JOURNAL OF BACTERIOLOGY, May 1991, p. 3252–3256
0021-9193/91/103252-05$02.00/0

Vol. 173, No. 10

Cotranscription of the Electron Transport Protein Genes *nifJ* and *nifF* in *Enterobacter agglomerans* 333

ROLAND KREUTZER,† SIDDAVATTAM DAYANANDA,‡ AND WALTER KLINGMÜLLER*

Lehrstuhl für Genetik, Universität Bayreuth, Universitätsstrasse 30, 8580 Bayreuth, Federal Republic of Germany

Received 7 September 1990/Accepted 4 March 1991

A nucleotide sequence showing extensive homology to the *nifF* gene, which codes for a flavodoxin involved in nitrogen fixation in *Klebsiella pneumoniae*, was localized on the plasmid pEA3 of *Enterobacter agglomerans* and determined. The analysis of transcriptional fusions, as well as transcript protection assays, indicated a novel *nif* gene organization, that is, the cotranscription of *nifJ* and *nifF*.

In the nitrogen-fixing *Enterobacter agglomerans* 333, the arrangement of *nif* genes within the plasmid-borne *nif* gene group is similar to that of *Klebsiella pneumoniae* (24). However, in *E. agglomerans* the gene *nifJ* was found to be located at the opposite end of the *nif* gene group as compared with the location in *K. pneumoniae* (Fig. 1). The gene product of *nifJ* is well characterized in *K. pneumoniae* and was identified as an oxido-reductase involved in the electron transport from pyruvate to a specific flavodoxin encoded by *nifF*, which itself mediates the electron transfer to dinitrogenase (9, 10, 18). In *K. pneumoniae*, both unlinked genes are transcribed from two separate promoters (1, 6) with the characteristic features of *nif* regulatory regions, namely the −24, −12 consensus sequence (2), which is recognized by the alternative σ factor σ^{54} (for a review, see reference 13), and the upstream activator sequence (UAS) (5), which represents a binding site for the transcriptional activator protein NifA (15, 17) required for efficient *nif* gene expression (3, 8, 22). For the induction of *nif* genes, NifA binds to the UAS and contacts the closed σ^{54}-RNA polymerase-promoter complex by looping out the DNA (4) supported by the integration host factor (7, 22). Recently, the *nifJ* promoter region of *E. agglomerans* was also characterized as a typical *nif* regulatory region with the characteristic features mentioned above (12). In the present work, we report that the gene *nifF* in *E. agglomerans* is located downstream of and organized in one operon with the *nifJ* gene. This is the first time, to our knowledge, that such an arrangement has been found.

The nucleotide sequence of the *E. agglomerans nifF* gene, which was identified on the basis of its homology to the *K. pneumoniae nifF* gene (see below), was determined by using the chain-termination method (21) with either recombinant M13mp18 and M13mp19 phage DNA or pUC18 plasmid DNA (Table 1). Also, the flanking regions of *nifF* were sequenced (Fig. 1). Comparison of the obtained sequence (Fig. 2) with the complete nucleotide sequence of the *K. pneumoniae nif* gene cluster (1) revealed obvious similarities to three *nif* genes of *K. pneumoniae* (*nifQ*, *nifF*, and *nifJ*). Thus, we conclude that *nifF* is located between the genes *nifQ* and *nifJ* in *E. agglomerans* identified earlier (24), as shown in Fig. 1. The position of *nifF* proposed earlier in a preliminary gene map of the *E. agglomerans nif* gene group (24) must therefore be revised. No *nifF*-specific hybridization probe had been used in the previous work. This may explain why the correct position of *nifF* had not been determined. Results from recently performed Southern hybridization experiments with *nifF*-specific probes (data not shown) clearly indicated that *nifF* and *nifJ* are adjacent in *E. agglomerans*. This remarkable gene arrangement has not been described for other nitrogen-fixing bacteria and may reflect a primitive organization of genes coding for presumably *cis*-acting proteins which later join together in a complex. Interestingly, the genes *nifF* and *nifJ* are oriented opposite to *nifQ* and to all other *nif* genes identified. A sequence motif which matches fairly well with the *nif* promoter consensus sequence (Table 2) was found 178

TABLE 1. Bacterial strains and plasmids

Escherichia coli strain or plasmid	Genotype or phenotype	Reference
CB454	F⁻ Δ*lacZ lacY*⁺ *galK thi rpsL recA56*	23
JM103	Δ(*lac-pro*) *thi rpsL supE endA sbcB hsdR* [F′ *traD36 proAB*⁺ *lacI*ᵠ *lacZ*ΔM15]	14
pUC18	Ap^r	26
pCB182	Ap^r promoter test plasmid	23
pCK3	Tc^r *nifA*(Con)$_{K.\ pneumoniae}$	11
pHP45Ω	Ap^r Sm^r Spc^r Ω fragment carrier plasmid	19
pMK182P1	Ap^r *nifJp*$_{E.\ agglomerans}$-*lacZ*	12
pMK38SB	Ap^r *Sal*I/*Bam*HI fragment subcloned in pUC18[a]	This work
pSD1	Ap^r transcriptional fusion[a]	This work
pSD2	Ap^r transcriptional fusion[a]	This work
pSD3	Ap^r transcriptional fusion[a]	This work
pSD4	Ap^r transcriptional fusion[a]	This work
pSD4Ω	Ap^r Sm^r Spc^r transcriptional fusion, Ω fragment inserted[a]	This work

[a] The construction of these plasmids is shown in Fig. 1.

* Corresponding author.
† Present address: Laboratorium für Biochemie, Universität Bayreuth, 8580 Bayreuth, Federal Republic of Germany.
‡ Present address: Department of Biochemistry, S. K. University, Anantapur-515003, India.

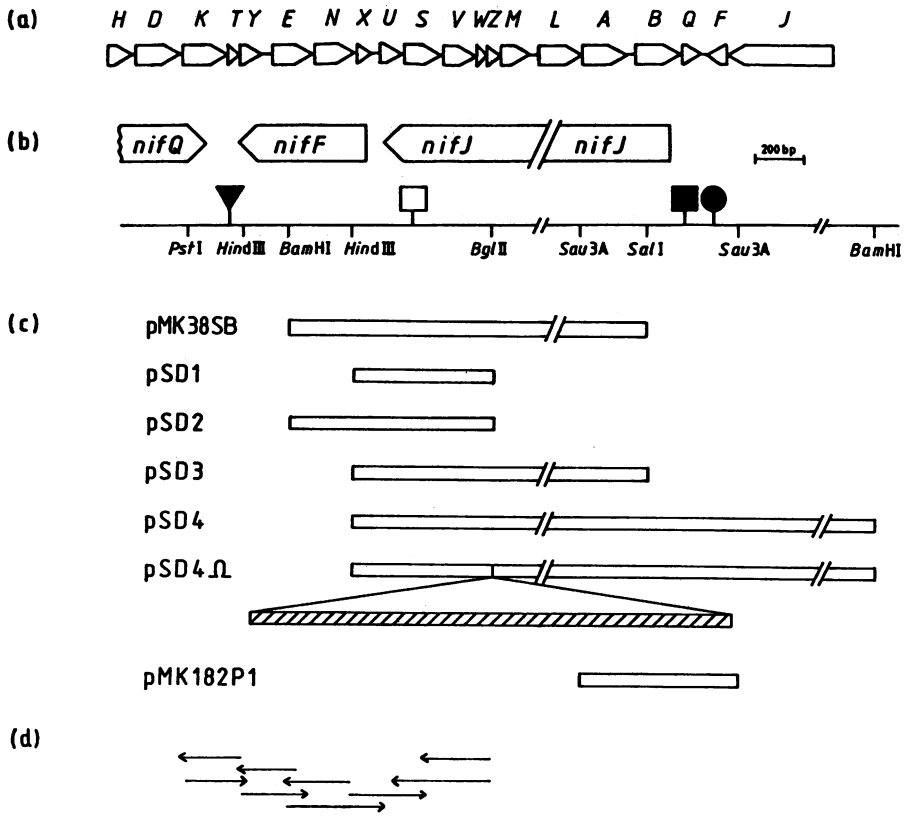

FIG. 1. (a) Organization of the *nif* gene group on the *E. agglomerans* plasmid pEA3. The open arrows indicate relative positions and orientations of the *nif* genes. (b) Detail of panel a. The positions of the genes *nifQ*, *nifF*, and *nifJ* are indicated by open arrows above the restriction map of the right end of the *nif* gene group. The positions of the *nifJ* promoter and of a promoterlike motif are indicated by filled and open boxes, respectively. The *nifJ* UAS is represented by a circle, and the putative transcriptional terminator is represented by a triangle. (c) The restriction fragment containing a 3.8-kb *Sal*I-*Bam*HI fragment with most parts of *nifJ* and *nifF* cloned into pUC18 (pMK38SB) and the restriction fragments cloned as transcriptional fusions with *lacZ* into the promoter test plasmid pCB182 (pSD plasmids) are indicated by rectangles corresponding to the map shown in panel b. The designations of the resulting plasmids are given on the left. The *Hin*dIII or *Bam*HI restriction sites positioned in the *nifF* coding region were used to construct the fusion. The 563-bp *Hin*dIII-*Bgl*II fragment of pSD1, the 838-bp fragment of pSD2, and the 3.5-kb *Hin*dIII-*Sal*I fragment of pSD3 were obtained from plasmid pMK38SB. The insert of pSD2 was ligated into the *Bam*HI restriction site of pCB182. To ensure the correct orientation, the resulting plasmids were hydrolyzed with endonuclease *Hin*dIII having an asymmetric recognition site on the insert. The 5.6-kb *Hin*dIII-*Bam*HI fragment of pSD4 was obtained from the recombinant cosmid peaMS2-16 (24). To construct pSD4Ω, the Ω fragment (hatched rectangle) was obtained as a 2.0-kb *Bam*HI fragment of pHP45Ω and ligated into the *Bgl*II restriction site of pSD4. (d) The extents of sequencing reactions are indicated by arrows in relation to the restriction map shown in panel b.

bp upstream of the *nifF* start codon in the 3′ portion of the *nifJ* coding region (at position 924 to 940 of the sequence in Fig. 2). However, no UAS-like element was detected as far as 400 bp upstream of *nifF*. An inverted repeat is present at position 162 to 185 in the sequence shown in Fig. 2 downstream of *nifF*; this inverted repeat may work as a transcriptional terminator of the proposed *nifJF* operon and also of *nifQ* because of its position in the intergenic region between *nifQ* and *nifF* and its dyad symmetry. The hairpin-loop structure in the mRNA formed by this inverted repeat has a free energy of −62.8 kJ/mol (25°C), as calculated by the computer method of Zuker and Stiegler (27).

The above-mentioned motif identified at position 924 to 940 in the sequence shown in Fig. 2, which is similar to the *nif* promoter consensus sequence (Table 2), was examined for its ability to act as a functional promoter. Four fragments of different sizes were cloned in the promoter test plasmid pCB182 in *Escherichia coli* CB454 (Fig. 1). Additionally, we inserted an Ω fragment (19), a strong transcriptional terminator, into the *Bgl*II restriction site located 309 bp upstream of the promoterlike motif in one of the fusion plasmids (pSD4) in order to terminate transcription from the *nifJ* promoter. β-Galactosidase activities of *Escherichia coli* CB454 clones containing both one of the fusion plasmids and the *nifA*-expressing plasmid pCK3, which constitutively expresses the *K. pneumoniae nifA* gene (11), were measured as described earlier (12) but by using a lysis mixture consisting of 1 part (vol/vol) 20 mM $MnSO_4$, 1 part 10% sodium dodecyl sulfate, 1 part toluene, and 5 parts β-mercaptoethanol. In each case, 10 μl of this lysis mixture was added to 0.5 ml of the cell suspensions. The results, presented in Table 3, clearly show that NifA-induced expression of *lacZ* occurs only with the transcriptional fusions of pMK182P1 and pSD4, which both carry the *nifJ* promoter on the inserted fragment, in the presence of pCK3. However, with pSD1, pSD2 and pSD3, all lacking the *nifJ* promoter, as well as with pSD4Ω, in which the Ω fragment was inserted downstream of the *nifJ* promoter, no *lacZ* expression was

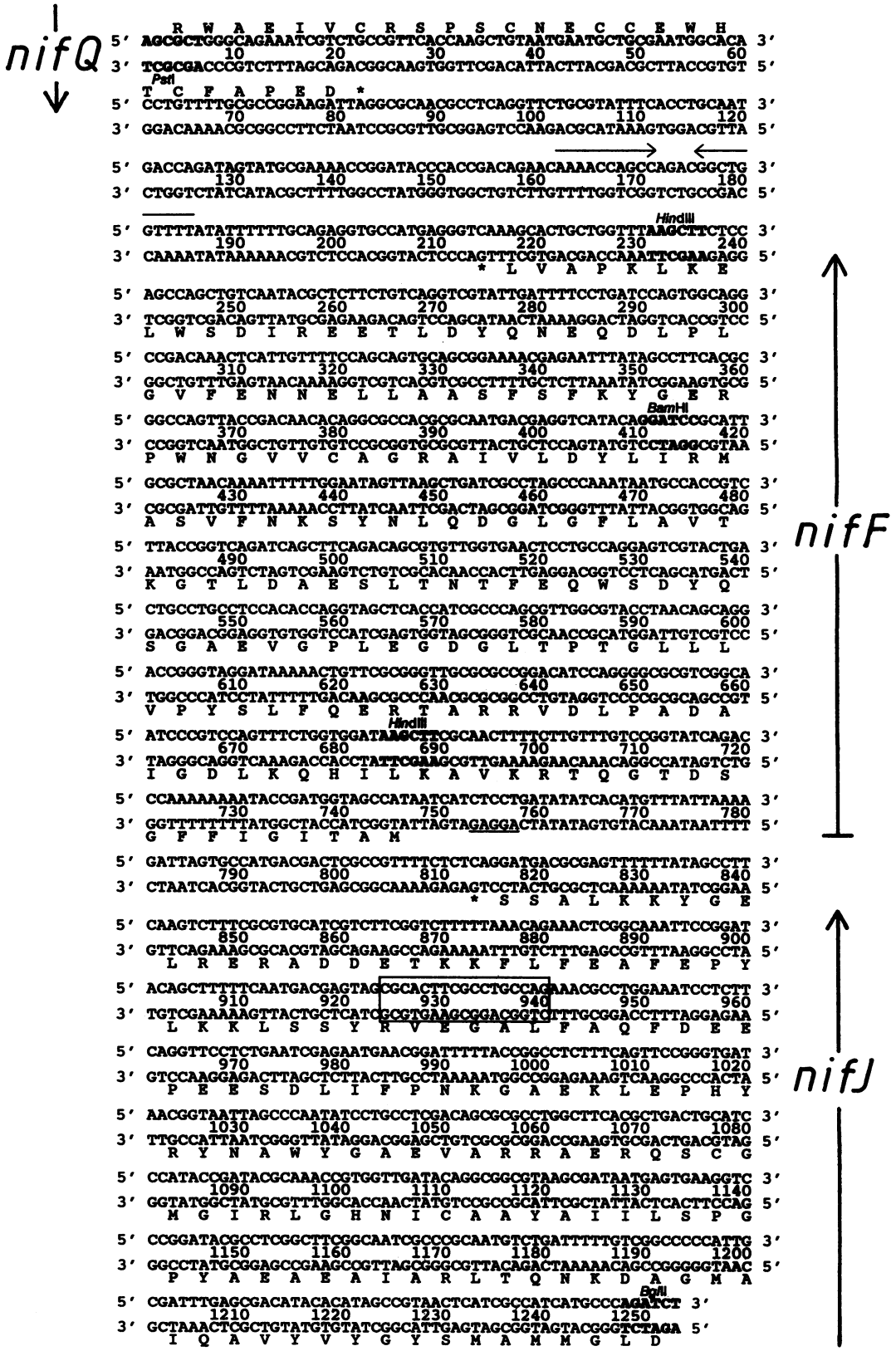

FIG. 2. Sequence of the *E. agglomerans nifF* gene and of the 3′ portions of the genes *nifQ* and *nifJ*. Both DNA strands and the amino acid sequences deduced from the nucleotide sequence are listed. The vertical arrows on the sides indicate the orientations of the genes *nifQ*, *nifF*, and *nifJ*. Relevant restriction sites are in boldface type. A promoterlike motif is boxed, a putative transcriptional terminator is indicated by oppositely oriented arrows, and the putative Shine-Dalgarno sequence of *nifF* is underlined.

TABLE 2. Comparison of the possible promoter motif upstream of *nifF* with the *nif* promoter consensus sequence

Sequence or motif	Nucleotide sequence	References
Consensus sequence	5′-YTGGCACRRNNNTTGCA	2, 16
Promoter motif in *nifJ*	5′-CTGGCAGGCGAAGTGCG[a]	This work

[a] Bases corresponding to conserved residues are underlined.

observed. Thus, the assumed promoter at position 924 to 940 in the sequence of Fig. 2 does not represent a functional promoter. Moreover, the possibility that the *nifJ* UAS substitutes in the activation of the assumed promoter was ruled out, because no promoter activity was measured when the Ω fragment was inserted between the *nifJ* UAS and the promoterlike motif, both present in *cis* in pSD4Ω.

We additionally used the S1 nuclease mapping technique to show that a *nifJF* cotranscript is present in *E. agglomerans* wild-type cells grown under nitrogen-fixing conditions in minimal medium free of combined nitrogen. A radioactively end-labeled 3.8-kb *Sal*I-*Bam*HI fragment covering most of the *nifJ* and *nifF* genes (corresponding to the insert of pMK38SB in Fig. 1) was hybridized in a liquid assay with total RNA prepared from either derepressed or repressed cells and subsequently treated with nuclease S1 (20). RNA from *E. agglomerans* was prepared by using Qiagen-pack 500 anion-exchange columns according to the manufacturer's protocol (Diagen Inc., Düsseldorf, Federal Republic of Germany). The protection assays were performed as reported previously (25). The autoradiogram of the reaction mixture run on a 1% agarose gel (Fig. 3) identified a hybridization signal of 3.8 kb when RNA from derepressed cells was used. The smear below the 3.8-kb band may be due to the hybridization of partially degraded *nifJF* mRNA to the DNA probe. No signal was observed when RNA from repressed cells was used. The result shows that RNA no smaller than 3.8 kb is transcribed from the *nifJF* genes, again indicating that both genes form a transcriptional unit.

Nucleotide sequence accession number. This nucleotide sequence has been submitted to GenBank and has been assigned accession number M38221.

TABLE 3. β-Galactosidase activities of transcriptional fusions in *Escherichia coli* CB454

Plasmid	Characteristics	β-Galactosidase activity (units)[a]	
		−pCK3	+pCK3
pCB182	Promoterless *lacZ*	<10	<10
pMK182P1	$nifJp_{E.\ agglomerans}$-*lacZ*	<10	3,862
pSD1	Transcriptional fusion[b]	<10	<10
pSD2	Transcriptional fusion[b]	<10	<10
pSD3	Transcriptional fusion[b]	<10	<10
pSD4	Transcriptional fusion[b]	<10	4,408
pSD4Ω	Transcriptional fusion, Ω fragment inserted[b]	<10	<10

[a] The data represent the average values of four independent experiments. Higher values than those previously published (12) were obtained with pMK182P1, probably because of the use of a modified lysis mixture enabling quantitative cell lysis.

[b] The construction of the transcriptional fusions is shown in Fig. 1.

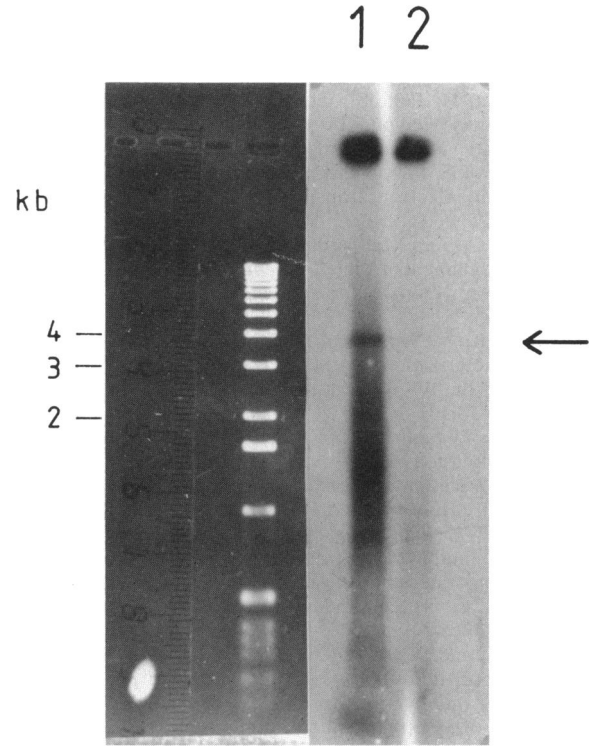

FIG. 3. Identification of the *nifJF* cotranscript by the nuclease S1 protection assay. (Left) A ruler was photographed together with the DNA length standard (kilobase ladder; Bethesda Research Laboratories) to enable correct assignment of the corresponding autoradiogram afterwards. (Right) Autoradiogram of the nuclease S1 reaction mixture run on a 1% agarose gel. The arrow indicates the position of the DNA-RNA hybrid. Lane 1, RNA from derepressed cells used; lane 2, RNA from repressed cells used.

Financial support from the German Academic Exchange Service to S. Dayananda is gratefully acknowledged.

We thank Nancy Schönbrunner for reading the manuscript.

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