Purification and Characterization of the *Pseudomonas aeruginosa* NfxB Protein, the Negative Regulator of the nfxB Gene

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The protein NfxB, involved in conferring resistance to quinolones in *Pseudomonas aeruginosa*, has a helix-turn-helix motif which is similar to that of other DNA-binding proteins. It appears to affect the membrane-associated energy-driven efflux of some antibiotics (H. Niki, Science 264:382–388, 1994). We constructed a plasmid that overproduced NfxB in *Escherichia coli* and purified the protein. Two species of NfxB (23 and 21 kDa), which are probably translated from different initiation codons, were isolated. Both proteins are also expressed in vivo in *P. aeruginosa*, with the 23-kDa NfxB being the major species. NfxB specifically binds upstream of the nfxB coding region as demonstrated by gel retardation and DNase I footprinting. Expression of the Φ(nfxB-lacZ′) (Hyb) gene was repressed in the presence of the nfxB gene product provided by a second compatible plasmid in *E. coli*. In the *P. aeruginosa* wild-type strain (PAO2142), NfxB was undetectable by immunoblotting; however, it was detected in the nfxB missense mutant (PK1013E). These results suggested that NfxB negatively autoregulates the expression of nfxB itself. Since the 54-kDa outer membrane protein (OprJ) (N. Masuda, E. Sakagawa, and S. Ohya, Antimicrob. Agents Chemother. 39:645–649, 1995) was overproduced in nfxB mutants, NfxB may also regulate the expression of membrane proteins that are involved in the drug efflux machinery of *P. aeruginosa*.

The intrinsic resistance of *Pseudomonas aeruginosa* to a large variety of antimicrobial agents was shown to be due mainly to efflux system effects and partly to the low-permeability outer membrane (10, 14).

The *P. aeruginosa* nfxB mutants, which show a 16-fold increase in resistance to norfloxacin, were isolated spontaneously (7). The mutants also show hypersusceptibility to β-lactam and aminoglycoside antibiotics (17). Some nfxB mutants overproduce the 54-kDa outer membrane protein, OprJ (11), little of which is produced in the wild-type strain (7, 17). Antibiotic resistance in the nfxB mutants is probably not due to altered outer membrane permeability but to multidrug efflux pump effects (14, 18).

The nfxB gene cloned from the wild type as well as a mutant (the nfx13E mutation) has been sequenced (16, 17). The amino acid sequence of NfxB revealed that it has a helix-turn-helix motif that might be responsible for its ability to bind in a sequence-specific manner to DNA, and it has no significant hydrophobic membrane-spanning regions (16). nfx13E is a missense mutation which replaces an arginine residue in the putative helix-turn-helix domain with a glycine residue in the mutant (16). The wild-type nfxB gene can restore susceptibility to norfloxacin in *P. aeruginosa* with the nfx13E mutation. This evidence suggests that NfxB binds to DNA and regulates the expression of genes that encode the outer membrane protein(s). It is possible that Nfx13E mutant protein is unable to bind to DNA and thus loses its regulatory function. In addition, nfxB expression might be autoregulated by NfxB protein itself. Like the LysR-type transcriptional regulators, many proteins that have helix-turn-helix motifs bind their regulatory region and negatively autoregulate their expression (4, 20).

To examine the DNA-binding activity and the regulatory function of NfxB, we constructed a plasmid system that overproduced the protein and then purified it. The protein specifically bound to DNA and thus loses its regulatory function. In addition, nfxB mutations (7) are present in the wild-type strain (PAO2142). NfxB was undetectable by immunoblotting; however, it was detected in the nfxB missense mutant (PK1013E). These results suggested that NfxB negatively autoregulates the expression of nfxB itself. Since the 54-kDa outer membrane protein (OprJ) (N. Masuda, E. Sakagawa, and S. Ohya, Antimicrob. Agents Chemother. 39:645–649, 1995) was overproduced in nfxB mutants, NfxB may also regulate the expression of membrane proteins that are involved in the drug efflux machinery of *P. aeruginosa*.
mids, pNF334 and pNF272, harbored the 343-bp fragment and the 272-bp fragment, respectively, in frame.

Luria-Bertani (LB) liquid medium and LB plates (19) were used throughout this study.

**Purification of NfxB protein.** *E. coli* BL21(DE3) (22) was the host for the NfxB-overproducing plasmid pNF7. The strain was grown in 3 liters of LB medium containing 100 μg of ampicillin per ml to an optical density at 600 nm of 0.5. NfxB overproduction was induced with a final concentration of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After incubation for a further 3 h, the cells were harvested by centrifugation and the cell pellet was suspended in 50 ml of buffer A (20 mM Tris-HCl [pH 7.5], 2 mM β-mercaptoethanol, 10% glycerol) containing 50 mM NaCl. After the cells were disrupted by sonication (six 10-s bursts), a clear supernatant was obtained by centrifugation at 32,000 g for 1 h (fraction I). Polymin-P (pH 8.0) was added to the supernatant to a final concentration of 0.135%. After being stirred for 1 h, the suspension was centrifuged at 25,000 × g for 20 min. The pellet was resuspended in buffer A containing 20 mM NaCl and dialyzed against buffer A containing 20 mM NaCl (fraction III). Fraction III was applied to a DEAE Bio-Gel A (Bio-Rad) column (bed volume, 50 ml) equilibrated with buffer A containing 20 mM NaCl. NfxB was passed through the column with buffer A containing 20 mM NaCl. The pooled flowthrough fraction (fraction IV) was dialyzed against buffer B (10 mM NaPO₄, buffer pH 7.0, 5 mM β-mercaptoethanol, 10% glycerol) containing 20 mM NaCl. The dialyzed fraction IV was applied to a CM Bio-Gel A (Bio-Rad) column (bed volume, 15 ml) equilibrated with buffer B containing 20 mM NaCl. The column was washed with 3 column volumes of buffer B containing 20 mM NaCl and proteins were eluted with a 150-ml linear gradient of 20 to 300 mM NaCl in buffer B. NfxB eluted at about 200 to 250 mM NaCl. These fractions were pooled (fraction V) and directly applied to a phosphocelulose column (bed volume, 8 ml) which had been equilibrated with buffer B containing 250 mM NaCl. The column was washed with 3 column volumes of buffer B containing 250 mM NaCl, and proteins were eluted with a 100-ml linear gradient of 250 to 1,000 mM NaCl. NfxB was eluted at about 400 to 450 mM NaCl and then was pooled and dialyzed against storage buffer C (50 mM NaPO₄, 80 mM NaCl, 5 mM β-mercaptoethanol, 50% glycerol) (fraction VI). The protein was stored at −20°C.

**DNA-binding experiments.** The interaction of NfxB with the regulatory regions of nfxB was studied by means of a gel retardation assay (24) and by a DNAase I protection experiment (8).

**β-Galactosidase assay.** *E. coli* CSH26 carrying appropriate plasmids was cultured to an optical density at 600 nm of 0.6, and β-galactosidase activity in the cells was measured as described by Miller (13).

**Separation of the 23- and 21-kDa NfxB proteins, and preparation of anti-NfxB serum.** Fraction VI (1.5 mg), which contained the 23- and 21-kDa NfxB proteins, was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and purified with a model 491 Prep Cell (Bio-Rad) as described by the manufacturer. The 21-kDa NfxB was injected into rabbits to raise antisera against NfxB (21).

**Other procedures.** All DNA manipulations were done by the methods in reference 19. SDS-PAGE was performed as described by Laemmli (9). Proteins were identified by immunoblotting (21), and primer extension experiments were performed as previously described (19). The amino termini of purified 23-kDa (fraction Vb) and 21-kDa (fraction VIs) proteins were sequenced by the Center for Instrumental Analysis (Hokkaido University) with a peptide sequencer (model ABI PRISM 491A; Applied Biosystems). The intensity of the bands of the sequenced protein was quantified with an ImageQuant (Molecular Dynamics). The protein concentrations were determined with bovine serum albumin as the standard, following the Bradford method (1).

**RESULTS**

**Overproduction and purification of NfxB.** *E. coli* BL21(DE3) carrying pNF7 overproduced a 23-kDa protein that constituted about 3% of the total protein after induction with IPTG (Fig. 1, lane 2). Since this protein was not found in the extract of the control cells that carried pT7-5 (the pNF7 parent vector without the nfxB insert [Fig. 1, lane 1]) and the size of the protein agreed with that of NfxB deduced from the DNA sequence (2,113 Da), the purified protein was probably the product of the nfxB gene.

The 23-kDa protein was purified from BL21(DE3) cells carrying pNF7 after IPTG induction. The purification steps of the protein were monitored by SDS-PAGE (Fig. 1, lanes 3 to 8).

The final purified fraction (fraction VI) contained two proteins (23 and 21 kDa) (Fig. 1, lane 8). These proteins were not separated by sequential chromatography, because the molecular masses are very similar. We separated these proteins with a model 491 Prep-Cell, as described in Materials and Methods, and analyzed their amino acid sequences. The sequence of the 21-kDa protein (fraction VIs) from the amino terminus was TLISHDERLI, which completely agreed with the sequence predicted from the nucleotide sequence of the nfxB gene (16).

The amino acid sequence of the 23-kDa protein (fraction Vb) was MRTIRK, which corresponded to the sequence translated from nucleotide (nt) 235 of nfxB (see Fig. 7). These results indicated that NfxB is translated from two initiation codons corresponding to nt 235 and 271. Putative Shine-Dalgarno sequences of both coding regions were identified (nt 216 to 219 for the 23-kDa NfxB, and nt 260 to 263 for the 21-kDa NfxB).

We also examined whether these two proteins were pro-
duced in *P. aeruginosa* by using anti-NfxB serum which was obtained from rabbits immunized with purified 21-kDa NfxB (fraction VIs). As shown in Fig. 2, the 23- and 21-kDa NfxB proteins were detected in *P. aeruginosa* PK1013E (lane 4). These two proteins in *P. aeruginosa* had exactly the same mobility as the purified NfxB fractions (lanes 1 to 3). The ratio of the 23- and 21-kDa proteins was 3.2:1.0 in *P. aeruginosa* (Fig. 2, lane 4). This value was obtained by measuring the intensity of the bands in Fig. 2 with an ImageQuant. This ratio was similar to that of pNF7 expression in *E. coli* (data not shown). The results suggested that there are two NfxB proteins in *P. aeruginosa* that are produced from two translation start sites. However, we cannot rule out the possibility that the 21-kDa species is a degradation product of the 23-kDa protein.

Since both NfxB proteins were found in *P. aeruginosa*, we used a purified mixture of these proteins (fraction VI) to characterize their functions.

**Identification of the mRNA initiation sites of nfxB.** To identify the promoter region of nfxB, we determined the transcription initiation site of this gene by primer extension. The mRNA was reverse transcribed with an oligonucleotide primer which is complementary to the sequence between nt 308 and 327 of nfxB. In this experiment, the initiation site is unclear; however, the cytosine at nt 140 is the most likely candidate (Fig. 3; also see Fig. 7). Since the initiation site was not accompanied by the −10 and −35 consensus sequences of the *E. coli* promoter, its structure probably differs from that of the general *E. coli* promoter. Although there were two minor start sites at nt 162 and 167, the intensities of these bands were eight- and sevenfold lower than that of the major band (nt 140), respectively. The major transcript of nfxB results from initiation at nt 140.

NfxB binds to the regulatory regions of nfxB itself. Since NfxB has an amino acid sequence similar to the DNA-binding domain of many well-characterized helix-turn-helix type DNA-
binding proteins (4, 16), we examined whether NfxB binds to the regulatory region of nfxB itself and regulates its own transcription. We used gel retardation to study the binding of purified NfxB to the DNA fragment containing the regulatory region of nfxB. The electrophoretic mobility of the DNA fragment that contained the regulatory region of nfxB was significantly retarded by NfxB (Fig. 4A, lane 2). To examine the sequence specificity of binding, a 5- to 100-fold excess of unlabeled DNA fragment containing the regulatory region was mixed with a [32P]DNA fragment as the competitor. The retardation was remarkably inhibited by the unlabeled DNA fragment, and the inhibition was dependent on the amount of the competitor DNA (Fig. 4A, lanes 3 to 6). On the other hand, there was no competition by excess salmon sperm DNA (lanes 8 to 11).

Binding to the DNA fragment containing the nfxB regulatory region was further analyzed by gel retardation by varying the protein concentration (Fig. 4B). As the concentration of NfxB increased, the retardation of the DNA fragment increased (Fig. 4B, lanes 5 to 8). It also appears that there are multiple bands in Fig. 4A, lane 3. This may reflect that NfxB binds to the DNA fragment at more than one site. Since some discrete bands are also observed in Fig. 4B, lanes 4 and 5, some NfxB may dissociate from DNA during the gel electrophoresis at these NfxB concentrations. These results suggest that NfxB specifically binds to the regulatory region of nfxB, possibly at multiple sites.

The region of NfxB binding was determined more precisely by DNase I protection. The 5' and 3'-end-labeled nfxB sequences were partially digested with DNase I in the presence or absence of NfxB and the digest was analyzed by gel electrophoresis. The protection pattern in Fig. 5 shows where protection and enhancement occurred. For a diagram of this pat-
tern, see Fig. 7. In both strands, NfxB protected nfxB from nt 121 to 223, which corresponds to -20 to +83 of the upstream region of nfxB relative to nt 140 (the putative transcription start site). The protection and enhancement were more obvious when over 0.65 pmol of NfxB (32 nM) was added in each strand (Fig. 6, lanes 3 to 5).

**Autoregulation of the nfxB gene.** To examine whether nfxB expression is regulated by NfxB in vivo, we used two Ph(nfxB-lacZ') (Hyb) fusion constructs, pNF334 and pNF272, that conferred the Lac' phenotype when introduced into a E. coli lac deletion strain. Expression of nfxB was measured in terms of β-galactosidase activity with E. coli CSH26 (13) as the host strain in the presence or absence of intact nfxB gene.

When the nfxB gene was supplied by the second compatible plasmid, pNF20, about 10 times less Ph(nfxB-lacZ') (Hyb) fusion products were expressed than in the absence of nfxB (Fig. 6A). Since the 23-kDa NfxB was detected by immunoblotting in E. coli CSH26 carrying pNF20 (data not shown), NfxB was expressed under the lac promoter in pNF20. In the presence of NfxB, nfxB expression under the nfxB promoter was repressed in E. coli. This phenomenon supports the notion that nfxB expression is negatively autoregulated.

We also examined the autoregulation of nfxB expression in P. aeruginosa by using anti-NfxB serum (Fig. 6B). Both the 23- and 21-kDa NfxB proteins were expressed by the nfxB mutant, PK1013E (Fig. 6B, lane 1), which has a point mutation at nt 394 (cytosine to guanine) in the nfxB coding region. This mutation causes an Arg-to-Gly substitution (16). On the other hand, neither the 23- nor 21-kDa NfxB protein was detected in the wild-type strain, PAO2142 (lane 2). The mutant PK1013E produced mutant NfxB, which should not be able to bind to the nfxB regulatory region, because the point mutation in the helix-turn-helix region of NfxB results in a loss of DNA-binding activity.

**DISCUSSION**

We purified NfxB from E. coli by using an overproduction system constructed with a T7 expression vector. Two species of
NfxB proteins were purified with this system. The molecular masses of the major and minor species are estimated by SDS-PAGE as 23 and 21 kDa, respectively. These two species were also identified in P. aeruginosa PK1013E, and the ratio of 23- to 21-kDa protein was 3.2:1.0. Although neither protein was detected in a wild-type strain, PAO2142, by immunoblotting, a trace amount of NfxB was apparently produced; this was sufficient to negatively regulate the expression of nfxB. These proteins might be expressed from different translation initiation sites, because two possible initiation codons with Shine-Dalgarno sequence exist. Further studies are necessary to determine whether both species have the same repressor functions, because only mixtures of these two proteins were studied. However, NfxB appears to negatively regulate its own expression.

In both strands, the region from −20 to +83 of nfxB was protected from DNase I digestion by purified NfxB. The protein masked over 100 bp of the nfxB regulatory region. It is not likely that only one NfxB protein masks over 100 bp. Furthermore, the protected regions in both strands contain some positions where the digestion was enhanced and some positions that were not protected from the digestion. These positions are thought to be gaps between two adjacent sites of several NfxB-binding sites. Possible multiple sites are consistent with the results obtained from gel retardation assays. As the concentration of the unlabeled DNA fragment was increased, two or more bands were found (Fig. 4A, lane 3). These bands may reflect the number of NfxB-binding sites of the regulatory region.

As shown in Fig. 7, the NfxB-binding region contains two 39-bp repeats (units 1 and 2) that are 59% homologous. Since each unit was almost completely protected from DNase I digestion, each probably contains NfxB-binding site(s). The homology between these units also supports the notion there are two or more NfxB-binding sites. The second unit (nt 164 to ca. 202) contains two inverted repeats (IR1 and IR2 in Fig. 7). These inverted repeats might play a crucial role in NfxB binding.

Interestingly, computer analyses revealed that the 5'-flanking region of the protein F gene in P. aeruginosa (5) also has a region highly homologous with the inverted repeat IR1 (6) (Fig. 7). Since protein F is one of the major outer membrane protein porin proteins (15), NfxB may bind to the regulatory region of the protein F gene and control its expression also. However, Hirai et al. (7) reported that there were no changes in protein F levels in nfxB mutants, which does not support NfxB regulation of the protein F gene. In any case, in vitro analysis (gel retardation or footprinting) will help to clarify this issue.

The gene encoding the 54-kDa outer membrane protein, OprF (11), is thought to be an outer membrane channel, which forms an integral component of the drug efflux system (14), since OprF is overproduced in nfxB mutants. After oprF is cloned and sequenced, it will be interesting to see if it has any of the 39-bp repeats in its regulatory region. If so, NfxB effects on its expression will also be of interest.

Poole et al. (18) cloned an operon, mexA-mexB-oprK, which confers resistance to a broad range of antimicrobial agents and is believed to function in the export of the siderophore poverdine in P. aeruginosa. mexA-mexB-oprK and nfxB mutants are susceptible to similar antimicrobial agents. MexA and MexB exhibit homology to previously described bacterial export proteins located in the cytoplasmic membrane, and OprK is thought to be an outer membrane channel like OprF (11, 14, 18). This gene organization suggests that the three proteins form a drug efflux complex. From these results, we suspect that nfxB regulates the production of an efflux complex that includes OprF.

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REFERENCES

1. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.
2. Casadaban, M. J., J. Chou, and S. N. Cohen. 1980. In vitro gene fusions that join an enzymatically active β-galactosidase segment to amino-terminal fragments of exogenous proteins: Escherichia coli plasmid vectors for the detection and cloning of transcriptional initiation signals. J. Bacteriol. 143:971–981.
3. Chandler, M. S. 1991. New shuttle vectors for Haemophilus influenzae and Escherichia coli. Plasmid 25:221–224.
4. Dodd, I. B., and J. B. Egan. 1990. Improved detection of helix-turn-helix DNA-binding motifs in protein sequences. Nucleic Acids Res. 18:5019–5026.
5. Duchene, M., A. Schweizer, F. Lottspeich, G. Krauss, M. Marget, K. Vogel, B.-U. Specht, and H. Domdey. 1988. Sequence and transcriptional start site of the Pseudomonas aeruginosa outer membrane porin protein F. J. Bacteriol. 170:155–162.
6. Green, A. A., and W. L. Hughes. 1955. Protein fractionation on the basis of solubility in aqueous solutions of salts and organic solvents. Methods Enzymol. 1:57–90.
7. Hirai, K., S. Suzue, T. Ikuzuka, S. Iyohe, and S. Mitsushashi. 1987. Mutations producing resistance to norfloxacin in Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 31:582–586.
8. Johnson, A. D., R. J. Meyer, and M. Ptashe. 1979. Interactions between DNA-bound repressors govern regulation by the β phage repressor. Proc. Natl. Acad. Sci. USA 76:5061–5065.
9. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.
10. Li, X., D. M. Livermore, and H. Nakaido. 1994. Role of efflux pump(s) in intrinsic resistance of Pseudomonas aeruginosa: resistance to tetracycline, chloramphenicol, and norfloxacin. Antimicrob. Agents Chemother. 38:1732–1741.
11. Masuda, N., E. Sakagawa, and S. Ohyu. 1995. Outer membrane proteins responsible for multiple drug resistance in Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 39:645–649.
12. Matsutomo, H., S. Ohta, R. Kohayashi, and Y. Terawaki. 1978. Chromosomal location of genes controlling the degradation of purines in Pseudomonas aeruginosa. Mol. Gen. Genet. 167:165–176.
13. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory. Cold Spring Harbor, N.Y.
14. Nakaido, H. 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science 264:382–388.
15. Nakaido, H., K. Nakaido, and S. Harayama. 1991. Identification and characterization of porins in Pseudomonas aeruginosa. J. Biol. Chem. 266:770–779.
16. Okazaki, T., and K. Hirai. 1992. Cloning and nucleotide sequence of the Pseudomonas aeruginosa nfxB gene, conferring resistance to new quinolones. FEMS Microbiol. Lett. 97:197–202.
17. Okazaki, T., S. Iyohe, H. Hashimoto, and K. Hirai. 1991. Cloning and characterization of a DNA fragment that complements the nfxA mutation in Pseudomonas aeruginosa PAO. FEMS Microbiol. Lett. 79:31–36.
18. Poole, K., K. Krebs, C. McNally, and S. Neshat. 1993. Multiple antibiotic resistance in Pseudomonas aeruginosa: evidence for involvement of an efflux operon. J. Bacteriol. 175:763–772.
19. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. Schell, M. A. 1993. Molecular biology of the LysR family of transcriptional regulators. Annu. Rev. Microbiol. 47:579–626.
21. Shinagawa, H., H. Iwasaki, T. Kato, and A. Nakata. 1988. RecA protein dependent cleavage of UmuD protein and SOS mutagenesis. Proc. Natl. Acad. Sci. USA 85:1806–1810.
22. Studier, F. W., A. H. Rosenberg, J. J. Dunn, and W. Dubendorff. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. 185:60–89.
23. Tabors, S., and C. C. Richardson. 1985. A bacteriophage T7 polymerase/promoter system for controlled expression of specific genes. Proc. Natl. Acad. Sci. USA 82:1074–1078.
24. Wang, L., J. D. Helmann, and S. C. Winnas. 1992. The tufA-meflacin transcriptional activator of E. coli causes a bend at a target promoter, which is partially relaxed by a plant tumor metastat cell. Cell 69:559–667.