Vol. 137, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1986 Pages 114-119

ISOLATION OF HYDROGEN-OXIDATION GENE FROM *ALCALIGENES HYDROGENOPHILUS* AND ITS EXPRESSION IN *PSEUDOMONAS OXALATICUS*

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Received March 27, 1986

A gene bank of a megaplasmid encoding the hydrogen-oxidizing enzyme system (Hox) in *Alcaligenes hydrogenophilus* was constructed using a broad host range cosmid vector pVK102, and established in *Escherichia coli*. Hybrid cosmids containing *hox* genes were identified by transferring the bank into *Pseudomonas oxalaticus* OX1 and screening colonies for the ability of H_2-dependent autotrophic growth. About 800 colonies were formed under autotrophic conditions. One of the Hox^+ transconjugants was isolated and its hydrogenases activities were measured. Although soluble hydrogenase was not detected, the Hox^+ transconjugant had four times the membrane-bound hydrogenase activity of *A. hydrogenophilus*.

Alcaligenes hydrogenophilus is a gram-negative facultative hydrogen bacterium isolated from soil in this laboratory(1); it has a megaplasmid (270 Md), pHG21-a, encoding a hydrogen-oxidizing enzyme system, Hox(2). Like *Alcaligenes eutrophus*(3) and *Alcaligenes ruhlandii*(4), cells of *A. hydrogenophilus* grown autotrophically with H_2, O_2, and CO_2 contain a soluble NAD^+-reducing hydrogenase as well as a membrane-bound hydrogenase linked to the respiratory chain and common to most H_2-oxidizing bacteria.

Unlike the H_2-independent synthesis of hydrogenases in *A. eutrophus*, those in *A. hydrogenophilus* are induced by H_2(2). Much less is known about the genetic regulation of Hox activity. The plasmid is too large to analyze, so we used recombinant DNA techniques to isolate gene segments specific for the Hox phenotype.

In a similar attempt to isolate *hox* genes from *A. eutrophus*, a gene bank of the total DNA was constructed using the broad host range cosmid vector pVK102, and the gene bank was screened for *hox* genes by analysis for complementation of Hox^- point mutants, but *hox* specific DNA was not isolated(5).

0006-291X/86 $1.50

We used a crude plasmid preparation as the DNA source for a gene bank to enrich for hox genes. Pseudomonas oxalaticus OX1, which does not have an H_2-oxidizing enzyme system, was used as a cloning host to screen hybrid cosmids containing all the determinants necessary for the expression of Hox activity. Here we report that the hybrid cosmid conferring Hox activity on P. oxalaticus OX1 has been isolated. The cosmid contained at least determinant necessary for the expression of membrane -bound hydrogenase.

MATERIALS AND METHODS

Bacterial strains and medium: Strains used are listed in Table 1. A. hydrogenophilus, P. oxalaticus OX1(pAH3), and P. oxalaticus OX1(pYM11) were grown at 30 C on mineral-salt medium(1) under an atmosphere of 80% H_2, 10% O_2, and 10% CO_2 for autotrophic growth. Plasmid pAH3 and hybrid cosmid pYM11 are described in RESULTS. Escherichia coli was grown at 37 C on LB medium.

Construction of plasmid pAH3 gene bank: Plasmid pAH3 was extracted from P. oxalaticus OX1(pAH3) by the methods of Yano et al.(9). The crude plasmid was treated with phenol-chloroform and partially digested with SalI to enrich for fragments in the 20 to 25 Kb range. The digested DNA was mixed with the SalI-digested cosmid vector pVK102 at a weight ratio of 10:1 and ligated with T_4 DNA ligase. The resulting hybrid cosmid mixture was treated with RNase for 1 hr and phenol-chloroform for 30 min. The hybrid cosmids were then packaged in vitro with lambda packaging extract purchased from Promega Biotec. The phage mixture was used to transduce E. coli LE392. The transduced cells were incubated for 1 hr at 37 C and plated on LB medium containing kanamycin(30 μg/ml). A pool of about 3600 clones was stored in glycerol at -70 C.

Triparental conjugation: E. coli HB101(pRK2013) was used to mobilize the recombinant pVK102. P. oxalaticus OX1 was grown at 30 C, E. coli HB101(pRK2013) at 37 C, and E. coli LE392(recombinant pVK102) at 37 C, all in LB medium. Cells at the logarithmic phase were mixed at a ratio of 1:1:1 and layed onto a 0.45 μm membrane filter. The filter was incubated on LB medium at

Table 1. Bacterial Strains

Strain	Relevant characteristics	Ref.
E. coli		
HB101	recA$^-$ hsdR hsdM Str^r pro leu thi	6
HB101(pRK2013)	Contains pRK2013	7
LE392	hsdR met trp	8
LE392(pYM11)	Contains pYM11	This paper
A. hydrogenophilus	Hox$^+$ contains pHG21-a, pHG21-b	1
P. oxalaticus		
OX1	Hox$^-$ wild-type	
OX1(pAH3)	Hox$^+$ contains pAH3	This paper
OX1(pYM11)	Hox$^+$ contains pYM11	This paper

Abbreviations: Str, streptomycin; Hox, H_2 uptake; ^{r}, resistant.

30 C overnight. Cells were removed from the filter and washed with phosphate buffer. Hox^+ transconjugants were identified by H_2-dependent autotrophic growth on mineral-salt medium under a gas mixture of 80% H_2, 10% O_2, and 10% CO_2 at 30 C.

Enzyme assays: The activity of the membrane-bound hydrogenase was assayed photometrically by measuring the H_2-dependent reduction of methylene blue at 570 nm in serum-stoppered cuvettes at 30 C. The activity of the soluble hydrogenase was assayed by measuring the H_2-dependent reduction of NAD^+ at 340 nm in serum-stoppered cuvettes at 30 C. One unit of hydrogenase activity was defined as the amount reducing 1 μmole of its electron acceptor per hour. Cells were incubated at 30 C under a gas mixture of 1% H_2, 5% O_2, and 94% N_2 to measure the activity of O_2-dependent H_2 uptake. The consumption of H_2 was measured with a gas chromatograph. One unit of the activity was defined as the amount consuming 1 μmole of H_2 per hour.

Protein measurement: Protein was measured by the method of Lowry(10).

RESULTS

Plasmid pHG21-a in A. hydrogenophilus could be transferred by conjugation into CO_2-fixing P. oxalaticus OX1. Although there was a deletion of about 40 Md, the transferred plasmid pAH3 could confer Hox activity on P. oxalaticus OX1. The plasmid pAH3 was extracted from the transconjugant P. oxalaticus OX1(pAH3). A gene bank of plasmid pAH3 was constructed in the cosmid vector pVK102 and established in E. coli, as described in MATERIALS AND METHODS. Kanamycin-resistant colonies were obtained at a frequency of 2.6×10^3 per microgram of DNA. P. oxalaticus OX1 was conjugated with the bank en masse, and Hox^+ transconjugants were identified by H_2-dependent autotrophic growth on mineral-salt medium at 30 C. Approximately 800 colonies arose after 4 weeks of incubation.

One of the Hox^+ transconjugants was isolated and incubated in liquid mineral-salt medium under a gas mixture of H_2:O_2:CO_2(8:1:1). As shown in Fig. 1, the Hox^+ transconjugant, P. oxalaticus OX1(pYM11), had H_2-dependent autotrophic growth, but P. oxalaticus OX1 without a cosmid did not grow. The generation time was 5.4 hr. A. hydrogenophilus, P. oxalaticus OX1(pAH3), and P. oxalaticus OX1(pYM11) were grown autotrophically to measure activities of O_2-dependent H_2 uptake and hydrogenases. The results are presented in Table 2. Soluble hydrogenase activity could not be detected in the Hox^+ transconjugant. However, the strain had much higher activities of O_2-dependent H_2 uptake and

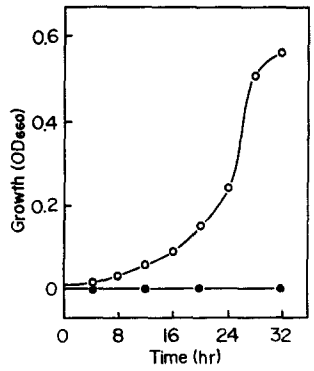

Fig. 1. Hydrogen-dependent autotrophic growth of Hox$^+$ transconjugant, P. oxalaticus OX1(pYM11). Cells of P. oxalaticus OX1(pYM11) (○) and P. oxalaticus OX1 wild-type(●) were incubated at 30 C in liquid mineral-salt medium under a gas mixture of 80% H_2, 10% O_2, 10% CO_2.

membrane-bound hydrogenase than those in A. hydrogenophilus and P. oxalaticus OX1(pAH3).

The hybrid cosmid pYM11 that conferred the ability of H_2-dependent autotrophic growth on P. oxalaticus OX1 was extracted from the Hox$^+$ transconjugant and isolated by ethidium bromide-cesium chloride gradient ultracentrifugation. Purified pYM11 was digested with several restriction endonucleases, and resulting DNA fragments were separated by agarose gel electrophoresis. The DNA fragment cloned in the SalI site of pVK102 was about 29 Kb. The restriction map is shown in Fig. 2.

Table 2. Activities of H_2 uptake and hydrogenases

Strain	Activities		
	O_2-dependent H_2 uptake (U/mg dry wt.)	membrane-bound hydrogenase (U/mg protein)	soluble hydrogenase (U/mg protein)
A. hydrogenophilus	6.47	28.3	1.03
P. oxalaticus OX1(pAH3)	7.72	18.2	1.45
P. oxalaticus OX1(pYM11)	19.6	105	N.D.

Cells were grown at 30 C in mineral-salt medium under a gas mixture of 80% H_2, 10% O_2, and 10% CO_2. N.D., not detected.

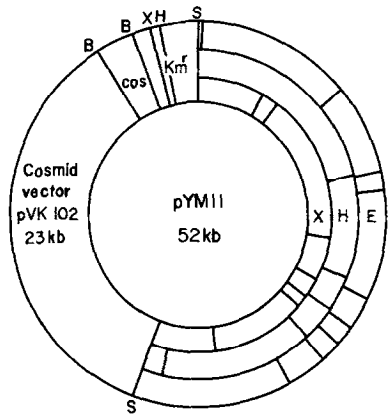

Fig.2. Restriction map of hox gene cosmid pYM11. The abbreviations for restriction endonucleases are: B, BglII; E, EcoRI; H, HindIII; S, SalI; X, XhoI.

DISCUSSION

We have described the isolation of A. hydrogenophilus gene segments that conferred the ability of H_2-dependent autotrophic growth on P. oxalaticus OX1. To demonstrate that the hybrid cosmid pYM11 stably maintained hox genes, we tried to reintroduce isolated pYM11 into P. oxalaticus OX1. After the transduction of E. coli with pYM11, P. oxalaticus OX1 was conjugated with E. coli(pYM11) by triparental conjugation. Hox^+ transconjugants were obtained at a frequency of 10^{-5}. Since the cosmid vector pVK102 has a broad host range(11), it is suggested that pYM11 could confer the ability of H_2-dependent autotrophic growth on various CO_2-fixing bacteria.

Isolated Hox^+ transconjugants, P. oxalaticus OX1(pYM11), had about 4 times more membrane-bound hydrogenase activity than A. hydrogenophilus. This increase appears to be due to gene amplification, because pVK102 is a derivative of plasmid pRK290 that is maintained at a copy number of 2 to 4 in Pseudomonas(12). Like most hydrogen bacteria lacking a NAD^+-reducing soluble hydrogenase, P. oxalaticus OX1(pYM11) could grow under autotrophic conditions without the soluble hydrogenase. We think that the function of soluble hydrogenase is the regeneration of NADH used to fix CO_2. The electron acceptor of the membrane-bound hydrogenase may have a higher electron potential than NAD^+, and the electron from H_2 can be transported by membrane-bound

hydrogenase to NAD^+. The generation times of A. hydrogenophilus, P. oxalaticus OX1(pAH3), and P. oxalaticus OX1(pYM11) were 2.8, 3.6, and 5.4 hr, respectively. It seems that the lack of the soluble hydrogenase influenced the autotrophic growth rate of P. oxalaticus OX1(pYM11).

ACKNOWLEDGEMENT

We thank E. W. Nester for the kind gift of pVK102, and D. R. Helinski for providing pRK2013.

REFERENCES

1. Ohi, K., Takada, N., Komemushi, S., Okazaki, M., and Miura, Y. (1979) J. Gen. Microbiol. 25, 53-58.
2. Friedrich, B., Friedrich, C. G., Meyer, M., and Schlegel, H. G. (1984) J. Bacteriol. 158, 331-333.
3. Wittenberger, C. L., and Repaske, R. (1961) Biochim. Biophys. Acta 47, 542-552.
4. Bone, D. H., Bernstein, S., and Vishniac, W. (1963) Biochim. Biophys. Acta 67, 581-588.
5. Andersen, K., and Wilke-Douglas (1984) J. Bacteriol. 159, 973-978.
6. Boyde, H. B., and Roulland-Dussoix (1969) J. Mol. Biol. 41, 459-472.
7. Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. R. (1980) Proc, Natl. Acad. Sci. U.S.A. 77, 7347-7351.
8. Murray, N. E., Brammar, W. J., and Murray, K. (1977) Molec. Gen. Genet. 150, 53-61.
9. Yano, K., and Nishi, T. (1980) J. Bacteriol. 143, 552-560.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
11. Knauf, V. C., and Nester, E. W. (1982) Plasmid 8, 45-54.
12. Bagdasarian, M., and Timmis, K. N. (1981) Current Topics in Microbiology and Immunology, Springer Verlag, pp 47-67.