Electron transfer to nitrogenase in *Klebsiella pneumoniae*

nifF gene cloned and the gene product, a flavodoxin, purified

Janet DEISTUNG, Frank C. CANNON,* Maura C. CANNON,* Susan HILL and Roger N. F. THORNELEY
A.F.R.C. Unit of Nitrogen Fixation, University of Sussex, Brighton BN1 9RQ, U.K.

The *nifF* gene of *Klebsiella pneumoniae* was cloned into a multicopy plasmid in order to construct a strain that synthesizes and retains an elevated concentration of the gene product relative to the wild-type strain. Characterization of the isolated flavodoxin, which serves as an electron donor to nitrogenase, shows unambiguously that it is the product of the *nifF* gene.

INTRODUCTION

The nitrogen-fixation (*nif*) gene cluster of *Klebsiella pneumoniae* comprises 17 genes that are required for the synthesis and activity of nitrogenase *in vivo* (Reidel *et al.*, 1979; Postgate & Cannon, 1981). *K. pneumoniae* nitrogenase comprises the MoFe protein (Kp1; M_r 220000, 32Fe and 2Mo atoms/molecule) and the Fe protein (Kp2; M_r 67000, 4Fe atoms/molecule). Kp1 is a tetramer of two non-identical subunits (α and β ; M_r 56000 and 60000 respectively), which are the products of *nifD* (α) and *nifK* (β). Kp2 is a dimer composed of two identical subunits specified by *nifH*. The *nifHDK* genes are contiguous and are transcribed from a promoter adjacent to *nifH*. The products from the nine genes *nifQ*, *nifB*, *nifA*, *nifL*, *nifM*, *nifV*, *nifS*, *nifN* and *nifE* are involved in post-translational processing (including the assembly of Fe–S and Fe–Mo–S centres) of the *nifHDK*-gene-product peptides or in the regulation of their synthesis and activity in response to, for example, NH_4^+ and O_2 concentrations (Kennedy *et al.*, 1981; Hill *et al.*, 1981). The function of the *nifX*, *nifY* and *nifU* gene products is not known. The 17 *nif* genes are arranged in seven operons, the expression of which is activated by the *nifA* gene product in concert with the *ntrA* gene product (Drummond *et al.*, 1983, and references cited therein). In the present context, the apparent high affinity of the *nifH* promoter relative to that of the *nifF* promoter for these activators is of particular relevance (Buchanan-Wollaston *et al.*, 1981a; Reidel *et al.*, 1983; Brown & Ausubel, 1984).

A flavodoxin, purported to be the *nifF* gene product, mediates electron transfer from the *nifJ* gene product, a pyruvate:flavodoxin oxidoreductase, to the Fe protein of nitrogenase (Kp2) (Scheme 1) (Hill & Kavanagh, 1980; Nieva-Gomez *et al.*, 1980; Shah *et al.*, 1983). Yates (1972)

showed that the fully reduced, hydroquinone, state of a flavodoxin isolated from *Azotobacter chroococcum* effected a single electron transfer to the Fe protein of *A. chroococcum* nitrogenase protein, the semiquinone state of the flavodoxin being a product of this reaction. Shah *et al.* (1983) also concluded that the hydroquinone–semiquinone states of Kp flavodoxin were operating in the electron-transport pathway shown in Scheme 1, in which pyruvate is the source of reducing equivalents.

In vitro, $\text{Na}_2\text{S}_2\text{O}_4$ will substitute for flavodoxins as an electron donor to nitrogenase (Bulen *et al.*, 1965). It has been used frequently in both equilibrium and kinetic studies because of its low potential, ready availability and comparative stability in solution at neutral pH. The component proteins of nitrogenase are usually prepared in the presence of approx. 1 mM- $\text{Na}_2\text{S}_2\text{O}_4$ under an atmosphere of N_2 or Ar with the use of a variety of techniques for minimizing damage by adventitious O_2 (Eady, 1980). The availability of glove-boxes filled with high-purity N_2 (less than 1 p.p.m. O_2) enables $\text{S}_2\text{O}_4^{2-}$ ions to be removed from solutions of Kp1 and Kp2 without loss of activity (Thorneley & Lowe, 1983). The installation of a stopped-flow spectrophotometer in an anaerobic glove-box made possible a kinetic study of the reduction of $\text{Kp2}_{\text{ox}}(\text{MgADP})_2$ by SO_2^- (formed by the predissociation of $\text{S}_2\text{O}_4^{2-} \rightleftharpoons 2\text{SO}_2^-$) and the modulation of this reaction by complex-formation with Kp1 (Thorneley & Lowe, 1983). It is now therefore technically feasible and desirable to study the kinetics and mechanism of electron-transfer reactions between Kp flavodoxin (the natural electron donor) and Kp2 protein. A source of purified flavodoxin in quantities of about 10–20 mg is required for a detailed characterization, redox-potential determination and kinetic investigation by stopped-flow spectrophotometry.

Previous attempts in this laboratory to isolate a

Scheme 1. Electron-transport chain to nitrogenase in *K. pneumoniae*

Abbreviation used: SDS, sodium dodecyl sulphate.

* Present address: Biotechnica International, 85 Bolton Street, Cambridge, MA 02140, U.S.A.

Table 1. Bacterial strains and plasmids

Abbreviations: Sm, streptomycin; Km, kanamycin; Cb, carbenicillin; Cm, chloramphenicol; Amp, ampicillin; Tc, tetracycline.

Strain or plasmid	Relevant genotype or phenotype	Drug-resistance	Reference of source
Strains			
<i>Klebsiella pneumoniae</i>			
UNF107	rpsL1 Δ 107 (gnd <i>his nif</i>)	Sm	Dixon <i>et al.</i> (1977)
UNF3001 = UNF107 (pRD1)	Nif ⁺ His ⁺	Sm, Km, Cb, Tc	Hill & Kavanagh (1980)
UNF1202 = UNF107 (pRD183)	<i>nifF2183::Tn7</i>	Sm, Km, Cb, Tc	Hill & Kavanagh (1980)
UNF1624 = UNF107 (pRD264)	<i>nifF2664::Tn7</i>	Sm, Km, Cb, Tc	Hill & Kavanagh (1980)
UNF926	<i>hisΔ nifΔ hsdRI</i>		This laboratory
UNF696	<i>nifF4066 muh P1 lysog. recA⁻</i>		This laboratory
Kp5022	Nif ⁺ <i>hisD2 hsdRI</i>		Streicher <i>et al.</i> (1974)
UNF5111 = UNF926 (pMC71A, pBCC12)		Cb, Cm	Present paper
UNF5112 = UNF926 (pMC71A, pBCC13)		Cb, Cm	Present paper
UNF2361 = UNF696 (pBCC12)		Cb, Amp	Present paper
UNF2362 = UNF696 (pBCC13)		Cb, Amp	Present paper
UNF2363 = UNF696 (pMC71A, pBCC13)		Cb, Cm	Present paper
UNF2364 = UNF696 (pMC71A, pBCC12)		Cb, Cm	Present paper
UNF2372 = UNF926 (pBCC13)		Cb, Amp	Present paper
Plasmids			
pRD1	Gnd His ⁺ Nif ⁺ ShiA Tra IncP	Km, Cb, Tc	Dixon <i>et al.</i> (1977)
pRD183	<i>nifF2183::Tn7</i> derivative of pRD1	Km, Cb, Tc	Merrick <i>et al.</i> (1978)
pRD264	<i>nifF2664::Tn7</i> derivative of pRD1	Km, Cb, Tc	Dixon <i>et al.</i> (1980)
pBCC12	<i>nifF⁺</i>	Cb	Present paper
pBCC13	<i>nifF⁺</i>	Cb	Present paper
pMC71A	<i>nifA^c</i>	Cm	Buchanan-Wollaston <i>et al.</i> (1981b)
pBR328	<i>nifHDKY</i>		Soberon <i>et al.</i> (1980)
pSA30	<i>nifHD</i>	Tc	Cannon <i>et al.</i> (1979)
pPM1		Cb, Amp	P. McClean (this laboratory)
pMC9	<i>nifF⁺</i>	Cb, Amp	M. Cannon (this laboratory)

flavodoxin from *K. pneumoniae* M5al (a wild-type *Nif*⁺ strain) failed (Hill & Kavanagh, 1980). Although Nieva-Gomez *et al.* (1980) and Yoch (1974) succeeded in isolating and partially characterizing Kp flavodoxin, no yields were reported, and from the nature of their experiments with purified material a low yield can be inferred. In addition, Nieva-Gomez *et al.* (1980) and Shah *et al.* (1983) provided no evidence that the flavodoxin that they isolated was the product of the *nifF* gene.

We therefore decided to clone the *nifF* gene into a multicopy plasmid and construct a strain of *K. pneumoniae* that would synthesize and retain an elevated concentration of active flavodoxin relative to strain M5al. In the present paper we describe the procedure adopted and a characterization of the flavodoxin obtained that unambiguously shows it to be the product of the *nifF* gene.

MATERIALS AND METHODS

Bacterial strains and plasmids

The strains of *Klebsiella pneumoniae* are listed in Table 1.

Media

Strains were grown in Nutrient Broth (NB), Luria Broth (LB) or Nitrogen-Free Davis and Mingioli Medium (NFD) as described by Dixon *et al.* (1977). These media were supplemented, when required, with filter-sterilized stock solutions to give final concentrations of: carbenicillin, 200 µg/ml; chloramphenicol, 20 or 40 µg/ml; kanamycin, 25 µg/ml; ampicillin, 200 µg/ml; L-histidine, 30 or 50 µg/ml; (NH₄)₂SO₄, 200 or 1000 µg/ml.

All biochemicals were purchased from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K., and salts from BDH Chemicals, Poole, Dorset, U.K.

Cloning procedures

Details of methods used for DNA restriction and ligation during cloning have been described by Cannon (1980) and Maniatis *et al.* (1982). Restriction enzymes were purchased from Boehringer Mannheim and used according to the manufacturer's instructions.

Plasmid purification

Plasmids were purified by the boiling method of Holmes & Quigley (1980) and also by poly(ethylene glycol) precipitation with subsequent CsCl-density-gradient centrifugation at 232 000 *g* for 40–60 h at 4 °C in a Pegasus 65 ultracentrifuge (MSE, Crawley, Sussex, U.K.) fitted with an 8 × 35 ml angle rotor (Humphrey *et al.*, 1975).

Transformation

K. pneumoniae strains UNF696 and UNF926 were transformed by the method of Cohen *et al.* (1972) with the following modifications. Cells (100 ml with approx. 2 × 10⁸ organisms/ml), which had been treated with 50 mM-CaCl₂, were incubated with 400 ng of DNA at 0 °C for 1–2 h. A heat shock was carried out for 3 min at 32 °C for UNF696 and for 3 min at 42 °C for UNF926. The cells of UNF696 and UNF926 were suspended in Luria Broth (5 ml) and grown for 3 h at 30 °C or 37 °C respectively. A series of dilutions were plated on to

selective plates and incubated at the appropriate temperature.

Agarose-gel electrophoresis

Digested DNA was fractionated by electrophoresis in horizontal 0.8 and 1.0% agarose gels in TAE buffer (40 mM-Tris/5 mM-sodium acetate/1 mM-disodium EDTA, adjusted to pH 7.8 with acetic acid) for 16 h with a current of 40 mA as described by Cannon (1980). The gels were stained and photographed by the procedure described by Cannon *et al.* (1977).

¹⁴C-pulse-labelling

This was performed essentially by the procedure of Cannon (1980), except that 200 ml cultures were labelled for 10 min with 10 µl of ¹⁴C-labelled amino acid mixture (50 µCi/ml) in screw-capped Eppendorf tubes (2 ml volume). Labelled samples were then subjected to polyacrylamide-gel electrophoresis as described below.

Polyacrylamide-gel electrophoresis

A modified Ortec gel system was used as described by Cannon (1980), with a 13% or 15% separating gel and a 5% stacking gel. The steady-state intracellular concentration of soluble protein was determined from Coomassie-Blue-R-250-stained polyacrylamide gels of denatured cell protein by using a Unicam SP.1800 spectrophotometer fitted with a scanning densitometer accessory. After electrophoresis of ¹⁴C-pulse-labelled samples, the gels were dried under vacuum and exposed to X-ray film (Fuji RX) for 7–14 days.

Growth of strains

Strains UNF3001, UNF1202 and UNF1624 were grown as described by Hill & Kavanagh (1980). Strain UNF926 was grown as follows: a 6 h aerobically grown culture in LB (50 ml) was used as an inoculum for 500 ml of NFD containing (NH₄)₂SO₄ (1 mg/ml) and histidine (50 µg/ml). After 16 h at 30 °C, with sparging with N₂, the cells were harvested by centrifugation at 31 770 *g* for 5 min at 4 °C. The supernatant was discarded, and the cells were resuspended in 500 ml of NFD containing histidine (50 µg/ml), and de-repressed by sparging with N₂ for a further 4 h at 30 °C. Organisms were harvested by centrifugation as described above.

Strain UNF5112 cells in 0.5-litre, 1.5-litre and 3.0-litre cultures were grown as for strain UNF926 but with carbenicillin (200 µg/ml) and chloramphenicol (20 µg/ml) in both LB inoculum and NFD containing (NH₄)₂SO₄ (1 mg/ml) and histidine (50 µg/ml). The 20-litre and 400-litre cultures were grown as follows. An N₂-sparged culture in 500 ml of LB containing carbenicillin (200 µg/ml) and chloramphenicol (20 µg/ml) was inoculated into 20 litres of NFD containing histidine (50 µg/ml), chloramphenicol (20 µg/ml), carbenicillin (200 µg/ml) and (NH₄)₂SO₄ (200 µg/ml). Cells were grown for 16 h at 30 °C under N₂ and then used as an inoculum for 400 litres of NFD supplemented with histidine (50 µg/ml) and (NH₄)₂SO₄ (200 µg/ml). After 13 h at 30 °C, cells were harvested by using a Westfalia SA7 continuous centrifuge.

Preparation of cell-free extracts

A 50% (w/v) suspension of cells in 25 mM-Tris/HCl buffer, pH 7.4, was disrupted by passage through a French pressure cell as described by Eady *et al.* (1972) and

centrifuged at 49 640 *g* for 1 h at 4 °C. Protein concentration was measured by the procedure of Lowry *et al.* (1951), with bovine serum albumin (Sigma Chemical Co.) as standard.

Acetylene-reduction assays

Whole-cell assays. A 0.1 ml portion of a 16 h aerobically grown culture in NB was added to 4 ml of NFD medium containing histidine (30 µg/ml) in 7 ml plastic vials and capped with Suba-Seal rubber closures. The cultures were incubated at 30 °C on a rotary shaker for 5–16 h. Acetylene (1 ml) was injected above the cultures, which were then shaken for a further 30 min. Ethylene production was measured in 0.5 ml gas samples by vapour-phase chromatography as described by Cannon *et al.* (1977).

Assay for the *nifF* gene product. These assays were carried out by the method described by Hill & Kavanagh (1980), with 26 mM-sodium pyruvate as the source of reducing equivalent. Crude extracts of strains UNF5112 and UNF926 (0.4 mg per assay) were assayed for activity by reconstituting the electron flow to nitrogenase in crude extracts of either an *nifF* mutant (UNF1202 or UNF1624) or wild-type strain UNF3001 (0.66 mg per assay). Ethylene production was assayed as described above.

Isolation of the *nifF* gene product

The *nifF* gene product from strain UNF5112 was isolated by using a combination of the methods described by Nieva-Gomez *et al.* (1980) and Shah *et al.* (1983). A crude extract of strain UNF5112 was prepared as described above. The pellet of broken cells, obtained after removal of the supernatant crude extract, was suspended in 200 mM-potassium phosphate buffer, pH 7.4 (1 g of cell pellet per 2 ml of buffer). Triton X-100 was added to 1% (v/v) and the suspension stored at –20 °C overnight. The suspension was thawed, diluted 6-fold with distilled water and centrifuged at 31 770 *g* for 40 min at 4 °C. The supernatant solution was combined with the crude extract and treated as described below. All operations were at 5 °C in air.

The crude extract supernatant mixture was applied to a DEAE-cellulose column (5 cm × 10 cm) that had been equilibrated with 25 mM-Tris/HCl buffer, pH 7.4. The column was washed with 100 ml of the same buffer and developed by successive 100 ml washes with buffer containing 100 mM-, 150 mM-, 200 mM- and 350 mM-NaCl. Fractions were assayed for flavodoxin activity by reconstituting electron flow to nitrogenase in an extract of an *nifF* mutant as described above. The flavodoxin was eluted in the 350 mM-NaCl fractions. The active fractions were combined, diluted with an equal volume of buffer and applied to a second DEAE-cellulose column (3 cm × 12 cm), which had been equilibrated with buffer containing 200 mM-NaCl. The flavodoxin was then eluted with buffer containing 350 mM-NaCl. The fractions containing active flavodoxin were pooled, diluted 3-fold with buffer and concentrated by absorption on DEAE-cellulose followed by elution with buffer containing 350 mM-NaCl. The concentrated flavodoxin was then applied to a Sephadex G-50 column (2.5 cm × 50 cm) equilibrated with 25 mM-Tris/HCl buffer, pH 7.4, and the column was eluted at a flow rate of 10 ml/h. The most active fractions were pooled and concentrated on

DEAE-cellulose as described above. The flavodoxin was subsequently purified to homogeneity by using a preparative gel-electrophoresis apparatus as described by Ludden *et al.* (1978). The gel was loaded with a solution of flavodoxin (4 ml; approx. 5 mg/ml) containing 10% (w/v) sucrose and electrophoresed through the stacking gel for 16 h at 40 V, 150 pulses/s. Electrophoresis was continued at 160 V, 150 pulses/s, until fractions had been collected by elution across the bottom of the running gel in 100 mM-Tris/acetate buffer pH 8.0. Fractions were concentrated on DEAE-cellulose as described above. The purity of the flavodoxin was ascertained after each purification step by analytical SDS/polyacrylamide-gel electrophoresis (Cannon, 1980).

Amino acid composition

Purified flavodoxin (100 µg) was hydrolysed in 1 ml of 6 M-HCl at 110 °C for 24 h in an evacuated sealed 8 mm × 100 mm rimless Pyrex test tube. The tube was then opened and the contents were dried in a vacuum desiccator containing NaOH pellets. The hydrolysed sample was then redissolved in 1 mM-HCl and the amino acid composition was determined by using a Locarte amino acid analyser.

N-Terminal amino acid analysis

The N-terminal amino acid residue was determined with dansyl chloride (5-dimethylaminonaphthalene-1-sulphonyl chloride) by using a modified version of the method described by Allen (1981*a,b*). Purified flavodoxin (100 µg) was precipitated with ice-cold 5% (w/v) trichloroacetic acid. The precipitated protein was resuspended in 50 µl of 0.1 M-NaHCO₃ in a borosilicate glass tube (5 mm × 50 mm), and an equal volume of dansyl chloride (5 mg/ml) in acetone was added. After gentle mixing the reaction mixture was left at 23 °C for 4–5 h and then evaporated to dryness in a vacuum desiccator containing NaOH pellets. After addition of 6 M-HCl (0.1 ml), the tubes were sealed and kept at 110 °C for 5 h. After cooling to room temperature, the tube was opened, the contents were dried *in vacuo* and the N-terminal dansylated amino acid residue was extracted with water-saturated ethyl acetate. The N-terminal amino acid residue was determined by two-dimensional t.l.c. on double-sided 5 cm × 5 cm polyamide layer plates with the mixed solvent systems described by Woods & Wang (1967).

C-Terminal amino acid analysis

The C-terminal amino acid determination was based on a procedure described by Allen (1981*a*). Carboxypeptidase A was suspended in water and centrifuged. The supernatant, which contains contaminating amino acids, was discarded. The pellet was then dissolved in a minimum volume (approx. 50 µl) of 2 M-NH₄HCO₃, added to the 400 µg of flavodoxin (220 nmol) in 0.5–1.0 ml of 0.2 M-N-ethylmorpholine, pH 8.5 (adjusted with acetic acid), at an enzyme/substrate ratio of 1:100 (w/w) and incubated at 25 °C. Portions containing 55 nmol of protein were removed after 0.5, 1.0, 4.0 and 16.0 h, and each was added to an equal volume of 1 M-acetic acid to stop the proteolysis. Precipitated protein was removed by centrifugation and the supernatants were dried down *in vacuo*. The amino acid composition of the samples was then determined by using a Locarte amino acid analyser.

Fig. 1. Cloning procedure used to construct plasmids carrying the *nifF* gene in two orientations

Plasmids pBCC12 and pBCC13 were constructed from pPM1, a plasmid derived by inserting the fragment RI(*nifJ*) to HindIII(*nifK*) of pSA30 into pBR328. For plasmid pBCC12 a BglII(*nifL*)-BamHI(*nifM*) fragment from pMC9 was inserted into pPM1, and for plasmid pBCC13 the fragment BglII(*nifL*)-Xho/SalI(*nifM*) from pMC9 was inserted into pPM1. Plasmids were selected by size after colony screening on agarose gels, and the orientation of the inserted fragment was determined by mapping restriction-enzyme sites. Cm, Amp and Tc refer to chloramphenicol-resistance, ampicillin-resistance and tetracycline-resistance genes; J, H, D, K, F, M and L refer to the corresponding *nif* genes, and RI, BglII, HindIII, BamHI and SalI refer to restriction sites.

Fig. 2. Restriction map of plasmids pBCC13 and pBCC12

This restriction and genetic map shows the *nif* genes that are present on plasmids pBCC13 and pBCC12. The restriction sites shown are those that are important for the construction of the *nifF* clones, plasmids pBCC13 and pBCC12 and for the assignment of the orientation of the *nifF*–*nifL*–*nifM* fragment in these plasmids. Abbreviations: Cm, chloramphenicol; Tc, tetracycline; Amp, ampicillin.

Table 2. Absence of genetic complementation by *nifF* plasmid in the presence and in the absence of *nifA* plasmids

These values were obtained from three independent experiments. Each assay was carried out in duplicate. Acetylene reduction (%) is defined as the nmol of ethylene produced given as a percentage of the Kp5022 (wild-type) value. A 5 h-de-repressed culture of *K. pneumoniae* 5022, with $A_{540} = 0.06$, gave 10.4 nmol of ethylene/0.5 ml of gas sample, and the 16 h-de-repressed culture, with $A_{540} = 0.12$, gave 22.6 nmol of ethylene/0.5 ml of gas sample. (A_{540} values were measured with an EEL spectrophotometer.) These values were used to normalize the data obtained for the different strains at the two de-repression times.

Strain	Acetylene reduction after 5 h de-repression (%)	Acetylene reduction after 16 h de-repression (%)
UNF696	12.5 ± 1.0	13.6 ± 1.0
UNF2361	18.8 ± 1.0	21.3 ± 1.0
UNF2362	20.6 ± 1.0	21.9 ± 1.0
UNF2363	21.2 ± 1.0	22.8 ± 1.0
UNF2364	20.9 ± 1.0	22.3 ± 1.0

RESULTS AND DISCUSSION

Cloning the *nifF* gene

Two plasmids that both carry a *nifL*–*nifF*–*nifM* fragment, but cloned in different orientations, have been constructed (Fig. 1). Plasmid pBCC12 carries a *Bgl*II (*nifL*)–(*nifM*) fragment, and plasmid pBCC13 carries a *Bgl*II(*nifL*)–*Sall*(*nifM*) fragment. These fragments have been individually cloned into pPMI, a plasmid derived by inserting an *Eco*RI/*Hind*III fragment carrying *nifJ*–*nifH*–*nifD*–*nifK* into plasmid pBR328. The different orientations of the *nifL*–*nifF*–*nifM* fragment in plasmids pBCC12 and pBCC13 were determined by restriction mapping (Fig. 2). When these plasmids were originally constructed the

direction of transcription of the *nifF* gene was uncertain. In an attempt to maximize expression of the *nifF* gene, plasmids pBCC12 and pBCC13 were constructed with the strong *nifH* promoter upstream from *nifF*. Studies by Beynon *et al.* (1983) and Drummond (1985) have shown that *nifF* is transcribed in the opposite direction to *nifL* (Fig. 2). It is therefore not surprising that strain UNF5112 carrying pBCC13 gave an 8-fold higher yield of the *nifF* gene product than did wild-type strain UNF3001 (see below). However, it is difficult to estimate the precise advantage of using a construct with a *nifH* promoter, for two reasons. Firstly, all the promoters associated with *nif* genes in Fig. 2 require *nifA* gene product for activation. Multiple copies of the *nifH*

Fig. 3. Autoradiograms of SDS/polyacrylamide-gel electrophoretograms of ^{14}C -pulse-labelled cultures of strains constructed for genetic complementation studies

Cultures were grown and pulse-labelled with a mixture of ^{14}C -labelled amino acids under conditions that de-repressed nitrogenase synthesis, as described by Cannon (1980). The ^{14}C -labelled samples were then subjected to SDS/polyacrylamide-gel electrophoresis, with 15000 c.p.m. being loaded for each sample. Subsequent procedure is described in the Materials and methods section. Lane 1, strain UNF2361 = UNF696 (pBCC12); lane 2, strain UNF2364 = UNF696 (pBCC12, pMC71A); lane 3, strain UNF2362 = UNF696 (pBCC13); lane 4, strain UNF2363 = UNF696 (pBCC13, pMC71A); lane 5, strain UNF696; lane 6, M_r markers, 30000, 18000 and 12500. The 19000- M_r and 22000- M_r polypeptides (indicated on the left) are products of strains UNF2362 and UNF2363 that are absent from the other strains.

promoter can titrate out *nifA* gene product (Buchanan-Wollaston *et al.*, 1981a; Reidel *et al.*, 1983). This may prevent the initiation of transcription of the *nifF* gene from its own promoter. This multicopy effect is discussed below, where a comparison of *nifF* gene expression from the two plasmids is made. Secondly, it is possible that *nifL* transcription from the opposite end of *nifH* in pBCC13 (as shown in Fig. 2) prevents significant read-through from *nifH* into *nifF*. Also the intergenic region between *nifL* and *nifF* might contain sequences that modulate enhanced transcription of *nifF* initiated from the *nifH* promoter.

Expression of the cloned *nifF* gene

The two plasmids pBCC12 and pBCC13 were transformed into strain UNF696, which carries a *nifF* point mutation, to give strains UNF2361 = UNF696 (pBCC12) and UNF2362 = UNF696 (pBCC13). No significant genetic complementation was detected (Table 2). A possible explanation is that the multiple copies of

Fig. 4. Autoradiogram of SDS/polyacrylamide-gel electrophoretogram of ^{14}C -pulse-labelled cultures showing the *nifA*-gene-dependence of the synthesis of the 19000- M_r and 22000- M_r polypeptides

De-repressed cultures were pulse-labelled with a mixture of ^{14}C -labelled amino acids as described by Cannon (1980). The ^{14}C -pulse-labelled samples were then subjected to SDS/polyacrylamide-gel electrophoresis, with 15000 c.p.m. being loaded for each sample. All subsequent procedures are described in the Materials and methods section. Lane 1, M_r markers, 30000, 18000 and 12500; lane 2, strain UNF926 (*his* $^{\Delta}$ *nif* $^{\Delta}$); lane 3, strain UNF5112 = UNF926 (pMC71A, pBCC13); lane 4, strain UNF2372 = UNF926 (pBCC13).

the *nifH* promoter present in these strains titrate out all the *nifA* gene product (see the preceding subsection). Strains UNF2363 and UNF2364 were therefore constructed carrying the *nifA* constitutive plasmid pMC71A in addition to plasmids pBCC12 and pBCC13. These strains did not show any enhanced acetylene-reduction activity (Table 2). We were thus unable to obtain genetic evidence for a copy number effect. However, decreased concentrations of *nifJ*-, *nifH*-, *nifD*- and *nifK*-gene-product polypeptides were observed even when the *nifA* constitutive plasmid was present (Fig. 3). The rate of acetylene reduction is a function of the concentration of these peptides as well as that of the *nifF*-gene-product peptide. We conclude that whole-cell acetylene-reduction activity is not related in a simple manner to the

Table 3. Assays for the *nifF* gene product, measured by acetylene-reducing activity in crude extracts of wild-type and mutant strains

Crude extracts of UNF5112 and UNF926 (0.66 mg of protein per assay) were assayed for acetylene-reducing activity by reconstituting electron flow to nitrogenase in crude extracts (0.66 mg of protein per assay) of either a *nifF*⁻ mutant (UNF1202 or UNF1624) or wild-type strain (UNF3001). The assays were performed at 30 °C for 10 min.

Strain no. or strain combination	Relevant genotype or phenotype	Sp. activity (nmol of C ₂ H ₂ reduced/min per mg of total protein)	
		Dithionite (26 mM)	Pyruvate (17 mM)
UNF926	Nif ^Δ	0.05 ± 0.01	0.09 ± 0.01
UNF926 UNF926		0.03 ± 0.01	0.21 ± 0.02
UNF926 UNF5112		0.09 ± 0.01	0.11 ± 0.01
UNF1202	NifF ⁻	41 ± 1	0.08 ± 0.01
UNF1202 UNF926		21 ± 1	0.12 ± 0.01
UNF1202 UNF5112		42 ± 1	13.9 ± 2.5
UNF1624	NifF ⁻	42 ± 1	0.22 ± 0.02
UNF1624 UNF926		29 ± 1	0.18
UNF1624 UNF5112		49 ± 1	15.2 ± 2.1
UNF3001	Nif ⁺	42 ± 1	1.5 ± 0.2
UNF3001 UNF926		30 ± 1	0.65 ± 0.06
UNF3001 UNF5112		51 ± 1	15.3 ± 1.3
UNF3001 UNF1202		80 ± 1	0.54 ± 0.02
UNF3001 UNF1624		76 ± 1	0.74 ± 0.02

Fig. 5. Coomassie-Blue-R-250-stained SDS/polyacrylamide-gel electrophoretogram showing crude extracts of strain UNF5112 and the purified protein obtained from it

Crude extract of strain UNF5112 (30 µg) and purified protein (5 µg) were subjected to SDS/polyacrylamide-gel electrophoresis and stained with Coomassie Blue R-250 as described in the Materials and methods section. Lane 1, purified protein; lane 2, crude extract of strain UNF5112; lane 3, *M_r* markers (values shown on Figure). The purified protein has *M_r* 19000, which, from scanning densitometry of strain UNF5112, is present as 3–4% of total soluble protein inside the cell.

concentration of the *nifF*-gene-product peptide and that acetylene-reduction data cannot be used to estimate the relative concentrations of the *nifF*-gene-product peptide in the relevant strains in Table 2. However, plasmids pBCC13 and pBCC12 do complement strain UNF696 for growth on nitrogen-free solid media, indicating that a functional *nifF* gene is present on these plasmids.

Fig. 3 shows two heavily labelled polypeptides in strains carrying plasmid pBCC13 (e.g. UNF2362), with *M_r* values of 19000 and 22000, that were absent from strains with plasmid pBCC12 (e.g. UNF2361). Kp flavodoxin has an *M_r* close to 20000 (Yoch, 1974; Nieva-Gomez *et al.*, 1980). We confirmed that these products were *nifF*-specific by showing that their formation was augmented by *nifA* gene product (from plasmid pMC71A) in an *nif*-deletion background (Fig. 4). Therefore plasmid pBCC13 was chosen in preference to plasmid pBCC12 for further characterization of the presumptive *nifF* gene product.

Assay of the presumptive *nifF* gene product

The presumptive *nifF* gene product was assayed for activity by biochemical complementation of pyruvate-supported nitrogenase activity (Hill & Kavanagh, 1980; Nieva-Gomez *et al.*, 1980). Table 3 shows that crude extracts of strain UNF5112 stimulated pyruvate-supported acetylene reduction about 100-fold in extracts of two *nifF*⁻ strains (UNF1202 and UNF1624) and augmented activity about 10-fold in extracts of the wild-type strain (UNF3001). This latter observation is consistent with the report by Hill & Kavanagh (1980) that pyruvate-supported acetylene-reduction activity with crude extracts of *K. pneumoniae* (M5al) is limited by the concentration of the *nifF* gene product. The crude extract of strain UNF5112 also enhanced the activity of a crude extract of strain UNF3001 in the presence of S₂O₄²⁻. The ability

Fig. 6. U.v.-visible-absorption spectrum of oxidized flavodoxin (*nifF* gene product) purified from *K. pneumoniae* strain UNF5112

The spectrum recording was made with 33 μM -flavodoxin in 25 mM-Hepes buffer, pH 7.4, in a 1 cm-pathlength cell.

of purified *Azotobacter vinelandii* flavodoxin to stimulate dithionite-supported acetylene reduction by purified *A. vinelandii* nitrogenase under certain conditions of component protein ratio has been reported by Hageman & Burris (1978) and explained by Thorneley & Lowe (1984).

Yield of the purified *nifF* gene product from strain UNF5112

The *nifF* gene product was purified as described in the Materials and methods section, monitored by the assay system described above. A single band corresponding to M_r 19000 after SDS/polyacrylamide-gel electrophoresis (Fig. 5) and a u.v.-visible-absorption spectrum for the oxidized protein with an absorption coefficient at 454 nm of $10.0 \pm 0.5 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ were used as criteria for peptide homogeneity and FMN content (Fig. 6). Table 4 shows that with soluble and membrane-bound cell protein from 100 g (wet wt.) of cells of strain UNF5112 (1.04 g of total protein), 12 mg of purified flavodoxin was obtained, corresponding to a 39% recovery of activity units. This recovery is more than twice that reported by Nieva-Gomez *et al.* (1980) for their purification of Kp flavodoxin from

wild-type *K. pneumoniae* (M5al). We have not attempted a purification of Kp flavodoxin from *K. pneumoniae* (M5al) in order to compare the absolute yields of purified flavodoxin with that from strain UNF5112. However, a comparison of Coomassie-Blue-R-250-stained SDS/polyacrylamide gels of wild-type strain UNF3001 and strain UNF5112 by scanning densitometry showed that Kp flavodoxin (M_r 19000) was present in UNF5112 at concentrations corresponding to 3–4% of the total soluble protein. The corresponding value for strain UNF3001 was only 0.5%.

Comparison of the M_r and amino acid composition of Kp flavodoxin from strain UNF5112 with that predicted from the *nifF* gene sequence

The M_r of the Kp flavodoxin purified from strain UNF5112 was determined by SDS/polyacrylamide-gel electrophoresis to be 19000 ± 500 . This is in agreement with a value of M_r 18950 determined from the DNA sequence for *nifF* gene (Drummond, 1985) and previous values of M_r approx. 20000 (Yoch, 1974; Nieva-Gomez *et al.*, 1980).

Table 5 shows a comparison of amino acid composition data determined for Kp flavodoxin from strain UNF5112 by using conventional amino acid analysis after protein hydrolysis (see the Materials and methods section) with that calculated from the DNA sequence data for the *nifF* gene obtained by Drummond (1985). The agreement between the two data sets is within the estimate of experimental error ($\pm 5\%$) inherent in the amino acid analysis procedure employed with the protein.

The *N*-terminal amino acid residue of Kp flavodoxin was determined to be alanine. The sequence at the *C*-terminus was shown to be Leu-Val-Thr-Arg. These findings are in precise agreement with DNA sequence data for the *nifF* gene (Drummond, 1985).

These data show unequivocally that the flavodoxin purified from UNF5112 is the product of the *nifF* gene. The co-migration of the purified flavodoxin from strain UNF5112 with the 19000- M_r polypeptide synthesized in the ^{14}C -labelling experiment with strain UNF5112 (Fig. 7) allows the assignment of this polypeptide as the *nifF* gene product. The 22000- M_r polypeptide, which could have been mistaken for the *nifF* gene product, is probably a *nifH*-*nifL*-gene-product hybrid protein that is transcribed from the *nifH* promoter.

Table 4. Purification of *nifF* gene product (flavodoxin) from *K. pneumoniae* UNF5112

The crude extract mixture assayed included the soluble and membrane-bound protein obtained from 100 g (wet wt.) of UNF5112. The *nifF* gene product was assayed at each stage of purification by reconstituting electron flow to nitrogenase in a crude extract of the *nifF*⁻ mutant UNF1202 (0.66 mg per assay). Assays were carried out at 30 °C over 10 min. The maximum specific activity obtained with a range of protein concentrations (24–660 μg per assay) is given at each stage of purification.

Fraction	Quantity of protein (mg)	Specific activity (nmol of C_2H_2 reduced/min per mg of protein)	Recovery (%)
Crude extract/supernatant mixture	1040	11	100
DEAE-cellulose (1st)	270	36	85
DEAE-cellulose (2nd)	180	46	73
Sephadex G-50	65	98	56
Preparative gel electrophoresis (1st)	18	279	44
Preparative gel electrophoresis (2nd)	12	372	39

Table 5. Amino acid composition analysis of *nifF* gene product

Two independent determinations were made on the purified 19000- M_r protein from strain UNF5112. These data (column 1) are compared with the amino acid composition of the *nifF* gene product (column 2) calculated from the gene sequence determined by Drummond (1985). Abbreviation: N.D., not determined.

Amino acid	Composition from purified protein	Composition from gene sequence
Aspartic acid	20.4	20
Threonine	8.6	10
Serine	11.2	12
Glutamic acid	18.4	18
Proline	7.6	7
Glycine	16.7	19
Alanine	15.6	15
Half-cystine + valine	8.8	9
Methionine	3.1	4
Isoleucine	10.2	8
Leucine	20.1	20
Tyrosine	3	3
Phenylalanine	9.1	10
Histidine	N.D.	1
Lysine	7.3	7
Arginine	8.0	8
Tryptophan	3	3
Total	174

Fig. 7. Assignment of the 19000- M_r polypeptide in ^{14}C -pulse-labelled cultures of strain UNF5112

The purified 19000- M_r polypeptide was mixed with a ^{14}C -pulse-labelled extract of strain UNF5112 and subjected to SDS/polyacrylamide-gel electrophoresis. After Coomassie-Blue-R-250 staining the gel was dried and exposed to X-ray film as described in the Materials and methods section. Lane 1, M_r markers (values shown on Figure); lane 2, purified 19000- M_r protein; lane 3, purified

The successful cloning of the *nifF* gene and purification of the gene product, Kp flavodoxin, in yields about 8-fold greater than those obtained from wild-type strains will greatly facilitate the detailed characterization of this flavodoxin. It will also enable the electron-transfer system to nitrogenase to be investigated by using spectroscopic, electrochemical and kinetic techniques that require relatively high concentrations of protein.

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19000- M_r protein with ^{14}C -labelled extract of strain UNF5112; lane 4, autoradiogram of lane 3. It is clear that the purified 19000- M_r polypeptide co-migrates with the 19000- M_r protein observed in ^{14}C -pulse-labelled extracts of strain UNF5112.

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