Genes encoding the NAD-reducing hydrogenase of Rhodococcus opacus MR11

Claudia Grzeszik, Meike Lübbers, Michael Reh and Hans G. Schlegel

Author for correspondence: Hans G. Schlegel. Tel: +49 551 39 3771. Fax: +49 551 39 3793. e-mail: hschlegl@gwdg.de

INSTITUT FUR MIKROBIOLOGIE, GEORG-AUGUST-UNIVERSITAT GOTTINGEN, GRISEBACHSTRASSE 8, D-37077 GOTTINGEN, GERMANY

The dissociation of the soluble NAD-reducing hydrogenase of Rhodococcus opacus MR11 into two dimeric proteins with different catalytic activities and cofactor composition is unique among the NAD-reducing hydrogenases studied so far. The genes of the soluble hydrogenase were localized on a 7.4 kbp Asnl fragment of the linear plasmid pHG201 via heterologous hybridization. Analysis of the nucleotide sequence of this fragment revealed the seven open reading frames ORF1, hoxF, -U, -Y, -H, -W and ORF7. The six latter ORFs belong to the gene cluster of the soluble hydrogenase. Their gene products are highly homologous to those of the NAD-reducing enzyme of Alcaligenes eutrophus H16. The genes hoxF, -U, -Y and -H encode the subunits α, γ, δ and β, respectively. The gene hoxW encodes a putative protease, which may be essential for C-terminal processing of the β subunit. Finally, ORF7 encodes a protein which has similarities to cAMP- and cGMP-binding protein kinases, but its function is not known. ORF1, which lies upstream of the hydrogenase gene cluster, encodes a putative transposase found in IS elements of other bacteria. Northern hybridizations and primer extensions using total RNA of autotrophically and heterotrophically grown cells of R. opacus MR11 indicated that the hydrogenase genes are under control of a σ70-like promoter located at the right end of ORF1 and are even transcribed under heterotrophic conditions at a low level. Furthermore, this promoter was shown to be active in the recombinant Escherichia coli strain LHY1 harbouring the 7.4 kbp Asnl fragment, resulting in overexpression of the hydrogenase genes. Although all four subunits of the soluble hydrogenase were shown via Western immunoblots to be synthesized in E. coli, no active enzyme was detectable.

Keywords: Rhodococcus opacus MR11, NAD-reducing hydrogenase, box genes

INTRODUCTION

Rhodococcus opacus strain MR11 (formerly Nocardia opaca 1b) is a Gram-positive, facultative chemolithoautotrophic bacterium which can grow on carbon dioxide and gaseous H₂ as the sole carbon and energy sources. Physiologically, it belongs to the knallgas bacteria, a group which is composed of phylogenetically diverse organisms. For the activation of H₂, all knallgas bacteria contain hydrogenases, which are of two basic types: the membrane-bound hydrogenases (MBHs), which are coupled to the electron transfer chain and are not capable of reducing NAD, and the soluble hydrogenases (SHs), which are localized in the cytoplasm and catalyse the transfer of electrons directly to NAD (Aragno & Schlegel, 1992; Schneider et al., 1984a; Zaborosch et al., 1989; Schink & Schlegel, 1979). The MBHs belong to the more common group of NiFe-hydrogenases consisting of one large and one small subunit, whereas the SHs belong to a family of less abundant multimeric NiFe-hydrogenases (Friedrich & Schwartz, 1993; Wu & Mandrand, 1993). The majority of the knallgas bacteria contain only the MBH, but a few, such as R. opacus MR11 and MR22, contain only the SH (Aggag & Schlegel, 1973; Grzeszik et al., 1997) or both enzymes, like Alcaligenes eutrophus H16 (Friedrich et al., 1984).

The SHs of R. opacus MR11 and A. eutrophus H16 have been investigated in some detail. Both enzymes are

Abbreviations: MBH, membrane-bound hydrogenase; SH, soluble hydrogenase (EC 1.12.1.2).

The GenBank accession number for the nucleotide sequence reported in this paper is U70364.
tetramers consisting of four non-identical subunits, \( \alpha, \beta, \gamma \) and \( \delta \). The corresponding subunits of the two organisms are immunologically related and comparison of their established N-terminal amino acid sequences revealed similarities of more than 60% (Zaborosch et al., 1989). In addition, the native enzymes are very similar with respect to many catalytic and molecular properties, for example metal content and cofactor composition (Schneider et al., 1984a, b). The unique property of the \( \text{R. opacus} \) hydrogenase of dissociating into two dimeric proteins with different enzymic activities under certain conditions made it possible to study this enzyme more closely (Zaborosch et al., 1995). The \( \beta \)-dimer, which contains nickel and one \([4\text{Fe}-4\text{S}]\) cluster, shows hydrogenase (\( \text{H}_2 \):acceptor oxidoreductase) activity with several electron acceptors but not with NAD. This dimer contains the \( \text{H}_2 \)-activating centre. Recent studies suggest that the \( \text{H}_2 \)-activating site of \( \text{NiFe}\)-hydrogenases consists of nickel and a second metal atom, probably iron, which form a binuclear metal cluster (Volbeda et al., 1993; Albracht, 1995). The second dimer consists of the \( \alpha \) and the \( \gamma \) subunit and has diaphorase (\( \text{NADH} \):acceptor oxidoreductase) activity. It contains two \([4\text{Fe}-4\text{S}]\), one \([3\text{Fe}-4\text{S}]\) and one \([2\text{Fe}-2\text{S}]\) cluster and one molecule of flavin mononucleotide. The genetic determinants of the SHs of \( \text{R. opacus} \) and \( \text{A. eutrophus} \) are located on the megaplasmids pHG201 and pHG1, respectively (Kalkus et al., 1990, 1993; Tran-Betcke et al., 1990). The genes of the \( \text{A. eutrophus} \) enzyme were cloned and sequenced and the comparison of the deduced amino acid sequences of the identified ORFs with the N-terminal amino acid sequences of the subunits determined by Zaborosch et al. (1989) made it possible to assign the genes to the subunits.

Genetic studies of the \( \text{A. eutrophus} \) hydrogenases and of \( \text{NiFe}\)-hydrogenases of several other \( \text{H}_2 \)-oxidizing bacteria indicated that for biosynthesis of active enzymes the gene products of several accessory genes are essential (Vignais & Toussaint, 1994; Friedrich & Schwartz, 1993). These are proteins which are involved in the uptake of nickel, incorporation of nickel and intracellular processing. For example, the nickel-containing subunits of hydrogenases of some bacteria were shown to be subject to post-translational processing at the C-terminus, consisting of a proteolytic cleavage of at least 15 amino acid residues (Thiemermann et al., 1996; Volbeda et al., 1995; Gollin et al., 1992; Sorgenfrei et al., 1993; Rossmann et al., 1994). The regulation of the hydrogenase genes studied so far also appears to be complex. In some organisms, the expression of hydrogenase is regulated by transcriptional activators belonging to the response-regulator family (Vignais & Toussaint, 1994; Friedrich & Schwartz, 1993).

While the SH of \( \text{R. opacus} \) MR11 has been studied in many respects at the biochemical level, nothing is known about its genetic determinants, and the regulation and expression of these genes. In this communication we report the cloning and sequencing of a 7.4 kbp \( \text{AsnI} \) fragment which contains the genes encoding the SH of \( \text{R. opacus} \) MR11. The complete nucleotide sequence was determined and analysed. Information about the regulation and transcription of the genes was obtained by mRNA analyses.

**METHODS**

**Bacterial strains and plasmids.** Strains and plasmids used in this study are listed in Table 1. \( \text{R. opacus} \) strain MR11, originally isolated by D. Siebert, is the type strain (Aggag & Schlegel, 1973; Klatte et al., 1994). The derivative strains MR2246 and MR1463 were described (Sensfuss et al., 1986; Reh & Schlegel, 1981) and studied in this laboratory. *Escherichia coli* BMH71-18, plasmid pMc5-8 and *A. eutrophus* HF14 were provided by W. Kramer, H.-J. Fritz and B. Friedrich, respectively.

**Growth conditions.** The \( \text{R. opacus} \) strains were grown heterotrophically or autotrophically as described by Sensfuss et al. (1986). For heterotrophic growth fructose/trypton/yeast extract (FTY) medium was used. For lithoautotrophic growth in liquid culture the composition of the mineral medium and the gas atmosphere were slightly modified: concentrations of ferric ammonium citrate, \( \text{NH}_4\text{Cl} \) and \( \text{NiCl}_2 \) were changed from 1.2 to 50 \( \mu \text{g} \cdot \text{mL}^{-1} \), 18.7 to 37.4 mM and 0.3 to 0.9 \( \mu \text{M} \), respectively. The gas atmosphere was composed of 5% \( \text{O}_2 \), 10% \( \text{CO}_2 \) and 85% \( \text{H}_2 \). Autotrophic cultures (up to 50 ml) were shaken in 300 ml Erlenmeyer flasks connected with the gas mixture contained in 10 l gasometer bottles. At intervals of 5–10 h the bottles were refilled with fresh gas mixture. In cultures used for RNA isolation, the high phosphate (36 mM) buffer of the media was replaced by 2.0 mM phosphate and 30 mM MOPS buffer (pH 7.0) to avoid precipitation of phosphate during RNA extraction.

*\text{A. eutrophus} \) strains were grown autotrophically in mineral medium and heterotrophically in nutrient broth at 30 °C as described previously (Kusian et al., 1995).

*\text{E. coli} \) strains were routinely grown in LB (Luria–Bertani) medium (Sambrook et al., 1989) under standard conditions. In order to prevent inclusion body formation in recombinant \( \text{E. coli} \) clones resulting from overexpression of the cloned SH genes, cells were cultivated at 25 °C and with a restricted oxygen supply. Growth was followed by measuring the optical density using a Shimadzu UV-1202 spectrophotometer and 1 cm cuvettes. Samples were diluted in the respective medium if necessary. The ODs of \( \text{R. opacus} \), \( \text{A. eutrophus} \) and \( \text{E. coli} \) were measured against the cell-free media at wavelengths of 550, 436 and 600 nm, respectively.

**Nucleic acid isolation and manipulation.** For isolation of the linear plasmid pHG201 of \( \text{R. opacus} \) strain MR2246, the cells were embedded in agarose and lysed according to McClelland et al. (1987) using lysozyme, \( \text{N-lauroylsarcosine, SDS and proteinase K} \) as described by Kalkus et al. (1993). Separation of linear DNA from chromosomal DNA was achieved by electrophoresis of agarose plugs containing high concentrations of genomic DNA. Large amounts of plasmid DNA from \( \text{E. coli} \) were prepared by using the Qiagen Plasmid Midi kit according to the manufacturer’s protocol. \( \text{E. coli} \) plasmids were detected according to Holmes & Quigley (1981).

For isolation of total RNA, \( \text{R. opacus} \) cells were grown in 30 ml of the respective low phosphate media to an OD of 3 of 5. Cultures of \( \text{E. coli} \) were grown in LB and harvested in the mid-exponential, late exponential and stationary phase at OD of values of 0.5, 0.9 and 3.0, respectively. Cells were harvested as described by Kusian et al. (1995). Total RNA was isolated according the method of Oelmüller et al. (1990a).
DNA and RNA were manipulated by standard methods (Sambrook et al., 1989). DNA fragments used in ligations or labellings were isolated from agarose gels either by using the Qiaex Kit (Qiagen) or by electroelution using an apparatus (HSB-elutor) obtained from Biometra, according to the manufacturers' protocols. Cells of Escherichia coli and Alcaligenes eutrophus were isolated from agarose gels either by using the Qiaex Kit (Qiagen) or by electroelution using rubidium chloride as described by Hanahan (1983).

The subcloning of SH structural genes of R. opacus on pHG201 was performed in heterologous hybridization studies using DNA fragments containing different parts of the A. eutrophus H16 SH locus as probes. As sources for the isolation of these fragments, the recombinant plasmids pCH233, pCH235, pGE22 and pAM10 were used, which were provided by B. Friedrich. [For references of the last three plasmids see Oelmüller et al. (1990b).] pCH233 and pCH235 were generated by cloning 1.1 kbp ClaI and 1.45 kbp KpnI fragments of the SH locus into the vector pTZ19R, pGE22 by cloning a 2.7 kbp HindIII–BamHI insert of pGE22 into the pBluescript II SK+ vector. pAM10 contains the structural genes hoxY, hoxU, as well as the upstream region of the SH locus including the SH promoter; the 1.2 kbp EcoRI fragment of pAM10 contains the structural genes hoxY and hoxU; the 1.1 kbp ClaI insert of plasmid pCH233 contains hoxH; and the 1.45 kbp KpnI insert of plasmid pCH235 contains hoxH and the region downstream of the SH structural genes.

**Table 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype and/or phenotype*</th>
<th>Source or reference</th>
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<tr>
<td><strong>Rhodococcus opacus</strong></td>
<td></td>
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<tr>
<td>MR11</td>
<td>Aut+; circular plasmids pHG31a and pHG31b; linear plasmids pHG201, pHG202 and pHG203</td>
<td>DSM 427; Aggar &amp; Schlegel (1973)</td>
</tr>
<tr>
<td>MR1463</td>
<td>Aut+, Sm+; linear plasmid pHG202</td>
<td>Reh &amp; Schlegel (1981)</td>
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<tr>
<td>MR2246</td>
<td>Aut+, Sm+; linear plasmid pHG201</td>
<td>Sensfuss et al. (1986)</td>
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<tr>
<td><strong>Alcaligenes eutrophus</strong></td>
<td></td>
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<tr>
<td>HFI4</td>
<td>SH+</td>
<td>Schlesier &amp; Friedrich (1982)</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacI8 lacZAM15 Tn10 (Te')]</td>
<td>Stratagene cloning systems</td>
</tr>
<tr>
<td>BMH71-18</td>
<td>supE \Lambda(lac–proAB) mutS::Tn10 (Te') [F'lacI8AM15]</td>
<td>W. Kramer, Georg-August-Universität Göttingen, Germany</td>
</tr>
<tr>
<td>S17-1</td>
<td>recA pro thi hsdR cbr::RP4-2, IncP</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pBluescript II SK+</td>
<td>lacPOZ′ ColE1 ori f1 ori, Ap′, T3 and T7 promoters</td>
<td>Stratagene</td>
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<tr>
<td>pBluescript II KS–</td>
<td>lacPOZ′ ColE1 ori f1 ori, Ap′, T3 and T7 promoters</td>
<td>Stratagene</td>
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<tr>
<td>pMc5-8</td>
<td>Cm′, Ap′ (in supE host strains)</td>
<td>Stannenss et al. (1989)</td>
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<td>pMP92</td>
<td>Te′</td>
<td>Spaink et al. (1987)</td>
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<td>pSQS01</td>
<td>pBluescript II SK+ ‖ 7.4 kbp AsnI fragment</td>
<td>This study</td>
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<td>pSQK01</td>
<td>pBluescript II KS– ‖ 7.4 kbp AsnI fragment</td>
<td>This study</td>
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* Aut, autotrophic growth; Ap′, ampicillin resistance; Cm′, chloramphenicol resistance; Sm′, streptomycin resistance; Te′, tetracycline resistance.

**Southern hybridization.** DNA samples were digested to completion with the appropriate endonucleases, and the resulting fragments were separated by agarose gel electrophoresis. According to the sizes of the fragments, separation was achieved by conventional gel electrophoresis or by pulsed-field gel electrophoresis as described previously (Kalkus et al., 1993). Treatment of the gel prior to the Southern transfer, the transfer procedure and fixation of the DNA to nylon membranes, as well as preparation of biotinylated DNA probes were done according to Kalkus et al. (1993). Hybridization and detection of biotinylated probes was carried out according to Oelmüller et al. (1990a).

**Northern hybridization.** Total RNA was separated by agarose gel electrophoresis in the presence of formaldehyde and transferred to nylon membranes as described by Sambrook et al. (1989). As probes, DNA fragments were used which had been labelled with [α-32P]dATP using the random primer technique (Gibco/BRL). Hybridization was performed as described by Oelmüller et al. (1990a).

**Nucleotide sequencing.** Sequencing was done by primer walking with the universal primer and primers deduced from...
the nucleotide sequence by the dyeoxy method of Sanger et al. (1977). All sequencing reactions were carried out with the Sequenase version 2.0 DNA sequencing kit (US Biochemical) and [35S]dATP instead of gGTP. Double-stranded as well as single-stranded DNA was used for sequencing. Single-stranded DNA was prepared according to Sambrook et al. (1989) with the aid of helper phage M13K07 (Vieira et al., 1987) isolated from E. coli MV1184. Sequence data were analysed with a Genetics Computer Group Sequence Analysis Package (version 6.2) on a Macintosh computer as described by Devereux et al. (1984). Sequence comparisons were done with the databases of the National Center of Biotechnology Information (Bethesda, MD, USA), including GenBank release 82.0, EMBL release 37.0, SWISS-PROT release 28.0 and PIR release 39.0.

**Primer extension.** Primer extension studies were performed as described by Kusian et al. (1995) except that SuperScript-plus reverse transcriptase (Gibco/BRL) was employed. As primers, three 17-mer oligonucleotides (see text), which were complementary to the 5' region of the SH transcript, were synthesized and radiolabelled at the 5' end with [γ-32P]ATP using T4 polynucleotide kinase. In order to determine the exact start point (5'-terminus) of the mRNA, the cDNA was electrophoresed next to a standard sequencing reaction which was prepared using the same 17-mer oligonucleotides. The densitometric evaluation of the autoradiograms was performed on a Macintosh computer using the programs Ofoto 2.0 and NIH Image 1.44.

**Preparation of cell extracts.** After harvesting of cells, the pellets were washed twice with 0.5% culture volumes of ice-cold 50 mM Tris/HCl (pH 8.0), 50 mM MgSO4, and 0.5 mM NiCl2, and resuspended in 1 mL of the same buffer. Cells were sonicated on ice using a Ultrasonic Disintegrator. The resultant sonicates were designated 'crude cell extracts'. Remaining intact cells, cell debris and membranes were sedimented by centrifugation at 100,000 g for 1 h at 4 °C. The supernatant was designated 'soluble extract'. The resulting pellets were resuspended in the wash buffer and designated 'particle fraction'.

**Western immunoblot analysis.** Proteins were fractionated by electrophoresis in 12% (w/v) SDS-polyacrylamide gels according to Laemml (1970). Transfer of the proteins onto PVDF membranes using a semidyed blotting device (Biometra) and detection of the immunoreactive proteins was performed according to Sambrook et al. (1989). For the detection of hydrogenase proteins, antibodies raised against the native NAD-reducing hydrogenase of R. opacus MR11 and against its native dimers were used, which were kindly provided by C. Zaborosch. They had been raised and prepared according to Schneider et al. (1994a).

**Enzyme assays and detection of catalytically active hydrogenase dimers in polyacrylamide gels.** Hydrogenase (H2:NAD oxidoreductase) activity of the native enzyme was assayed by measuring the reduction of NAD photometrically in 1 cm cuvettes at 365 nm and at 30 °C (Schneider et al., 1984a) using an Eppendorf filter photometer. For determination of hydrogenase activity in the soluble extract, the reaction mixture (3 mL) contained 50 mM H2-saturated Tris/HCl (pH 8.0), 50 mM MgSO4, 0.5 mM NiCl2, 0.9 mM NAD and an appropriate amount of enzyme. In the case of A. eutrophus strains SH activity was determined using permeabilized whole cells at the same wavelength and temperature according to Oelmüller et al. (1990b). For detection of hydrogenase (H2; acceptor oxidoreductase) activity and diaphorase (NADH:acceptor oxidoreductase) activity in native polyacrylamide gels, proteins of the soluble extracts were separated by electrophoresis in gels containing 7.5% (w/v) acrylamide according to Jovin et al. (1964). Hydrogenase activity-staining with methyl viologen and triphenyltetrazolium chloride as acceptors was carried out as described by Fiebig & Friedrich (1989). Incubation for diaphorase-activity-staining was done in a nitrogen atmosphere, and NADH was used as electron donor.

**RESULTS**

**Cloning and localizing of hydrogenase genes**

Previous work had shown that the genes of the hydrogenase of R. opacus are localized at the right end of the linear plasmid pHG201 (Kalkus et al., 1990). Further localization was done by heterologous hybridization, using DNA fragments of the gene cluster of the soluble hydrogenase (SH locus) of A. eutrophus H16 as DNA probes (Tran-Betcke et al., 1990). In this way the four structural genes of the R. opacus hydrogenase were localized on a 7.4 kbp AsnI fragment (Fig. 1). This was cloned into the single AsnI cleavage site of the vector plasmid pMC5-8 and transferred to E. coli BMH71-18. In order to test for the functionality of the cloned hydrogenase genes, the AsnI fragment was cloned into the broad-host-range vector pMP92 and transferred to E. coli S17-1. The plasmid was transferred by conjugation to the mutant HF14 of A. eutrophus. This mutant contains the membrane-bound hydrogenase of the wild-type but a defective SH which consists of only a single subunit (Hornhardt et al., 1990) and cannot reduce NAD. The transconjugants obtained were able to grow under autotrophic conditions much faster (doubling time 7-1 h) than the mutant HF14 (doubling time 10-2 h), and the permeabilized cells were able to reduce NAD with H2. This result indicated that the cloned genes were functional in A. eutrophus.

For sequencing, the 7.4 kbp AsnI fragment was cloned into the pBluescript vectors SK+ and KS−. The overlapping ends of the AsnI fragments were replenished, and the fragments were inserted into the EcoRV cleavage site of the pBluescript vectors via their blunt ends. From E. coli XL1-Blue, two clones, LHY1 and LHY2, were obtained which contained the hybrid plasmids pSQS01 and pSQK01, respectively (Table 1). In both hybrid plasmids the AsnI fragment was cloned in the same orientation with respect to the orientation of the lacZ′ gene. Both E. coli clones showed slow growth and formed inclusion bodies. The Western immunoblot analyses indicated that the inclusion bodies consisted of SH proteins which were expressed by the cloned fragment. The proteins were not enzymically active.

**Subclones and DNA sequence**

For sequencing, the AsnI fragment was shortened by subcloning. Ten subclones were obtained from pSQS01 and nine subclones from pSQK01 (Fig. 1). Using these clones and applying primer walking both strands of the AsnI fragment were completely sequenced. The nucleotide sequence comprised 7388 bp, and the G + C content
Soluble hydrogenase locus of *Rhodococcus opacus*

Fig. 1. Map of the 7.4 kbp *AsnI* fragment. (a) Localization of the 7.4 kbp *AsnI* fragment on the linear plasmid pHG201. (b) Restriction map of the 7.4 kbp *AsnI* fragment constructed using ten different endonucleases. This coincides with the map obtained from the nucleotide sequence data except for the second *BglII* and the second *ApaI* sites. (c) Organization and orientation of the ORFs. (d) Plasmid inserts of the subclones. (e) Synthetic primers used for primer walking sequencing.

amounted to 56.98 mol%. Seven open reading frames, ORF1–ORF7, were detected, all with the same orientation. ORF7 is not complete on the cloned fragment. All ORFs are preceded by potential ribosome-binding sites. ORF2–ORF7 were identified as genes of the SH locus of *R. opacus* and their nucleotide sequence is shown in Fig. 2. On the basis of the high homology of the derived gene products to those of the SH locus of *A. eutrophus* H16, the ORFs were given the same designations used for the genes of strain H16 (Tran-Betcke et al., 1990). The four sequential genes *hoxF*, *-U*, *-Y* and *-H* code for the α, γ, δ and β subunits of the SH, respectively. The amino acid sequences of the derived gene products (Fig. 2) exactly coincide with the N-terminal sequences of the subunits purified by Zaborosch et al. (1989). With the exception of the δ subunit, the subunits lack the initial methionine residue, which is probably removed by post-translational cleavage. Comparison of the molecular masses of the proteins determined by SDS-PAGE (Schneider et al., 1984a) with those determined by sequence analysis revealed relatively large deviations in the case of the small molecules of the γ and δ subunits (64.0 and 66.6 kDa for the α subunit, 56.0 and 51.6 kDa for the processed β subunit, 31.0 and 25.9 kDa for the γ subunit, 27.0 and 23.1 kDa for the δ subunit, respectively). These differences are probably due to the limited precision of the SDS-PAGE molecular mass determinations. When calculating the molecular masses of the four subunits from the derived amino acid sequences the post-translational modification of the N-terminal methionine residue was taken in consideration. In the case of the β subunit, C-terminal processing, which has been described for hydrogenases of other bacteria (Thiemermann et al., 1996) and obviously occurs with the *R. opacus* MR11 enzyme as well (see below and Figs 4, 5) was also taken into account.

The two genes *hoxW* (165 amino acid residues, 18.6 kDa) and ORF7 (122 amino acid residues, incomplete) are located downstream of the structural genes of the SH locus of *A. eutrophus* H16, and organized as an operon. The stop and start codons of the genes *hoxF*, *-U* and *-Y* overlap. The genes *hoxY* and *hoxH* are separated by an intergenic region of 16 nucleotides. The start codon of *hoxW* lies within *hoxH*, 14 nucleotides in front of its stop codon. ORF7 and *hoxW* are separated by 21 nucleotides. A weakly conserved consensus sequence for σ 54-dependent promoters was found in the intergenic region between *hoxF* and ORF1 in position 1834–1850. A well-conserved consensus sequence for
Fig. 2. Nucleotide sequence of the region of the 7.4 kbp Asnl fragment carrying the ORFs homologous to \( \text{hoxF, } -\text{U, } -\text{Y, } -\text{H, } -\text{E} \), \( \text{m} \) and \( \text{ORF7} \). Derived amino acids are given in the one-letter code. Potential ribosome-binding sites (SD) and putative \( \sigma^{70} \)-dependent promoters are underlined. The start sites of the hydrogenase mRNA in \( R. \text{opacus} \) MR11 (R) and in \( E. \text{coli} \) LHY1 (E) as determined from primer extension analyses are indicated by arrows below the nucleotide sequence. N-terminal amino acids of the isolated hydrogenase proteins and their degradation (Zaborosch et al., 1989) are underlined. The initiator methionines absent in the protein sequences of the subunits are in parentheses.

\( \sigma^{70} \)-dependent promoters is located at the right end of ORF1 in position 1689–1718. ORF1 codes for a putative transposase. As it has no relationship to the SH operon it will be described elsewhere.

Although the G + C content of the sequenced fragment is relatively low (56-98 mol\%) in comparison to the total DNA from \( R. \text{opacus} \) MR11 (64-8 mol\% ; Reh & Schlegel, 1973) codon preference for cytosine or guanine in the third position is recognizable. Among the codons of the seven protein-encoding sequences, the G + C content in the third position is 69 mol\%, in the first position 60.7 mol\% and in the second position 41.1 mol\%. These relationships correspond to the rule...
of Bibb et al. (1984) for G + C-rich DNA: position 3 > 1 > 2.

Amino acid similarity

The amino acids sequences that are encoded by the seven genes were compared with those in the NCBI databanks using the program TBLASTN (Altschul et al., 1990).

Structural genes. The highest homologies of the four SH subunits of R. opacus MR11 were obtained with the corresponding subunits of the SH from A. eutrophus H16 (Tran-Betcke et al., 1990) and Anabaena variabilis (Schmitz et al., 1995). The similarity values against the A. eutrophus gene products of boxF, -U, -Y and -H amounted to 89.9, 92.3, 91.4 and 93.7%, and against the corresponding Ab. variabilis gene products 57.0, 54.8, 57.1 and 66.6%. The similarities for the subunits of the hydrogenase dimer against the corresponding subunits of the methyl-viologen- and F₄₃0-reducing hydrogenases of methanogenic bacteria were also high. All hydrogenases mentioned so far are assigned to class IV according to Wu & Mandrand (1993). The homologies against the corresponding subunits of the membrane-bound and periplasmic NiFe(Se)-hydrogenases belonging to classes I and II sequenced to date were significantly lower. While the similarity values of the β subunit against the large subunits of the membrane-bound and periplasmic hydrogenases were still significant, the similarities of the δ subunit against the small subunits of the corresponding hydrogenases were beyond significance or were not recognized as homologous.

The subunits of the diaphorase dimer had high homologies with the products of the gene cluster of the NADP-reducing hydrogenase from Desulfovibrio fructosovorans (Malki et al., 1995). High homologies were also found against three peripheral subunits of the NADH:ubiquinone oxidoreductases of some bacteria (from E. coli (Weidner et al., 1993), Salmonella typhimurium (Archer et al., 1993) and Paracoccus denitrificans (Xu et al., 1991a, b, 1992)) and from the mitochondrial complex I of some higher organisms (from cattle (Pilkington et al., 1991; Pilkington & Walker, 1989; Runswick et al., 1989) and human (Pilkington & Walker, 1989; Ali et al., 1993; Chow et al., 1991) and the filamentous fungus Neurospora crassa (Preis et al., 1991; Azevedo et al., 1994)). These relationships have been repeatedly pointed out for the diaphorase dimer of A. eutrophus (Walker, 1992; Pilkington et al., 1991; Preis et al., 1991; Patel et al., 1991).

boxW and ORF7. The highest homologies for the gene products of boxW and ORF7 of R. opacus were found against the gene products of boxW and ORF2 (formerly boxI) from the SH locus of A. eutrophus. The similarities to these gene products amounted to 84%. Whereas the boxW gene of A. eutrophus was shown to code for a protease involved in hydrogenase maturation, the function of the ORF2 gene product is not clear (Thiemermann et al., 1996). For the boxW gene product, no additional homologies have been detected. The ORF7 gene product showed significant homologies with eukaryotic cAMP- and cGMP-dependent protein kinases as well as with the cAMP-receptor-protein (CRP) from E. coli. The highest similarity (51%) was that against the bovine cGMP-dependent protein kinase (Wernet et al., 1989). Further significant homologies were found with the human cGMP-dependent protein kinase (Sandberg et al., 1989) and with the regulatory subunit of the cAMP-dependent protein kinase from Aplysia californica (Bergold et al., 1992), and lower homologies with the regulatory subunits of cAMP-dependent protein kinases from mammals (human, cattle, pig and rat).

Formation of mRNA

The transcription of the SH genes in R. opacus MR11 and E. coli LHY1 was studied by Northern blot analyses.

R. opacus. Total RNA was isolated from strain MR11 grown under autotrophic conditions and, as a negative control, from heterotrophically grown cells of MR11 and MR1463. The latter is a MR11 mutant lacking the autotrophy plasmid pHG201 and therefore does not contain hydrogenase genes at all. MR1463 was included since in heterotrophically growing cultures of strain MR11 after entering the stationary phase a low hydrogenase activity is detectable (unpublished data). All cultures were harvested in the mid-exponential phase as this is when the specific hydrogenase activity in autotrophically growing cultures of MR11 is highest. A 3.64 kbp Apal fragment (position 2443–6083), which contains the four SH structural genes except the beginning of boxF and the end of boxH, was used as DNA probe. The Northern blot analysis showed that the hydrogenase genes are transcribed in MR11 under autotrophic conditions (Fig. 3a) and, as indicated by the signal intensity, under heterotrophic conditions at a fairly low level. The signals appeared as blackening of the total lane. The blackening started at about 7.5 kb and ended at 0.2 kb. This suggests that a relatively long polycistrionic transcript with maximal size of about 7.5 kb is formed. The blackening is probably due to synthesis and processing/degradation of the large SH mRNA. This appears to be typical for many prokaryotic operon structures (Newbury et al., 1987; Oelmüller et al., 1990b). No hybridization signal was detected in the total RNA of MR1463.

E. coli. For examining the transcription of the SH genes in E. coli LHY1, the total RNA of this strain was isolated from cells harvested in three different growth phases: mid-exponential, late exponential and stationary phase. Northern hybridization using the Apal probe described above revealed the presence of hydrogenase-specific mRNA in all three preparations (Fig. 3b). The signal patterns were the same as those obtained with the R. opacus RNA. The three preparations gave nearly the same signal intensity. This result indicates that the transcription of the genes in E. coli was constitutive. No hybridization signals were detected with the total RNA.
of strain XL1-Blue(pBluescript SK+* ) harvested in the corresponding growth phases mentioned above.

**Start of transcription**

The start point of the hydrogenase-specific mRNA was determined by primer extension analysis. Three different oligonucleotides were used, which differ with respect to the position of the ATG codon of the first gene within the SH operon, *hoxF*. P1 (5′ CCACCTTGCAACCAATAAG 3′) lies 47 nucleotides upstream of the ATG, while P2 (5′ CGAGATTGCCTTGATG 3′) and P3 (5′ CAGATGCTGAACATCCC 3′) map 11 and 70 nucleotides, respectively, downstream of this ATG. For the hydrogenase-specific mRNA of the autotrophically and heterotrophically grown MR11 cells, as well as that of *E. coli* LHY1 (mid-exponential-phase cells), the transcription start point was determined with all three oligonucleotides 170 nucleotides upstream of the ATG codon of the *hoxF* gene (Fig. 3c). The start point of the primary transcript in MR11 was the guanine in position 1726 and in *E. coli* LHY1 a thymine in position 1725. Immediately in front of the transcription start a nucleotide sequence...
was localized which resembles the consensus sequence of σ70-dependent promoters. It lies at the right end of ORF1; this is a perfect -35 region (TTGACA) with an almost perfect -10 region (TAAAGT) separated by a distance of 18 nucleotides (Figs 2, 3c). This highly conserved promoter sequence explains the constitutive transcription of the hydrogenase genes in E. coli LHY1. The ratio of hydrogenase-specific mRNA from autotrophically to heterotrophically grown MR11 cells was estimated by densitometric evaluation of the primer extension signals and was 100:1. This result suggests that the SH genes are not constitutively transcribed in strain MR11. The primer extension analyses of the total RNA of the controls (MR1463 and E. coli XL1-Blue with pBluescript SK+) did not reveal any signals.

**SH in heterotrophically grown cells of R. opacus**

**MR11 and in cells of E. coli LHY1**

**R. opacus.** Western immunoblot analyses showed that the hydrogenase-specific mRNA, which is formed in MR11 under heterotrophic conditions, gave rise to the synthesis of hydrogenase proteins. Soluble crude extracts were prepared from a batch of those R. opacus cells which had been grown for the isolation of total RNA. The proteins were separated by SDS-PAGE and studied by Western blot analyses using polyclonal antibodies which were raised against the native SH of strain MR11. The signals indicated that in heterotrophically grown cells of MR11 the four subunits of the SH had been formed. However, in the position of the β subunit a double band was detected; one of these bands was in the position of the β subunit detected in the soluble extract of autotrophically grown cells and the other was located immediately above this band (data not shown). The double band indicated that heterotrophically grown cells contained the processed as well as the unprocessed form of the β subunit. The extract of heterotrophically grown cells of MR1463 was free from SH proteins. Measurements of the hydrogenase activities in the soluble extracts indicated, however, that the proteins formed in the heterotrophically grown MR11 cells of the exponential phase did not possess any detectable catalytic activity. Further experiments with heterotrophically growing cultures of strain MR11 indicated that the H2-dependent reduction of NAD becomes detectable after the culture entered the stationary phase. The highest specific hydrogenase activity amounted to only 12% of that of autotrophically grown exponential-phase-cells.

**E. coli.** To find out whether the inclusion bodies formed in E. coli LHY1 consist of SH proteins, extracts of this strain and of the necessary controls were also studied by Western immunoblot. Strain LHY1 was grown under standard conditions and harvested in the stationary phase. Strain XL1-Blue (pBluescript SK+) served as a control. The crude cell extracts of these strains and a soluble extract of autotrophically grown cells of R. opacus MR11 were subjected to SDS-PAGE and studied by immunoblot. Five hydrogenase-specific bands were detected in the extract of LHY1 (Fig. 4); the extract of XL1-Blue did not show any signal. Three of the five bands coincided with the α, γ and δ subunits of the hydrogenase of autotrophically grown cells of MR11. The band corresponding to the β subunit moved more slowly than the β subunit of MR11 cells, indicating that the precursor of the β subunit is not processed in E. coli. The fifth subunit was located slightly below the γ subunit. The identical mobilities of the α, γ and δ subunits and the different mobilities of the latter two subunits was confirmed by Western blot with a mixture of equal amounts of the extracts from MR11 and LHY1. The role of the band which ran below the γ subunit has not yet been elucidated. Further Western immunoblots with the crude cell extracts of E. coli LHY1 and antibodies raised against the native βδ dimer and the αγ dimer indicated that the protein is related to the αγ dimer.

The ready expression of the SH genes in E. coli LHY1 raised the question of whether a catalytically active enzyme can also be formed. Since proteins aggregated in inclusion bodies are catalytically inactive and Western immunoblots with the soluble extract and the particle fraction prepared from the whole cell extract of LHY1 indicated that the SH proteins synthesized were quantitatively aggregated, other conditions for cultivation had to be chosen. When the cells were grown under conditions resulting in slow growth (low temperature, impaired oxygen supply), aggregation of the hydrogenase proteins was suppressed, and in the soluble fraction of the crude extract the hydrogenase proteins became detectable. However, only the α subunit and the unprocessed form of the β subunit became detectable as distinct bands. At the position of the small subunits only a diffuse band was detected. Obviously these proteins were degraded. When the proteins of the soluble fraction were separated by native PAGE and tested for hydrogenase and diaphorase activity in the gel none were detectable, while the bands obtained with the MR11 extract showed significant activities.

**DISCUSSION**

When the amino acid sequences of the subunits of the R. opacus MR11 and A. eutrophus H16 SHs were aligned (Fig. 5) no striking differences were found which could explain the dissociating character of the R. opacus SH and the non-dissociating character of the A. eutrophus SH. Amino acids of the R. opacus subunits substituted by amino acids with different or similar properties in the A. eutrophus subunits are not clustered in certain regions but are distributed throughout the sequences. Additional comparative studies using computer programs to characterize the peptides with respect to their secondary structures and their hydrophobic and hydrophilic regions did not reveal striking differences either. With respect to the primary structure, the molecular masses of the corresponding subunits are almost identical (66.6 and 66.7 kDa for the α subunits, 51.6 and 52.2 kDa for the processed β subunits, 25.9 and 26.0 kDa for the γ subunits, 23.1 and 22.9 kDa for the δ subunits of R. opacus).
opacus and A. eutrophus (Tran-Betcke et al., 1990), respectively. This suggested identical electrophoretic mobilities of the corresponding subunits in SDS-PAGE. However, recent studies showed that the identity concerns only the α and the δ subunits (Grzeszik et al., 1997). The β subunit of the R. opacus enzyme migrates faster and the γ subunit more slowly than the subunits from A. eutrophus indicating the β and the γ subunits from R. opacus to be lighter and heavier, respectively. The difference between the electrophoretic mobilities of these subunits could be due to different chain lengths, as a consequence of different post-translational processing, or simply due to abnormal electrophoretic mobilities, as has been described for other Fe–S proteins (Schmitz et al., 1995). Regarding the β subunit, a post-translational processing different from that of the A. eutrophus β subunit seems to be very unlikely. Since the N-terminal amino acid sequence determined by Edman degradation coincides with the derived amino acid sequence (Fig. 2), the N-terminus is not processed. In contrast, the results of the Western immunoblot analyses with extracts of E. coli LH11 and heterotrophically grown cells of R. opacus MR11 indicate that processing occurs at the C-terminus as has been described for the nickel-containing subunits of other hydrogenases (Gollin et al., 1992; Volbeda et al., 1995; Sorgenfrei et al., 1993), including the β subunit of the soluble enzyme of A. eutrophus (Thiemermann et al., 1996). The processing site was determined as C-terminal to a histidine residue localized at the end of the consensus motif DPCx5Cx3H. In the large subunit of the E. coli hydrogenase 3 this histidine is substituted by an arginine residue, but processing occurs at the same site (Rossmann et al., 1994). The motif DPCx5Cx3H is also fully conserved in the β subunit of R. opacus MR11. Since amino acids of this motif were shown to participate in nickel coordination and therefore are essential for catalytic activity (Volbeda et al., 1995; Albracht, 1994; Przbyla et al., 1992), processing of the R. opacus β subunit in front of this motif is unlikely. Therefore, the different mobilities of the β subunits of R. opacus and A. eutrophus are apparently not due to different chain lengths. It is unclear at present whether the same is true for the γ subunits.

High homologies do not only concern the gene products of the structural genes but also those between the accessory genes boxW and ORF7 from R. opacus MR11, and boxW and ORF2 from A. eutrophus H16. Even with these genes comparison of the proteins by alignment did not reveal marked differences in the primary structures (Fig. 5). In strain H16, HoxW was recognized to be a protease of high specificity which removes a 24 amino acid residue peptide chain from the C-terminus of the β subunit before assembly of the four subunits occurs (Thiemermann et al., 1996). However, the function of the ORF2 gene product in H16, the so-called B protein (Kärst et al., 1987), has not yet been elucidated. An alignment of the ORF7 and ORF2 gene products of R. opacus and A. eutrophus with the cGMP- and cAMP-dependent kinases of cattle and Ap. californica and the E. coli cAMP-receptor-protein (CRP) showed that the central region of the ORF7 and ORF2 gene products between positions 46 and 115 were homologous to the cAMP/cGMP-binding regions of the other proteins. This result, and the property of the B protein of H16 to form dimers in the native state (Kärst et al., 1987), like the E. coli CRP and the kinases, suggests that this protein may be involved in signal transduction pathways. But interestingly, ORF2 deletion mutants of H16 revealed no recognizable phenotype (Thiemermann et al., 1996). The fact that no homologies to other accessory or pleiotropic proteins of NiFe(Se)-hydrogenases have been found allows the conclusion that these gene products are only involved in biosynthesis and maturation of the NAD-reducing hydrogenases.

The high homology between the corresponding gene products is reflected by the high homology between the nucleotide sequences. Not only is the arrangement of the four structural and the two accessory genes the same, but also the relative positions of the start and stop codons of the genes. The long inverted repeat, which
was found in H16 11 nucleotides downstream of the stop codon of boxH (Tran-Betcke et al., 1990) does not exist in MR11. The comparison of the nucleotide sequences of the SH loci of the two bacteria showed that the sequences including the coding regions are 84% identical. The high coincidence ends immediately in front of the start codon of boxF. This high degree of identity suggests that the SHs of the two bacteria have a common ancestor. However, the signal sequences, which are essential for gene regulation, are apparently different.

In alignments of the SH subunits of R. opacus MR11 with the corresponding subunits of other NiFe(Se)-hydrogenases, diaphorases and NADH:ubiquinone oxidoreductases, conserved sequence motifs were identified, the amino acids of which are thought to participate in the coordination of the metal centres and cofactors (Fig. 5).

**Hydrogenase dimer**

The β subunit contains four conserved motifs common to all nickel-containing subunits of uptake hydrogenases: R̂GxF̂E₁₆₋1₉RX̂CGx̂Cx₅₇H (1L-2L), Hx₅L (3L), Gx₉PRGx₅₇H (4L) and DPCx₅₇Cx̂₂H (5L), and the δ subunit 1 motif present in the N-terminal region of all small subunits of NiFe(Se)-hydrogenases: Cx₂₅₈₋₆₁Gx₅₇Cx₉θ₄₋₅θGCPP (1S-3S; designations of the motifs are according to Albracht, 1994). The C-terminal region of the small subunits of the membrane-bound and periplasmic enzymes is missing in the δ subunit of R. opacus MR11 as is the case for the corresponding subunits of the NAD-dependent hydrogenases of A. eutrophus H16 and Ab. variabilis, and hydrogenase 3 of E. coli (Albracht, 1994; Schmitz et al., 1995). Information about the involvement of the amino acids of the motifs in the coordination of nickel and Fe-S centres was obtained by spectroscopic studies of NiFe(Se)-hydrogenases (Albracht, 1994) and by crystallographic studies on the periplasmic hydrogenase of Desulfovibrio gigas (Volbeda et al., 1995). The large subunit contains the binuclear metal centre which is coordinated by the cysteine residues of the motifs 1L-2L and 5L. The small subunit coordinates the Fe-S centres; as shown in the small subunit of the D. gigas hydrogenase a [4Fe-4S] cluster is coordinated by the four cysteines of the motif 1S-3S. Besides a nickel and a [4Fe-4S] centre no isolated metal atom has been found in the βδ dimer of the R. opacus MR11 enzyme (Zaborosch et al., 1995), but recent studies suggest the presence of an additional metal atom in the active centres of NiFe(Se)-hydrogenases (Albracht, 1995). The conserved sequence motifs present in the β and the δ subunits indicate that the binuclear metal centre and the [4Fe-S] centre no isolated metal atom has been found (Zaborosch et al., 1995). Cysteine residues of this motif may be involved in the coordination of the metal centres and cofactors (Fig. 5).

**Diaphorase dimer**

The δ subunit is obviously a fusion product consisting of the homologous 24 kDa and 51 kDa subunits of the NADH:ubiquinone oxidoreductases of the mitochondrial complex I of higher organisms and the corresponding subunits of the NADH:ubiquinone oxidoreductases of the bacteria (Walker, 1992; Weidner et al., 1993). The N-terminal region of the δ subunits (residues 1-188) of R. opacus and A. eutrophus exhibits homologies to the 24 kDa subunit of the NADH:ubiquinone reductases. These subunits of the reductases probably contain a [2Fe-2S] centre (Walker, 1992) which presumably is coordinated by four highly conserved cysteine residues. A [2Fe-2S] centre is also present in the δ subunits of both knallgas bacteria (Friedrich & Schwartz, 1993; Zaborosch et al., 1995), but the δ subunit of MR11 contains only one of the four cysteines at the corresponding positions (75, 80, 116 and 120). The δ subunit of strain H16 contains, in contrast, two of these four cysteines (at positions 75 and 116), and the corresponding gene products of the NADH-reducing hydrogenase of D. fructosovorans and the NAD(P)-dependent hydrogenase of Ab. variabilis all four (Malki et al., 1995; Schmitz et al., 1995). With regard to the coordination of the [2Fe-2S] centres, in those of some proteins up to two, but not three, non-sulfur ligands are thought to be involved (for further citations see Albracht, 1994). The question as to whether the [2Fe-2S] centre in the δ subunit of MR11 is bound via the N-terminal region remains open.

The C-terminal region of the δ subunits shows high homology to the 51 kDa subunits of the NADH:ubiquinone reductases of complex I and the corresponding subunits of the bacterial enzymes. Here, consensus motifs for the binding sites of NAD(H) and flavin mononucleotide as well as cysteine motifs were detected which may serve as ligands for a [4Fe-4S] centre. The glycine-rich motif A (GxGx₅G; designations of motifs according to Preis et al., 1991) is considered to be the binding site of the adenine nucleotide unit of NAD(P). Motif B, also a glycine-rich conserved region, is probably the binding site for flavin mononucleotide. Motif C, which contains three cysteine residues as a cluster (Cx₅CxC), together with a conserved cysteine located 40 amino acids downstream, probably provides the ligands for a [4Fe-4S] cluster (Walker, 1992). According to Zaborosch et al. (1995) the δ subunit from MR11 contains a [3Fe-4S] cluster beside the [2Fe-2S] cluster. The conserved cysteine motif may be involved in binding the [3Fe-4S] cluster. Whether the [2Fe-2S] cluster in this part of the δ subunit can be coordinated remains unclear as the sequence lacks further typical clustered cysteine residues which are characteristic of this type of binding.

The γ subunit of the SH is homologous to the 75 kDa subunits from complex I and the corresponding subunits of the bacterial NADH:ubiquinone reductases. In all sequences compared, the motif Cx₅₁₋₅₂CRx₅₋₅₁Cx₅₁₋₅₂Cxx₅₋₅₁CXX₅₋₅₁CXX₅₋₅₁CXX₅₋₅₁CXX₅₋₅₁CXX₅₋₅₁CP has been found (Schmitz et al., 1995). Cysteine residues of this motif may be involved in the coordination of the two [4Fe-4S] centres which are present in the γ subunit of strain MR11 (Zaborosch et al., 1995). The three clustered cysteine residues CxxCxxC, to-
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Fig. 5. For legend see facing page.
Soluble hydrogenase locus of \textit{Rhodococcus opacus}

![Figure 5. Comparison of the deduced amino acid sequences of the four subunits \(\alpha\), \(\beta\), \(\gamma\) and \(\delta\) of the NAD-reducing hydrogenases of \textit{R. opacus} MR11 (R.o.) and \textit{A. eutrophus} H16 (A.e.) as well as the gene products of the corresponding accessory genes (the GenBank accession numbers of \textit{A. eutrophus} HoxUYH and HoxW and ORF2 are M55230 and X92988, respectively). For the comparison the alignment program \textit{GAP} based on the method of Needleman & Wunsch (1970) was used. The symbol ‘1’ between the sequences indicates identity; ‘*’ and ‘.’ indicate closer and further evolutionary distance between similar amino acids, respectively. Highly conserved amino acids present in the subunits of the hydrogenases and NADH:ubiquinone oxido-reductases are marked by +. Conserved sequence motifs are marked by \textit{HoxW}.

As shown by mRNA analyses, the \(\alpha7'\)-dependent promoter sequence upstream of the \(\alpha\) subunits of \textit{R. opacus} MR11 and MR22, requires further investigation.

As shown by mRNA analyses, the \(\sigma^{70}\)-dependent promoter at the right end of ORF1 which codes for a putative transposase and we therefore conclude that it primarily belongs to a transposable element. This assumption is confirmed by results of current studies on the identification of a transposable element in \textit{R. opacus} MR11. The main question of why much less SH mRNA is detectable in \textit{R. opacus} MR11 than in autotrophically grown cells leads to extensive speculation. This question is now being investigated with strains of \textit{R. opacus}, which at the position described (ORF1) do not contain a transposable element. The regulatory control of hydrogenase formation in both strains of \textit{R. opacus}, MR11 and MR22, requires further investigation.
enzyme (Zuber et al., 1986; Voordouw et al., 1985; Karube et al., 1983). In cells of E. coli LHY1 grown under standard conditions, all four SH subunits were detectable but were present in the form of inclusion bodies, and under conditions which avoided the formation of inclusion bodies the small subunits were exposed to proteolytic degradation. However, the Western immunoblots with the LHY1 crude cell extracts led to the detection of a fifth hydrogenase-specific band which is smaller than the γ subunit and is immunoreactive with antibodies raised against the native diaphorase dimer. Considering its size, this protein may be a modified product of the γ subunit.

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