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# Two-Component Regulatory Proteins ResD-ResE Are Required for Transcriptional Activation of *fnr* upon Oxygen Limitation in *Bacillus subtilis*

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***Bacillus subtilis*** **can grow anaerobically in the presence of nitrate as a terminal electron acceptor. The two component regulatory proteins, ResD and ResE, and an anaerobic gene regulator, FNR, were previously shown to be indispensable for nitrate respiration in *B. subtilis*. Unlike *Escherichia coli fnr*, *B. subtilis fnr* transcription was shown to be highly induced by oxygen limitation. *fnr* is transcribed from its own promoter as well as from a promoter located upstream of *narK*, the first gene in the *narK-fnr* dicistronic operon. DNA fragments containing the *narK* promoter, the *fnr* promoter, and both of the promoters were used to construct three *lacZ* fusions to examine the transcriptional regulation of the *narK-fnr* operon. ResDE was found to be required for transcriptional activation of *fnr* from the *fnr*-specific promoter, and FNR was required for activation of *narK-fnr* transcription from the FNR-dependent *narK* operon promoter under anaerobiosis. In order to determine if the requirement for ResDE in nitrate respiration is solely to activate *fnr* transcription, *fnr* was placed under control of the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter, P*spac*. The observed defect in anaerobic growth of a P*spac-fnr* Δ*resDE* mutant in the presence of IPTG indicated that *resDE* has an additional role in *B. subtilis* anaerobic gene regulation.**

The gram-positive bacterium *Bacillus subtilis* can grow anaerobically in the presence of nitrate as a terminal electron acceptor (2, 4, 8, 26, 28, 29, 35). Two distinct operons specifying two nitrate reductases have been isolated; one (*narGHJI*) encoding respiratory nitrate reductase, which is required for nitrate respiration under anaerobic conditions (2, 8) and, the other encoding assimilatory nitrate reductase (*nasBC*), which is involved in the assimilation of nitrogen from nitrate under aerobic conditions (19, 22). Respiratory nitrate reductase activity which is induced under anaerobic conditions was shown to be dependent on *fnr*, a gene encoding a protein homologous to *Escherichia coli* FNR (2). *B. subtilis fnr* was found (2) to be the second gene of a dicistronic operon also containing a gene homologous to *E. coli narK* (21), which is required for nitrite extrusion (3, 27). Two unknown genes, sharing a putative FNR-binding site in their promoter regions, and the *narGHJI* operon were identified downstream of the *narK-fnr* operon (2). *B. subtilis* FNR is structurally distinct from that of *E. coli* in that it contains a cysteine cluster within its C-terminal end instead of the N terminus and the putative FNR-binding sequence of *B. subtilis* is more similar to the *E. coli* catabolite activator protein site than the *E. coli* FNR site (2). Furthermore, two anaerobically induced *fnr* transcripts can be detected in *B. subtilis* (2), unlike the situation in *E. coli* in which *fnr* is autorepressed under anaerobic conditions (24, 32). The synthesis of the longer transcript, containing *narK-fnr*, is initiated from the *narK* promoter and is dependent on *fnr* itself, in agreement with the presence of a putative FNR-binding site centered at position −41.5 in the *narK* promoter. The shorter transcript is *fnr* specific and is transcribed from a promoter within the *narK-fnr* intergenic region. It was proposed that *fnr* induction by anaerobiosis involves two steps: first, the activation of *fnr* transcription by an unknown FNR-independent mechanism, and, second, the induction of *fnr* transcription at the FNR-dependent promoter (2).

Another gene, *resD* (formerly *orfX17*), originally isolated and sequenced as a part of the *Bacillus* genome project (31), was found to be essential for anaerobic nitrate respiration and aerobic respiration in *B. subtilis* (9, 35). *resD* and its downstream gene, *resE*, are members of a two-component signal transduction system. *resD* and *resE* encode a response regulator and a histidine protein kinase, respectively. *resD* and *resE* belong to an operon along with upstream genes *resABC*, which encode proteins similar to cytochrome *c* biogenesis proteins. *resD* and *resE* are also transcribed from a promoter upstream of *resD*. ResD is required for transcription of *resA*, as well as for *ctaA*, which is essential for heme A synthesis, and the expression of the *petCDE* operon encoding subunits of the cytochrome *bf* complex. In addition to a defect in aerobic respiration, the *resD* mutant can not grow anaerobically on medium containing nitrate (35).

In this paper, we examined the epistatic relationship between *fnr* and *resDE* in the regulation of anaerobic nitrate respiration in *B. subtilis*. We show that *resDE* is required for the transcriptional activation of *fnr* upon a shift to an anaerobic environment.

## MATERIALS AND METHODS

**Strains, plasmids, and culture methods.** Cells were grown aerobically in 2× YT (yeast extract-tryptone) medium (18) supplemented with 1% glucose and 0.2% $KNO_3$ with vigorous shaking (300 rpm). For anaerobic cultures, screw-cap tubes were filled to the top with cell suspension in the same medium and

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TABLE 1. Characteristics of *B. subtilis* strains used in this study

| Strain | Relevant characteristic | Source |
|---|---|---|
| JH642 | *trpC2 pheA1* | J. Hoch |
| ZB307A | SPβ*c2del2*::Tn*917*::pSK10Δ6 | P. Zuber |
| LAB2135 | *trpC2 pheA1* Δ*resDE*::Tet$^r$ | This work |
| LAB2136 | *trpC2 pheA1 fnr*::Spc$^r$ | This work |
| LAB2143 | *trpC2 pheA1 narG-lacZ* (Cm$^r$) | This work |
| LAB2251 | *trpC2 pheA1* SPβ*c2del2*::Tn*917*::pMMN287 | This work |
| LAB2252 | *trpC2 pheA1* SPβ*c2del2*::Tn*917*::pMMN288 | This work |
| LAB2253 | *trpC2 pheA1* SPβ*c2del2*::Tn*917*::pMMN289 | This work |
| LAB2256 | *trpC2 pheA1 fnr*::Spc$^r$ SPβ*c2del2*::Tn*917*::pMMN287 | This work |
| LAB2257 | *trpC2 pheA1 fnr*::Spc$^r$ SPβ*c2del2*::Tn*917*::pMMN288 | This work |
| LAB2258 | *trpC2 pheA1 fnr*::Spc$^r$ SPβ*c2del2*::Tn*917*::pMMN289 | This work |
| LAB2262 | *trpC2 pheA1* Δ*resDE*::Tet$^r$ SPβ*c2del2*::Tn*917*::pMMN287 | This work |
| LAB2263 | *trpC2 pheA1* Δ*resDE*::Tet$^r$ SPβ*c2del2*::Tn*917*::pMMN288 | This work |
| LAB2264 | *trpC2 pheA1* Δ*resDE*::Tet$^r$ SPβ*c2del2*::Tn*917*::pMMN289 | This work |
| LAB2311 | *trpC2 pheA1 fnr*::pMMN297 | This work |
| LAB2313 | *trpC2 pheA1* Δ*resDE*::Tet$^r$ *fnr*::pMMN297 | This work |
| LAB2324 | *trpC2 pheA1 fnr*::pMMN297 SPβ*c2del2*::Tn*917*::pMMN289 | This work |
| LAB2326 | *trpC2 pheA1* Δ*resDE*::Tet$^r$ *fnr*::pMMN297 SPβ*c2del2*::Tn*917*::pMMN289 | This work |
| LAB2334 | *trpC2 pheA1 fnr*::pMMN297 SPβ*c2del2*::Tn*917*::pMMN287 | This work |
| LAB2335 | *trpC2 pheA1 fnr*::pMMN297 SPβ*c2del2*::Tn*917*::pMMN288 | This work |
| LAB2336 | *trpC2 pheA1* Δ*resDE*::Tet$^r$ *fnr*::pMMN297 SPβ*c2del2*::Tn*917*::pMMN287 | This work |
| LAB2337 | *trpC2 pheA1* Δ*resDE*::Tet$^r$ *fnr*::pMMN297 SPβ*c2del2*::Tn*917*::pMMN288 | This work |
| LAB2349 | *trpC2 pheA1 narG-lacZ* (Spc$^r$) | This work |
| LAB2350 | *trpC2 pheA1* Δ*resDE*::Tet$^r$ *narG-lacZ* (Spc$^r$) | This work |
| LAB2351 | *trpC2 pheA1 fnr*::pMMN297 *narG-lacZ* (Spc$^r$) | This work |
| LAB2352 | *trpC2 pheA1* Δ*resDE*::Tet$^r$ *fnr*::pMMN297 *narG-lacZ* (Spc$^r$) | This work |

incubated without shaking. Anaerobic growth on Luria-Bertani agar plates supplemented with 1% glucose and 0.2% $KNO_3$ was performed in an anaerobic jar with a gas-generating system (Becton Dickinson, Cockeysville, Md.). When necessary, chloramphenicol (5 μg/ml), spectinomycin (75 μg/ml), or tetracycline (10 μg/ml) was also added. The *B. subtilis* strains used in this study are listed in Table 1. The construction of *resDE* (35) and *fnr* mutations (2) was performed as previously described. These mutations were introduced by transformation into JH642 to construct LAB2135 (Δ*resDE*) and LAB2136 (*fnr*).

**Construction of *lacZ* fusions.** DNA fragments containing either the *narK* promoter (between 298 bp upstream and 77 bp downstream of the *narK* transcription start site), the intergenic *fnr* promoter (between 493 bp upstream and 26 bp downstream of the *fnr* transcription start site), or both promoters were isolated from pDIA5352 (2) and cloned in front of a promoterless *lacZ* gene in the plasmid pTKlac (11). The resultant plasmids were named pMMN287, 288, and 289, respectively (Fig. 1). The plasmids were then used to transform ZB307A cells to introduce the *lacZ* fusions into the SPβ prophage locus as previously described (20, 40). In order to examine the effect of the *resDE* deletion and *fnr* mutation on *narK-fnr* expression, SPβ phages carrying *narK-*, *fnr-*, and *narK-fnr-lacZ* fusions were introduced by specialized transduction into the *fnr* and Δ*resDE* strains as well as the wild-type strain.

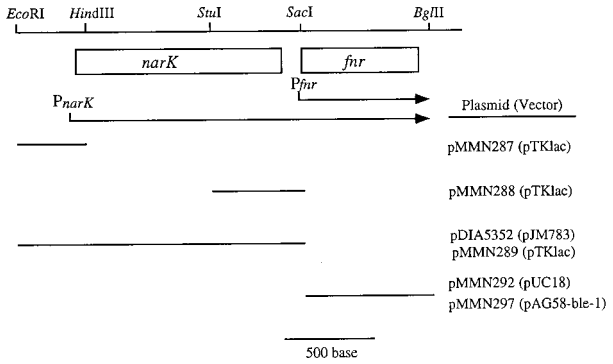


FIG. 1. Physical map of the *narK-fnr* operon. The locations of the *narK* and *fnr* genes are shown by boxes. Transcription from two promoters (P*narK* and P*fnr*) is shown by arrows. Shown above the gene is a restriction enzyme map of the region. The plasmids used in this work are shown on the right, with vectors in parentheses. The DNA insert in each plasmid is indicated as a bar to the left of the plasmid name.

To obtain a *narG-lacZ* strain, pDIA5360 (1) was constructed by cloning a 990-bp fragment containing the *narG* promoter in front of promoterless *lacZ* in pJM783 (25). JH642 cells were transformed with pDIA5360 with selection for chloramphenicol resistance (Cm$^r$) (LAB2143). The integration of the plasmid into the *narG* locus by Campbell-type integration does not cause disruption of *narG*. The Cm$^r$ marker was replaced by a spectinomycin-resistant (Spc$^r$) marker after transformation of pJL62 (14) with selection of Spc$^r$ and Cm$^s$ to obtain strain LAB2349. The chromosomal DNA from LAB2349 was used to transform mutant strains.

Strains carrying *lacZ* fusions were precultured overnight and 100-fold diluted into 2× YT with 1% glucose and 0.2% $KNO_3$ (optical density at 600 nm [$OD_{600}$] is around 0.03 to 0.05). Cells were incubated aerobically until reaching an $OD_{600}$ of 0.15 to 0.3 and then transferred to anaerobic conditions as described above. Cells were harvested at 30-min or 1-h intervals for measurement of β-galactosidase activity as described elsewhere (16), except that the specific activity was calculated as activity per milligram of protein instead of in Miller units. The calculation is $1{,}000 \times (OD_{420} - OD_{550} \times 1.75)$/reaction time (min) × mg of protein.

**Construction of a P*spac-fnr* strain.** A plasmid, pMMN249, bearing DNA flanking the *narG* gene (12) was propagated in and purified from *E. coli* cells. The plasmid contains a 6.3-kb insert encompassing the 5′ end of *narG* and the 3′ end of *narK* as well as an intact *fnr* gene. pMMN249 was digested with *Sac*I, located immediately upstream of the *fnr* translation start codon, and *Bgl*II, which was positioned in the *fnr* coding sequence. The resultant 600-bp fragment was inserted into pUC18 to generate pMMN292. pMMN292 was digested with *Sac*I, and the resulting ends were rendered flush by reaction with T4 DNA polymerase and deoxynucleotide triphosphates. The linear plasmid was further digested with *Sph*I. The *fnr* fragment, thus released, was inserted downstream of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter P*spac* (38) in plasmid pAG58-ble-1 (39), which had been digested with *Xba*I, T4 DNA polymerase to make the *Xba*I ends flush, and then *Sph*I. The plasmid pMMN297 was used to transform wild-type (JH642), Δ*resDE* (LAB2135), and *fnr* (LAB2136) cells.

**Measurement of nitrate reductase activity and nitrite extrusion activity.** Measurement of respiratory nitrate reductase activity was described in a previous paper (4). Nitrite extrusion activity was determined as the concentration of nitrite in culture media after removal of cells which were also used to examine nitrate reductase activity and *narG-lacZ* expression. Nitrite content was determined by a method previously reported (4).

## RESULTS

**Effect of *resDE* and *fnr* mutations on expression of transcriptional fusions of *narK* and *fnr*.** Both ResDE and FNR are required for nitrate respiration (2, 35). Since the ResDE signal transduction system is also required for aerobic respiration

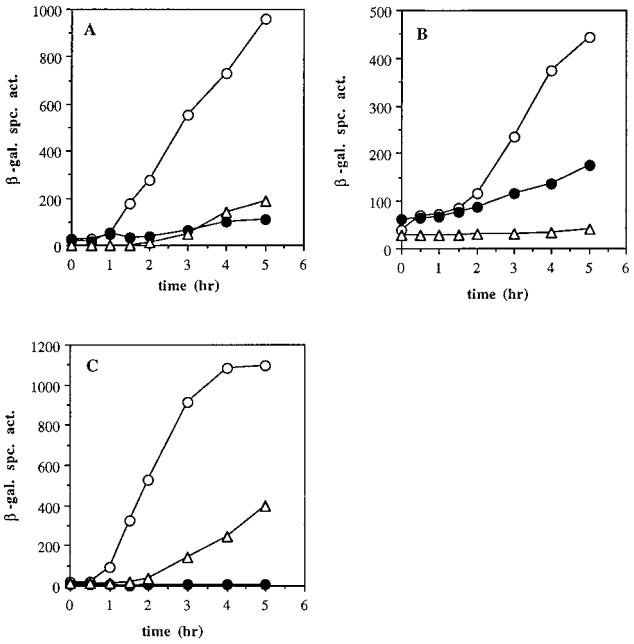


FIG. 2. Effect of mutations on expression of *lacZ* from both the *narK* and *fnr* promoters (A), the *fnr* promoter (B), and the *narK* promoter (C). The wild-type and mutant strains are lysogenized with SPβ phage carrying the *lacZ* fusions as described in Materials and Methods. β-Galactosidase specific activity (spc. act.) was measured from cells transferred to anaerobic conditions at zero hour. (A) ○, LAB2253 (wild type); ●, LAB2258 (*fnr*); △, LAB2264 (Δ*resDE*). (B) ○, LAB2252 (wild type); ●, LAB2257 (*fnr*); △, LAB2263 (Δ*resDE*). (C) ○, LAB2251 (wild type); ●, LAB2256 (*fnr*); △, LAB2262 (Δ*resDE*).

(35) and *fnr* transcription is induced by oxygen limitation (2), it is reasonable to speculate that ResDE function lies upstream of *fnr* in the anaerobic regulatory pathway. To address this possibility, *fnr* expression was examined with Δ*resDE* and *fnr* mutants. Our preliminary results with a *fnr-lacZ* fusion in the *fnr* gene showed that anaerobic expression of *fnr* is strongly dependent on *fnr* and *resDE* (data not shown). Since *fnr* is the second gene in a dicistronic operon, *narK-fnr*, and is transcribed from two promoters, one upstream of *narK* and the other upstream of *fnr* (2), it was necessary to examine the two promoters individually in order to understand how *fnr* expression is regulated. For this purpose, three transcriptional *lacZ* fusions, one transcribed from the *fnr*-specific promoter in the *narK-fnr* intergenic region (*fnr-lacZ*), another transcribed from the *narK-fnr* operon promoter (*narK-lacZ*), and a third transcribed from both promoters (*narK-fnr-lacZ*), were constructed as described in Materials and Methods (Fig. 1). Each was inserted into the SPβ prophage locus and subsequently introduced by specialized transduction into the wild-type and mutant cells.

First, we determined if *lacZ* transcription initiated from both the *narK* and *fnr* promoters, which reflects expression of the normal *fnr* gene, is similar during anaerobic induction when the *narK-fnr-lacZ* operon fusion is introduced into the SPβ prophage locus or placed in the *fnr* locus. Figure 2A shows that the expression of *lacZ* fused to a fragment containing both the *narK* and *fnr* promoters is induced by oxygen limitation in the wild-type strain (37-fold) and that the induction is severely reduced in Δ*resDE* and *fnr* mutant strains, indicating that the *narK-fnr-lacZ* expression when the fusion is in the SPβ site is similar to that of the same fusion located in the *fnr* locus.

The previous work showed that *fnr* has its own oxygen-regulated promoter that is distinct from the *narK* promoter, as was suggested by Northern (RNA) blot analysis (2). The *fnr*-specific promoter activity was further characterized to examine the anaerobic regulation of *fnr* (Fig. 2B). *fnr*-directed β-galactosidase activity was slight but detectable in the presence of oxygen (zero hour) but further induced upon a shift to anaerobiosis (11-fold) in the wild-type cells. The *fnr* mutation resulted in slightly increased aerobic *lacZ* expression, but anaerobic induction of *fnr* was significantly reduced. *resDE* had little effect on aerobic expression of *fnr*; however, no induction was observed in the *resDE* mutant after a shift to the anaerobic condition. This result indicates that ResDE is required for activation of *fnr* transcription from the *fnr* promoter upon oxygen limitation.

*narK-lacZ* expression was barely detectable in cells of aerobic cultures but was strongly induced after a shift to anaerobic conditions (65-fold) (Fig. 2C). The induction was completely dependent on *fnr* itself, as previously shown (2). *narK* transcription was barely detectable in the *resDE* mutant until 2 h after a shift to anaerobic conditions, after which time it gradually increased during prolonged incubation under anaerobiosis. Nitrite extrusion activity by NarK was also examined. Wild-type cells began to excrete nitrite at 1 h after a shift to anaerobiosis, and nitrite content in the media was 80 μmol/mg of cellular protein at 5 h after an anaerobic shift. Very little or no detectable nitrite was accumulated in the media for cultures of *resDE* and *fnr* cells, which corresponds well with the observed *narK-lacZ* expression.

These results suggest that the signal transduction pathway mediated by *resDE* is required for anaerobic induction of *fnr* transcription initiating at the *fnr*-specific promoter. FNR thus produced then stimulates *narK-fnr* expression from the operon promoter.

**Expression of *fnr*- and *narK-lacZ* fusions in a P*spac-fnr* strain.** The results described above show that at least one of the roles of *resDE* in nitrate respiration is to induce *fnr* expression from the *fnr*-specific promoter. In order to determine if the function of *resDE* in anaerobiosis is solely to activate *fnr*, the *fnr* gene was placed under control of the IPTG-inducible promoter P*spac* (*narK* is still transcribed from its own promoter in this strain) (Fig. 3), and the effect of the *resDE* mutation on anaerobic growth was tested in the cells carrying P*spac-fnr* in the absence and presence of IPTG. If *resDE* is required solely for induction of *fnr* in anaerobic gene regulation, the cells will grow anaerobically in the presence of IPTG. In order to verify that *fnr* expression in the P*spac-fnr* strain is dependent on IPTG, we constructed strains carrying P*spac-fnr* (LAB2311) and P*spac-fnr* Δ*resDE* (LAB2313), and these strains were lysogenized with SPβ phage carrying P*fnr-lacZ*, P*narK-lacZ*, and P*narK-fnr-lacZ*, which were constructed as described above.

Figure 4B confirms that *fnr-lacZ* expression was not dependent on *fnr* itself, since addition of IPTG for P*spac-fnr* cells (LAB2335) did not have any significant effect. The requirement of ResDE for P*fnr-lacZ* expression as shown in Fig. 2B was reconfirmed by the low level of β-galactosidase activity observed in LAB2337 regardless of the presence of IPTG.

P*narK-lacZ* expression in Fig. 4C shows that very little expression was detected both in P*spac-fnr* (LAB2334) and in P*spac-fnr* Δ*resDE* (LAB2336) mutants in the absence of IPTG, indicating again the requirement of *fnr* for *narK* expression. In the presence of IPTG, LAB2334 and LAB2336 strains showed almost equal levels of β-galactosidase activity. This clearly demonstrates that the requirement of *resDE* for *narK* expression is solely to activate *fnr* and when *fnr* expression is independent of *resDE*, as is the case in LAB2336 upon P*spac-fnr*

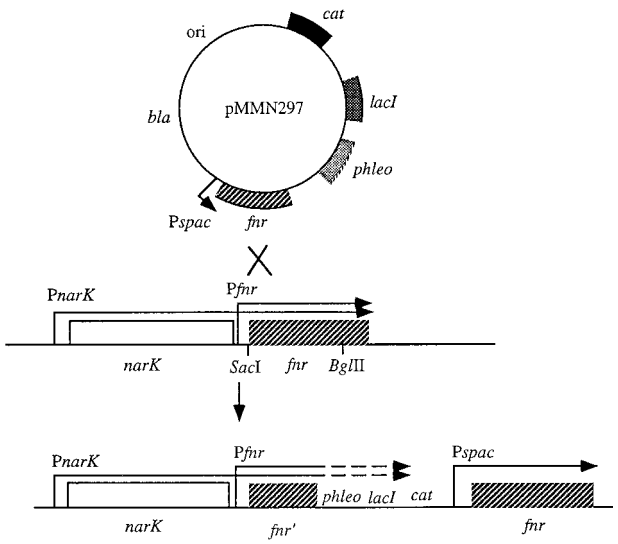


FIG. 3. Construction of a strain carrying P*spac-fnr*. A plasmid, pMMN297, was constructed by cloning the 5′ portion of the *fnr* (*Sac*I-*Bgl*II) fragment into pAG58-ble-1. P*spac* is a hybrid promoter, constructed by Yansura and Henner (38), composed of an early promoter of the *B. subtilis* phage SPO-1 and the *lac* operator. The *lacI* gene, encoding the *lac* repressor, was placed under the transcriptional and translational control of the *Bacillus licheniformis* penicillinase gene. *bla*, β-lactamase gene; *cat*, chloramphenicol acetyltransferase gene; *phleo*, phleomycin resistance gene; *ori*, replication origin. pMMN297 was integrated into the *fnr* gene by homologous recombination. In the recombinant, *narK* is transcribed from the *narK* promoter (P*narK*), *fnr* is transcribed from the P*spac* promoter, and a truncated copy of *fnr* (shown as *fnr′*) is transcribed from the P*fnr* promoter (P*fnr*).

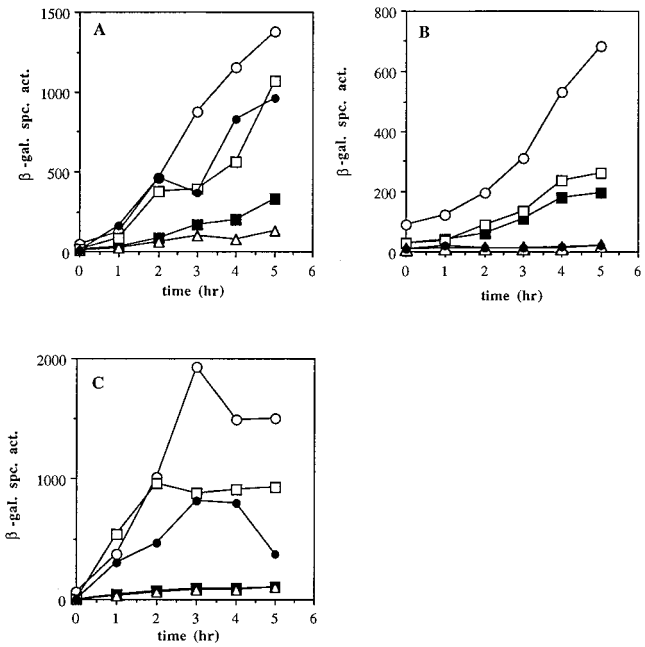


FIG. 4. Expression of *lacZ* in P*spac-fnr* strains from both the *narK* and *fnr* promoter (A), the *fnr* promoter (B), and the *narK* promoter (C). β-Galactosidase specific activity (spc. act.) was measured as described in the legend to Fig. 2. (A) ○, LAB2253 (wild type); ■, LAB2324 (P*spac-fnr*) without IPTG; □, LAB2324 with 1 mM IPTG; △, LAB2326 (P*spac-fnr* Δ*resDE*) without IPTG; ●, LAB2326 with IPTG. (B) ○, LAB2252 (wild type); ■, LAB2335 (P*spac-fnr*) without IPTG; □, LAB2335 with 1 mM IPTG; △, LAB2337 (P*spac-fnr* Δ*resDE*) without IPTG; ●, LAB2337 with IPTG. (C) ○, LAB2251 (wild type); ■, LAB2334 (P*spac-fnr*) without IPTG; □, LAB2334 with 1 mM IPTG; △, LAB2336 (P*spac-fnr* Δ*resDE*) without IPTG; ●, LAB2336 with IPTG.

induction by addition of IPTG, the effect of *resDE* on *narK* expression was not observed.

Figure 4A shows that *lacZ* expression dependent on transcription from both the *narK* and *fnr* promoters requires *fnr* and *resDE* and the requirement of *resDE* is bypassed by *resDE*-independent *fnr* expression. These results strongly argue that the requirement of *resDE* for expression of the *narK-fnr* operon is solely to activate *fnr* transcription from the *fnr*-specific, intergenic promoter upon oxygen limitation.

***narG-lacZ* expression and nitrate reductase activity in P*spac-fnr* strains.** Expression of nitrate reductase as well as *narK* was shown to be dependent on *fnr* (2). We show here that the level of *narG-lacZ* activity was also very low in the Δ*resDE* mutant (LAB2350), as demonstrated by introduction of *narG-lacZ* into Δ*resDE* mutant cells and examination of *narG*-directed β-galactosidase activity (Fig. 5A). By using P*spac-fnr* strains constructed as described above, we asked whether the requirement of *resDE* for *narG* expression is only to activate *fnr* expression as in the case of *narK* expression. The *narG-lacZ* transcriptional fusion was introduced into the wild type and P*spac-fnr* and P*spac-fnr* Δ*resDE* mutants. As expected, *narG-lacZ* expression in P*spac-fnr* cells (strain LAB2351) grown in the absence of IPTG was significantly reduced compared with the activity observed in wild-type cells, in keeping with the finding that *narG-lacZ* is inactive in an *fnr* mutant (data not shown). The low level of activity that is observed may be due to leaky expression of *fnr* in the P*spac-fnr* construct. In the presence of IPTG, the levels of *narG* expression in P*spac-fnr* (LAB2351) and wild-type (LAB2349) cells were almost identical (Fig. 5A). In P*spac-fnr* Δ*resDE* mutant cells (strain LAB2352), no expression was detected in the absence of IPTG. The lower level of activity in LAB2352 than that in LAB2351 is suggestive of an additional effect of the *resDE* mutation on *narG*, indicating that *resDE* may be required for *narG* expression, not only for activation of *fnr*, but also for FNR-independent activation. In fact, the level of *narG-lacZ* expression in LAB2352 cells grown in the presence of IPTG was much lower than that observed in LAB2351 cells under the same condition. This is in contrast with the result in which the expression of P*narK-lacZ* as well as that of P*narK-fnr-lacZ* was indistinguishable in P*spac-fnr* and P*spac-fnr* Δ*resDE* mutants in the presence of IPTG (Fig. 4). To confirm this result, the activity of respiratory nitrate reductase encoded by the *narG* operon was

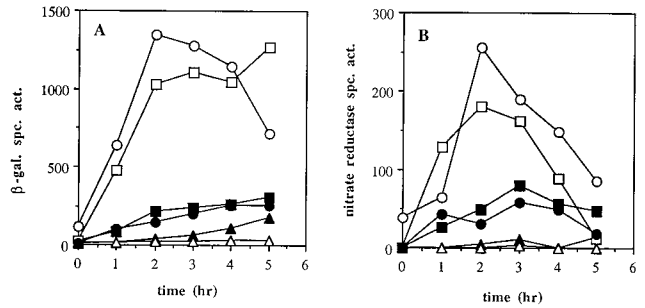


FIG. 5. Expression of *narG-lacZ* (A) and nitrate reductase (B) activity in P*spac-fnr* strains. β-Galactosidase specific activity (spc. act.) was measured as described in the legend to Fig. 2. Respiratory nitrate reductase activity was measured as described in Materials and Methods with cells transferred to anaerobic conditions at zero hour and is shown as nanomoles of nitrite produced per milligram of protein per minute. ○, LAB2349 (wild type); ▲, LAB2350 (Δ*resDE*); ■, LAB2351 (P*spac-fnr*) without IPTG; □, LAB2351 with IPTG; △, LAB2352 (P*spac-fnr* Δ*resDE*) without IPTG; ●, LAB2352 with IPTG.

also measured in the same cell cultures used for the assay of *narG-lacZ* expression shown in Fig. 5A. Figure 5B shows that the data about nitrate reductase activity were similar to those obtained in *narG-lacZ* expression.

The anaerobic growth of P*spac-fnr* and P*spac-fnr* Δ*resDE* mutant cells was examined in the absence and presence of IPTG. The growth rate of P*spac-fnr* strains in the presence of IPTG was equal to that of wild-type cells (doubling time of around 2 h). In the absence of IPTG, P*spac-fnr* strains still grew anaerobically, even though their doubling time was twice as long as that of the $fnr^+$ cells, indicating the leaky expression of *fnr* in the P*spac-fnr* construct. A very slight but reproducible improvement in the growth of P*spac-fnr* Δ*resDE* cells was observed by the addition of IPTG, because the cell density, measured as $A_{600}$, increased by around 40% after 5 h of incubation in the presence of IPTG compared with a less than 10% increase in the absence of IPTG. However, the strain was still unable to grow anaerobically to the wild-type level, indicating that *resDE* plays a role in nitrate respiration besides activation of *fnr* transcription.

## DISCUSSION

FNR is a global anaerobic gene regulator in many bacteria, including *E. coli* and *B. subtilis*. *fnr* was originally identified as a mutation that conferred a defect in fumarate and nitrate reduction in *E. coli* (13). The *fnr* gene product, which has significant primary structure similarity to the cyclic AMP receptor protein (catabolite activator protein) (30), except for the presence of a cysteine-rich N-terminal region, acts as a transcriptional regulator controlling the expression of its target genes in response to anaerobiosis. A study with a translational *fnr-lacZ* fusion has shown that the *fnr* gene in *E. coli* is expressed under both aerobic and anaerobic conditions and is subject to autorepression under anaerobic conditions (24, 32). This indicates that the activity of *fnr* must be modulated by oxygen availability. The presence of a cysteine cluster in its N terminus suggests that reduction of bound iron to the cysteine residues from the $Fe^{3+}$ state to the $Fe^{2+}$ state allows FNR to activate the DNA binding function in response to anaerobic conditions (37). In fact, studies with chelating agents demonstrated that deprivation of divalent metals, including iron was correlated with the loss of FNR-dependent gene regulation (34, 36), and it was further shown that a ferrous iron chelator inhibits open promoter complex formation in vivo (5). Site-directed mutagenesis showed that three of the four N-terminal cysteines (Cys-20, Cys-23, and Cys-29 but not Cys-16), as well as Cys-122, are all essential for the normal regulation of FNR-dependent promoters (6, 15). In contrast to *E. coli fnr*, as reported in earlier work (2) and studies described herein, transcription of *B. subtilis fnr* is strongly activated by oxygen limitation. In this paper, we demonstrated that transcription of *fnr* is first activated upon anaerobiosis by the ResD-ResE two-component signal transduction pathway. Continued synthesis of FNR stimulates its own transcription as well as that of *narK* by activating initiation from the *narK* promoter. A previous study has shown that ResD and, to a lesser extent, ResE caused defective nitrate respiration (35). All of the experiments presented in this paper were done with the mutant bearing a deletion of both *resD* and *resE*. We have also examined *resD* and *resE* mutations individually and have shown that the effect of each mutation on *fnr-lacZ* expression was almost identical to that of *resDE* (data not shown). This indicates that although the *resD* mutant showed more a severe defect in anaerobic growth than the *resE* mutant (35), the effects of *resD* and *resE* mutations on the expression of *fnr-lacZ* are indistinguishable, suggesting that ResE is a major, if not the only, kinase controlling ResD-dependent *fnr* transcription initiation.

The reason for the requirement of FNR for full anaerobic induction of *fnr-lacZ* (Fig. 2B) is unclear at this moment. The putative FNR-binding site deduced from the comparison of the promoter regions of four anaerobically induced genes, including *narK* and *narG*, was proposed to be $TGTGAN_2TAN_2$ TCACA, centered at position −41.5 (2). The *fnr* promoter region has a sequence ($TGTTAN_2TTN_2TCTCG$) which shows weak homology (8 matches out of 12 bases) centered at −42.5. Involvement of the sequence in transcriptional activation by FNR remains to be studied, although the four previously identified FNR sites, all of which are centered at position −41.5, are highly conserved (2).

Although the *resDE* mutation completely abolished anaerobic *fnr* induction (Fig. 2B), the effect of *resDE* on *narK* expression was modest (Fig. 2C), indicating that ResDE controls anaerobic induction of *fnr* but that induction contributes to a smaller extent to *narK* expression. It also suggests that a low level of FNR in aerobic cells is sufficient for most of the induction at the *narK* promoter after a shift to anaerobiosis and therefore that FNR activity is also modulated by oxygen limitation. This is confirmed by the studies with the P*spac-fnr* construct (Fig. 3 and 4). In this experiment, in which *fnr* was placed under the oxygen-independent P*spac* promoter, FNR was shown to transcriptionally stimulate *narK* only when oxygen was limiting. The presence of a cysteine cluster in *B. subtilis* FNR, which is indispensable for FNR's activity in *E. coli*, raises a possibility that the activity of *B. subtilis* FNR is also regulated by anaerobiosis by a mechanism similar to that observed in the case of *E. coli* FNR.

In the strains in which *fnr* is placed under the control of an IPTG-inducible promoter, *resDE* was shown to have a role in nitrate respiration besides the activation of *fnr* transcription. The defect in anaerobic growth exhibited by the *resDE* mutant is probably not related to the role of ResDE in heme A biosynthesis, since a mutation in *ctaA* does not affect anaerobic growth, even though transcription of *ctaA* requires ResDE (35). An additional role of ResDE in anaerobiosis may be the activation of *narGHJI* transcription, as suggested by the finding that the P*spac-fnr* Δ*resDE* mutant (LAB2352) has much lower levels of nitrate reductase activity and *narG-lacZ* expression than P*spac-fnr* cells (strain LAB2351) grown in the presence of IPTG. However, an argument against this can be raised from the observation that LAB2351 cells can grow anaerobically with a reduced rate in the absence of IPTG, while LAB2352 cells cannot grow in the presence of IPTG, although nitrate reductase activity in LAB2351 in the absence of IPTG is almost identical to that of LAB2352 in the presence of IPTG, as shown in Fig. 5B. One possible explanation for these observations is that although nitrate reductase activity in these mutant strains may not be enough to support anaerobic growth under normal conditions, the P*spac-fnr* strain in the absence of IPTG has no nitrite extrusion activity (data not shown), a situation which might cause the nitrite produced to be reduced more efficiently. However, the P*spac-fnr* Δ*resDE* strain can excrete nitrite in the presence of IPTG (data not shown) in spite of a low level of nitrate reductase activity, which may cause inefficient nitrite reduction. The other possibility is that although the levels of nitrate reductase activities in both strains are high enough for nitrate respiration, *resDE* may have another function required for anaerobic growth. One possible ResDE target is respiratory nitrite reductase, which has yet to be identified in *B. subtilis*. In *E. coli*, there are two nitrite reductases: a soluble NADH-dependent enzyme (Nir), which functions in detoxifying nitrite, and a membrane-bound formate-dependent

enzyme (Nrf), which provides energy during anaerobiosis (23). The latter enzyme is known to be a *c*-type cytochrome ($c_{552}$) complex. *B. subtilis* has nitrite reductase encoded by *nasDE* in the *nasB* operon (22), which has a high degree of homology to Nir in *E. coli*. Mutations of the *nasDE* genes resulted in a defect in nitrate and nitrite assimilation (22) but not in nitrate respiration (17). If respiratory nitrite reductase in *B. subtilis* contains cytochrome $c_{552}$, the requirement of *resDE* for expression of *resABC*, encoding proteins with similarity to those that function in cytochrome *c* biogenesis, may explain the defect of anaerobic growth of the *resDE* cells expressing *fnr*.

It was previously shown that ResDE functions in the regulation of aerobic respiration as well as that of anaerobic respiration (35). Since the ResDE signal transduction pathway is not specifically activated by oxygen limitation, the question that remains to be answered is where the oxygen limitation signal enters the ResDE-*fnr* regulatory pathway. One possibility is that ResDE, responding to a signal which is created by different environmental stimuli, including oxygen limitation, activates *fnr* transcription. Once FNR is produced, it is activated as a functional transcriptional regulator under anaerobic conditions, as in the case with *E. coli* FNR. An alternative possibility is that ResDE activates transcription of a gene which encodes an anaerobic gene regulator required for transcription initiated at the *fnr* promoter. One may question whether *fnr* expression is under the control of stationary-phase induction, since ResDE may function as a stationary-phase regulator. As far as has been tested, *fnr* expression is induced only by oxygen limitation, while other potential stimuli such as entry into the stationary phase of growth were not able to induce *fnr* expression (data not shown). We do not yet know if *resDE* is required for expression of a gene essential for both aerobic and anaerobic respiration or if ResD itself acts directly as a transcriptional activator for the genes known to be regulated by *resDE*. No common sequence features have been identified in the promoter regions of the *resDE*-regulated genes, *ctaA*, *petCBD*, *resABCDE*, and *fnr*, suggesting that transcription of most of these genes, if not all, is not activated directly by ResD.

Our results, together with those of other studies, showed that anaerobic gene regulation in *B. subtilis* clearly differs from that in *E. coli*. Aside from the difference in the transcriptional regulation of *fnr* as described above, *B. subtilis* has a pleiotropic regulator, ResD, which is required both for aerobic and anaerobic respiration. In *E. coli*, aerobic-anaerobic regulators such as ArcA/B and FNR function oppositely for aerobic and anaerobic respiration (7, 10, 33). For example, ArcA functions as an anaerobic repressor of aerobically expressed genes, such as those encoding tricarboxylic acid cycle enzymes, and serves as an activator for the *pfl* gene encoding pyruvate formate lyase that catalyzes pyruvate cleavage under anaerobic conditions. FNR also functions as an activator of genes that function in anaerobic respiration and acts as a transcriptional repressor for the aerobic NADH dehydrogenase II gene. Further study of anaerobic respiration and metabolism in *B. subtilis* will provide insight into this unique system.

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