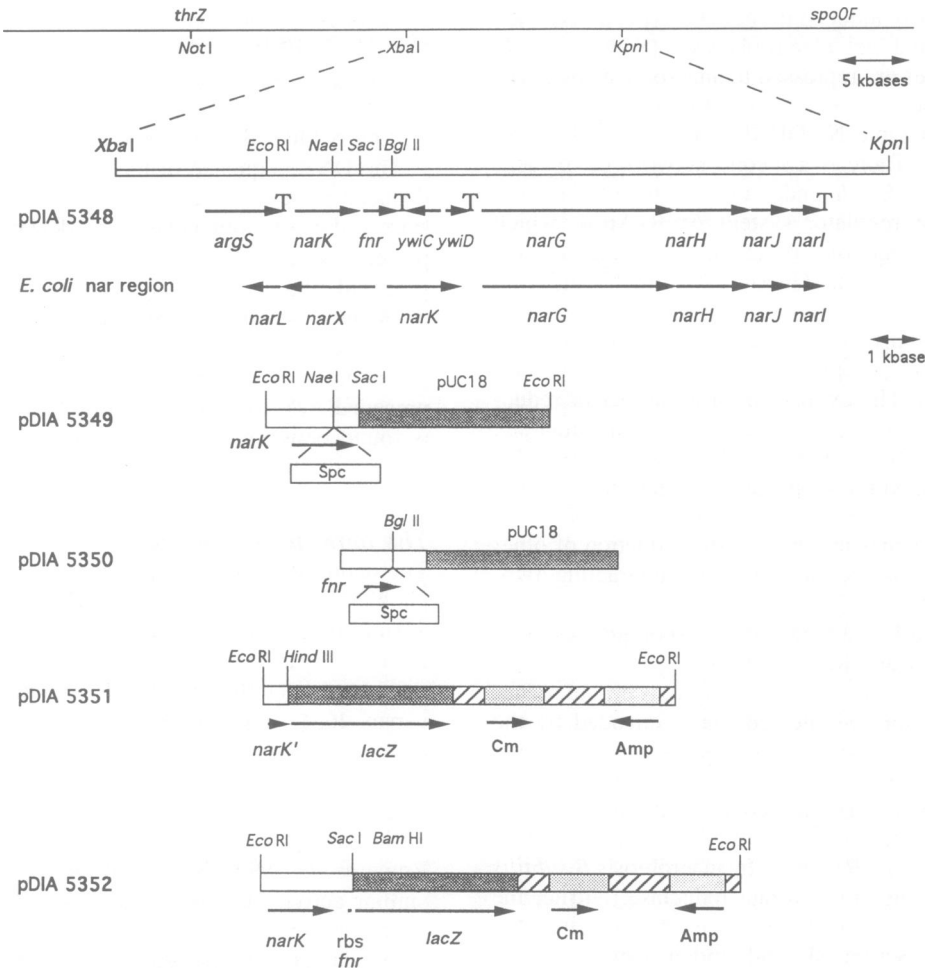

Fig. 1. Restriction maps of plasmids pDIA5348, pDIA5349, pDIA5350, pDIA5351 and pDIA5352. The *B.subtilis* DNA inserts are presented as open bars. Genes are represented by arrows. The four Ts indicate the four putative transcription terminators. The arrangement of *nar* genes in *E.coli* is shown beneath that of *B.subtilis*. The initiation codon and the RBS of *lacZ* gene originate from the *B.subtilis spoVG* gene (Perego, 1993). Amp, Cm and Spc refer to the ampicillin resistance gene from pBR322 (Sambrook *et al.*, 1989), the chloramphenicol resistance gene from pC194 (Horinouchi and Weisblum, 1982) and the spectinomycin resistance gene from transposon Tn554 (Murphy, 1985).

The fnr gene product

Protein sequence comparisons suggest that the *fnr* gene product (Bs-FNR) is a member of the *E.coli* CAP, FNR (Ec-FNR) activator family. The deduced protein sequence of *B.subtilis* Bs-FNR was multi-aligned with the protein sequences of Ec-FNR and CAP (Figure 4). The similarity between these proteins is low (20% identical residues between Bs-FNR and Ec-FNR, 24% between Bs-FNR and CAP and 21% between Ec-FNR and CAP). However, the conservation extends over the entire length of the proteins and several conserved features were revealed by the alignment (see Discussion). The three proteins may have similar secondary structures. The three-dimensional structure of CAP has been determined by X-ray diffraction (Weber and Steitz, 1987) and used to predict the three-dimensional structure of the *E.coli* FNR monomer (Bell and Busby, 1994). Similarly, we used this structure to propose a model for both Bs-FNR and Ec-FNR (Figure 5).

To confirm that Bs-FNR is a regulatory protein equivalent to *E.coli* FNR we disrupted the *fnr* gene in the *B.subtilis* chromosome by a double cross-over event between the chromosome and plasmid pDIA5350 (Figure 1). Growth of the *fnr* mutant and the wild-type strain were compared in aerobiosis and in anaerobiosis, with alternative electron acceptors, nitrate or fumarate, in the medium as indicated (Table II). *Bacillus subtilis* growth in aerobiosis was not affected by the *fnr* disruption (not shown), but was affected in anaerobiosis (Table II). *Bacillus subtilis* was able to grow in LBc medium in anaerobiosis and without the addition of an alternative electron acceptor. The terminal electron acceptor in this complex medium is not known and we cannot rule out the possibility that growth involves a fermentative type metabolism. However, the growth rate was strongly stimulated by the presence of nitrate or fumarate in the medium. In the *fnr* strain the growth rate was similar to that of the wild-type in LBc, but was not affected by the addition of nitrate or fumarate. Induction of nitrate reductase activity by anaerobiosis was abolished in the mutant. Because inactivation of this gene led to a large reduction in the use of nitrate or fumarate as electron acceptor, we called this gene, as in *E.coli*, *fnr* (fumarate nitrate reduction).

Transcription of the narK–fnr locus

To investigate the expression of these two genes we first analysed transcription of the locus by Northern experiments. Two different probes were used, one specific for the *narK* gene and the other for the *fnr* gene (Figure 6).

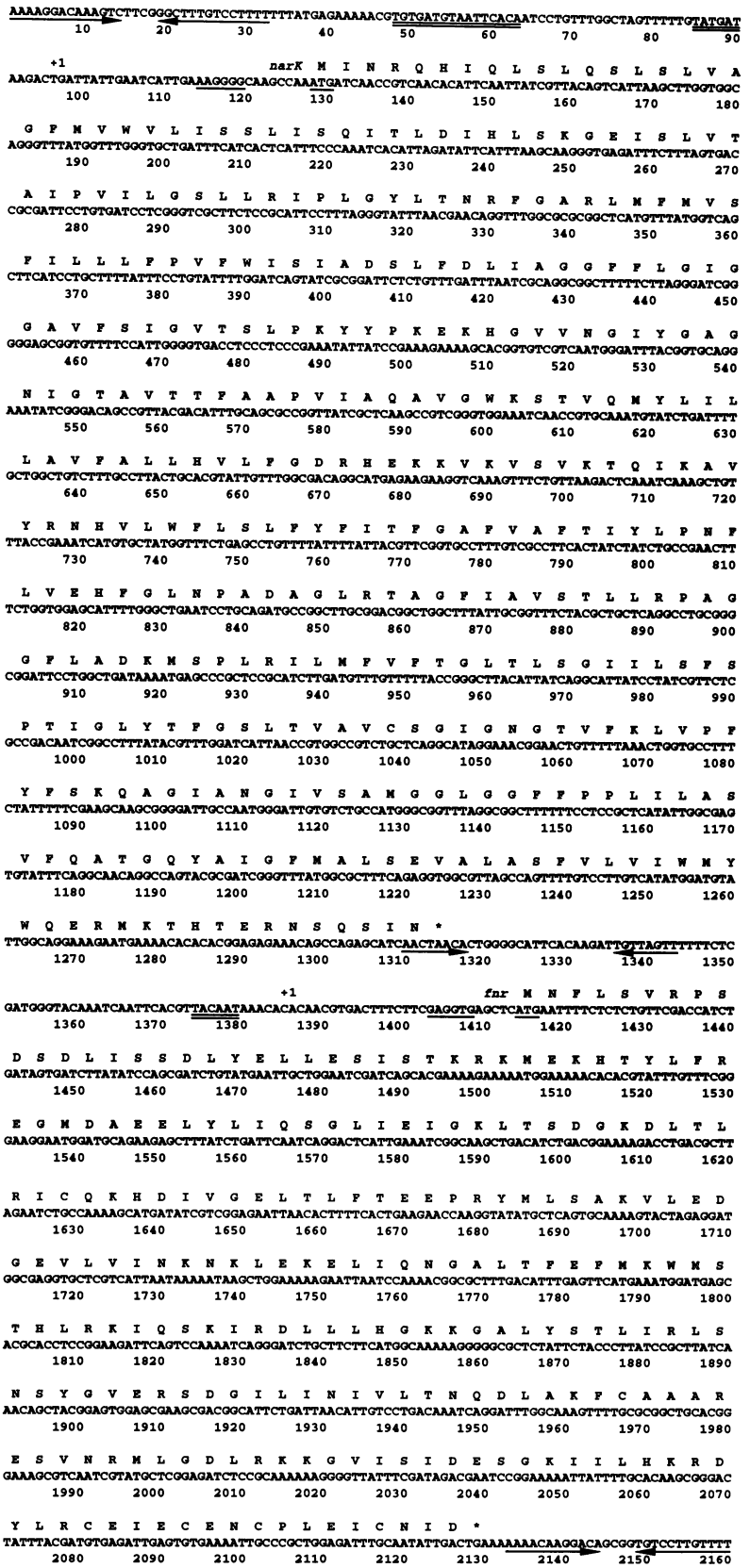

Fig. 2. Nucleotide sequence of the *B.subtilis narK* and *fnr* genes and deduced amino acid sequences of NarK and FNR. Nucleotides are numbered from the 5′-end. Putative transcription termination signals and the palindromic sequence in the *narK–fnr* intergenic region are represented by arrows. The proposed ribosome binding sites and initiation codons from *narK* and *fnr* are underlined. The putative FNR binding site and the –10 promoter regions are underlined twice. The mapped transcription starts positions are indicated by +1. The GenBank TM/EMBL accession number is Z49884.

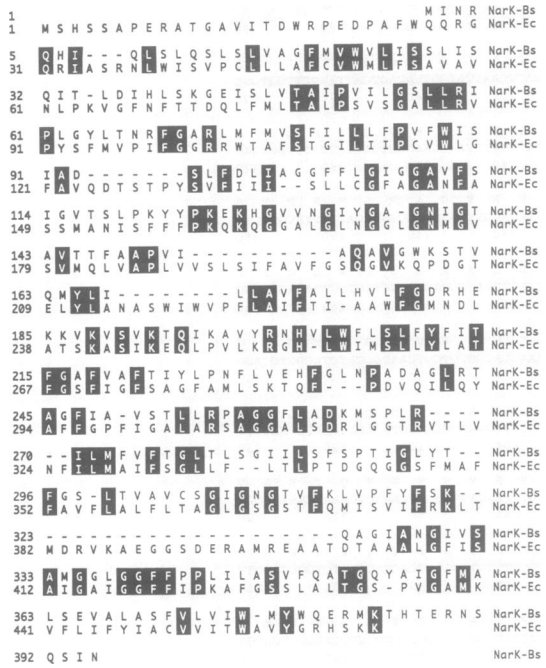

Fig. 3. Deduced protein sequence alignment of NarK from *B.subtilis* (NarK-Bs) with that from *E.coli* (NarK-Ec) (Noji *et al.*, 1989). Identical residues are in black boxes.

Table I. Growth characteristics of wild-type and *narK* mutant strains

B.subtilis strain	Nitrate reductase activity[a]	Nitrite excretion[b]	Doubling time in MMc (min)	
			Fumarate	Nitrate
168	0.3	14	105	70
168 *narK*	0.4	<0.1	105	120

All experiments were performed in anaerobiosis.
[a]In mid-exponential phase in LBc medium supplemented with 20 mM nitrate; expressed as μmol nitrite produced per min per ml of culture at an OD of 1.
[b]In early exponential phase in MMc nitrate medium; expressed as μmol of nitrite excreted into the medium per ml of culture at an OD of 1.

RNA was extracted after bacterial growth in the presence and absence of oxygen, nitrate and fumarate. A 2 kb messenger RNA was detected by the *narK* probe. This RNA was detected only in anaerobiosis and was induced by nitrate. This observation was substantiated by expression studies performed using a *narK–lacZ* gene fusion (see below). The size of this mRNA suggests that *narK* and *fnr* are co-transcribed. No shorter RNA was detected with this probe. Thus although there is an inverted repeat sequence in the DNA region between *narK* and *fnr* (indicated by arrows in Figure 2), transcription does not appear to terminate between the two genes or, if transcription does indeed stop at this structure, the shorter transcript is degraded very rapidly and is not detectable.

As expected, this 2 kb transcript was also detected by the *fnr* probe, which also revealed a second shorter transcript, ~700 bases long. This short transcript was presumably a *fnr*-specific transcript, starting in the *narK–fnr* intergenic region and ending at the transcription terminator located after the *fnr* gene. As for the long transcript, its synthesis was strongly induced in anaerobiosis. After longer exposure the short transcript was detected when bacteria were grown in rich medium in the presence of oxygen (data not shown).

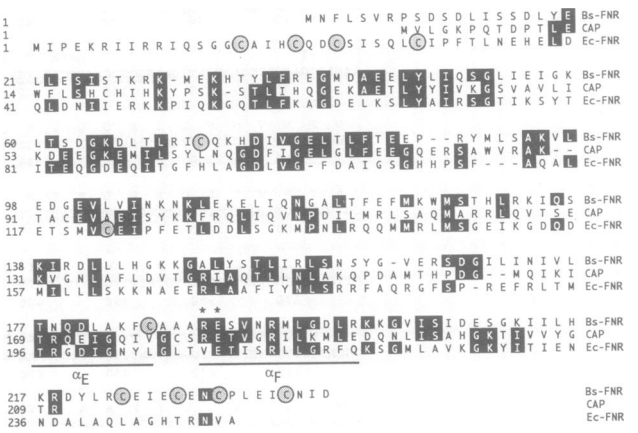

Fig. 4. Multiple alignment of deduced amino acid sequences of *B.subtilis* FNR (Bs-FNR), *E.coli* CAP (Cossart and Gicquel-Sanzey, 1982) and *E.coli* FNR (Ec-FNR) (Spiro and Guest, 1987b). Residues conserved in at least two sequences are in black boxes. The two helices from the CAP DNA binding motif are indicated by solid lines. Residues important for DNA binding are indicated by asterisks. Bs-FNR and Ec-FNR cysteine residues are in gray circles.

Promoter mapping and the FNR regulatory site

The two mRNA 5′-ends were mapped by primer extension experiments (Figure 6). Analysis of the signal intensities in these experiments are in good agreement with the Northern hybridization results; both promoters were strongly activated during anaerobiosis, the promoter upstream of *narK* is further induced by nitrate and only the promoter upstream of *fnr* has a detectable activity in aerobiosis.

A typical –10 region is found six bases upstream of the transcription start point of the bi-cistronic *narK–fnr* mRNA (Figure 6). There is no obvious –35 sequence, although there is a palindromic sequence centred at position –41.5 (indicated in Figures 2 and 7). In this sequence the two core 5 bp motifs of the *E.coli* CAP consensus binding sequence (Kolb *et al.*, 1993) are strictly conserved. There is a similar sequence upstream of the *narGHJI* operon and between the *ywiC* and *ywiD* genes. We have also searched for similar sequences in all published sequences from *B.subtilis* using the SubtiList database (Moszer *et al.*, 1995). This signal was found upstream of *ywcJ* (*ipa48r*; Glaser *et al.*, 1993). The *ywcJ* gene product is similar to the *E.coli nirC* gene product (Peakman *et al.*, 1990). *nirC* is the fourth gene of the *E.coli* soluble NADH-dependent nitrite reductase operon. Its product is not essential for nitrite reductase activity and its function is not known, although it has been shown that its expression is activated by FNR in anaerobiosis (Harborne *et al.*, 1992). An alignment of these four sequences together with *E.coli* FNR and CAP consensus binding sequences is presented in Figure 7. Comparison of the four sequences allowed us to predict a putative –10 region downstream of the conserved inverted repeat detected in the *narGHJI*, *ywiD* and *ywcJ* promoter regions (Figure 7). In all cases this sequence is centred at position –41.5. Furthermore, preliminary transcriptional analysis indicates that transcription

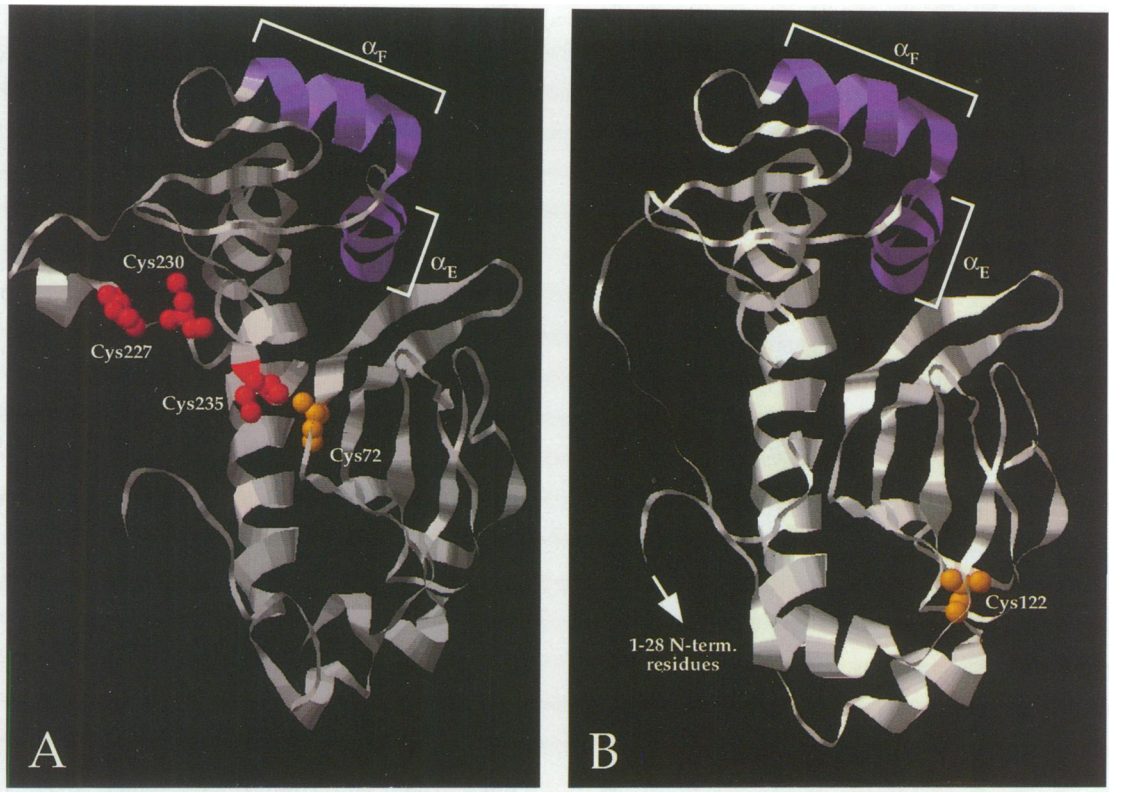

Fig. 5. Predicted three-dimensional structures of Bs-FNR (**A**) and Ec-FNR (**B**) monomers, based on the known structure of *E.coli* CAP protein. The Bs-FNR C-terminal cysteine residues presumably involved in the binding of an iron–sulfur centre are shown as red spacefill spheres (A). The central cysteine residue is shown as yellow spacefill spheres (A and B). The N-terminal end (residues 1–28) of the Ec-FNR structure could not be predicted, but its putative location is consistent with the proposed model. The two helices constituting the helix–turn–helix motif involved in DNA binding are indicated in violet.

of the *narGHJI* operon, *ywiD* and *ywcJ* is indeed induced during anaerobiosis (data not shown). It is therefore probable that this conserved inverted repeat sequence is the Bs-FNR binding site. This site is highly reminiscent of the *E.coli* CAP site. In order to test whether CAP could recognize this sequence and activate transcription of the *B.subtilis narK* promoter, plasmid pDIA5351 harboring the *narK–lacZ* gene fusion (see below and Figure 1) was introduced into *E.coli crp* (TP2139), *cya* (TP2006) and the isogenic wild-type strain for these two loci (TP2100). β-Galactosidase activity was measured in the three strains, in the presence and absence of cAMP for the *cya* strain (Table III). Expression of the *B.subtilis narK* promoter in *E.coli* was activated 50-fold by the CAP–cAMP complex.

The DNA sequence of the *fnr* promoter is also presented in Figure 7 and a consensus −10 sequence is predicted. No other significant similarities with the former four sequences were found. The only striking feature of the *fnr* region is an eight base inverted repeat with an 18 base loop centred at position −59.5, as indicated by arrows in Figure 2. This structure could be involved in regulation of *fnr* gene expression.

Expression analysis

To further investigate expression of the *B.subtilis fnr* and *narK* genes we constructed transcriptional fusions with the *E.coli lacZ* reporter gene (see Materials and methods and Figure 1). We measured β-galactosidase activities in extracts from bacteria grown in LBc in aerobiosis or in anaerobiosis, with alternative electron acceptors, fumarate or nitrate, in the medium as indicated. The effect of *fnr* inactivation on expression of the *narK–lacZ* and *fnr–lacZ* fusions was also investigated (Table IV).

Table II. Growth characteristics of wild-type and *fnr* mutant strains in LBc medium in anaerobiosis at 37°C

B.subtilis strain	Nitrate reductase activity[a]	Doubling time (min)		
		LBc	LBcN[b]	LBcF[c]
168	0.3	120	60	90
168 *fnr*	<0.005	120	120	130

[a]As in Table I.
[b]LBc supplemented with 20 mM nitrate.
[c]LBc supplemented with 30 mM fumarate.

narK expression was induced >200-fold during anaerobiosis. During anaerobic growth the expression of the *narK* gene in a wild-type background was further increased 3-fold by addition of nitrate to the medium. The level of expression of the *narK* fusion was not increased during anaerobiosis in the *fnr* mutant. The anaerobic induction of *narK* is therefore FNR dependent.

β-Galactosidase expression by the *fnr–lacZ* fusion was assumed to reflect expression of the normal *fnr* gene. It corresponds to combined transcription from the *fnr* promoter and *narK* promoter. *fnr* gene expression was strongly induced by anaerobiosis (200-fold). Similarly, in the *fnr* mutant, expression of the *fnr–lacZ* fusion was strongly induced by anaerobiosis, although at a lower level (100-

fold). This weaker induction could be due to a reduction in *narK* promoter activity in this background. In agreement with the absence of a detectable FNR target consensus in the vicinity of the *fnr* promoter, activation of the *fnr* promoter in anaerobiosis was FNR independent. Induction by anaerobiosis presumably, therefore, involves a second mechanism.

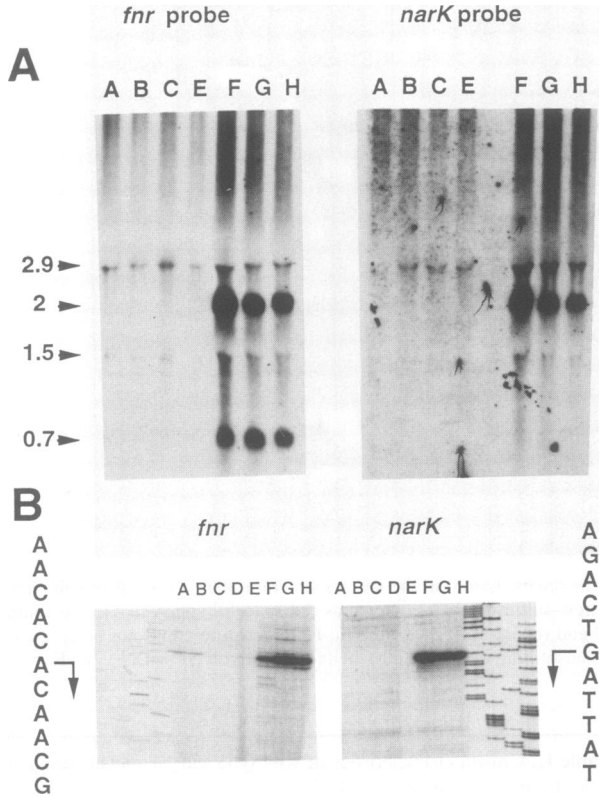

Fig. 6. mRNA analysis of the *narK–fnr* locus. (**A**) Northern blot analysis. The *narK* probe was the 660 bp DNA insert, from codon 7 to codon 227 within *narK*, from a pUC18 derivative obtained by shotgun cloning of the pDIA5348 insert. The *fnr* probe was the 476 bp *Eco*RI–*Bgl*II fragment internal to *fnr*. 16S (1553 bases) and 23S (2928 bases) rRNA were detected by non-specific hybridization and used as molecular weight markers. (**B**) Primer extension mapping. Location of the transcription start points was deduced from the lengths of the cDNA bands. The lengths were obtained by comparison with the sequencing reaction products (in the order A, C, G and T) of pDIA5348. Each set of sequence reactions were performed with the 5′-end-labelled oligonucleotide used for the corresponding primer extension experiment. mRNA were extracted from *B.subtilis* grown under the following conditions: A, LB in aerobiosis; B, LB nitrate aerobiosis, C, MM ammonium as nitrogen source in aerobiosis; D, MM ammonium and nitrate in aerobiosis; E, MM nitrate in aerobiosis; F, LBc nitrate in anaerobiosis; G, LBc fumarate in anerobiosis; H, LBc in anaerobiosis. Aliquots of 10 μg RNA were used in each track.

Discussion

Bacillus subtilis is a soil bacterium. In its environment oxygen availability can fluctuate rapidly and bacteria have to adapt their metabolism to these variations. However, very little is known about anaerobic metabolism and its regulation in *B.subtilis*. Systematic sequencing of the genome of *B.subtilis* uncovered genes involved in nitrate respiration and this was the starting point of the understanding of anaerobic regulation in this bacterium. Anaerobic metabolism and its regulation has been well characterized at a molecular level in the enteric bacterium *E.coli* (Spiro and Guest, 1990; Iuchi and Lin, 1993). During its life cycle *E.coli* encounters both anaerobic conditions in the mammalian gut and aerobic conditions in the external medium. For both bacteria transition from aerobiosis to anaerobiosis or vice versa can be very fast. Both *B.subtilis* and *E.coli* have regulatory mechanisms to adapt their metabolisms to anaerobic conditions of growth and to use alternative electron acceptors. Comparison of the two bacteria is interesting from evolutionary and physiological points of view.

Organization of the five *nar* genes (*narK* and *narGHJI*) is conserved between *B.subtilis* and *E.coli*; the three subunits of nitrate reductase are encoded by a single operon, *narGHJI*, together with a fourth polypeptide essential for assembly of the complex, the product of *narJ*. There are similar *narK* genes upstream of the *narGHJI* operon in both species. These genes are in the same orientation and transcribed independently (Figure 1). Despite sequence divergence between the two NarK proteins, they have a similar function in nitrite excretion and *B.subtilis* NarK is functional in *E.coli* and its structural gene complements an *E.coli narK* mutation. In *E.coli narK* transcription is activated by nitrate, mediated by dual interacting two-component regulatory systems (Stewart and Rabin, 1995); nitrate signalling is mediated by the homologous sensor proteins NarX and NarQ. Of the two response regulator proteins NarL and NarP, only

Table III. Activation of *B.subtilis narK* expression in *E.coli* by the cAMP–CAP complex

E.coli strain	LB	LB + cAMP (2 mM)
TP2100	2200	ND
TP2006 (*cya*)	45	1500
TP2139 (*crp*)	45	ND

β-galactosidase activities of the *narK–lacZ* fusion in *E.coli* strains during exponential growth in LB medium at 37°C (Miller units/OD_{600} unit).
ND, not determined.

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                      *****   **   *****                          +1
narK          AACG TGTGA TGTAAT TCACA atcctgtttggctagtttttg TATGATaagactG
ywiD          AGGC TGTGA AATACA TCACT gctgtacaggctcagtcgagc TACACTaaagctA
narG          AGTG TGTGA CATAGT TCACA aggaaacacgctgtctctgtt TATAGTgaacttA
ywcJ (nirC)   TAAT TGTGA AATACT TCACA atatcgtgccatactatgctc AATCATgaaagaA
fnr           acaagattgttagtttttctcgatgggtacaaatcaattcacgt TACAATaaacacA
Cons. CAP      AA- TGTGA --T--- TCACA -T-
Cons. Fnr      A-A -TTGA T--A-A TCAAT ---
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Fig. 7. Promoter regions of four *B.subtilis* genes induced during anaerobiosis and of *fnr*. The −10 promoter regions are underlined. Conserved residues in the putative Bs-FNR binding sequences are indicated by asterisks. *Escherichia coli* CAP and FNR binding consensus sequences are also shown (from Spiro and Guest, 1991).

Table IV. Effect of growth conditions and of the *fnr* mutation on *narK* and *fnr* gene expression

B.subtilis strain	LBc		LBc + nitrate[a]		LBc + fumarate[b]	
	Aero.[c]	Anaero.[d]	Aero.	Anaero.	Aero.	Anaero.
narK–lacZ	<10	1900	13	7000	<10	1800
narK–lacZ fnr::spc	<10	20	<10	20	<10	20
fnr–lacZ	15	4200	20	4300	20	2900
fnr–lacZ fnr::spc	10	1200	16	1400	20	1900

β-galactosidase activities of *narK–lacZ* and *fnr–lacZ* fusion strains during exponential growth at 37°C (Miller units /mg protein).
[a]LBc supplemented with 20 mM nitrate.
[b]LBc supplemented with 30 mM fumarate.
[c]Aerobic growth condition.
[d]Anaerobic growth condition.

NarL binds DNA to control nitrate induction of *narK*. The *narL* and *narX* genes are organized in a single operon upstream of *narK* (Figure 1; Stewart *et al.*, 1989). In *B.subtilis* three additional genes are found between *narK* and *narG*: *fnr*, *ywiC* and *ywiD*. However, apart from *fnr* we found no genes encoding proteins similar to regulatory proteins in the vicinity of the newly discovered *B.subtilis nar* genes. The amino acid sequences of the predicted products of the two other CDS, *ywiC* and *ywiD*, are not similar to any known protein sequence. Furthermore, we have not identified in the *narK* and *narG* promoter regions sequences similar to the consensus *E.coli* NarL binding sites (Tyson *et al.*, 1993).

Regulation of *narK* gene expression in *B.subtilis* and *E.coli* is similar. They are strongly induced by anaerobiosis and more weakly by nitrate. Furthermore, induction by nitrate in both bacteria requires an active FNR (Kolesnikow *et al.*, 1992).

FNR is an activator member of the CAP family (Kolb *et al.*, 1993) and is required for anaerobic induction of *narK* in both organisms. FNR is a pleiotropic regulator sensing anaerobiosis. More than 14 target sites for FNR have been identified in *E.coli* and we describe four sites for FNR in *B.subtilis*. There is a palindromic sequence very similar to the CAP consensus upstream of the *narK* promoter and we found sequences similar to this sequence upstream of three other anaerobically induced *B.subtilis* promoters. These four sequences allow us to predict the Bs-FNR binding site on the DNA sequence (Figure 7). The four identified sites are much more conserved than known *E.coli* CAP sites and FNR sites; 12 bases are conserved, except for the *ywiD* site, for which 11 bases are conserved.

In *E.coli* FNR sites are reminiscent of CAP sites. However, the two palindromic consensuses differ at two symmetry-related positions (underlined twice in Figure 7; Spiro and Guest, 1990). Active CAP and Ec-FNR proteins are dimers and each subunit interacts with one half of the binding site (Kolb *et al.*, 1993). The CAP DNA binding helix–turn–helix motif has been extensively studied and amino acid residues involved in the binding of CAP to its targets on the chromosome have been identified using several methods, essentially site-directed mutagenesis (Zhang and Ebright, 1990) and X-ray crystallography of the CAP–DNA complex (Schultz *et al.*, 1992). The differences in the CAP and Ec-FNR DNA binding sites have been correlated with differences in the DNA binding motif (Bell *et al.*, 1989; Spiro *et. al.*, 1990; Zhang and Ebright, 1990). In CAP Arg180 contacts a GC base pair at position 5 of the DNA half-site (underlined twice in Figure 7). The residue at the corresponding position in Ec-FNR is a valine (Figure 4) and, instead of a GC base pair, a TA base pair is found in the FNR half-site (Figure 7). The specificity of CAP and FNR have been changed by site-directed mutagenesis in such a way that one activator recognizes the binding site specific for the other activator (Spiro and Guest, 1987a). As in CAP, an arginine residue is found at the corresponding position in Bs-FNR and a GC base pair in the predicted Bs-FNR binding site (Figure 7). Furthermore, Spiro *et al.* (1990) have proposed that specificity of Ec-FNR for its target is due to Ser212 and that this residue might interact with the TA base pair at position 5 of the DNA half-site (Figure 7). This residue is conserved neither in CAP nor in Bs-FNR, a Gly and a Gln residue respectively being found at the corresponding positions of these two proteins. These observations are in complete agreement with the model for DNA recognition by members of this regulatory protein family. It raises the question of co-evolution of the regulator DNA binding domain and its targets on the chromosome. It should be noted that immunological studies have shown that *B.subtilis* does not synthesize a protein homologous to CAP (Biville and Guiso, 1985), allowing Bs-FNR to recognize a target sequence identical to a CAP site.

The four identified Bs-FNR sites are all at exactly the same position with respect to the putative –10 promoter region (Figure 7); the palindromic sequence is centred on –41.5. This is identical to the majority of known *E.coli* FNR binding sites (Spiro and Guest, 1990). However, in the case of CAP numerous sites are located further upstream of the –10 region. In the *lac* promoter it is centred at –61.5 for example (Kolb *et al.*, 1993). The DNA binding site for Bs-FNR is identical to the *E.coli* CAP site. We have also shown that Bs-FNR site can also be recognized by CAP and that the *narK* promoter is activated in *E.coli* by the cAMP–CAP complex (Table III). Thus the activation mechanism is well conserved between these two bacteria, which diverged ~2000 million years ago.

The molecular mechanism of regulation of Ec-FNR activity by oxygen is still largely hypothetical, *in vitro* experiments using this protein being difficult. It is assumed that in anaerobiosis FNR is subject to conformational changes essential for its activator property and that iron is required for this regulation (Spiro and Guest, 1990). It has been shown by Green *et al.* (1993), using site-directed

mutagenesis, that four of the five cysteine residues in Ec-FNR (C20, C23, C29 and C122) are involved in transcriptional activation by FNR and that the replacement of C122 by alanine substantially decreases the iron content of FNR. A model of the modulation by oxygen has been proposed: (i) the oxygen concentration affects the redox state of the iron, FeIII in the presence of oxygen and FeII in its absence; (ii) the redox state of the iron (FeII or FeIII) affects the structure of the activator and its activity. Khoroshilova *et al.* (1995) studied a modified FNR protein, with increased DNA binding and increased dimerization in the presence of oxygen, and suggest that the iron in FNR is in the form of a [4Fe–4S] centre.

None of the five Ec-FNR cysteine residues are conserved in Bs-FNR, although there is a cluster of four cysteine residues at the C-terminal end of the protein and two other cysteine residues: C72 in the nucleotide binding-like domain and C185 in the DNA binding domain. Thus possibly a metal, presumably iron in an iron–sulfur centre, is also involved in activation of Bs-FNR in anaerobiosis. We have predicted the three-dimensional structure of both Bs-FNR and Ec-FNR monomers according to the three-dimensional structure of CAP (Figure 5). These models are compatible with the binding of an iron–sulfur centre by cysteine residues. This centre would be on the protein face opposite to the dimerization face. In Bs-FNR, C72, but not C185, would bind the iron–sulfur centre. Not only the type of regulator, but also the mechanism of its activation by oxygen, appears to be conserved between *B.subtilis* and *E.coli*. We propose two evolutionary models for this activator. In the first model one regulator evolved from the other by a translocation event leading to shuffling of the cysteine terminal motif to the other extremity of the protein and the subsequent substitution of the internal cysteine residue. In the second model both regulators derive from the same ancestral activator and have gained the cysteine motif and the associated regulation independently.

In *E.coli* the *fnr* gene is expressed constitutively, but with a 2-fold repression in anaerobiosis (Spiro and Guest, 1987b). Similarly, the CAP–cAMP complex represses expression of the *crp* gene (Okamoto *et al.*, 1988). In *B.subtilis* the situation is exactly opposite; *fnr* gene expression being weak in aerobiosis and strongly induced in anaerobiosis. This induction is largely independent of FNR itself. The *fnr* gene is expressed from two promoters, the *narK* and the *fnr* promoters. The slight reduction in induction of *fnr–lacZ* fusion in an *fnr* mutant could reflect the absence of activation of the *narK* promoter, the *fnr* promoter being still fully induced. In *B.subtilis* induction of genes involved in anaerobic respiration requires a cascade of at least two steps: one FNR-independent and affecting FNR synthesis, the other is activation by FNR. This activation mechanism could allow fine tuning of the response of *B.subtilis* to a shift from aerobiosis to anaerobiosis.

Materials and methods

Bacterial strains and growth conditions

All *B.subtilis* strains used are derivatives of strain 168. The *E.coli* strain used for DNA sequencing was XL1 blue (Bullock *et al.*, 1987). The *E.coli* strain TP611 (*pcnB*) was used for plasmid rescue cloning (Glaser *et al.*, 1993). To test complementation of *narK* we used *E.coli* strain RK5266 (Stewart and MacGregor, 1982). Plasmids integrated by Campbell-type recombination were prepared in the *E.coli* recombination-proficient strain TG1 (Gibson, 1984). Three isogenic *E.coli* strains, TP2006 (*cya*), TP2139 (*crp*) and TP2100 (Roy and Danchin, 1982), were used for analysis of activation of *narK* expression by CAP.

Luria-Bertani (LB) medium was used for standard cultures of *B.subtilis* and *E.coli* (Miller, 1972). Sporulation medium (SP) was prepared as described by Schaeffer *et al.* (1965). For anaerobic growth of *B.subtilis* a supplemented LB medium was used (LBc). In this rich medium, 1% glucose, 0.1 mM tryptophan, 2 mM magnesium sulfate, 0.1 mM ferric ammonium citrate and 20 mM potassium phosphate buffer, pH 7, were added to the LB medium. The defined medium (MMc) was that of Anagnostopoulos and Spizizen (1961) supplemented with 1% glucose, 0.1 mM ferric ammonium citrate and a mixture of 18 amino acids (5 mM each), but no methionine or cysteine. Nitrate was added to 20 mM and fumarate to 30 mM. Anaerobic growth was performed in Erlenmeyer flasks with rubber stoppers and with shaking. Anoxia was achieved by bubbling N_2 for 1 min. Antibiotics were added when necessary at the following concentrations: 100 mg/l ampicillin; 5 mg/l chloramphenicol; 5 mg/l kanamycin; 60 mg/l spectinomycin. Bacteria were grown at 37°C in all experiments. Optical density (OD) of bacterial cultures was measured at 600 nm using a Hitachi U-1100 spectrophotometer.

Plasmids

DNA sequences were determined from sub-clones in plasmid pUC18 (Yanisch-Perron *et al.*, 1985). Plasmid rescue cloning was performed after sub-cloning in the integrative vector pDIA5304 (Glaser *et al.*, 1993). Transcriptional fusions with the *E.coli lacZ* gene were constructed using integrative plasmids pJM783 (Perego, 1993) and pDIA5307 (Calogero *et al.*, 1994).

DNA and RNA manipulations and genetic techniques

Escherichia coli was transformed as described by Chung and Miller (1988). Shotgun libraries in pUC18 were introduced into *E.coli* XL1 blue by transformation as described by Hanahan (1983). Recombinant plasmids were introduced into *E.coli* TP611 by calcium chloride transformation (Sambrook *et al.*, 1989). *Bacillus subtilis* cells were transformed as described by Kunst and Rapoport (1995).

RNA was extracted as described by Hagen and Young (1978) with one minor modification: before addition of the lysis solution the bacterial pellet was resuspended in 200 µl lysozyme solution (40 mg/ml in 12 mM Tris, 5 mM EDTA, 10% glucose). Southern blotting, colony transfers and Northern blotting were performed as described by Sambrook *et al.* (1989). Membranes were further hybridized with non-radioactively labelled probes (Boehringer; DIG-UTP). Primer extensions with reverse transcriptase were as decribed by Piekielny and Rosbash (1985). The oligonucleotides 5′-CTAAAGAAATCTCACCCTTGC-3′ and 5′-TCCC-GAAACAAATACGTGTG-3′ were used as primers for mapping the *narK* and the *fnr* promoters respectively.

The sequencing strategy was as previously described (Glaser *et al.*, 1993; Moszer *et al.*, 1991). DNA sequences were compiled using the program XBAP of Staden (Dear and Staden, 1991). Sequences were analysed using DNA Strider 1.1 software (Marck, 1988). Similarity searches using the BLAST program (Altschul *et al.*, 1990) were performed at the NCBI in the non-redundant protein library from the NCBI. Sequences were compared using the Wisconsin Genetics Computer Group sequence analysis software package version 6.0 (University of Wisconsin Biotechnology Center, Madison, WI). The CLUSTAL method (Higgins *et al.*, 1992) was used for initial multiple alignment and refined manually. The SubtiList database (Moszer *et al.*, 1995) was used to search for sequence patterns in *B.subtilis* sequences. The molecular modeling of Bs-FNR and Ec-FNR was performed using the Swiss Model Automated Protein Modelling service at the GLAXO Institute for Molecular Biology SA, which makes use of the ProMod (PROtein MODelling tool) software (Peitsch and Jongeneel, 1993). The GCG package was used for preliminary alignments between the *E.coli* CAP sequence and each FNR sequence.

Construction of fusion and mutant strains

Bacillus subtilis strains containing a transcriptional fusion between the *E.coli lacZ* gene and the *narK* or *fnr* genes were constructed as follows. The 1.6 kb *Eco*RI–*Sac*I DNA fragment from plasmid pDIA5348, encompassing the 3′-end of the *argS* gene the complete *narK* gene and the RBS sequence from the *fnr* gene, was first inserted between the *Eco*RI and *Sac*I sites in pUC18 to give pDIA5356. The 370 bp long *Eco*RI–*Hin*dIII fragment from pDIA5356 was then inserted between

*Eco*RI and *Hin*dIII sites in pDIA5307, leading to plasmid pDIA5351 (Figure 1). The *lacZ* gene was thus placed after the 15th codon of the *narK* gene, creating a transcriptional fusion. To construct a transcriptional fusion with the *fnr* gene the chromosomal insert from plasmid pDIA5356 was excised as an *Eco*RI–*Bam*HI fragment and inserted between *Eco*RI and *Bam*HI sites in plasmid pJM783, leading to plasmid pDIA5352 (Figure 1). The *lacZ* gene in this last construction was placed just after the putative *fnr* RBS, creating a transcriptional fusion. Both transcriptional fusions were introduced into the *B.subtilis* chromosome by Campbell-type recombination events.

Two *B.subtilis* strains in which *narK* or *fnr* were interrupted by a spectinomycin resistance gene (Murphy, 1985) were constructed by homologous recombination using pDIA5349 and pDIA5350 respectively. pDIA5349 was obtained by insertion of a spectinomycin cassette at the single *Nae*I site in plasmid pDIA5356, interrupting the *narK* gene after the 240th codon. pDIA5350 was constructed by insertion of a spectinomycin cassette at the single *Bgl*II site in a pUC18 derivative obtained in the pDIA5348 shotgun cloning experiment. In pDIA5350 the *fnr* gene was interrupted after the 198th codon. Plasmids pDIA5349 and pDIA5350 were linearized and used to transform *B.subtilis*. Strains in which the wild-type *narK* or *fnr* genes were replaced by the disrupted copies, *narK*::*spec* and *fnr*::*spec*, were selected as spectinomycin-resistant transformants.

Southern blotting was used to confirm the appropriate substitution of the wild-type gene by the mutated copy in mutant strains and to verify that only a single copy of the *lacZ* fusion was integrated.

Enzymatic assays

Nitrate reductase activity was determined by using reduced MVH as electron donor as previously described (Glaser *et al.*, 1995). Nitrite concentration was measured using the method of Shinn (Allport, 1947).

β-Galactosidase was assayed as previously described (Msadek *et al.*, 1990). β-Galactosidase activity was expressed in Miller units/milligram protein (Miller, 1972). Protein concentrations were determined using the Bio-Rad Protein Assay kit.

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