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Oxygen Sensitivity of the *nifLA* Promoter of *Klebsiella pneumoniae*

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Oxygen sensitivity of the *nifLA* promoter of *Klebsiella pneumoniae* has been demonstrated. Studies on the oxygen regulation of *nifB-lacZ* and *nifH-lacZ* fusions in the presence of the *nifLA* operon, which contains either an intact or a deleted *nifL* gene, indicate that possibly both the *nifL* promoter and the *nifL* product are responsible for *nif* repression by oxygen.

In *Klebsiella pneumoniae*, the expression of *nif* genes is regulated at the transcriptional level by the products of four genes, i.e., the *ntrC* (*glnG*), *ntrA* (*glnF*), *nifA*, and *nifL* genes (1, 2, 10, 11, 13, 16). The regulatory genes *nifL* and *nifA* make up a single operon which is activated by the product of *ntrA* and *ntrC* (8), while the product of *nifA* in conjunction with that of *ntrA* activates all other *nif* operons (14). In contrast, the *nifL* product has been shown to act as a repressor for *nif* genes in the presence of oxygen or an excess of fixed nitrogen (9, 10, 12).

Previous investigation showed that the introduction of a plasmid carrying a constitutive *nifA* gene enhanced the transcription of nitrogenase genes and that the oxygen and ammonium repression of *nif* genes was readily relieved by an abundance of the *nifA* product (17). Thus, a deficiency of available *nifA* product under oxygen and ammonium has been suggested as the cause of oxygen and ammonium repression of *nif* genes in *K. pneumoniae*. Our current study provides evidence that the repressive action of the *nifL* product is in the inactivation of the *nifA* product or with the *nifA*-specific transcriptional complex (D. W. Ow, Y. Xiong, G. Gu, J. B. Zhu, and S. C. Chen, *in Proceedings of the Sixth International Symposium on Nitrogen Fixation*, in press).

Recently, the results of studies on *nif* expression in *K. pneumoniae* at the level of transcription, translation, and nitrogenase activity during derepression and repression by NH_4^+ and O_2 revealed that the transcription profile of *nifLA* had two peaks during derepression, (the first peak was at 1 h before those of other operons, and the second coincided with the peaks of other operons) and that oxygen rapidly repressed the transcription of all *nif* operons but not the transcription of the *nifLA* operon (6). These results thus substantiate the evidence that *nif*-specific repression by O_2 is independent of transcriptional regulation of the *nifLA* operon. In this paper we report the high sensitivity of the *nifLA* promoter to oxygen, and we also report preliminary results that show the oxygen repression of *nifB-lacZ* and *nifH-lacZ* fusions to be mediated by inhibition of transcription at the *nifLA* promoter as well as by the *nifL* product already present.

For the construction of *lac* fusions, plasmid pRZ5202 (7.7 kilobases), a derivative of ColE1 containing an intact *lacZ* gene but no promoter region upstream of this gene, was used as a promoter search vector. The restriction nuclease and genetic maps of this plasmid and its derivatives are shown in Fig. 1. Plasmid pCRA37, which contains *nifQBALFMVSU'* (4, 15), was digested with *Eco*RI and *Bam*HI, and a fragment 6.1 kilobases long, coding for *nifLABQ* genes, was cloned at the *Eco*RI and *Bam*HI sites of vector plasmid pRZ5202. The recombinant plasmid pNC230 thus obtained contains the *nifLABQ-lacZ* fusion. This plasmid was partially digested with *Sal*I and religated, yielding pNC262, which contains a *nifLA-nifB-lacZ* fusion. Likewise, when pNC230 was completely digested with *Sal*I and then religated, plasmid pNC220, which contains a *nifL-lacZ* fusion, was obtained. Since the sticky ends generated by *Bam*HI and *Bgl*II cleavage are identical in sequence, digestion of pNC230 with these enzymes, followed by religation, yielded plasmid pNC247, which contains the *nifLA* promoter-*lacZ* fusion with only the promoter-proximal portion of the *nifL* gene (15). For construction of the *nifB-lacZ* fusion, a *nifLAB* fragment (3.0 kilobases) was isolated by *Sal*I restriction of plasmid pA4 (10), which was a derivative of pACYC184 carrying this *nif* fragment in the *tet* gene, and then recombined with the *Sal*I-linearized pRZ5202. By making use of its *pst*I restriction sites presented asymmetrically in this inserted *nifLAB* fragment and its very high β-galactosidase activity in the presence of the *nifA* product in transformants, we easily selected the desired *nifB-lacZ* fusions with the correct orientation. The resultant plasmid, pNC276, contains *lacZ* under the *nifB* promoter flanked by *nifLA*. Plasmid pNC276 was digested with *Bam*HI and *Hin*dIII, and a fragment of about 3.3 kilobases containing *nifLAB* was obtained and then subcloned into the *Bgl*II and *Hin*dIII sites of plasmid pNC220. The resultant plasmid, pNC304, contains a Δ*nifLA-nifB-lacZ* fusion. Plasmid pNC124, which carries a *nifH-lacZ* fusion, was constructed by subcloning the *Eco*RI-*Bgl*II restriction fragment, which contains the *nifH* promoter region from plasmid pSA30 (5, 14), into the *Eco*RI and *Bam*HI sites of plasmid pRZ5202. For the construction of plasmid pNC136, carrying the *tet* promoter-*dacZ* fusion, the *tet* promoter fragment was isolated from *Bam*HI- and *Eco*RI-digested pBR322 and cloned into plasmid pRZ5202 at the corresponding sites.

Oxygen repression of *nifL-lacZ*. To test the oxygen repression of the *nifLA* promoter, the plasmid bearing the *nifL-lacZ* fusion (pNC247) was introduced into the appropriate strains of *Escherichia coli* and *K. pneumoniae* for testing β-galactosidase activity in cells grown under anaerobic and aerobic depression conditions. Derepression was achieved by 200-fold dilution of an exponentially growing nutrient

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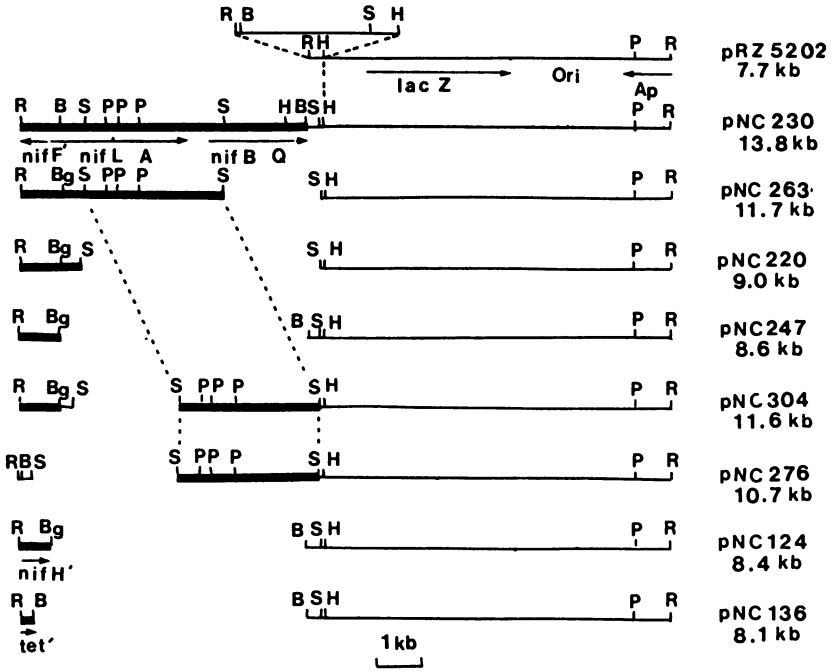

FIG. 1. Restriction nuclease and genetic map of *nif-lac* fusions. The restriction nuclease sites shown are only those used for construction of the fusions. Thick lines indicate *nif* or *tet* DNA; thin lines indicate vector pRZ5202 DNA. Sites of cleavage by restriction enzymes are as follows: P, *Pst*I; S, *Sal*I; H, *Hin*dIII; B, *Bam*HI; *Bgl*II; R, *Eco*RI. kb, Kilobase.

broth culture into freshly prepared nitrogen-free minimal media (3) supplemented with 100 μg of Casamino Acids and 5 μg of biotin per ml in addition to the required amino acids and antibiotics. Cultures were incubated for 20 h under anaerobic conditions at 30°C. For oxygen repression experiments, the cultures (2 ml) were incubated with shaking for 20 h at 30°C in test tubes (15 by 150 mm) with cotton plugs and set at a slant. The data given in Table 1 show that the *lacZ* gene under the control of the *nifL* promoter was markedly repressed by oxygen in both *E. coli* and *K. pneumoniae*, though in the case of the *nif*$^+$ wild-type strain of *K. pneumoniae*, the oxygen repression of the *nifL* promoter was somewhat weaker than that of other hosts. Similar results have been reported elsewhere (7, 11, 13). When the *tet* promoter-*lacZ* fusion plasmid (pNC136) instead of the *nifL-lacZ* fusion plasmid was introduced into these strains, the *lacZ* gene was not repressed at all by oxygen. Therefore, the oxygen effect on *nifL-lacZ* expression is apparently due to the high sensitivity of the *nifL* promoter to oxygen. Furthermore, the *nifL-lacZ* fusion in a Δ*glnALG* strain, yMC11, containing both plasmids pNC247 and pST1021 (17) was also repressed by oxygen, indicating that oxygen repression of *nifL* is probably not mediated by the product of the *glnC* gene.

Oxygen regulation of *nif-lacZ* coupled with *nifLA*. The *nifLA* promoter showed high sensitivity to oxygen although the molecular mechanism of its sensitivity to oxygen is unknown. Now the question arises: how can the role of the *nifLA* promoter be differentiated from that of the *nifL* product in the oxygen regulation of *nif* genes? The following three *nifB-lacZ* fusions were constructed in plasmids: pNC276 contains the *nifB-lacZ* fusion flanked by a promoterless *nifA* gene and lacks *nifL*, pNC263 contains *nifLA* and the *nifB-lacZ* fusion, and pNC304 is pNC263 with the *nifL* gene deleted. Each of these plasmids was introduced into appropriate test strains, and the expression of *lacZ* was assayed in cells grown aerobically or under an atmosphere of nitrogen. Since the expression of *nifB-lacZ* depends on the activation by the *nifA* and *ntrA* (*glnF*) products, the expression of the *nifLA* operon plays a key role in the regulation of the *lacZ* gene in this system. Consequently, the differential expression of *lacZ* should, in a *nifL*-background, be a measure of the affect of oxygen on the *nifA* promoter directly. The data given in Table 2 show the following. (i) Strain SY203 harboring plasmid pNC276 exhibited low β-galactosidase levels irrespective of the presence of oxygen, indicating that the *nifA* gene is weakly expressed, presumably from a nearby vector promoter in this plasmid. (ii) When the bacterial strain was introduced with plasmid 263, which carries the *nifB-lacZ* fusion as the plasmid pNC276 does but with an intact *nifLA* operon as supplement, the β-galactosidase level was markedly repressed by oxygen, and the extent of repression was comparable to that of the *nifL* promoter under oxygen; deletion of the *nifL* gene in the case of the Δ*nifLA-nifB-lacZ* fusion reduced expression in air to 16% of that under N_2, while the *nifL*$^+$ strain (*nifLA-nifB-lacZ* fusion) showed activity in air only 6% that under N_2. (iii) When the constitutive *nifA* (*tet-nifA* fusion in plasmid pST1021) was introduced to the strain which harbors the plasmid containing *nifLA-nifB-lacZ* or Δ*nifLA-nifB-lacZ* fusion fragment, 70 or 34% of the β-galactosidase activity was restored. The unrestored oxygen repression here might be due to the oxygen sensitivity of the *nifB* promoter itself or to the physiological conditions of the cultures. (iv) When plasmid pNC276, which contains the *nifB-lacZ* fusion, was introduced together with a compatible plasmid, pDO503, which contains the Δ*nifLABQ*, into the same strain of *E. coli*, oxygen regulation of *nifLA* was then assayed with the *nifLA* genes in the *trans* configuration relative to the *nifB-lacZ* fusion. Again, expression of the *lacZ* gene controlled by the *nifLA* promoter was repressed by oxygen to different degrees according to whether the *nifL*

TABLE 1. Oxygen repression of the *nifL* promoter in *E. coli* and *K. pneumoniae* strains[a]

Strains (plasmid)	Genotype of relevant host/plasmid(s)	Sp act of β-galactosidase in culture		Activity ratio, $+O_2/-O_2$
		$-O_2$	$+O_2$	
E. coli SY203(pRZ5202)[b]	*gln*$^+$/*lacZ*[c]	0.7	1.3	1.8
E. coli SY203(pRZ5202 + pST1021)[d]	*gln*$^+$/*lacZ*/Φ(*tet-nifA*)	1.3	1.6	1.2
E. coli SY203(pNC247)	*gln*$^+$/Φ(*nifL-lacZ*)	450	29	0.06
E. coli SY203(pNC247 + pST1021)	*gln*$^+$/Φ(*nifL-lacZ*)/Φ(*tet-nifA*)	510	31	0.06
E. coli YMC11(pNC247)[e]	Δ*glnALG*/Φ(*nifL-lacZ*)	6.7	7.7	1.1
E. coli YMC11(pNC247 + pST1021)	Δ*gln(ALG)*/Φ(*nifL-lacZ*)/Φ(*tet-nifA*)	250	30	0.12
K. pneumoniae M5al(pNC247)	*nif*$^+$ *gln*$^+$/Φ(*nifL-lacZ*)	500	120	0.24
K. pneumoniae 107-3(pNC247)	Δ*nif gln*$^+$/Φ(*nifL-lacZ*)	690	49	0.07
K. pneumoniae UN1770(pNC247)	*nifL*$^+$ *nifA*$^-$ *gln*$^+$/Φ(*nifL-lacZ*)	670	75	0.11
E. coli SY203(pNC136)	*gln*$^+$/Φ(*tet* promoter-*lacZ*)	260	270	1.0
E. coli YMC11(pNC136)	Δ*gln(ALG)*/Φ(*tet* promoter-*lacZ*)	550	550	1.0
K. pneumoniae M5al(pNC136)	*nif*$^+$ *gln*$^+$/Φ(*tet* promoter-*lacZ*)	620	980	1.6
K. pneumoniae 107-3(pNC136)	Δ*nif gln*$^+$/Φ(*tet* promoter-*lacZ*)	730	870	1.2
K. pneumoniae UN1770(pNC136)	*nifL*$^+$ *nifA-gln*$^+$/Φ(*tet* promoter-*lacZ*)	660	950	1.4

[a] Bacteria were grown in nitrogen-free minimal media (see text) (5) aerobically or anaerobically for 20 h (see text). For testing oxygen repression, bacteria were shaken at an angle in ordinary cotton-plugged test tubes (15 by 150 mm) containing 2 ml of nitrogen-free minimedicine.

[b] Relevant genotype: F$^-$ *lac proXIII argE*(Am) *araD thi gyrA rpo hsdR* (Michael Syvanen).

[c] No promoter for *lacZ* expression.

[d] See reference 15.

[e] See reference 12.

TABLE 2. Repressive effect of *nifL* product on the *nifB* and *nifH* promoters in *E. coli* SY203[a]

Plasmid(s)	Relevant plasmid genotype	Sp act of β-galactosidase in culture		Activity ratio, $+O_2/-O_2$
		$-O_2$	$+O_2$	
pNC276[b]	*nifA* Φ(*nifB-lacZ*)	110	100	0.91
pNC263	*nifLA* Φ(*nifB-lacZ*)	4,950	320	0.06
pNC263 + pST1021	*nifLA* Φ(*nifB-lacZ*)/Φ(*tet-nifA*)	4,400	3,100	0.70
pNC304	Δ*nifLA* Φ(*nifB-lacZ*)	5,200	840	0.16
pNC304 + pST1021	Δ*nifLA* Φ(*nifB-lacZ*)/Φ(*tet-nifA*)	9,400	3,200	0.34
pNC276 + pD0503	*nifA* Φ(*nifB-lacZ*)/*nifLABQ*	3,400	180	0.05
pNC276 + pD0504	*nifA* Φ(*nifB-lacZ*)/Δ*nifLABQ*	4,100	960	0.23
pNC124	Φ(*nifH-lacZ*)	3	3	1.00
pNC124 + pD0503	Φ(*nifH-lacZ*)/*nifLABQ*	520	14	0.03
pNC124 + pD0504	Φ(*nifH-lacZ*/Δ*nifLABQ*	580	370	0.63

[a] The culture conditions (aerobic and anaerobic) of bacteria were as described for Table 1.

[b] No promoter for *nifA* expression.

gene was intact (5%) or not (23%). (v) When the plasmids that carried *nifLABQ* or Δ*nifLABQ* were introduced into bacterial strains which harbored the compatible *nifH-lac* fusion plasmid (pNC124), the *nifH-lacZ* fusion was as severely repressed as the *nifB-lacZ* when the *nifLA* operon was present. However, deletion of the *nifL* gene restored about 70% of the expression of *nifH-lacZ*. This result indicates that the *nifL* product is involved in oxygen repression of *nifH-lac*. How can the effect of high oxygen sensitivity of the *nifLA* promoter be explained here? A possible explanation is that the *nifH* promoter requires less *nifA* product for activation than do other *nif* promoters, presumably, due to the high affinity of this promoter for *nifA* product (2, 9). When *nifLA* is strongly repressed by oxygen, even the small amount of *nifA* product produced from this *nifLA* operon would be sufficient to activate the *nifH* promoter. More *nifA* product, though, would be required to activate the *nifB* promoter.

In view of these results, we speculate that *nif* regulation by oxygen is possibly mediated at two different levels. First, the oxygen sensitivity of the *nifLA* promoter ensures that the *nifLA* promoter, hence all other *nif* promoters, is repressed by oxygen; second, the *nifL* product acts as a repressor in the presence of oxygen, modulating the transcription of other *nif* operons.

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