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Cloning and sequencing the genes encoding uptake-hydrogenase subunits of *Rhodocyclus gelatinosus*

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Summary. *Rhodocyclus gelatinosus* grew photosynthetically in the light and consumed H_2 at a rate of about 665 nmol/min per mg protein. The uptake-hydrogenase (H_2ase) was found to be membrane bound and insensitive to inhibition by CO. The structural genes of *R. gelatinosus* uptake-H_2ase were isolated from a 40 kb cosmid gene library of *R. gelatinosus* DNA by hybridization with the structural genes of uptake-H_2ase of *Bradyrhizobium japonicum* and *Rhodobacter capsulatus*. The *R. gelatinosus* genes were localized on two overlapping DNA restriction fragments subcloned into pUC18. Two open reading frames (ORF1 and ORF2) were observed. ORF1 contained 1080 nucleotides and encoded a 39.4 kDa protein. ORF2 had 1854 nucleotides and encoded a 68.5 kDa protein. Amino acid sequence analysis suggested that ORF1 and ORF2 corresponded to the small (HupS) and large (HupL) subunits, respectively, of *R. gelatinosus* uptake-H_2ase. ORF1 was approximately 80% homologous with the small, and ORF2 was maximally 68% homologous with the large subunit of typical membrane-bound uptake-H_2ases.

Key words: Uptake-hydrogenase structural genes – *Rhodocyclus gelatinosus* – *hup* genes – *hup*S, *hup*L sequences – Hydrogenase

Introduction

Rhodocyclus gelatinosus is a purple, nonsulphur photosynthetic bacterium and a member of the metabolically diverse Rhodospirillaceae family. *R. gelatinosus* carries out anoxygenic photosynthesis and, like certain other anaerobic photosynthetic microorganisms (cf. Vignais et al. 1985 for a review), the cells can also grow in the dark anaerobically (Uffen 1976) or aerobically. During growth with or without light, reversible hydrogen metabolism is an essential activity. H_2-uptake serves to provide the reducing power needed to fix CO_2 during photolithoautotrophic growth, or it may operate during diazotrophic development in cells to recapture reducing potential lost as H_2 during dinitrogen fixation. In addition to H_2 consumption, H_2-output reactions also exist in photosynthetic bacteria, such as the one induced in *R. gelatinosus* which operates to produce H_2 when cells grow with CO according to the equation $H_2O + CO \rightarrow H_2 + CO_2$ (Uffen 1976, 1981, 1983).

Knowledge about the mode of action and the regulation of uptake-hydrogenase (H_2ase) in photosynthetic bacteria is becoming available from studies with *Rhodobacter capsulatus* (Colbeau and Vignais 1983; Colbeau et al. 1983, 1986; Xu et al. 1989). Information is limited about H_2ases in other photosynthetic bacteria. However, like *R. capsulatus*, *R. gelatinosus* provides an excellent model system to examine the mode of action and regulation of uptake-H_2ase in cells growing anaerobically with H_2 and CO_2. Enzyme regulation is especially interesting in *R. gelatinosus* since growth with CO as sole carbon source (Uffen 1976, 1981, 1983) requires that H_2-uptake and H_2-output operate together to support growth. Unidirectional output-H_2ase functions during oxidation of CO to CO_2 (Champine and Uffen 1987), while uptake-H_2ase operates with ribulose-1,5-bisphosphate carboxylase during synthesis of cell material from the CO oxidation products, H_2 and CO_2 (Uffen 1983).

To learn about *R. gelatinosus* reversible hydrogenase(s), a 40 kb cosmid gene library was constructed and the presumptive structural hydrogen uptake (*hup*) genes were isolated by hybridization with *hup* structural uptake-H_2ase genes from *Bradyrhizobium japonicum* (Cantrell et al. 1983) and *R. capsulatus* (Leclerc et al. 1988). The DNA sequence of *R. gelatinosus* genes was determined and analysed. Physiological studies on the uptake-H_2ase in photoautotrophically grown cells were also performed. Results showed that the uptake-H_2ase reaction was membrane associated and was totally insensitive to inactivation by CO.

Preliminary oral reports of this work have been presented at the Congrès de la Société de Chimie Biologique in Grenoble, France, April 1989, at the FEMS Symposium on "Molecular biology of membrane-bound complexes in phototrophic bacteria" in Freiburg, FRG, August 1989 and at the Sixth International Symposium on Microbial Growth on C1-Compounds in Göttingen, FRG, August 1989.

Materials and methods

Bacterial strains, plasmids and growth conditions. Bacteria and plasmids used are described in Table 1. *R. gelatinosus* strain 1 was cultured in CO or PYE medium (Champine and Uffen 1987). Photoautotrophic cell growth was under H_2:CO_2 (95.5: v/v) in the mineral salts medium (RCV) of Weaver et al. (1975) containing 7 mM $(NH_4)_2SO_4$ as N source. *Escherichia coli* was grown aerobically in TYN

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Table 1. Bacterial strains and plasmids

Strains and plasmids	Relevant genotype or phenotype	References
Rhodocyclus gelatinosus		
Strain 1	Wild type	Uffen and Wolfe (1970)
Escherichia coli		
HB101	F$^-$, *rec*A$^-$, *hsd*S, *pro*A, Str^r	Boyer and Roulland-Dussoix (1969)
TB1	*Δ(lac pro)*, *str*A, *ara, thi, ϕ*80, *lac*Z *Δ*M15, *hsd*R	Gibco BRL (host for pUC plasmids)
BHB2688	N205 *rec*A$^-$ (*λimm434, cIts, b2 red3 Eam4 Sam7*)/*λ*	Hohn (1979)
BHB2690	N205 *rec*A$^-$ (*λimm434, cIts b2 red3 Dam15 Sam7*)/*λ*	Hohn (1979)
Plasmids		
pHC79	Ap^r, Tc^r, cosmid vector	Hohn and Collins (1980)
pUC18	pBR322 derivative	Yanisch-Perron et al. (1985)
pRA411A	Ap^r (pHC79 40 kb *Sal*I insert)	This work
pRA22	Ap^r (pUC18 4.6 kb *Xho*I insert)	This work
pRA31	Ap^r (pUC18 3.2 kb *Bam*HI insert)	This work

medium composed of 10 g/l yeast extract, 10 g/l peptone, 5 g/l NaCl, or in Luria-Bertani (LB) medium. Occasionally, LB medium also contained 0.4% maltose. Antibiotics were filter-sterilized and added separately to sterile growth media wherever indicated. Final antibiotic concentration was 100 μg/ml for streptomycin (Str) and ampicillin (Ap), and 10 μg/ml for tetracycline (Tc). Solid medium was prepared with 1.5% agar (BioMérieux, Charbonnières-les-Bains, France).

Liquid aerobic cultures were incubated on a New Brunswick gyratory shaker operating at 300 rpm (New Brunswick, NJ, USA). Anaerobic photoautotrophic cultures were grown in light (about 2500 lx) under H_2:CO_2 (95:5, v/v) in Fernbach flasks sealed with sleeve-type rubber serum bottle stoppers. A constant positive H_2:CO_2 gas pressure was maintained. Fernbach flasks were incubated on a GFL reciprocating shaker (type 3016; Gesellschaft für Labortechnic, Burgwedel, FRG). In one experiment cells were grown anaerobically in the dark in Blake-type culture bottles under a stream of CO (Champine and Uffen 1987). Growth of *R. gelatinosus* was at 30° C and of *E. coli* at 37° C.

Cell preparation. Enzyme activities were measured using anaerobically grown *R. gelatinosus* whole cells, sphaeroplasts, or membrane vesicles. Cells were harvested by centrifugation (6000 *g*, 10 min, 4° C), washed and resuspended under argon in 20 mM TRIS-HCl buffer, pH 8.0, with 0.3 mM dithiothreitol (DTT). Previously described conditions (Champine and Uffen 1987) were used to prepare sphaeroplasts by treatment with lysozyme and membrane vesicles by osmotic lysis.

Enzyme reactions. H_2 consumption was estimated spectrophotometrically with methylene blue (MB) as electron acceptor (Colbeau and Vignais 1981). H_2-output activity was measured using sodium dithionite reduced methylviologen (MV) as described earlier (Champine and Uffen 1987) only with 20 mM TRIS-HCl, pH 6.0 and 5 mM MV. Anaerobic oxidation of CO was estimated spectrophotometrically at 578 nm by MV reduction (Uffen 1976, 1983). One unit equals 1 nmol of gas consumed or produced per minute.

Isolation of R. gelatinosus *DNA.* After overnight aerobic growth (12–14 h), cells from 1 l of PYE-grown culture containing 1.8×10^{10} cells/ml were collected by centrifugation (5000 *g*, 5 min, 4° C), washed once in 40 ml and then resuspended in 25 ml of a buffered solution containing 10 mM TRIS-HCl, pH 8.0, 100 mM EDTA, 150 mM NaCl. Cells were disrupted with 8% (v/v) sodium dodecyl sulphate (SDS) for 10 min at 50° C before addition of proteinase K to obtain a final concentration of 100 μg/ml. After 1.5 h at 50° C, the proteinase K cell digest was diluted with a further 20 ml of the Tris-EDTA-NaCl buffer mixture described above, and the supernatant solution was obtained after centrifugation (20000 *g*, 20 min, 15° C). The disrupted cell mass was washed two additional times, each time with 20 ml of buffered mixture. Finally, supernatant solutions were mixed with 2 vol. absolute ethanol. After 2 h at −20° C, the precipitated material was collected by centrifugation (20000 *g*, 10 min, 4° C), washed with a 70:30 (v/v) mixture of ethanol: TE buffer (10 mM TRIS-HCl, pH 8.0, 1 mM EDTA), and dried in vacuo. The material was redissolved in TE buffer and treated with 100 μg/ml DNase-free RNase (Maniatis et al. 1982). After 30 min at 37° C, the solution was rinsed twice with 1:1 (v/v) phenol:chloroform to remove protein. NaCl was then added to a concentration of 0.1 M and DNA was precipitated with ethanol, washed and dried as above. The DNA yield was 7.8 mg. To remove additional protein the DNA was once again solubilized, this time in saline-citrate (SSC) buffer containing 0.15 M NaCl and 0.015 M trisodium citrate, pH 7.0 according to the method of Marmur (1961). After rinsing twice with 24:1 (v/v) chloroform:isoamyl alcohol each time using gentle agitation for 15 min, precipitating DNA, and drying, a stock DNA solution was prepared in TE buffer. The final DNA yield was 4.5 mg.

Preparation of plasmid DNA. Large scale preparation of native (pHC79) and recombinant (pRA411A) cosmid DNA and recombinant pUC18 (pRA22, pRA31) plasmid DNA was as outlined by Maniatis et al. (1982) using caesium chloride gradient centrifugation. The method of Ish-Horowicz and Burke (1981) was used to prepare small amounts of plasmid DNA for routine restriction enzyme and gel electrophoresis analysis.

Construction of cosmid library. R. gelatinosus genomic DNA was partially digested with *Sal*I to achieve an average fragment size of 40 kb. Restriction fragments were purified by

10%–40% preparative sucrose gradient centrifugation and DNA was precipitated in sucrose solution with 2 vol. ethanol at −20° C. Use of glass vessels and dialysis tubing was avoided. Ligation of 40 kb *R. gelatinosus* DNA fragments into the dephosphorylated *SalI* site of pHC79 (Hohn and Collins 1980) was accomplished using the procedure of Leclerc et al. (1988) with a ratio of fragment DNA to plasmid DNA of 4. Total DNA concentration in the ligation solution was 0.3 mg/ml. Encapsidation of the ligation mixture was accomplished with the cell mixture system of Hohn (1979) using *E. coli* BHB2688 and BHB2690. Encapsidation strains were grown separately in LB medium, and then induced for λ by treatment at 65° C for 2 to 3 min followed by 20 min at 45° C. After heat shock, the cells were returned to 37° C for 2.5 h. The cell mix was prepared and stored for use at −70° C. Recombinant DNA was encapsidated by thoroughly mixing 3 µl of pHC79 ligation solution with 40 µl of frozen *E. coli* encapsidation mixture. To improve λ transduction efficiency, recipient *E. coli* HB101 was grown and infected in LB (Str) medium supplemented with maltose (Maniatis et al. 1982). Infected HB101 was allowed to express Ap resistance during growth at 37° C for 1 h in LB (Str) medium before spreading onto LB agar plates containing both Ap and Str antibiotics. Recombinant (Str^r, Ap^r, Tc^s) clones were stored in 40% glycerol.

Localization of uptake-H_2ase structural genes. Both colony and filter hybridization methods were used as described by Leclerc et al. (1988). In this study, however, the cosmid gene library was grown directly on Hybond nylon membrane (Amersham, UK) placed onto LB (Str, Ap) medium. After overnight growth, colonies were solubilized with 0.5 M NaOH and filters with combined denatured DNA were prepared for hybridization analysis. In other experiments, DNA digested by restriction endonuclease was resolved first by electrophoresis on a 1% agarose gel, and then transferred for hybridization onto Hybond membrane by electroblotting using an IBI apparatus (Genofit, Geneva, Switzerland), or by transfer with the Vacugene blotting system (Pharmacia LKB Biotechnology, Sweden). Vacugene blotting was performed according to the manufacturer's method, except that the time to denature DNA was increased to 6 or 7 min.

DNA hybridizing probes were labelled with α-[^{32}P]dCTP (ca. 110 TBq/mmol, Amersham, UK) by nick-translation using a kit according to the manufacturer's instructions (Boehringer-Mannheim, FRG). High stringency Southern hybridization was performed with 50% (v/v) formamide at 42° C (Maniatis et al. 1982). The hybridization period varied for different experiments. Afterwards, membranes were washed twice in 2 × SSC, 0.1% SDS at room temperature and twice in 0.2 × SSC, 0.1% SDS at 65° C; they were wrapped without drying in Saran wrap, and exposed to Amersham Hyperfilm-MP type X-ray film at −70° C.

Construction of subclones. Restriction fragments were subcloned into pUC18 (Yanisch-Perron et al. 1985) after purification of single DNA fragments. DNA was electroeluted from agarose gels onto DEAE-cellulose paper (Whatman DE81) and isolated as described by Dretzen et al. (1981). Purified, fully digested DNA fragments were ligated into linearized, dephosphorylated plasmid DNA under the conditions of Maniatis et al. (1982) except that the final concentrations of ATP and dithioerythritol (DTE) were 0.5 mM and 5 mM, respectively. Ligation occurred at 16° C with a 1:1 ratio between fragment and plasmid DNA. The final DNA concentration was 0.01 mg/ml.

Recombinant plasmid DNA was used to transform *E. coli* TB1. TB1 was made competent before each experiment by treatment with calcium ions according to the method of Morrison (1979). To obtain a competency level of 1 to 2×10^6 transformants/µg pUC18, cells were used after 16 h incubation at 4° C in 50 mM $CaCl_2$ solution buffered with 10 mM TRIS-HCl, pH 8.0.

E. coli TB1 was transformed by the method of Leclerc et al. (1988). Cells were incubated for 1 h at 37° C in TYN (Str) medium to allow expression of Ap resistance before spreading onto TYN (Str, Ap) solid medium with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal; approx. 40 µg/ml of medium). TB1 with recombinant pUC18 plasmid DNA formed colourless colonies.

DNA sequence analysis. DNA sequencing on both strands was performed by the dideoxy sequencing method of Sanger et al. (1977). Specific oligonucleotide primer DNA (10 ng) was annealed to 2 to 3 µg template DNA. Sequencing reactions were performed using the reaction mixtures and conditions of United States Biochemical Corp. (Ohio, USA) with α-[^{35}S]dATP (ca. 37 TBq/mmol; Amersham, UK). ^{35}S-labelled samples were loaded onto 8.3 M urea polyacrylamide (5%) gels (0.4 mm thick) for electrophoresis in the TRIS-borate (TBE) buffer of Maniatis et al. (1982). Gel electrophoresis was at 1850 V using a Sequi-Gen nucleic acid sequencing cell (38 × 50 mm) (Bio-Rad Chemical Division, Richmond, Calif, USA). Afterwards, gels were rinsed in a 10% glacial acetic acid, 10% methanol solution and dried.

Autoradiography was performed at about 20° C (room temperature) for 2 days using Amersham Hyperfilm-β max type X-ray film.

Computer-assisted sequence analysis was performed on a Prime computer connected to B.I.S.A.N.C.E. (Base Informatique sur les Séquences d'Acides Nucléiques pour les Chercheurs Européens; Centre Inter Universitaire de Traitement Informatique, Paris, France) where sequence data banks and data processing programs are accessible.

Miscellaneous operations. Methods for determining DNA concentration and for routine gel electrophoresis were as described by Maniatis et al. (1982). Conditions for restriction enzyme reactions were those described by the manufacturer (Boehringer-Mannheim, FRG). Protein was estimated using the BCA protein assay reagent developed by Pierce Chemical Co. (Rockford, Ill, USA) with bovine serum albumin as protein standard.

Results

Hydrogenase reactions

R. gelatinosus exhibited rapid growth under an H_2:CO_2 atmosphere and reached a cell density of 3 to 5×10^9 cells/ml after 36 to 48 h of culture. During photoautotrophic growth ribulose-1,5-bisphosphate carboxylase (Uffen 1983) operated with uptake-H_2ase to synthesize cell material. In a typical experiment during late logarithmic growth, cells exhibited H_2-uptake activity of 665 units/mg whole cell protein. Subsequent treatment of cells suggested that uptake-

H_2ase was only associated with the cell membrane. As a result, enzyme activity scarcely changed after treatment with lysozyme to convert 85% to 90% of the cells to osmotically fragile sphaeroplasts (Champine and Uffen 1987). Likewise, after osmotic disruption of *R. gelatinosus* sphaeroplasts, the resulting membrane fraction contained H_2-uptake activity of 458 units/mg protein. Although loss of enzyme activity occurred during the experiment, uptake-H_2ase was only found in membrane fractions. No uptake-H_2ase was detected in the soluble protein fraction after osmotic lysis of sphaeroplasts. In separate experiments CO had no inhibitory influence on uptake-H_2ase in cell membrane preparations or in whole cells. In a typical experiment, when 100 µl of pure CO was added to a 2 ml reaction vessel containing whole cells with H_2 and MB, dye reduction continued unchanged at a rate of 408 units/mg of cell protein.

The reversibility of the uptake-H_2ase was tested using photoautotrophically grown *R. gelatinosus* whole cells and membrane material prepared from osmotically disrupted sphaeroplasts. In experiments with whole cells and with reduced MV as reductant no H_2 release could be detected although dithionite-reduced MV was reoxidized at an apparent rate of 60 units/mg protein. Failure to evolve H_2 might result from competing reactions inside cells. However, it was possible to observe the reverse action of uptake-H_2ase with cell membranes. In these cell-free reactions, the rate of H_2 evolution from reduced MV was about 14% of the rate of H_2-uptake. In contrast, CO-grown whole cells which oxidized CO at a rate of 614 units/mg protein at pH 6.0 with MB as oxidant, evolved H_2 at 480 units/mg protein from reduced MV. The reversibility of the H_2-output reaction was low and cells only consumed H_2 at 1.6 units/mg protein with MB as oxidant. Virtually identical results had been reported earlier using whole cells and membrane preparations under slightly different reaction conditions (Champine and Uffen 1987).

Preparation of R. gelatinosus *genomic DNA and construction of the gene bank*

The method of Marmur (1961) was used first to prepare genomic DNA. The results using this purification process, however, were unsatisfactory because the DNA was broken into pieces with an average size of 50 to 60 kb. Subsequently, a method was devised to obtain large (100 kb) DNA fragments using proteinase K (Maniatis et al. 1982). This DNA, however, still contained a substantial amount of protein as suggested by a low 260/280 nm absorbancy ratio of 1.4, and 40 kb DNA restriction fragments prepared from it could not be successfully ligated into pHC79. DNA deproteinization was continued using chloroform:isoamyl alcohol (Marmur 1961). This additional treatment increased the 260/280 nm absorption ratio of the sample to 1.7. [The 260/280 nm absorption ratio for pure DNA is 1.8 (Maniatis et al. 1982)].

Purified DNA was readily hydrolysed by *Sal*I and *Sau*3A restriction endonucleases. Digestion by *Bam*HI (see Fig. 2, lane 6) and *Pst*I was slow and incomplete, and there appeared to be no restriction sites at all for *Eco*RI or *Hin*dIII. Thus *Sal*I was used to prepare DNA for use with pHC79. After partially digesting genomic DNA with *Sal*I, 40 kb restriction fragments were isolated, ligated into pHC79, and then used successfully to form a cosmid gene bank in *E. coli* HB101. With the 627 recombinant *E. coli* (Str^r, Ap^r, Tc^s) colonies, the probability that the gene bank contained the entire *R. gelatinosus* chromosome was 99.9% (Maniatis et al. 1982).

Identification and cloning of DNA homologous to the hup *structural genes of* B. japonicum *and* R. capsulatus

The *R. gelatinosus* gene bank was grown on Hybond nylon membrane and colony hybridization was used to screen for clones harbouring presumptive *hup* structural genes. A ^{32}P-labelled 5.9 kb *Hin*dIII fragment of *B. japonicum* from cosmid pHU1 (Cantrell et al. 1983) containing the authentic *hup* structural genes (Sayavedra-Soto et al. 1988) was used as the probe. Four colonies of the gene bank hybridized strongly with the *B. japonicum* DNA fragment. The same clones also strongly hybridized with the 3.5 kb DNA fragment containing the *hup* structural genes of *R. capsulatus* (Leclerc et al. 1988).

Recombinant plasmid DNA was obtained from each clone using caesium chloride gradient centrifugation and samples were prepared for restriction analysis on agarose gels. DNA was digested with *Bam*HI, *Bgl*II, *Pst*I, *Sal*I, *Stu*I, or *Xho*I. There were no *Eco*RI or *Hin*dIII restriction sites in any of the 40 kb cloned DNA fragments of *R. gelatinosus* as expected from previous studies with genomic DNA. Fully digested DNA was transferred onto Hybond nylon membrane, and DNA bands were screened by Southern hybridization with the ^{32}P-labelled 3.5 kb *Hin*dIII fragment containing the *R. capsulatus hup* structural genes.

Plasmid pRA411A DNA fully digested with *Xho*I is shown in Fig. 1 A. Only a single 4.6 kb band of DNA exhibited strong hybridization with the *R. capsulatus* DNA probe (Fig. 1 B, lane III). The same 4.6 kb *Xho*I band was also observed in fully digested plasmid DNA from each of the other three clones obtained from the gene library. Plasmid pRA411A was used to obtain the 4.6 kb *Xho*I DNA fragment of *R. gelatinosus* for subcloning, since the band in this recombinant plasmid migrated widely separated from the other restriction fragments on agarose gels (Fig. 1 A).

Initial experiments to ligate the *R. gelatinosus Xho*I restriction fragment into either *Sal*I-linearized pUC18 or pUC19 were unsuccessful. Subsequently, studies were undertaken to test reaction conditions. Results based upon observation of agarose gels suggested that the efficiency of ligation by the T4 DNA ligase preparation (Boehringer-Mannheim, FRG) could be improved using only 0.5 mM and 5 mM of ATP and DTE, respectively, and by diluting the 10 × concentrated buffer solution, prepared by Boehringer-Mannheim, 20-fold in the final ligation reaction mixture. The reason for the improved enzyme efficiency was not understood. Nevertheless, these changes made it possible to ligate the 4.6 kb *Xho*I DNA fragment of *R. gelatinosus* into *Sal*I-digested pUC18 DNA and transform *E. coli* TB1. After overnight culture 269 transformant colonies had developed on LB (Str, Ap) solid medium containing X-gal. Of the clones 22 showed non-pigmented growth with X-gal, but only 5 of these contained the 4.6 kb *R. gelatinosus* DNA fragment. Recombinant plasmid pRA22 was selected for use. The reason for the small number of cells with pUC18 containing a 4.6 kb *Xho*I insert may have been a lowered ligation efficiency between the *Xho*I and *Sal*I termini. When the same reaction conditions were used to subclone a 3.2 kb *R. gelatinosus Bam*HI fragment into *Bam*HI-treated pUC18

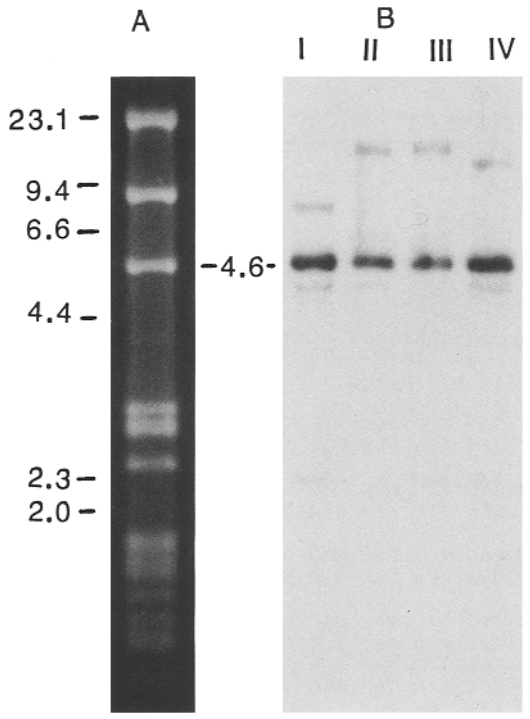


Fig. 1 A and B. Localization of the *Rhodocyclus gelatinosus* 4.6 kb *Xho*I DNA fragment containing the structural uptake-hydrogenase (uptake-H_2ase) genes. **A** Recombinant plasmid pRA411A fully digested with *Xho*I and fractionated on an agarose gel. **B** Southern blots of *Xho*I-digested plasmid DNA transferred to Hybond nylon membrane and then hybridized with the ^{32}P-labelled 3.5 kb *Hin*dIII DNA fragment from *Rhodobacter capsulatus* containing the *hup* uptake-H_2ase structural genes. Lane III represents pRA411A. Additional recombinant plasmids containing the same 4.6 kb *Xho*I DNA fragment are shown in lanes I, II and IV

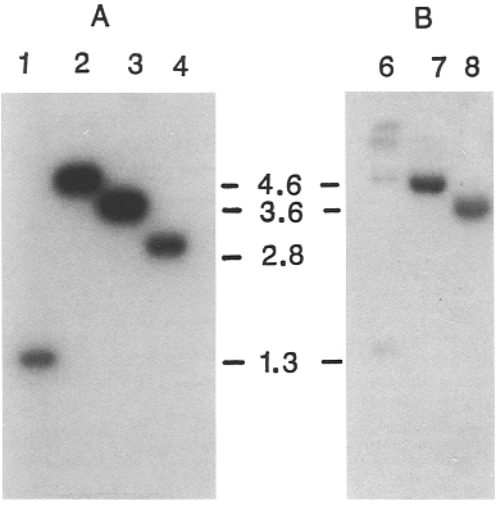


Fig. 2 A and B. Southern blots of **A** the recombinant plasmid pRA411A and **B** *Rhodocyclus gelatinosus* genomic DNA. DNA was digested with *Bam*HI (lanes 1 and 6), *Xho*I (lanes 2 and 7), *Sal*I (lanes 3 and 8) and *Pst*I (lane 4), fractionated on an agarose gel, transferred to Hybond nylon membrane and hybridized using the ^{32}P-labelled 1.3 kb *Bam*HI DNA fragment from pRA22 as a probe

(this 3.2 kb piece of DNA overlapped the *Xho*I fragment; cf. Fig. 3), 43 out of 196 colonies showed non-pigmented growth on LB medium with X-gal, and 90% of these colonies contained recombinant pUC18 DNA with a 3.2 kb *Bam*HI insert. Plasmid pRA31 was selected for further studies.

Genomic Southern blots

Hybridization analysis was performed to determine if the hydrogenase genes of *R. gelatinosus* were localized in the same genomic environment on the 40 kb DNA from the gene bank as on the cell chromosome. Both pRA411A and genomic DNA were digested with restriction endonucleases, separated on agarose gels, and transferred onto Hybond nylon membrane for hybridization with the ^{32}P-labelled 1.3 kb *Bam*HI DNA fragment (cf. Fig. 3) purified from pRA22.

Bands of hybridizing pRA411A DNA produced by treatment with selected restriction endonucleases are shown in Fig. 2A. Incubation with *Bam*HI produced the expected 1.3 kb restriction fragment. Likewise, *Xho*I treatment resulted in the typical 4.6 kb DNA fragment (see also Fig. 1), and *Sal*I treatment gave rise to a 3.6 kb fragment. A single 2.8 kb *Pst*I restriction fragment from pRA411A also hybridized with the ^{32}P-labelled probe.

Chromosomal DNA showed the same hybridization pattern (Fig. 2B) as observed with pRA411A. *R. gelatinosus* genomic DNA fully digested with *Xho*I or *Sal*I showed single hybridizing bands of 4.6 kb and 3.6 kb, respectively. Hybridizing zones of 1.3 and 2.8 kb in genomic DNA partially digested by *Bam*HI and *Pst*I respectively (not shown) were also observed. In separate experiments virtually identical hybridization sites were identified on digested *R. gelatinosus* genomic DNA hybridized with DNA containing the *hup* structural genes of *B. japonicum* or *R. capsulatus* (not shown).

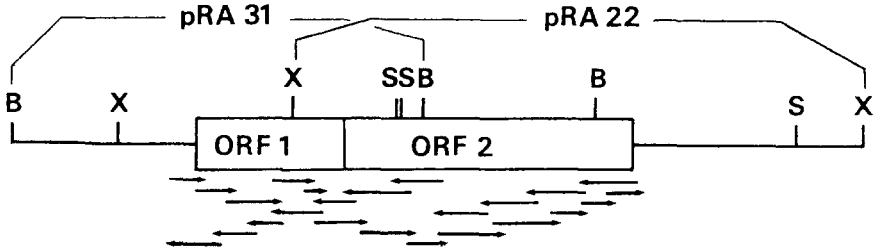
Nucleotide sequencing

R. gelatinosus uptake-H_2ase structural subunit genes were located on overlapping recombinant plasmids pRA22 and pRA31. A restriction map used for cloning and subcloning DNA is shown in Fig. 3A. Two open reading frames (ORF1 and ORF2) were present in this region. Nucleotide and derived amino acid sequences are shown in Fig. 3B. By homology with the amino acid sequences of the uptake-H_2ase subunits of *R. capsulatus* (Leclerc et al. 1988), and of *B. japonicum* (Sayavedra-Soto et al. 1988), it was concluded that ORF1 and ORF2 represented *hup*S and *hup*L, respectively, and thus encoded the small and large subunits of the *R. gelatinosus* uptake-H_2ase.

ORF1 (*hup*S) contained 1080 nucleotides including the initiation codon ATG at position 290 and ended with a TGA stop codon at position 1370. The gene was capable of encoding a protein of 39.4 kDa with 360 amino acids including a putative signal peptide of 42 residues. Without the leader sequence, the calculated size of the ORF1 protein was 34.6 kDa. A consensus Shine-Dalgarno sequence (AGGAG) was present 9 bases upstream from the leader peptide start codon.

ORF2 (*hup*L) started 33 bases downstream from the TGA stop codon of the ORF1. A second AGGAG ribosome binding site was present 5 bases upstream from the

A



B

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ggacggccgctcggtggtttccctagatcaaggaaaagcgggcacgcatcgtgggtatgacacagctcaaagaccgaacccacggcgaggagcc   282

cgatccgATG GAG ACC TTC TAC GAA GTC ATG CGC CGC CAG GGC ATC TCG CGG CGC AGC TTC CTC AAG TAC TGC   355
       Met Glu Thr Phe Tyr Glu Val Met Arg Arg Gln Gly Ile Ser Arg Arg Ser Phe Leu Lys Tyr Cys    22

                                                                                1
   TCG CTG ACG GCC ACC TCG CTC GGG CTC GCG CCG TCG TTC GTG CCG CAG ATC GCG CAC GCG ATG GAG ACC   424
   Ser Leu Thr Ala Thr Ser Leu Gly Leu Ala Pro Ser Phe Val Pro Gln Ile Ala His Ala Met Glu Thr    45

   AAG CCG CGC ACC CCG GTG CTC TGG CTG CAC GGC CTG GAA TGC ACC TGC TGC AGC GAG AGC TTC ATC CGC   493
   Lys Pro Arg Thr Pro Val Leu Trp Leu His Gly Leu Glu Cys Thr Cys Cys Ser Glu Ser Phe Ile Arg    68

   AGC GCG CAC CCG CTG GCG AAG GAC GCC GTG CTG TCG ATG ATC TCG CTG GAC TAC GAC GAC ACG CTG ATG   562
   His Pro Leu Ala Lys Asp Ala Val Leu Ser Met Ile Ser Leu Asp Tyr Asp Asp Thr Leu Ser Ala Met    91

   GCC GCC GCC GGC CAC CAG GCC GAG GCC ATC CTC GAC GAG ATC ATG GCC AAG TAC AAG GGC AAC TAC ATC   631
   Ala Ala Ala Gly His Gln Ala Glu Ala Ile Leu Asp Glu Ile Met Ala Lys Tyr Lys Gly Asn Tyr Ile   114

   CTG GCC GTC GAG GGC AAC CCG CCG CTC AAC GAG GAC GGG ATG TTC TGC ATC CAG CGG CAA GCC CTT CCT   700
   Leu Ala Val Glu Gly Asn Pro Pro Leu Asn Glu Asp Gly Met Phe Cys Ile Gln Arg Gln Ala Leu Pro   137

   CGA GAA GCT CAA GCC GTC GCC GCC GAC TGC AAG GCC GTC ATC GCC TGG GGC TCC TGC GCC TCC TGG GGT   769
   Arg Glu Ala Gln Ala Val Ala Ala Asp Cys Lys Ala Val Ile Ala Trp Gly Ser Cys Ala Ser Trp Gly   160

   TGC GTG CAG GCC GCC AAG CCG AAT CCG ACG CAG GCC ACG CCG ATC CAC AAG GTG ATC ACC GAC AAG CCG   838
   Cys Val Gln Ala Ala Lys Pro Asn Pro Thr Gln Ala Thr Pro Ile His Lys Val Ile Thr Asp Lys Pro   183

   ATC ATC AAG GTG CCC GGC TGC CCG CCG ATC GCC GAG GTG ATG ACC GGC GTC ATC ACC TAC ATG CTG ACC   907
   Val Pro Gly Cys Pro Pro Ile Ala Glu Val Met Thr Gly Val Ile Thr Tyr Ile Ile Lys Met Leu Thr   206

   TTC GAC CGC ATC CCC GAG CTC GAC CGC CAG GGC CGG CCG AAG ATG TTC TAC AGC CAG CGC ATC CAC GAC   976
   Phe Asp Arg Ile Pro Glu Leu Asp Arg Gln Gly Arg Pro Lys Met Phe Tyr Ser Gln Arg Ile His Asp   229

   AAG TGC TAC CGC CGG CCG CAC TTC GAC GCC GGC CAG TTC GTC GAG AGC TTC GAC GAC GAG AAC GCG CGC 1045
   Lys Cys Tyr Arg Arg Pro His Phe Asp Ala Gly Gln Phe Val Glu Ser Phe Asp Asp Glu Asn Ala Arg   252

   AAG GGC TTC TGC CTC TAC AAG GTC GGC TGC AAG GGC CCG ACG ACC TAC AAC GCC TGC TCC ACG GTG ATG 1114
   Lys Gly Phe Cys Leu Tyr Lys Val Gly Cys Lys Gly Pro Thr Thr Tyr Asn Ala Cys Ser Thr Val Met   275

   TGG AAC GAA GGC ACG AGC TTC CCG ATC AAG GCC GGC CAC GGT GCA CGG CGC TCC GAG GAC GGC TTC TGG 1183
   Trp Asn Glu Gly Thr Ser Phe Pro Ile Lys Ala Gly His Gly Ala Arg Arg Ser Glu Asp Gly Phe Trp   298

   GAC AAG GGC TCG TTC TAC GAC CGC CTG ACC AAC ATC CAC CAG TTC GGC ATC GAG GCC AGC GCC GAC AAG 1252
   Asp Lys Gly Ser Phe Tyr Asp Arg Leu Thr Asn Ile His Gln Phe Gly Ile Glu Ala Ser Ala Asp Lys   321

   GTC GGC GGC ACC GCG GCC GGC GTC GTC GGT GCG GCC ATC GCC GCG CAC GCC GCC GCG TCG GTG ATC AAG 1321
   Val Gly Gly Thr Ala Ala Gly Val Val Gly Ala Ala Ile Ala Ala His Ala Ala Ala Ser Val Ile Lys   344

CGC CTG TCG CAC GAC CCG GAC GCC GCC GCG CGC GCC GAG TCG CGC AGC tgaccccacacccgaacgagaacac   1394
Arg Leu Ser His Asp Pro Asp Ala Ala Ala Arg Ala Glu Ser Arg Ser ***                        360

                1
aggagcaccc  ATG GGC GCC ATC GAA ACC CAA GGC TTC AAG CTC GAC GAC AGC GGC CGC CGC ATC GTC GTC 1464
            Met Gly Ala Ile Glu Thr Gln Gly Phe Lys Leu Asp Asp Ser Gly Arg Arg Ile Val Val   19

GAC CCC GTC ACG CGC ATC GAG GGC CAC ATG CGC TGC GAG GTG AAC GTC GAC GCC AAC AAC GTC ATC CGC 1533
Asp Pro Val Thr Arg Ile Glu Gly His Met Arg Cys Glu Val Asn Val Asp Ala Asn Asn Val Ile Arg   42

AAC GCC GTT TCC ACC GGC ACG ATG TGG CGC GGG CTG GAG GTC ATC CTC AAG GGC CGC GAC CCG CGC GAC 1602
Asn Ala Val Ser Thr Gly Thr Met Trp Arg Gly Leu Glu Val Ile Leu Lys Gly Arg Asp Pro Arg Asp   65

GCC TGG GCC TTC GTC GAG CGC ATC TGC GGC GTC TGC ACC GGC TGC CAT GCG CTG ACC AGC GTG CGC GCC 1671
Ala Trp Ala Phe Val Glu Arg Ile Cys Gly Val Cys Thr Gly Cys His Ala Leu Thr Ser Val Arg Ala   88

GTC GAG GAC GCG CTG GGC ATC AGG ATC CCG AAG AAC GCG CAC CTC ATC CGC GAG ATG ATG GCC AAG ACG 1740
Val Glu Asp Ala Leu Gly Ile Arg Ile Pro Lys Asn Ala His Leu Ile Arg Glu Met Met Ala Lys Thr  111
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CTG CAG GTG CAC GAC CAC GCG GTG CAC TTC TAC CAC CTG CAC GCG CTG GAC TGG GTG GAC GTC GTC TCG 1809
Leu Gln Val His Asp His Ala Val His Phe Tyr His Leu His Ala Leu Asp Trp Val Asp Val Val Ser  134

GCG CTG AAG GCC GAC CCG AAG AAG ACC AGC GAG CTG CAG CAC CTG GTG TCG CCC TCG CAC CCG CTG TCC 1878
Ala Leu Lys Ala Asp Pro Lys Lys Thr Ser Glu Leu Gln His Leu Val Ser Pro Ser His Pro Leu Ser  157

TCG CCG GGC TAC TTC CCG CGA CGT GGC AGA ACC GGG CTG AAG AAG TTC GTC GAG AGC GGC CAG CTC GGG 1947
Ser Pro Gly Tyr Phe Pro Arg Arg Gly Arg Thr Gly Leu Lys Lys Phe Val Glu Ser Gly Gln Leu Gly  180

CCC TTC ATG AAC GGC TAC TGG GGC AGC AAG GCC TAC GTG CTG CCG CCC GAG GCC AAC CTG ATG GCC GTC 2016
Pro Phe Met Asn Gly Tyr Trp Gly Ser Lys Ala Tyr Val Leu Pro Pro Glu Ala Asn Leu Met Ala Val  203

ACC CAC TAC CTG GAG GCG CTG GAC CTG CAG AAG GAA TGG GTG AAG GTG CAC GCC ATC TTC GGC GGC AAG 2085
Thr His Tyr Leu Glu Ala Leu Asp Leu Gln Lys Glu Trp Val Lys Val His Ala Ile Phe Gly Gly Lys  226

AAC CCG CAC CCG AAC TAC CTC GTC GGC GGC GTT CCC TGC GCG ATC AAC CTC GAC GGC AAC GGC GCC GCC 2154
Asn Pro His Pro Asn Tyr Leu Val Gly Gly Val Pro Cys Ala Ile Asn Leu Asp Gly Asn Gly Ala Ala  249

GGG CGC ATC AAC ATG GAG CGG CTG AAC TTC GTC AAG GCT CGC ATC GAC GAG ATG ATC GAG TTC AAC AAG 2223
Gly Arg Ile Asn Met Glu Arg Leu Asn Phe Val Lys Ala Arg Ile Asp Glu Met Ile Glu Phe Asn Lys  272

AAC GTC TAC CTG CCC GAC GTG CTG GCC ATC GGC ACG ATC TAC AAG CAG GCC GGC TGG CTG CAC GGC GGC 2292
Asn Val Tyr Leu Pro Asp Val Leu Ala Ile Gly Thr Ile Tyr Lys Gln Ala Gly Trp Leu His Gly Gly  295

GGC CTG TCG GCG CTG AAC GTC GCC GAC TAC GGC ACC TAC GAC AAG GTG GCC TAC GAC CAC GCC ACG CAC 2361
Gly Leu Ser Ala Leu Asn Val Ala Asp Tyr Gly Thr Tyr Asp Lys Val Ala Tyr Asp His Ala Thr His  318

CAG CTG CCC GGC GGC GTC ATC CTC GAC GGC AAC TGG GAC GAG ATC CAC GCC ATC GAC CCG CGC GAC CCC 2430
Gln Leu Pro Gly Gly Val Ile Leu Asp Gly Asn Trp Asp Glu Ile His Ala Ile Asp Pro Arg Asp Pro  341

GAG CAG GTG CAG GAG TTC GTC GCC CAC AGC TGG TAC CAG TAC GCC GAC GAG AGC AAG GGC CTG CAC CCC 2499
Glu Gln Val Gln Glu Phe Val Ala His Ser Trp Tyr Gln Tyr Ala Asp Glu Ser Lys Gly Leu His Pro  364

TGG GAC GGC GTC ACC GAG CCG AAG TTC GAG CTC GGC GCC AGG ACC AAG GGC ACA CGC ACC GCG ATC GAA 2568
Trp Asp Gly Val Thr Glu Pro Lys Phe Glu Leu Gly Ala Arg Thr Lys Gly Thr Arg Thr Ala Ile Glu  387

CAC ATC GAC GAG AGC GCC AAG TAC TCG TGG ATC AAG TCG CCG CGC TGG CGC GGC CAC GCC GTC GAG GTC 2637
His Ile Asp Glu Ser Ala Lys Tyr Ser Trp Ile Lys Ser Pro Arg Trp Arg Gly His Ala Val Glu Val  410

GGC CCG CTG TCG CGC TAC ATC CTC GGC TAC GCC CAT GCG CTG AAG GGC AAC AAG TAC TGC CAG CGC GTC 2706
Gly Pro Leu Ser Arg Tyr Ile Leu Gly Tyr Ala His Ala Leu Lys Gly Asn Lys Tyr Cys Gln Arg Val  433

AAG GAG CAG GTG GAC TTC GCC GCC GAG GCG ATC AAC CAC GCC ATC CCC AAG GCC CTG GGC CTG CCG GAG 2775
Lys Gly Gln Val Asp Phe Ala Ala Glu Ala Ile Asn His Ala Ile Pro Lys Ala Leu Gly Leu Pro Glu  456

ACG CAG TAC ACC TTG AAG CAG CTG CTG CCG ACG ACC ATC GGC CGC ACG CTG GCG CGC TGC CTC GAA GGC 2844
Thr Gln Tyr Thr Leu Lys Gln Leu Leu Pro Thr Thr Ile Gly Arg Thr Leu Ala Arg Cys Leu Glu Gly  479

CAG TAC TGC GGC GAG ATG ATG CTG GCC GAC TAC CAC GAG CTG GTG GCC AAC ATC CGC GCC GGC GAC ACC 2913
Gln Tyr Cys Gly Glu Met Met Leu Ala Asp Tyr His Glu Leu Val Ala Asn Ile Arg Ala Gly Asp Thr  502

GCC ACC GCC AAC GTC GAG AAG TGG GAC CCG GCG ACC TGG CCC AAG GAA GCC AAG GGC GTC GGC ACC GTC 2982
Ala Thr Ala Asn Val Glu Lys Trp Asp Pro Ala Thr Trp Pro Lys Glu Ala Lys Gly Val Gly Thr Val  525

GCC GCG CCG CGC GGC ATG CTG GGC CAC TGG ATC CGC ATC AAG GAC GGC AAG ATC GAG AAC TAC CAG TGC 3051
Ala Ala Pro Arg Gly Met Leu Gly His Trp Ile Arg Ile Lys Asp Gly Lys Ile Glu Asn Tyr Gln Cys  548

GTC GTG CCG ACC ACC TGG AAC GGC AGC CCG CGC GAC GCC AAG GGC CAG ATC GGC GCC TTC GAG GCC AGC 3120
Val Val Pro Thr Thr Trp Asn Gly Ser Pro Arg Asp Ala Lys Gly Gln Ile Gly Ala Phe Glu Ala Ser  571

CTG CTG GGC ACG CCG ATG GTC AAC CCG GAA CAG CCG GTC GAG ATC CTG CGC ACG CTG CAC TCC TTC GAC 3189
Leu Leu Gly Thr Pro Met Val Asn Pro Glu Gln Pro Val Glu Ile Leu Arg Thr Leu His Ser Phe Asp  594

CCC TGC CTG GCC TGC TCG ACC CAC GTG ATG AGC GAA GAC GGC CGC GAA CTG ACG ACC GTG AAG GTG CGC 3258
Pro Cys Leu Ala Cys Ser Thr His Val Met Ser Glu Asp Gly Arg Glu Leu Thr Thr Val Lys Val Arg  617

tgatagcgccgcccgacctgaaaggacacccatgaagaagctcgtcccgccgtcctgctgccgccgccgcgacgcccgcgttcgcccaccc 3349
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Fig. 3A and B. Restriction map, sequencing strategy and nucleotide sequence of *Rhodocyclus gelatinosus* uptake-H_2ase. **A** Restriction map of pRA31 and pRA22. B, *Bam*HI; S, *Sal*I; X, *Xho*I. Sequencing strategy is illustrated by *arrows* showing the orientation and extent of sequencing. Uptake-H_2ase subunit open reading frames ORF1 and ORF2 are represented by *boxes*. **B** Nucleotide and deduced amino acid sequences for the small and large subunits of *R. gelatinosus* uptake-H_2ase. Coding regions and the putative leader peptide are in *capital letters*. Shine-Dalgarno sequences are *underlined*. The first amino acid of each mature proteins is indicated by 1

ATG initiation codon at position 1405. ORF2 contained 1854 nucleotides and was capable of encoding a protein of 68.5 kDa with 618 amino acids including the first Met residue.

The structural uptake-H_2ase genes contained 24 Cys residues. One Cys residue occurred in the signal sequence with 12 others distributed on ORF1. The remaining 11 residues were located in ORF2.

The polarity index, based upon anticipated amino acid composition, indicated that about 58% of the amino acids

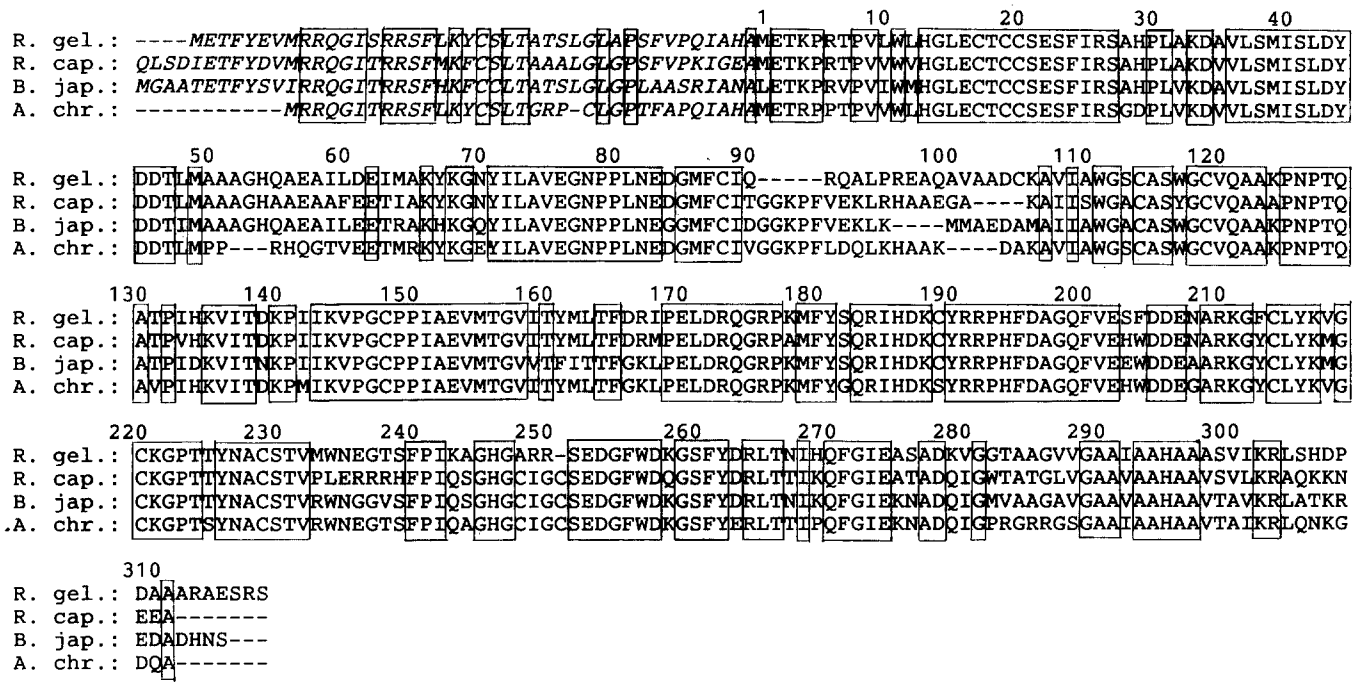

Fig. 4. Comparison of the deduced polypeptide sequence of the small subunit including the signal peptide (*italics*) of the uptake-H_2ase from *Rhodocyclus gelatinosus* (R. gel.), with those of *Rhodobacter capsulatus* (R. cap.), *Bradyrhizobium japonicum* (B. jap.) and *Azotobacter chroococcum* (A. chr.). Residues conserved between the four sequences are *boxed*

in ORF1 (small) and ORF2 (large) subunit proteins were hydrophobic. The hydropathy plots (not shown) for the *R. gelatinosus* uptake-H_2ase subunit proteins were virtually identical to their counterparts in *R. capsulatus* (Leclerc et al. 1988).

Sequence comparison

The modified Needleman-Wunsch algorithm was used to compare the amino acid sequences of the small (ORF1) and large (ORF2) subunits of the membrane-bound uptake-H_2ases of *R. gelatinosus* with those of *R. capsulatus* (Leclerc et al. 1988), *B. japonicum* (Sayavedra-Soto et al. 1988) and *Azotobacter chroococcum* (M.G. Yates, personal communication). The degree of similarity between small subunit proteins was 75% to 80%. However, between the large subunits it was somewhat lower, being 68% with *R. capsulatus* and *B. japonicum* and 53% with *A. chroococcum*.

Cys residues were conserved in the same relative positions in *R. gelatinosus* as in other membrane-associated uptake-H_2ases. However, *R. gelatinosus* lacked two Cys groups because of a shorter consensus sequence in the small subunit protein. In comparison with the *R. capsulatus hup*S sequence $GXCX_4GCKGPTXYXXCX_{18}\underline{CIXC}$ (positions 213 to 252 starting from Met1 of *R. capsulatus hup*S; Leclerc et al. 1988) the amino acid residues 249 to 252 (underlined in the above sequence) were absent in *R. gelatinosus*. All four uptake-H_2ases were membrane associated and exhibited typical leader peptides. The amino acid sequence of the leader peptide of *R. gelatinosus* compared with those of *R. capsulatus* and *B. japonicum* are very similar (cf. Leclerc et al. 1988) (Fig. 4).

Discussion

Molecular biology studies among the anoxygenic photosynthetic bacteria have been mostly limited to *R. capsulatus* and *Rhodobacter sphaeroïdes*, with a few studies also on *Rhodospirillum rubrum* (Scolnik and Marrs 1987). On the basis of 16 S rRNA patterns, these cells are grouped together in the α-purple bacterial subdivision of Woese (1987). This is the first report of a molecular biological system for *R. gelatinosus* which is classified in the separate β-purple bacterial subdivision on the basis of 16 S rRNA sequence differences. Furthermore, when compared with α-purple bacterial counterparts, *R. gelatinosus* contains a different type of cytochrome *c* and an alternative photoreaction centre which may lack the H-subunit in certain strains (Clayton and Clayton 1978; Fukushima et al. 1988). Additional differences have been recognized in the present molecular biological study.

R. gelatinosus genomic DNA appeared to be tightly associated with cell protein material as reported in some other bacteria (Mayer and Friedrich 1986) and attempts to purify the DNA were unsatisfactory using methods successful in most other bacteria (Marmur 1961). After treatment of DNA with proteinase K, however, it was finally possible to obtain protein-free material.

Purified genomic *R. gelatinosus* DNA exhibited unusual properties when treated with restriction endonucleases. Although it was readily hydrolysed by *Sal*I and *Sau*3A, genomic DNA resisted total digestion by other restriction endonucleases such as *Bam*HI or *Pst*I. Meeks et al. (1988) observed that some preparations of cyanobacterial DNA also resisted complete digestion, but this could be remedied by ion-exchange chromatography treatment of DNA. Similar treatment of *R. gelatinosus* genomic DNA might be useful.

However, it was judged unnecessary in the present study since 40 kb DNA restriction fragments from the cosmid gene library appeared to be completely susceptible to endonuclease action. On the other hand, restriction sites for both *Eco*RI and *Hin*dIII seemed to be totally absent in *R. gelatinosus* DNA. No evidence of digestion by either enzyme was observed with genomic DNA or with DNA fragments produced by the action of other endonucleases.

The structural genes of *R. gelatinosus* uptake-H_2ase were isolated using DNA probes from *R. capsulatus* and *B. japonicum*. The nucleotide sequence of the *R. gelatinosus* uptake-H_2ase structural gene exhibited two open reading frames. Protein sequences were deduced using a codon usage table established from other sequenced Rhodospirillaceae genes. ORF1 and ORF2 were identified as the genes encoding the small and large uptake-H_2ase subunits, respectively, based on amino acid sequence alignment with the uptake-H_2ase structural genes of *R. capsulatus* (Leclerc et al. 1988).

R. gelatinosus uptake-H_2ase appeared to be closely related to sequenced membrane uptake-H_2ases in other bacteria when analysed on the basis of its presumptive amino acid sequence composition. The small structural subunit protein of *R. gelatinosus* exhibited more that 75% identity with the analogous subunit proteins in *R. capsulatus, B. japonicum* and *A. chroococcum*. The identity index between the large subunit proteins was somewhat lower. The principal reason for this difference may in part be attributed to an amino acid sequence between positions 416 and 440 in *R. gelatinosus* that does not appear in the other three large subunit uptake-H_2ase proteins. Cys residues were largely conserved in the different membrane uptake-H_2ases with the exception that two Cys residues corresponding to those in positions 249 and 252 in the *R. capsulatus* small subunit protein were missing in *R. gelatinosus*. None of the membrane uptake-H_2ases exhibited the typical [4Fe-4S] ferredoxin pattern $CX_2CX_2CX_3C$.

The role of H_2 metabolism in bacteria is well known and among the anoxygenic photosynthetic bacteria, membrane-associated uptake-H_2ase is especially important (Vignais et al. 1985). In accord with this, *R. gelatinosus* was found to produce an inducible membrane-associated uptake-H_2ase which closely resembled the enzyme in *R. capsulatus*. As in *R. capsulatus*, the H_2-uptake enzyme reached a high rate of activity and supported rapid photoautotrophic cell growth. The uptake-H_2ase in *R. gelatinosus*, however, differed somewhat from the *R. capsulatus* enzyme by being totally resistant to inhibition by CO. In addition to H_2-uptake, *R. gelatinosus* also evolves H_2 during growth with CO (Uffen 1976, 1983). The CO-dependent, unidirectional, H_2-output reaction is likewise membrane bound (Champine and Uffen 1987). Unidirectionality may depend on the proximity of redox components tightly bound to the H_2ase protein so that electrons can either be transferred from H_2ase to an electron acceptor of higher potential as is the case for uptake-H_2ases, or can be provided by redox components of low potential as may be the case for CO-dependent output-H_2ase in *R. gelatinosus*. In other words, the same H_2ase protein could work unidirectionally in a complex where an appropriate additional redox component contributed to the driving of electrons in one direction or the other. On the other hand, unidirectional H_2-output H_2ases have been identified in *Clostridium pasteurianum* and in *Desulfovibrio* species; the output-H_2ases often belong to the class of [Fe]H_2ases and differ from the uptake-H_2ases which are [NiFe]H_2ases (Fauque et al. 1988). Consequently, in studies on *R. gelatinosus* H_2ase, the important question now remains whether a single set of H_2ase structural genes exists which encodes a single enzyme analogous to the H_2ase of *R. capsulatus* but which may then operate in association with the cytoplasmic membrane either as an uptake- or as an output-H_2ase, or if two different H_2ase enzymes may be synthesized in *R. gelatinosus* depending on the growth conditions. An answer may be found using genetic recombinant methods (Sistrom 1977) once appropriate *R. gelatinosus* H_2ase mutants have been obtained.

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