Volume 283, number 1, 109–112 FEBS 09750 May 1991

ADONIS 001457939100459C

Construction and properties of a triprotein containing the high-affinity nickel transporter of *Alcaligenes eutrophus*

Lutz Wolfram, Thomas Eitinger and Bärbel Friedrich

Institut für Pflanzenphysiologie und Mikrobiologie der Freien Universität Berlin, Königin-Luise-Str. 12-16 a, W-1000 Berlin 33, Germany

Received 25 February 1991; revised version received 28 March 1991

The high-affinity nickel transporter of *Alcaligenes eutrophus* H16 is encoded by gene *hoxN*, which maps within the hydrogenase gene cluster of megaplasmid pHG1. A tripartite gene fusion was constructed, consisting of (i) the *Escherichia coli lacZ* gene for β-galactosidase, (ii) a segment encoding an endoproteolytically cleavable peptide, and (iii) the *A. eutrophus* gene *hoxN*. An *E. coli* strain harboring this construct (plasmid pCH307) efficiently produced the corresponding triprotein upon induction. A broad-host-range derivative of pCH307 was shown to complement an *A. eutrophus* HoxN⁻ mutant.

Nickel transporter; Gene fusion; Fusion protein; *Alcaligenes eutrophus*

1. INTRODUCTION

In *Alcaligenes eutrophus*, a Gram-negative hydrogen bacterium, nickel uptake is mediated by a non-specific high-capacity magnesium transport system and by a high-affinity low-capacity nickel transporter. The latter was shown to be encoded by the megaplasmid-borne locus *hoxN* [1]. Recently we reported the subcloning of the *hoxN* region and the nucleotide sequence analysis of gene *hoxN*. Evidence was presented that the *hoxN* gene product is an integral membrane protein with a molecular mass of 33.1 kDa [2].

To elucidate the mechanism of high-affinity nickel transport at the molecular level it is necessary to purify and to reconstitute the carrier. However, purification of transport proteins in general is rather difficult because of their conformational instability in the solubilized form and their tendency to form aggregates. Gene fusion techniques provide strategies for overcoming these problems. A major proline carrier, the *putP* gene product of *Escherichia coli*, for example, was successfully purified after fusing *putP* to *lacZ* via a linker sequence encoding a collagenolytically cleavable peptide [3,4]. Expression of this construct resulted in a fusion protein which was subsequently purified by affinity chromatography using anti-β-galactosidase IgG-sepharose. The carrier protein could be released from the hybrid by collagenolysis.

In this letter we describe the construction and properties of a triprotein consisting of the nickel transporter, a peptide linker containing recognition sites for collagenase and endoproteinase Xa, and β-galactosidase.

Correspondence address: T. Eitinger or B. Friedrich, Institut für Pflanzenphysiologie und Mikrobiologie der Freien Universität Berlin, Königin-Luise-Str. 12–16a, W-1000 Berlin 33, Germany. Fax: (49) (30) 8383118.

2. EXPERIMENTAL

The bacterial strains and plasmids used in this study are listed in Table 1. Recombinant DNA techniques as described by Sambrook et al. [7] were applied. Site-directed mutagenesis without phenotypic selection was carried out by the method of Kunkel [8]. Vector and hybrid plasmids were transferred from *E. coli* to *A. eutrophus* by conjugation as described previously [9]. Proteins were separated by sodium dodecyl sulfate polyacrylamid gel electrophoresis (SDS-PAGE) according to Laemmli [10].

3. RESULTS AND DISCUSSION

3.1. *Construction of the gene fusion*

The strategy for construction of the gene fusion between *lacZ'* and *hoxN*, linked by a DNA fragment encoding a proteolytically cleavable peptide, is illustrated in Fig. 1. Plasmid pCH231 [2] was subjected to site-directed mutagenesis: a recognition site for restriction endonuclease *Bam*HI was generated by an adenine-to-thymine exchange, 22 base pairs upstream of the *hoxN* initiation codon. The plasmid containing the modified sequence was designated pCH289. A 1.2-kb *Bam*HI/*Pst*I fragment of pCH289 was inserted between the *Bgl*II and *Pst*I sites of vector pAX4b+ yielding plasmid pCH307. pCH307 should encode a proteolytically cleavable fusion protein (the relevant segment is shown in Fig. 1). Thus, proteolysis by endoproteinase Xa should yield a nickel transport protein with 16 additional amino acid residues at its amino terminus. It is unlikely that the additional peptide will interfere with

Table 1

Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference or source
Alcaligenes eutrophus		
H16	HoxN^{+}[b]	ATCC17699
HF260	*hoxN*::Tn*5*-B21, HoxN^{-}[b]	[2]
Escherichia coli		
WM1704	Δ*lac*U169 Δ*lon*	W. Messer, Max-Planck-Inst. für molekulare Genetik, Berlin, Germany
pCH231	*hoxN*, f1*ori*, Ap^{r}	[2]
pCH289	*hoxN*, f1*ori*, Ap^{r}	This study
pAX4b+	*lacZ* ', Ap^{r}	*medac* GmbH, Hamburg
pCH307	*hoxN* in pAX4b+	This study
pFDX500	p15A*ori*, Km^{r}, *lacI*^{q}	[5]
pJRD215	RSF1010*ori*, *mob*, Km^{r}, Sm^{r}	[6]
pGE190	pCH307 in pJRD215	This study
pGE191	a 2.2-kb *Hin*dIII fragment containing the intact *hoxN* gene inserted into pJRD215	[2] and this study

[a] Ap, ampicillin; Km, kanamycin; Sm, streptomycin; ', resistant
[b] The HoxN phenotype was formerly designated Nic^{+} or Nic^{-} [1,2]

Fig. 2. Expression of the *lacZ'-hoxN* fusion in *E. coli* WM1704. Strains were grown in NZCYM medium; where indicated, the *lac* promoter was induced by addition of IPTG. Cells were suspended in SDS-containing buffer, and proteins were separated by SDS-PAGE. Lane 1, WM1704(pFDX500; pAX4b+); 2, WM1704(pFDX500; pAX4b+) +IPTG; 3, WM1704(pFDX500; pCH307); 4, WM1704(pFDX500; pCH307) +IPTG; 5, protein standard. The triangles in lanes 2 and 4 indicate β-galactosidase and the fusion protein, respectively.

nickel binding of protein HoxN, since the amino-terminal end of the multi membrane-spanning nickel transporter was shown to be located in the cytoplasm [2].

3.2. *Expression of the gene fusion in E. coli*

Plasmid pCH307 was transformed into *E. coli* strain WM1704 (Δ*lon* Δ*lac*) harboring plasmid pFDX500 (*lacI*^{q}). The deletion in *lon* strongly reduces degradation of artificial proteins. Transformants were grown in 10 ml NZCYM medium [11] to late exponential phase at 37°C. For induction isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1.5 mM and incubation was continued for 2 h. Cells were harvested by centrifugation, resuspended in buffer containing 1% SDS, and proteins were separated by SDS-PAGE. The results of the expression studies are shown in Fig. 2. Upon induction, plasmid pCH307 directed the synthesis of a fusion protein with a molecular mass of approximately 150 kDa (Fig. 2, lane 4). For proteolysis of the fusion protein cells were grown in 100 ml NZCYM medium and disrupted by sonication. The crude extract was treated with collagenase or endoproteinase Xa as recommended by the manufacturers. Preliminary results indicated that the fusion protein was cleavable (data not shown).

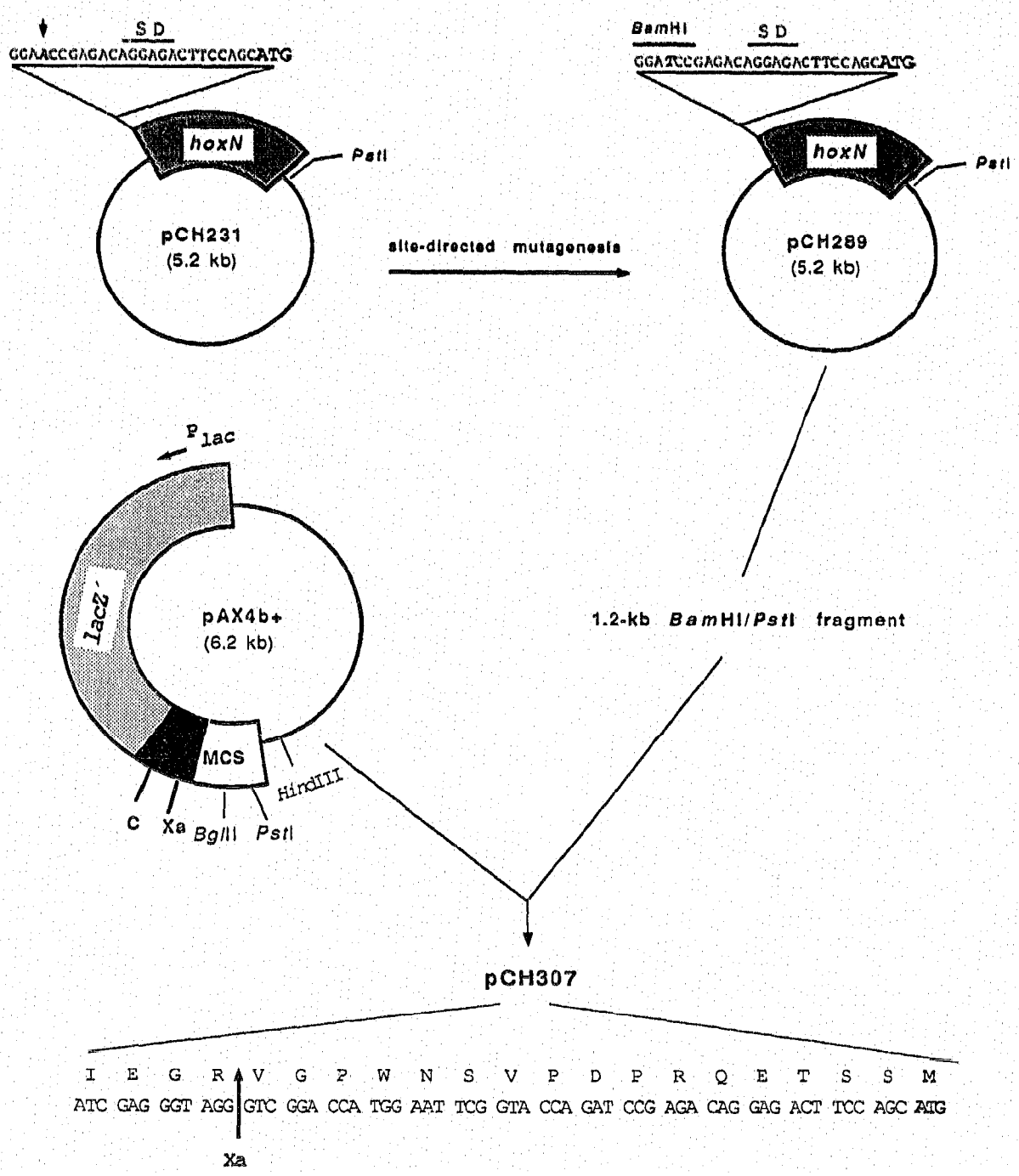
3.3. *The gene fusion complements nickel deficiency in an A. eutrophus HoxN^{-} mutant*

Previously we reported the construction of a HoxN^{-} insertion mutant of *A. eutrophus* (strain HF260). In this mutant both growth on hydrogen as the energy source and wild-type levels of hydrogenase activity (*A. eutrophus* hydrogenases are nickel-containing enzymes) were dependent on high concentrations of nickel in the medium [2]. We used mutant HF260 to test the *lacZ'-hoxN* fusion for complementation of nickel deficiency. Plasmid pCH307 was inserted into the broad-host-range vector pJRD215 taking advantage of a unique *Hin*dIII site in pCH307 (Fig. 1). The resulting co-integrate, designated pGE190, was transferred into mutant HF260 by conjugation. Growth of the resulting transconjugants on hydrogen under nickel limitation and nickel excess was monitored on agar plates incubated under an atmosphere of hydrogen, oxygen, and carbon dioxide at a ratio of 8:1:1 (*vol/vol/vol*). As shown in Fig. 3 pGE190 restored normal growth under nickel limitation, while the plasmid-free control grew only in the presence of high concentration of nickel. It

Fig. 1. Construction of a *lacZ'-HoxN* fusion. See text for details. ATG, initiation codon of gene *hoxN*; SD, Shine-Dalgarno sequence; MCS, multiple cloning site; C, recognition site for collagenase; Xa, recognition site for endoproteinase Xa. The sequence of the proteolytically cleavable linker encoded by plasmid pCH307 is shown in the lower part of the figure; the amino acid sequence is given in single letter code.

cannot be excluded, however, that the native nickel transporter, rather than the fusion protein, restored the HoxN* phenotype since the *lacZ'-hoxN* fusion contains the Shine-Dalgarno sequence of gene *hoxN*. This ribosomal binding site could allow internal initiation of translation.

The high-level production of a proteolytically cleavable fusion protein is the first step in the purification of the *A. eutrophus* nickel ion carrier. Reconstitution of the carrier in liposomes must await large-scale purification of the fusion protein and optimization of the conditions for proteolysis by endoproteinase Xa.



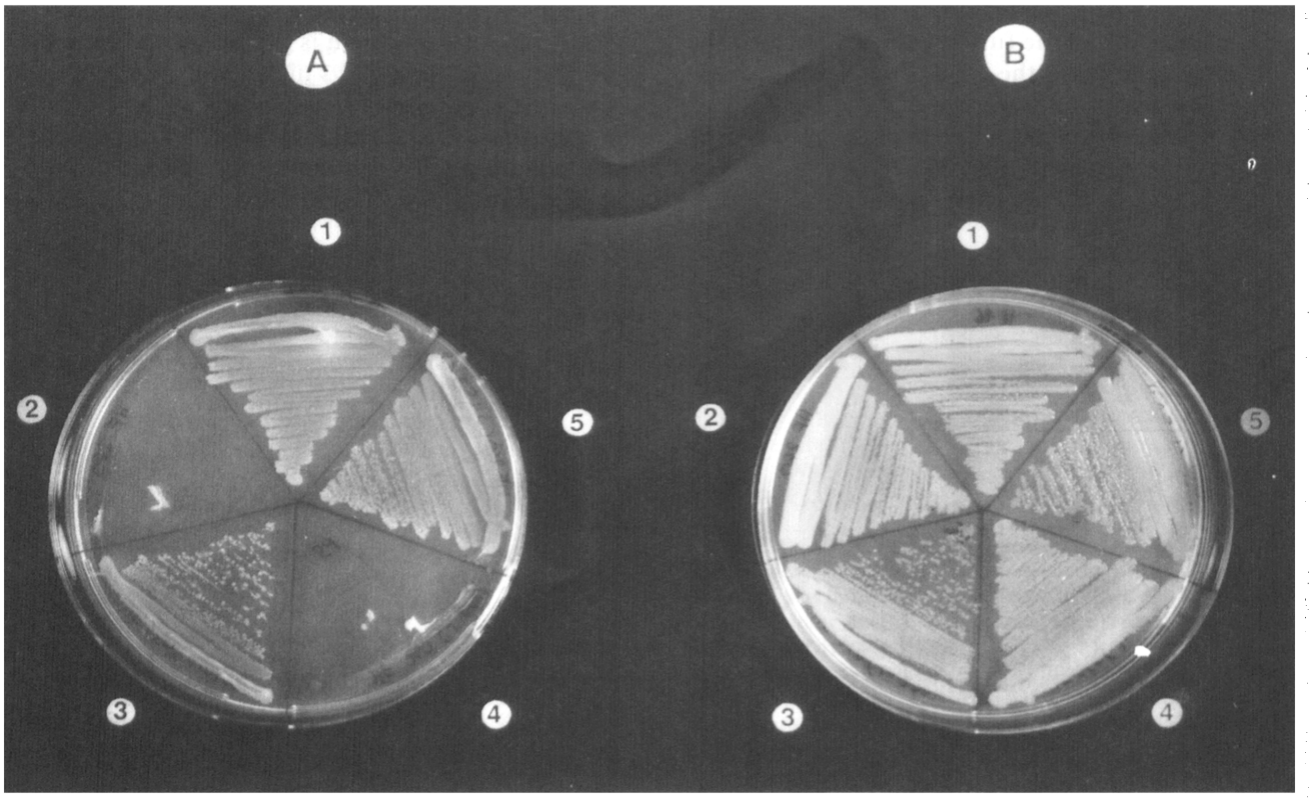


Fig. 3. Complementation of nickel deficiency in an *A. eutrophus* HoxN⁻ mutant by the *lacZ'-hoxN* fusion. Growth on agar plates incubated under an atmosphere composed of H_2, O_2, and CO_2 is shown. **A**, plate without added $NiCl_2$; **B**, plate supplemented with 1 μM $NiCl_2$. **1**, H16 (wild type); **2**, HF260 (HoxN⁻), **3**, HF260(pGE191); **4**, HF260(pJRD215 :: pAX4b+); **5**, HF260(pGE190).

Acknowledgements: We thank Dr. Edward Schwartz for helpful discussions and critical reading of the manuscript. This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

REFERENCES

[1] Eberz, G., Eitinger, T. and Friedrich, B. (1989) J. Bacteriol. 171, 1340–1345.
[2] Eitinger, T. and Friedrich, B. (1991) J. Biol. Chem. 266, 3222–3227.
[3] Hanada, K., Yamato, I. and Anraku, Y. (1987) J. Biol. Chem. 262, 14100–14104.
[4] Hanada, K., Yamato, I. and Anraku, Y. (1988) J. Biol. Chem. 263, 7181–7185.
[5] Schnetz, K., Sutrina, S.L., Saier, M.H. and Rak, B. (1990) J. Biol. Chem. 265, 13464–13471.
[6] Davison, J., Heusterspreute, M., Chevalier, N., Vinh, H.-T. and Brunel, F. (1987) Gene 51, 275–280.
[7] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual. 2nd ed. Cold Spring Harbor, New York.
[8] Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. USA 82, 488–492.
[9] Eberz, G., Hogrefe, C., Kortlüke, C., Kamienski, A. and Friedrich, B. (1986) J. Bacteriol. 168, 636–641.
[10] Laemmli, U.K. (1970). Nature 227, 680–685.
[11] Scholtissek, S. and Grosse, F. (1988) Gene 62, 55–64.