JOURNAL OF BACTERIOLOGY, May 1985, p. 852–854
0021-9193/85/050852-03$02.00/0

Vol. 162, No. 2

Denitrification by *Alcaligenes eutrophus* Is Plasmid Dependent

DETLEF RÖMERMANN AND BÄRBEL FRIEDRICH*

Institut für Mikrobiologie der Universität Göttingen, D-3400 Göttingen, Federal Republic of Germany

Received 28 November 1984/Accepted 31 January 1985

Curing of the hydrogenase-specifying megaplasmid pHG indigenous to strains of the facultative lithoautotrophic bacterium *Alcaligenes eutrophus* was correlated with a loss of denitrifying ability (Nitd). The retransfer of plasmid pHG1 reconstituted the Nitd phenotype. Plasmid-free mutants were still capable of converting some nitrate to nitrite, but they did not metabolize nitrite under anaerobic conditions.

The self-transmissible 450-kilobase-pair plasmid pHG1 of *Alcaligenes eutrophus* H16 encodes regulatory and structural genes for hydrogen-oxidizing ability (Hox) of the host (1, 6, 8) and genes associated with autotrophic CO_2 fixation (2, 3). We previously described pleiotropic Hox^- mutants which lack the cytoplasmic and particulate hydrogenases and are impaired in assimilatory as well as in dissimilatory nitrate metabolism (Hox^- Nit^-). We presented evidence that the Hox^- Nit^- phenotype results from a single mutation in the chromosome (8).

In the current study, we ask whether mutants bearing plasmid-borne Hox mutations are affected in nitrate metabolism. *A. eutrophus* H16 grows anaerobically with fructose or gluconate as the carbon source and nitrate or nitrite, respectively, as the electron acceptor. The cells first utilize nitrate or nitrite as the electron acceptor (Table 1). The culture conditions have been described previously (8). Hydrogenase-deficient mutants carrying plasmid-located point mutations, such as strain HF18, transposon Tn*5* insertions, such as strain HF148, or a 70-kilobase-pair deletion in plasmid pHG1, such as strain HF47, were unimpaired in nitrate and nitrite respiration (Table 1). However, curing of the Hox-encoding plasmid was accompanied by the loss of denitrifying ability (Nitd). Identical growth behavior was observed with plasmid-free derivatives of other *A. eutrophus* strains, for example, N9A and H20 (Table 1). The plasmid-free mutants represented by *A. eutrophus* HF33 grew poorly for one doubling anaerobically with nitrate (Fig. 1A). Nitrite accumulated in the medium, although at a lower concentration than in the wild-type culture (Fig. 1B). Nitrite levels

TABLE 1. Denitrification by wild-type *Alcaligenes* strains and hydrogenase-deficient mutants

Strain	Reference or source	Relevant phenotype[a]	Plasmid[b]	Growth[c] on:	
				Nitrate	Nitrite
A. eutrophus					
H16	ATCC 17699	Hox Nitd	pHG1	+	+
HF33	8	Hox^- $Nitd^-$	pHG$^-$	(−)	−
HF18	7	Hox $Nitd^+$	pHG1●	+	+
HF148	5	Hox^- $Nitd^+$	pHG1::Tn*5*	+	+
HF47	5	Hox $Nitd^+$	ΔpHG1	+	+
N9A	DSM518	Hox Nitd	pHG3	+	+
N9AF06	8	Hox^- $Nitd^-$	pHG3$^-$	(−)	−
H20	ATCC 17700	Hox Nitd	pHG7	+	+
H20F01	3	Hox^- $Nitd^-$	pHG7$^-$	(−)	−
A. hydrogenophilus					
M50	4, 10	Hox $Nitd^-$	pHG21-a pHG21-b	−	−
M55	4	Hox^- $Nitd^-$	pHG21-a$^-$ pHG21-b	−	−

[a] Hox, Ability to use H_2 as energy source; Nitd, ability to denitrify.
[b] −, Loss of the plasmid; ●, point mutation; ::, insertion; Δ, deletion.
[c] +, Growth; −, no growth; (−), poor growth. The medium contained gluconate and ammonium, pH 7.5, with 0.2% (wt/vol) potassium nitrate or 0.1% (wt/vol) sodium nitrite in the absence of oxygen.

nitrate, and nitrite accumulates in the medium; then nitrite is taken up and reduced to dinitrogen (11).

Most of the wild-type strains examined here, with the exception of *Alcaligenes hydrogenophilus*, grew well anaerobically in gluconate-ammonium minimal medium with were determined by the method of Lowe and Evans (9) as previously described (8). When incubated with nitrite as the electron acceptor, the pHG1-free strain did not grow at all (Fig. 2A), and no nitrite disappeared from the medium (Fig. 2B). With respect to anaerobic metabolism of nitrate or nitrite, the plasmid-cured strains behaved exactly like the pleiotropic Hox^- Nit^- mutants. Strain HF09, a representative of the latter class, is shown in Fig. 1 and 2. Neverthe-

* Corresponding author.

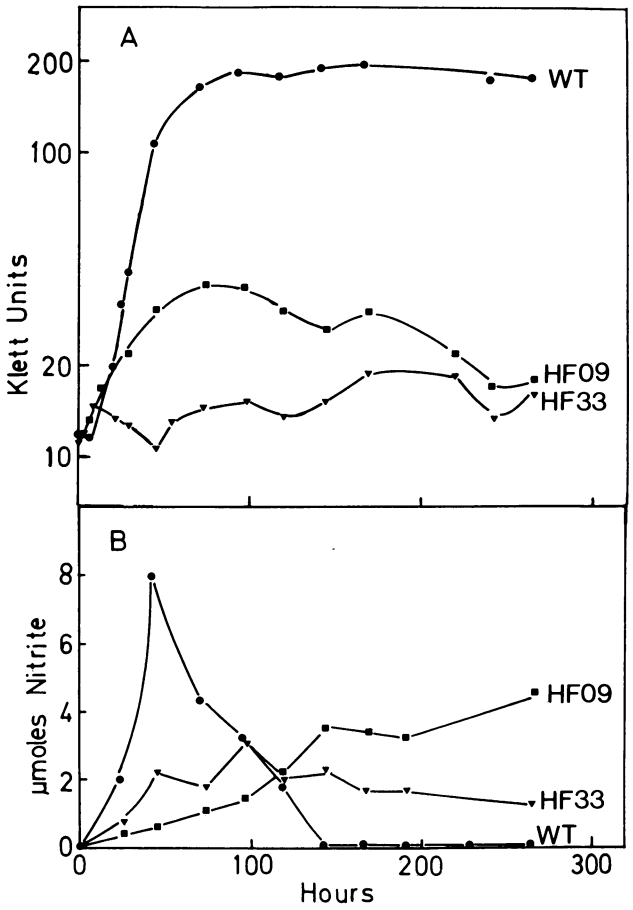

FIG. 1. Anaerobic growth on nitrate (A) and accumulation of nitrite in the medium (B) by *A. eutrophus* wild-type (WT), the Hox Nitd⁻ mutant HF33, and the Hox⁻ Nit⁻ mutant HF09. The optical density of the cells is given in Klett units. The nitrite concentration refers to 1 ml of culture volume.

less, in contrast to the Hox⁻ Nit⁻ mutants, plasmid-free isolates were still able to use nitrate or nitrite aerobically as a nitrogen source. Thus, their phenotype is clearly different from that of chromosomally defective Hox⁻ Nit⁻ mutants. The observation that various mutations in plasmid pHG1 that led to the loss of catalytic and immunological activity of the cytoplasmic and particulate hydrogenases (5, 7, 8) did not affect denitrification (Table 1) indicates that the plasmid-borne Nitd marker is not linked genetically to Hox.

The conjugal transfer of plasmid pHG1 to plasmid-cured recipients of various *Alcaligenes* wild-type species (4, 8) by agar mating (6) resulted in transconjugants which were not only restored in Hox but also in denitrifying ability (Nitd). Moreover, *A. hydrogenophilus*, a naturally Nitd⁻ wild-type strain (Table 1) gained the Nitd function by exchanging plasmid pHG1 from *A. eutrophus* H16 for its indigenous Hox plasmid pHG21-a (Table 2).

These preliminary results permit no conclusion about the nature of a plasmid-encoded Nitd gene(s). The absence of this gene(s) led to a complete loss of nitrite-respiring activity and a marked decrease in the amount of nitrite produced from nitrate. At present we do not know whether plasmid pHG1 encodes structural genes or regulatory components or both for nitrate respiration or even determinants of denitrification-specific electron transport. Plasmid-free strains grew heterotrophically with oxygen, as does the wild type, indicating that they suffered no lesion in oxygen-dependent respiration. Interestingly, there is evidence that the expression of a cytoplasmic flavohemoprotein in *A. eutrophus* is encoded by plasmid pHG1 (K. Schmidt and V. Weihs, personal communication). The physical properties of this *b*-type cytochrome are well characterized, but its physiological function remains unresolved (12). It is tempting to speculate that this flavohemoprotein is involved in nitrate respiration. The supposition gains support from the observation that the concentration of the flavohemoprotein increases 20-fold in cells grown under limited oxygen supply (12). The isolation and characterization of specifically plasmid-defective Nitd⁻ mutants may help to substantiate this hypothesis.

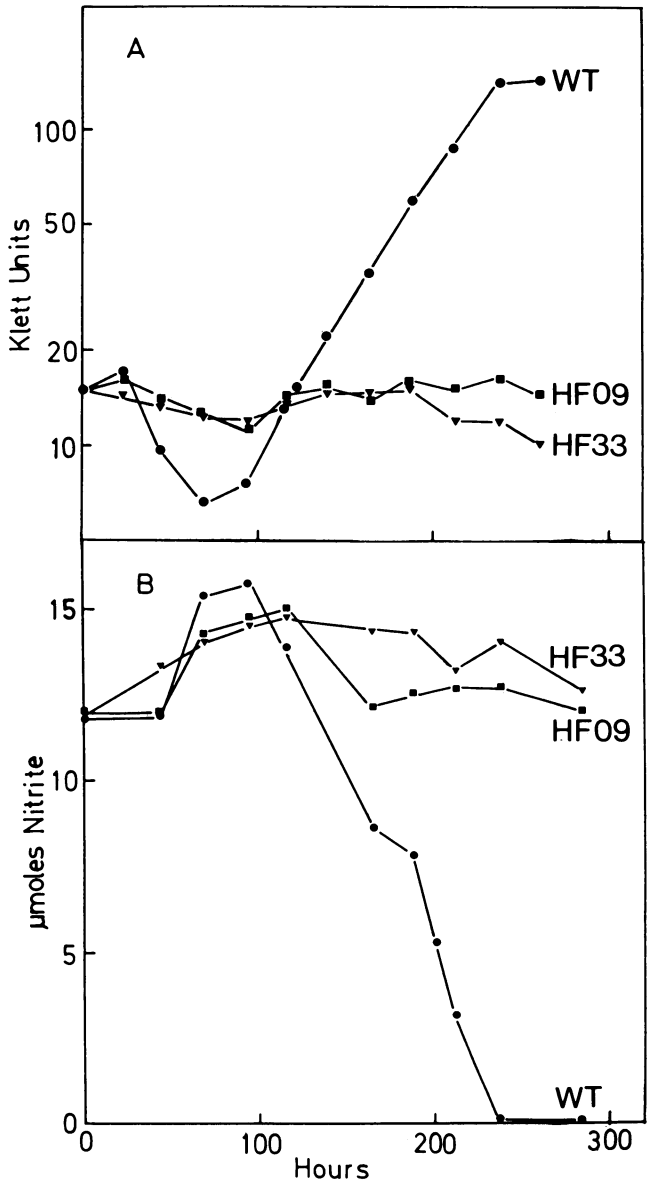

FIG. 2. Anaerobic growth on nitrite (A) and nitrite dissappearance from the culture medium (B) for *A. eutrophus* wild-type (WT), the Hox⁻ Nitd⁻ mutant HF33, and the Hox⁻ Nit⁻ mutant HF09. The optical density of the cells is given in Klett units. The concentration of nitrite refers to 1 ml of culture medium.

TABLE 2. Denitrification by Hox$^+$ transconjugants of *Alcaligenes* strains

Recipient strain[a]	Plasmid content	Growth[b] on:	
		Nitrate	Nitrite
HF33	pHG1	+	+
N9AF06	pHG1	+	+
H20F01	pHG1	+	+
M55	pHG21-b pHG1	+	+

[a] Transconjugants arose from agar mating as previously described (6), with *A. eutrophus* H16 as the donor. Recipient strain characteristics are given in Table 1.

[b] See Table 1; footnote *c*.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

LITERATURE CITED

1. **Andersen, K., R. C. Tait, and W. R. King.** 1981. Plasmids required for utilization of molecular hydrogen by *Alcaligenes eutrophus*. Arch. Microbiol. **129**:384–390.
2. **Andersen, K., and M. Wilke-Douglas.** 1984. Construction and use of a gene bank of *Alcaligenes eutrophus* in the analysis of ribulose bisphosphate carboxylase genes. J. Bacteriol. **159**: 973–978.
3. **Bowien, B., B. Friedrich, and C. G. Friedrich.** 1984. Involvement of megaplasmids in heterotrophic derepression of the carbon-dioxide assimilating enzyme system in *Alcaligenes* spp. Arch. Microbiol. **139**:305–310.
4. **Friedrich, B., C. G. Friedrich, M. Meyer, and H. G. Schlegel.** 1984. Expression of hydrogenase in *Alcaligenes* spp. is altered by interspecific plasmid exchange. J. Bacteriol. **158**:331–333.
5. **Friedrich, B., and C. Hogrefe.** 1984. Genetics of lithoautotrophic metabolism in *Alcaligenes eutrophus*, p. 244–247. *In* R. L. Crawford and R. S. Hanson (ed.), Microbial growth on C_1 compounds. American Society for Microbiology, Washington, D.C.
6. **Friedrich, B., C. Hogrefe, and H. G. Schlegel.** 1981. Naturally occurring genetic transfer of hydrogen-oxidizing ability between strains of *Alcaligenes eutrophus*. J. Bacteriol. **147**:198–205.
7. **Friedrich, C. G., B. Bowien, and B. Friedrich.** 1979. Formate and oxalate metabolism in *Alcaligenes eutrophus*. J. Gen. Microbiol. **115**:185–192.
8. **Hogrefe, C., D. Römermann, and B. Friedrich.** 1984. *Alcaligenes eutrophus* hydrogenase genes (Hox). J. Bacteriol. **158**:43–48.
9. **Lowe, R. H., and H. J. Evans.** 1964. Preparation and some properties of a soluble nitrate reductase from *Rhizobium japonicum*. Biochim. Biophys. Acta **85**:377–389.
10. **Ohi, K., N. Takada, S. Komemushi, M. Okazaki, and Y. Miura.** 1979. A new species of hydrogen-utilizing bacterium. J. Gen. Appl. Microbiol. **25**:53–58.
11. **Pfitzner, J., and H. G. Schlegel.** 1973. Denitrifikation bei *Hydrogenomonas eutropha* Stamm H16. Arch. Mikrobiol. **90**:199–211.
12. **Probst, I., G. Wolf, and H. G. Schlegel.** 1979. An oxygen-binding flavohemoprotein from *Alcaligenes eutrophus*. Biochim. Biophys. Acta **576**:471–478.