JOURNAL OF BACTERIOLOGY, Oct. 1997, p. 6053–6060 Vol. 179, No. 19
0021-9193/97/$04.00+0

Purification and Molecular Characterization of the H_2 Uptake Membrane-Bound NiFe-Hydrogenase from the Carboxidotrophic Bacterium *Oligotropha carboxidovorans*

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Received 14 March 1997/Accepted 22 July 1997

The membrane-bound hydrogenase of *Oligotropha carboxidovorans* was solubilized with *n*-dodecyl-β-D-maltoside and purified 28-fold with a yield of 29% and a specific activity of 173 to 178 μmol of $H_2 \cdot min^{-1} \cdot mg^{-1}$. It is the first hydrogenase studied in a carboxidotrophic bacterium. The enzyme acts on artificial electron-accepting dyes, such as methylene blue, but is ineffective with pyridine nucleotides or other soluble physiological electron acceptors. Hydrogenase of *O. carboxidovorans* belongs to class I of hydrogenases and is a heterodimeric 101,692-Da NiFe-protein composed of the polypeptides HoxL and HoxS. Molecular cloning data revealed, that HoxL comprises 604 amino acid residues and has a molecular mass of 67,163 Da. Pre-HoxS comprises 360 amino acid residues and is synthesized as a precursor protein which is cleaved after alanine at position 45, thus producing a mature HoxS of 33,767 Da. The leader sequence corresponds to the signal peptide of small subunits of hydrogenases. The hydropathy plots of HoxL and HoxS were indicative for the absence of transmembranous helices. HoxZ has four transmembranous helices and is considered the potential membrane anchor of hydrogenase in *O. carboxidovorans*. Hydrogenase genes show the transcriptional order 5′ *hoxV* → *hoxS* → *hoxL* → *hoxZ* 3′. The *hox* gene cluster as well as the clustered CO dehydrogenase (*cox*) and Calvin cycle (*cbb*) genes are arranged within a 30-kb DNA segment of the 128-kb megaplasmid pHCG3 of *O. carboxidovorans*.

The carboxidotrophic bacterium *Oligotropha carboxidovorans* (formerly *Pseudomonas carboxydovorans* [35]) is characterized by the chemolithoautotrophic utilization of CO or H_2 plus CO_2 as sole sources of carbon and energy under aerobic conditions (31, 32). Compared to CO (generation time of about 16 h), H_2 plus CO_2 (generation time of about 7 h) supported faster growth (32). Experiments using ultracentrifugation or sucrose density gradient centrifugation identified hydrogenase of *O. carboxidovorans* as firmly bound to the cytoplasmic membrane (CM) (33). The enzyme was shown to reduce oxidized methylene blue, thionine, and other artificial electron acceptors (8). A soluble hydrogenase was absent. All of these studies were done at the level of subcellular fractions, and attempts to study purified hydrogenase from *O. carboxidovorans* have so far not been undertaken.

It was shown recently that the CO dehydrogenase structural genes (*cox*) and *orf4* reside on the 128-kb megaplasmid pHCG3 of *O. carboxidovorans* in the transcriptional order 5′ *coxM* → *coxS* → *coxL* → *orf4* 3′ (41). The *O. carboxidovorans* mutant OM5-12, cured from pHCG3, could neither grow with H_2 plus CO_2 nor oxidize CO (24). Mutant OM5-24 carries a 15-kb, and mutant OM5-29 carries a 54-kb, deletion in pHCG3 which renders them incapable of utilizing H_2 (24). The genes $cbbP_p$ and $cbbL_p$ encode phosphoribulokinase and the large subunit of ribulosebisphosphate carboxylase on plasmid pHG1 of *Ralstonia eutropha* (20) (formerly *Alcaligenes eutrophus* [46]). Heterologous gene probes identified $cbbP_p$ and $cbbL_p$ on pHCG3 of *O. carboxidovorans* (19). Apparently the genes encoding the key enzymes of chemolithoautotrophy in *O. carboxidovorans*, which are CO dehydrogenase, hydrogenase, ribulosebisphosphate carboxylase, and phosphoribulokinase, are pHCG3 encoded. In *R. eutropha*, the genes encoding ribulosebisphosphate carboxylase and membrane-bound or soluble hydrogenase are arranged on a 100-kb segment of the 540-kb plasmid pHG1 (22). We were therefore interested in the molecular characterization of the H_2 uptake hydrogenase of *O. carboxidovorans* and the analysis of the arrangement of *cox*, *cbb*, and *hox* genes on pHCG3.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used are listed in Table 1. *O. carboxidovorans* OM5 was grown chemolithoautotrophically in a 50-liter fermentor, using the mineral salts medium described previously (32) and a gas mixture composed of 50% (vol/vol) air, 40% (vol/vol) H_2, and 10% (vol/vol) CO_2 at a flow rate of 0.5 liter/min. Bacteria were grown heterotrophically in the presence of 0.3% pyruvate and 0.2% nutrient broth. *Escherichia coli* DH5α was grown in LB medium (3).

Assay of hydrogenase. The oxidation of H_2 by hydrogenase was monitored spectrophotometrically at 30°C and pH 7.0 as described before (33). In routine testing and if not otherwise indicated, methylene blue (50 μM) was used as the electron acceptor. All other acceptor concentrations were also 50 μM except that of 2-(4-iodo-phenyl)-3-(4-nitrophenyl)-2H-tetrazolium chloride (123 μM)–1-methoxyphenazinemethosulfate (17 μM).

Protein determination. The methods of Beisenherz et al. (4) and Bradford (6) were used for protein estimation.

Enzyme purification. All purification steps were carried out at 4°C at air. Chromatographic purification was done by fast protein liquid chromatography (Pharmacia, Freiburg, Germany). Frozen cells (50 to 70 g [wet weight]) were suspended in 150 ml of 20 mM HEPES-NaOH buffer) (pH 7.0) with the addition of few crystals of DNase I and disrupted in a high-pressure homogenizer (Rannie AS, Copenhagen, Denmark). Intact bacteria and bacterial fragments were removed from crude extracts by low-spin centrifugation. CM were obtained from crude extracts by ultracentrifugation for 3 h at 100,000 × *g*. The pelleted CM were washed by centrifugation in 0.25 M sucrose containing 0.1 M NaCl. Hydrogenase was solubilized by stirring suspensions of CM for 2 h in HEPES buffer containing 1 mg of *n*-dodecyl-β-D-maltoside · mg of protein^{-1}. After centrifugation at 100,000 × *g* for 1 h, the supernatant (170 ml) was loaded onto a hydroxylapatite column (20 by 5 cm) equilibrated with HEPES buffer. The HEPES buffer used for the purification of solubilized hydrogenase contained 0.05% Triton

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype or description	Reference or source
Bacteria		
O. carboxidovorans		
OM5	Wild type	32
OM5-12	Cox^- Cbb^+ Hox^-	24
OM5-24	Cox^+ Cbb^+ Hox^-	24
OM5-29	Cox^- Cbb^+ Hox^-	24
E. coli DH5α	φ80d *lacZ*ΔM15 *endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44 rpsL20 thi-1* λ^- *gyrA96 relA1* F^- Δ(*lacZYA-argF*)*U169*	21
Plasmids		
pHCG3	128-kb plasmid of *O. carboxidovorans* OM5	24
Δ^1 pHCG3	15-kb deletion derivative of pHCG3	24
Δ^2 pHCG3	54-kb deletion derivative of pHCG3	24
pUC18	Amp^r	Pharmacia
pBluescript I KS+/SK+	Amp^r	Stratagene
pBS19	19-kb *Sal*I fragment of pHCG3 in pUC18	This study
pBS2.2	2.2-kb *Eco*RV fragment of pBS19 in pBluescript I SK+	This study
pBSHL2.2	2.2-kb *Pst*I-*Hin*dIIII fragment of pBS19 in pBluescript I SK+	This study
pBS0.5	0.5-kb *Pst*I fragment of pBS2.2 in pBluescript SK+	This study
pBS1.4	1.4-kb *Pst*I fragment of pBS2.2 in pBluescript I SK+	This study
pBS5.7	5.7-kb *Sal*I fragment of pHCG3 in pBluescript I KS+	This study
pBS7.7	7.7-kb *Sal*I fragment of pHCG3 in pBluescript I KS+	This study
pBS7.6	7.6-kb *Sal*I fragment of pHCG3 in pBluescript I KS+	This study

X-100. After washing of the column with 400 ml of HEPES buffer, bound proteins were eluted with 300 ml of a linear gradient of 0 to 0.5 M NaH_2PO_4 in the same buffer. Hydrogenase was eluted at 0.05 M P_i. Fractions containing hydrogenase activity were pooled and adjusted to 30% (wt/vol) ammonium sulfate. After gentle stirring for 60 min, precipitated protein was removed by low-spin centrifugation. The supernatant, containing hydrogenase, was adjusted to 60% (wt/vol) ammonium sulfate. The hydrogenase-containing precipitate was resuspended in HEPES buffer and subjected to gel filtration on a Sephacryl G200 (Pharmacia) column (75 by 1.6 cm) equilibrated with HEPES buffer. Fractions with hydrogenase activity were concentrated by ultrafiltration and stored at −20°C until use.

Gel electrophoresis. Analytical polyacrylamide gel electrophoresis (PAGE) was carried out in a discontinuous system (26). For nondenaturing PAGE, a 5% (wt/vol) acrylamide stacking gel and a 7.5% (wt/vol) acrylamide running gel were used. For sodium dodecyl sulfate (SDS)-PAGE, a 7.5% (wt/vol) acrylamide stacking gel and a 12% (wt/vol) running gel were used. Protein staining was performed with Coomassie brilliant blue G250. Staining for H_2-oxidizing activity was by incubating gels in H_2-saturated buffer containing benzyl viologen and nitroblue tetrazolium chloride (10 mM each). Isoelectric focusing was performed with commercial IsoGel agarose plates (FMC Bio-products, Rockland, Maine).

Analysis of metals and acid-labile sulfur. Metal contents were estimated by inductively coupled plasma mass spectroscopy (model VG Plasmaquad PQ2 Turbo Plus; Fisons Instruments/VG Elemental, Wiesbaden, Germany). Acid-labile sulfur was determined by methylene blue formation from *p*-dimethylaniline (12).

N-terminal amino acid sequencing. The hydrogenase subunits were blotted from SDS-gels onto poly(vinylidene difluoride) membranes. Staining of blotted proteins was with amino black 10B. After destaining and drying of membranes, the blotted proteins were subjected to automated Edman degradation in a gas-liquid phase sequenator (model 477 A; Applied Biosystems) hooked to a phenylthiohydantoin amino acid analyzer (model 120; Applied Biosystems).

Preparation of DNA. The method of Marmur (27) for the preparation of total bacterial DNA was used. Plasmid DNA was prepared from *O. carboxidovorans* as described previously (24). Plasmid DNA from *E. coli* was isolated with Qiagen tip 100 (Qiagen, Hilden, Germany).

Oligonucleotides. OligoS was the 258-fold degenerate 17-mer GCNGGYTT NGTYTCCAT derived from the N-terminal sequence METKPR of HoxS. OligoL was the 2,048-fold degenerate 20-mer TGNACNGGRTCNACNACNAC derived from the sequence VVVDPVT of HoxL.

Labeling and hybridization of oligonucleotides or gene probes. Deoxyoligonucleotides were 3′ end labeled with digoxigenin-11-ddUTP. Restriction fragment gene probes were labeled with digoxigenin-11-dUTP by use of random primers (11). Hybridizations with oligonucleotides were carried out at room temperature and washes with 0.5 × SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 38°C (OligoS) and with 0.2× SSC–0.1% SDS at 42°C (OligoL), respectively. Restriction fragment gene probes were hybridized at 68°C and washed with 0.2× SSC–0.1% SDS at 58°C.

Sequencing. Sets of nested deletions were introduced bidirectionally into the cloned regions of pBS1.4 and pBSHL2.2 by treatment with exonuclease III-S1 nuclease (16) of an Erase-a-Base kit (Promega, Madison, Wis.). Sequencing of both strands of cloned DNA with the reverse and universal primers was performed with the AutoRead sequencing kit in the ALF sequencing system (Pharmacia). The HUSAR program package (Deutsches Krebsforschungszentrum, Heidelberg, Germany) was used for sequence analysis.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession no. Y11916.

RESULTS

Purification, subunit structure, and sequences of amino termini. Hydrogenase was purified from *O. carboxidovorans* OM5 grown with H_2 plus CO_2 to maximal activity (8 μmol of H_2 oxidized $min^{-1} \cdot$ mg of crude extract $protein^{-1}$) in the late exponential growth phase. Hydrogenase was membrane bound and solubilized with *n*-dodecyl-β-D-maltoside. The enzyme was purified 28-fold with a yield of 29% and a specific activity of 173 μmol of H_2 oxidized $min^{-1} \cdot$ mg of $protein^{-1}$ and amounted to nearly 4% of the entire bacterial protein (Table 2).

Purified hydrogenase revealed a single protein band upon gel filtration (105 kDa), sucrose density centrifugation (102 kDa), or native PAGE (128 kDa), which showed hydrogenase activity (Fig. 1). Denaturing PAGE revealed noncovalently bound subunits of 69 kDa (HoxL) and 30 kDa (HoxS), which occurred in a 1:0.94 molar ratio (Fig. 1), indicating a heterodimeric subunit structure of hydrogenase. Hydrogenase was a weak anion with an isoelectric point (pI) of 6.0. The sequences of the amino termini of the hydrogenase subunits were SVIQT PNGYKLDNSGRRVVVDPVTRIE (HoxL) and METKPRT PVLWLHGLEET (HoxS).

Hydrogenase contains Ni, Fe, and acid-labile sulfur. Analysis of hydrogenase by inductively coupled plasma mass spectroscopy revealed the presence 0.725 ± 0.025 mol of Ni and 7.3 ± 0.3 mol of Fe per mol of hydrogenase (six determinations on two independent enzyme preparations). One mole of hydrogenase contained 7.2 ± 0.2 mol of labile sulfide. Co, Cu, Mg, Mn, Mo, Se, V, and Zn did not occur in stoichiometric amounts since they showed molar ratios below 0.12 mol of metal per mol of hydrogenase.

The UV/visible light absorption spectrum of hydrogenase was very similar to the spectrum of hydrogenase from *Clostridium pasteurianum* (1). It revealed a constant weak increase of

TABLE 2. Representative purification scheme of hydrogenase (n = 5) from *O. carboxidovorans*

Step	Protein (mg)	Sp act ($U^a \cdot mg^{-1}$)	Purification (fold)	Yield (%)
Crude extract	2,946	6.3	1	100
CM	1,252	14.3	2.2	95
Washed CM	753	21.7	3.5	89
Solubilized CM	292	39.2	6.2	62
Hydroxylapatite chromatography	105	69.9	11.1	40
Ammonium sulfate fractionation	74	90.8	14.4	37
Gel filtration on Sephacryl G200	31	173.4	27.5	29

[a] 1 U = 1 μmol of H_2 oxidized min^{-1}.

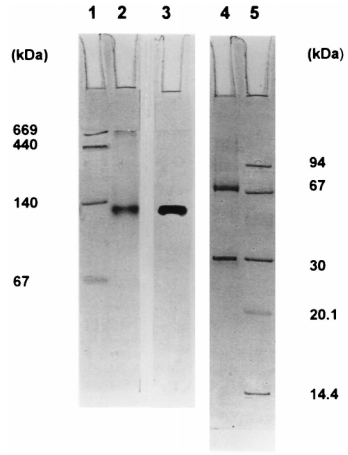

FIG. 1. Analysis of hydrogenase by nondenaturing PAGE (lanes 1 to 3) and SDS-PAGE (lane 4 and 5). Lanes 2 to 4 contained 65 μg of hydrogenase. Gels were stained for H_2-oxidizing activity (lane 3) or for protein (lanes 1, 2, 4, and 5). The molecular mass standards used were thyroglobulin (669 kDa), ferritin (440 kDa), lactate dehydrogenase (140 kDa), and bovine albumin (67 kDa) in lane 1 and phosphorylase *b* (94 kDa), bovine albumin (67 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) in lane 5.

absorption from 590 to 320 nm, a steep increase from 320 nm to lower wavelengths, and a protein absorption maximum at 280 nm. The spectrum revealed no indications for the presence of flavins, cytochromes, or other chromophores.

The specific activities of hydrogenase with different electron acceptors relative to the activity with methylene blue (173 μmol of H_2 $min^{-1} \cdot mg^{-1}$, set at 100%) were as follows: phenazine methosulfate, 15%; thionine, 15%; and 2-(4-iodophenyl)-3-(4-nitrophenyl)-2H-tetrazoliumchloride–1-methoxyphenazinemethosulfate, 2%. Oxidized benzyl viologen, methyl viologen, NAD, flavin adenine dinucleotide, cytochrome *c*, and ubiquinone Q10 were ineffective as electron acceptors under the experimental conditions applied. Hydrogenase activity was optimal with methylene blue at pH 5.8 and 52°C. The enzyme showed Michaelis-Menten kinetics with a K_m of 73.5 μM H_2 and V_{max} of 178.3 μmol of H_2 $min^{-1} \cdot mg^{-1}$ (pH 7.0, 30°C).

***hoxS* and *hoxL* reside on plasmid pHCG3.** Hybridizations of digested total DNA or plasmid DNA from *O. carboxidovorans* OM5 with the oligonucleotide probes OligoL and OligoS identified the *hox* genes on plasmid pHCG3. Chromosomal DNA of *O. carboxidovorans* OM5 or total DNA of the plasmid cured mutant *O. carboxidovorans* OM5-12 did not hybridize.

Cloning, DNA sequencing, and molecular organization of *hox* genes. A 19-kb *Sal*I fragment of pHCG3 which hybridized with OligoL and OligoS was inserted into the vector pUC18, yielding plasmid pBS19 (Fig. 2). A 2.2-kb *Eco*RV subfragment of pBS19 which hybridized with both oligonucleotides was subcloned in pBluescript, yielding pBS2.2.

Southern hybridizations with OligoS and OligoL identified the 5′ coding region of *hoxS* on a 1.4-kb *Eco*RV-*Pst*I fragment and the 5′ coding region of *hoxL* on a 0.5-kb *Pst*I fragment of pBS2.2 (Fig. 2). Both fragments were cloned and completely sequenced in both directions. The 2.2-kb *Pst*I-*Hin*dIII fragment directly located downstream of the 0.5-kb *Pst*I fragment was also subcloned from pBS19, and the resulting plasmid, pBSHL2.2, was also sequenced (Fig. 2).

The nucleotide sequence included three open reading frames arranged as a gene cluster in the transcriptional order 5′ *hoxS* → *hoxL* → *hoxZ* 3′ (Fig. 2 and 3). The ribosomal binding sites

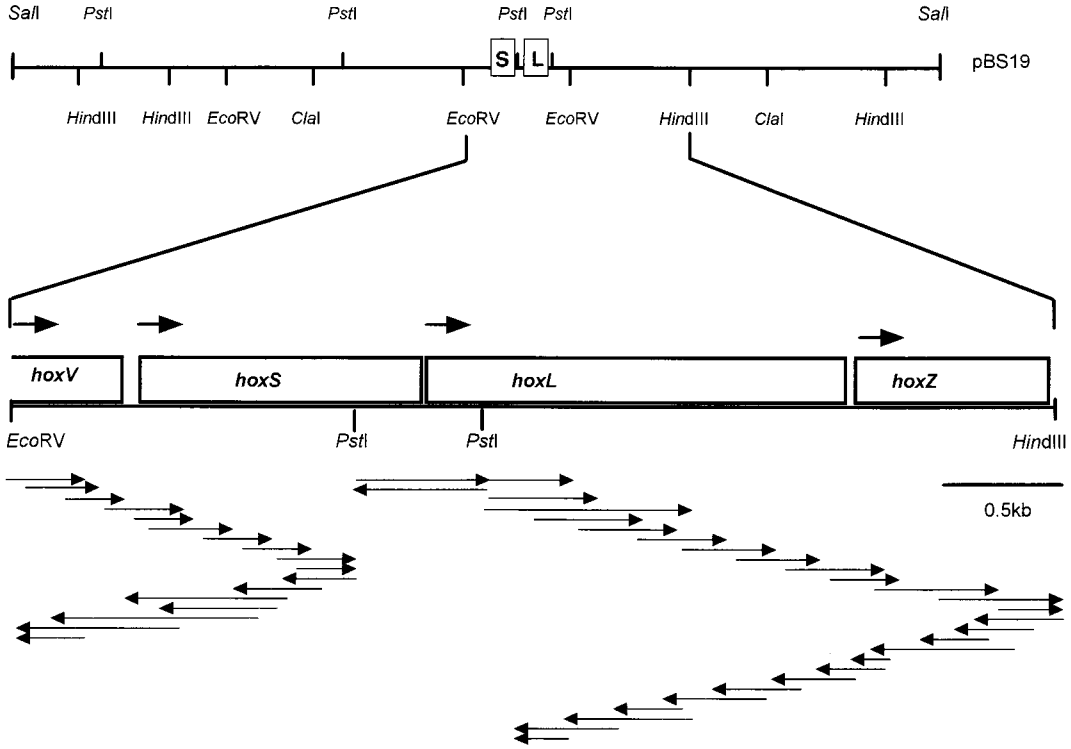

FIG. 2. Physical and genetic maps of the *hox* region on pHCG3 of *O. carboxidovorans*. Depicted are the 19-kb *Sal*I fragment, which hybridized with OligoL (L) and OligoS (S), the subcloned fragments, the lengths and orientations of the sequences obtained from the subcloned fragments and their deletion derivatives (thin arrows), and the transcriptional arrangement of *hoxSLZ* (heavy arrows).

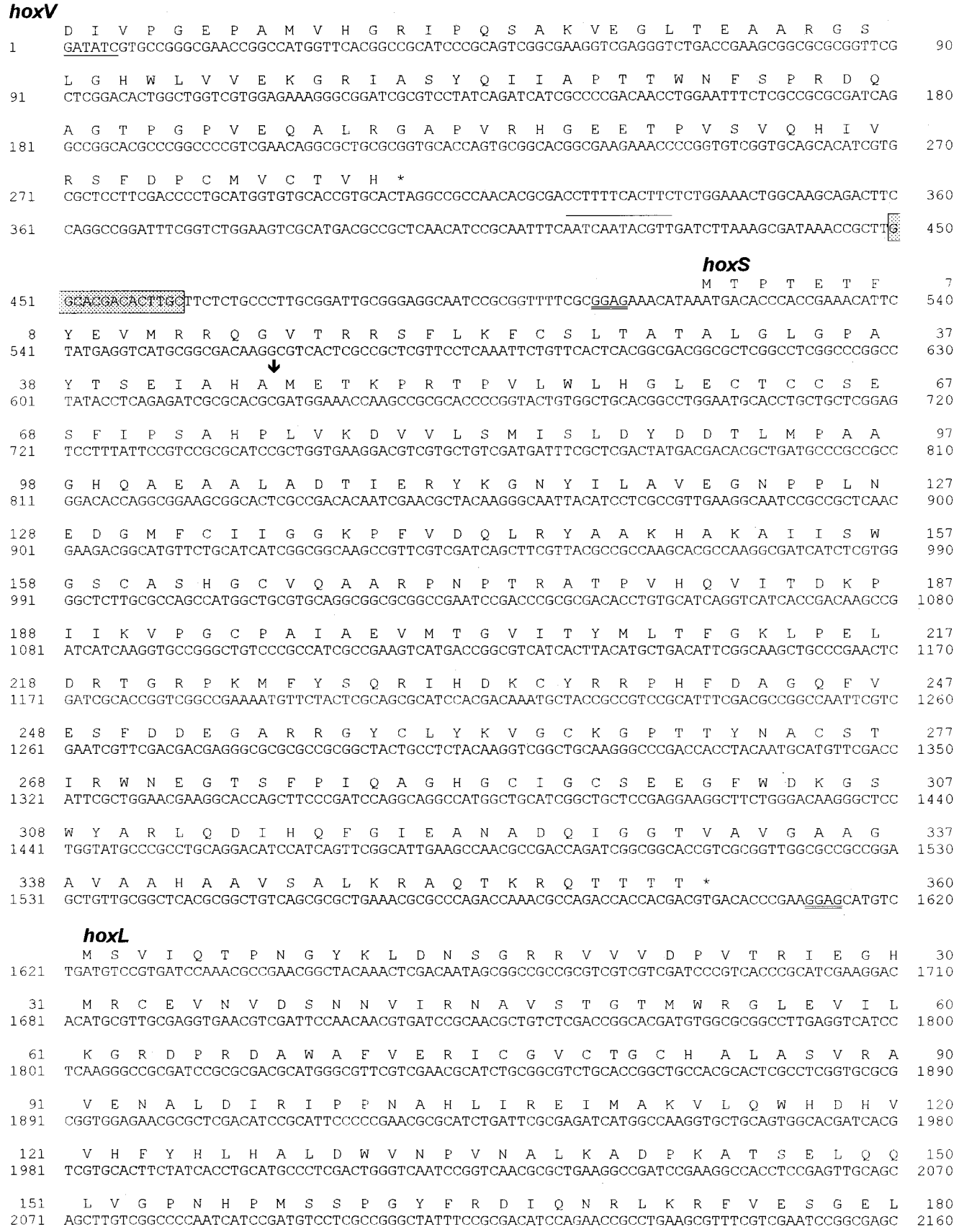

FIG. 3. Nucleotide sequence of *hoxVSLZ*. The DNA sequence is continuously numbered from the *Eco*RV restriction site to the *Hin*dIII site (both underlined). Deduced amino acid sequences are shown above the corresponding DNA sequences and are separately numbered. Stop codons are indicated by asterisks. The putative ribosomal binding sites of the *hox* genes complementary to the 3′ terminus of the 16S rRNA of *O. carboxidovorans* are double underlined. The putative −24/−12 promoter sequence is boxed, and the putative integration host factor-binding site is overlined. ↓ indicates putative cleavage sites.

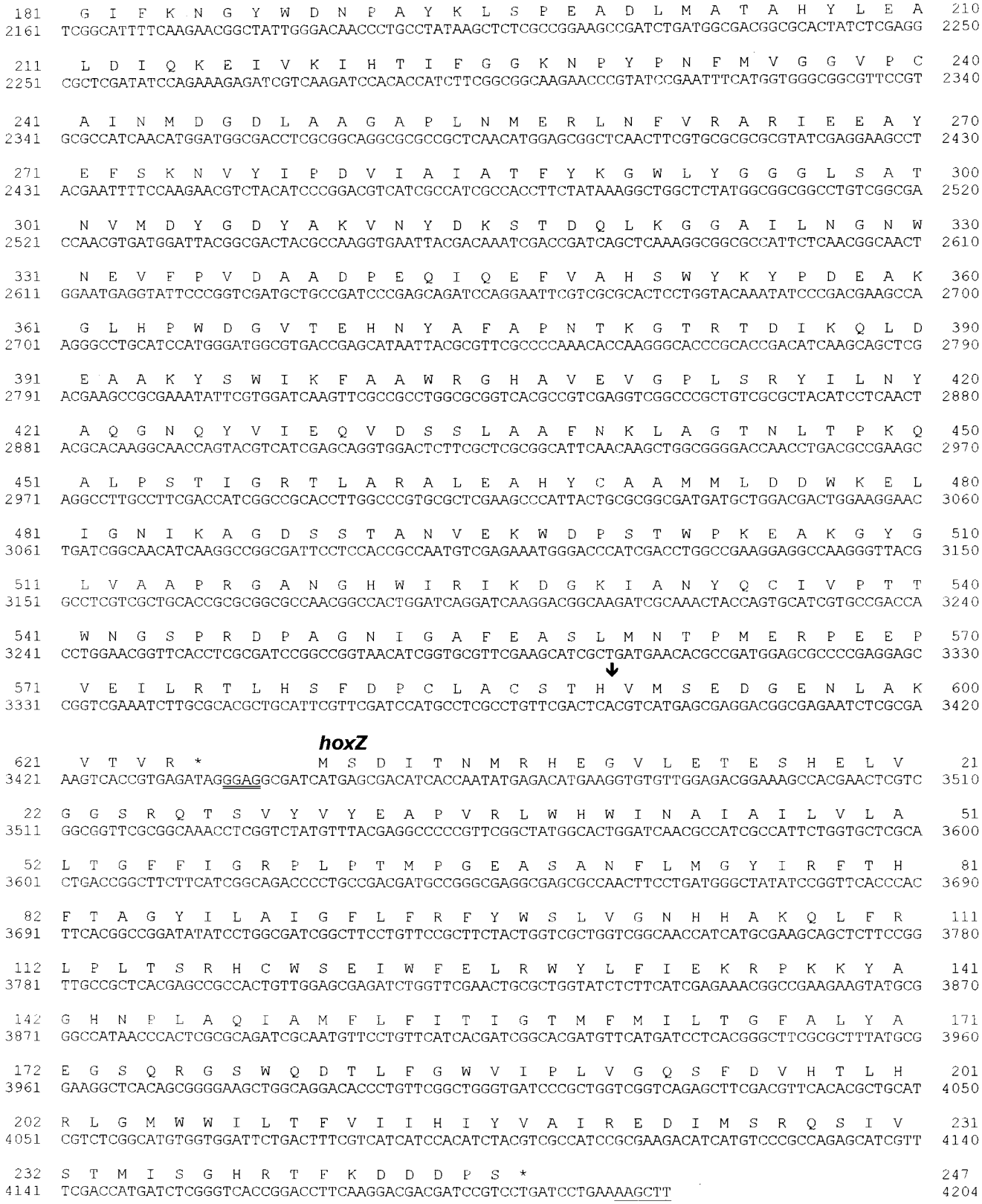

FIG. 3—*Continued.*

of *hoxS*, *hoxL*, and *hoxZ* could be identified on the nucleotide sequence (Fig. 3), since they are complementary to the 3′ terminus of the 16S rRNA of *O. carboxidovorans* (2). A plausible −24 (GG)/−12 (GC)-specific promoter sequence is present between nucleotides 450 and 463 (Fig. 3), and the sequence extending from nucleotides 415 to 427 (Fig. 3) corresponds to the consensus sequence (A/TATCAAN$_4$TTA/G) of an integration host factor-binding site (15).

(i) *hoxS*. *hoxS* (1,080 bp) starts 520 nucleotides downstream of the *Eco*RV restriction site (Fig. 3). The corresponding protein encodes the small subunit of hydrogenase and is composed of 360 amino acids with a molecular mass of 38,760 Da. The N-terminal amino acid sequence of the HoxS polypeptide starts at position Met^{46} of the deduced amino acid sequence, indicating that the first 45 amino acids are modified posttranslationally. HoxS is a hydrophilic protein on the basis of a hydropathy index of −0.20 (moving segment of seven amino acids [25]).

(ii) *hoxL*. *hoxL* (1,863 bp) starts 18 nucleotides transcriptionally downstream of the 3′ end of *hoxS* (Fig. 3). The corresponding HoxL protein contains 604 amino acids. Its molecular mass of 67,163 Da matches that of the large hydrogenase subunit (69 kDa). The N terminus of the peptide inferred by *hoxL* matches that of HoxL except for the methionine at position 1, which is absent in mature HoxL. HoxL is a hydrophilic protein on the basis of a hydrophathy index of −0.32 (moving segment of seven amino acids [25]).

(iii) *hoxZ*. *hoxZ* (741 bp) starts 11 nucleotides downstream of *hoxL*. The deduced HoxZ polypeptide contains 247 amino acids and has a molecular mass of 28,637 Da. HoxZ contains four transmembranous helices (moving segment of seven amino acids [25]).

(iv) *hoxV*. The 3′ region (303 nucleotides) of *hoxV* was identified 217 nucleotides upstream from *hoxS*.

Refined molecular mass of hydrogenase. Assuming an LS subunit structure of hydrogenase, the molecular masses of the deduced polypeptides HoxS and HoxL add up to 100,930 Da. Considering the presence of one Ni atom, eight Fe atoms, and eight labile sulfide atoms per enzyme molecule leads to a M_r of 101,692 of catalytically competent hydrogenase.

DISCUSSION

Hydrogenase in *O. carboxidovorans* is an H_2 uptake membrane-bound NiFe-enzyme. The H_2 uptake membrane-bound hydrogenase from *O. carboxidovorans* is the first hydrogenase studied in a carboxidotrophic bacterium. The presence of hydrogenase introduces hydrogenotrophy as a complementary chemolithoautotrophic capability into *O. carboxidovorans* and, in addition, enables the bacterium to utilize CO as a sole carbon source under hydrogenotrophic conditions.

The contents of nearly one Ni atom, eight Fe atoms, and eight labile sulfide atoms per enzyme molecule characterize the enzyme as a typical NiFe-hydrogenase. The Fe/labile sulfide ratio of 1:1 in combination with the conserved sequence of the [4Fe-4S_{dist}] (distal) and [4Fe-4S_{prox}] (proximal) centers of *Desulfovibrio gigas* (43) in HoxS also identify these FeS centers in *O. carboxidovorans* hydrogenase (Fig. 4). In addition, HoxS contains the membrane-bound NiFe-hydrogenase-specific motif (amino acids 317 to 350) (Fig. 3), which presumably anchors hydrogenase to the CM (29). The Ni-binding motif of *D. gigas* hydrogenase (43) was also identified on HoxL (Fig. 4).

Processing of hydrogenase. The amino acid sequence of HoxS showed that the first 45 amino acids (4,993 Da) are subject to posttranslational modification. The mass of HoxS (38,760 Da) determined from the deduced amino acid sequence exceeds the mass of the small hydrogenase subunit (30 kDa) analyzed by PAGE. In addition, the N-terminal amino acid sequence of the HoxS polypeptide starts at position Met^{46} (Fig. 3). The 45-amino-acid leader sequence at the N terminus corresponds to the highly conserved (40% identity) signal peptide of the small subunit of hydrogenases (45). The sequence contains the strictly conserved RRXFXK consensus element (37, 44), as well as an alanine at the putative signal peptidase cleavage site (Fig. 3). Maturation of membrane-bound hydrogenase involves processing of the large subunit at the C terminus. The conserved cleavage site (DPCXXCXXH↓V) of *E. coli* and *D. gigas* (39, 30) is also present on HoxL of *O. carboxidovorans* (Fig. 3).

Ni-binding-ligands

D. gigas	$C_{65}GVC_{68}$ – X_{461} – $C_{530}IAC_{533}$
O. carboxidovorans	$C_{75}GVC_{78}$ – X_{506} – $C_{585}LAC_{588}$

[4Fe-4S_{prox}]

D. gigas	$C_{17}TGC_{20}$ – X_{91} – C_{112} – X_{35} – C_{148}
O. carboxidovorans	$C_{62}TCC_{65}$ – X_{94} – C_{160} – X_{33} – C_{194}

[4Fe-4S_{dist}]

D. gigas	$H_{185}DNC_{188}$ – X_{24} – $C_{213}LYELGC_{219}$
O. carboxidovorans	$H_{232}DKC_{235}$ – X_{24} – $C_{260}LYKVGC_{266}$

FIG. 4. Comparison of the structurally identified Ni-binding ligands and the proximal and distal FeS clusters of hydrogenase from *D. gigas* and the potential ligands in hydrogenase from *O. carboxidovorans*. Ligands are in boldface.

NiFe-hydrogenase of *O. carboxidovorans* is grouped in class I of hydrogenases. The clustered arrangement and the transcriptional order of *hoxVSLZ* in *O. carboxidovorans* (Fig. 2 and 3) agree with the structure of hydrogenase gene clusters of other bacteria (42). The sequences of *O. carboxidovorans* hydrogenase subunits contain the motif blocks a to f (HoxS) and A to G (HoxL) characteristic of class I hydrogenases (45). The sequences of HoxS and HoxL reveal high percentages of identity to those of the corresponding polypeptides of *Azotobacter vinelandii* (83 and 75%) (28, 29), *R. eutropha* (81 and 74%) (23), and *Rhizobium leguminosarum* (76 and 68%) (17, 19). The structurally characterized periplasmic hydrogenase of *D. gigas* is representative of class II hydrogenases (45). The overall identities of HoxS (23%) and HoxL (34%) to the small or large polypeptide of the *D. gigas* hydrogenase were significant but low. HoxS and HoxL were not homologous to any of the hydrogenases classified in classes III to VI. The CO-induced hydrogenase of *Rhodospirillum rubrum* showed good sequence similarity with hydrogenase 3 (formate hydrogenlyase) of *E. coli* and somewhat less similarity to the periplasmic hydrogenase from *D. gigas* (13, 14). The enzyme is involved in the formation of H_2 by *R. rubrum* during the anaerobic utilization of CO. It is neither structurally nor functionally related to the hydrogenase from the aerobe *O. carboxidovorans* described here.

According to these considerations, the H_2 uptake membrane-bound NiFe-hydrogenase from *O. carboxidovorans* can be grouped into the class I of hydrogenases defined by Wu and Mandrand (45).

Products of the accessory genes *hoxZ* and *hoxV*. HoxZ of *O. carboxidovorans* (Fig. 2 and 3) is homologous to HoxZ from *A. vinelandii* (66% identity, 91% similarity) (28), *R. capsulatus* (60% identity, 89% similarity) (7), and *Wolinella succinogenes* (27% identity, 66% similarity) (9). HoxZ of *O. carboxidovorans* (248 amino acids; M_r, 28,637) has the same M_r and contains a similar number of amino acids as its counterparts from other bacterial sources (42). The HoxZ equivalents in *W. succinogenes* (9) and *R. capsulatus* (7) are *b*-type cytochromes functioning as electron acceptor and membrane anchor of hydrogenase. Additional evidence for HoxZ of *O. carboxidovorans*

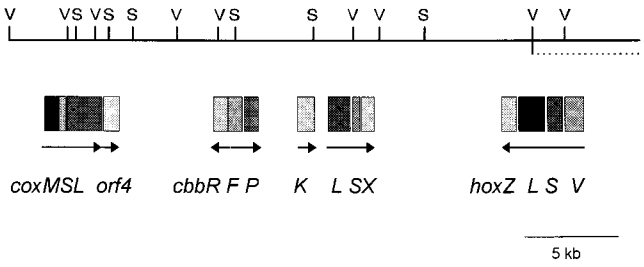

FIG. 5. Arrangement of the *hox*, *cbb*, and *cox* gene clusters on a 30-kb segment of the 128-kb megaplasmid pHCG3 from *O. carboxidovorans*. The arrangement of *coxMSL orf4* is from reference 41. The *cbb* genes have been identified on different cloned fragments (pBS5.7 and pBS7.7) of pHCG3 (36). The *hox*, *cbb*, and *cox* genes were localized by chromosome walking. The dotted lane at the *hox* site of the 30-kb segment refers to the deletion in *O. carboxidovorans* OM5-24. V, *Eco*RV; S, *Sal*I.

being a cytochrome comes from the signatures ^{37}RLWHWI NAIAILVLALTG^{54} and ^{198}HTLHRLGMWWILTFVIIH^{215} that correspond to the consensus patterns I and II which have been developed for these proteins (7, 9, 18). The conserved histidines in these motifs are believed to bind the heme iron group.

The gene product of the fragmentary open reading frame *hoxV* (Fig. 2 and 3) was homologous to HupV (66% identity, 88% similarity) from *Bradyrhizobium japonicum* (5) or *R. capsulatus* (63% identity, 82% similarity) (10). Like *hoxV* in *O. carboxidovorans*, *hupV* in these bacteria is located upstream of the hydrogenase structural operon (*hupSLC*). In *R. capsulatus*, HupV participates in negative regulation of hydrogenase expression in concert with HupT, a sensor histidine kinase involved in repression (10).

The *hox* gene cluster is arranged within a 30-kb DNA segment of the megaplasmid pHCG3, which also contains the clustered *cox* and *cbb* genes. The 30-kb DNA segment from plasmid pHCG3 of *O. carboxidovorans* containing the *cbb* genes is flanked by *cox* and *hox* genes (Fig. 5). The *cox* and *hox* genes encode CO dehydrogenase and hydrogenase, respectively, which are the key enzymes for the chemolithoautotrophic generation of energy through the oxidation of CO or H_2. The *cbb* genes encode the enzymes required for CO_2 fixation and reside between *cox* and *hox* (Fig. 5). Hydrogenase gene clusters are very conserved in different bacteria (42). Their sizes extend from 15 to 20 kb. Considering the organization of hydrogenase gene clusters from different bacteria (42), genes involved in the regulation of hydrogenase synthesis and maturation in *O. carboxidovorans* can be assumed in the 7-kb region extending from *hoxZ* to *cbbX*. In *O. carboxidovorans*, H_2 chemolithoautotrophic growth conditions induced the formation of mRNA specific for *hoxSL*, *cbbLS*, and *cbbR* (40). Under CO chemolithoautotrophic conditions, mRNA specific for *coxMSL*, *orf4*, the DNA region extending from *orf4* to *cbbR*, *cbbR*, and *cbbLS* was formed, whereas *hoxSL*-specific mRNA was absent (40). The mutant *O. carboxidovorans* OM5-12 is cured from pHCG3 and cannot utilize CO or H_2 (24). Mutant OM5-29, which carries a 54-kb deletion in pHCG3, lacks the entire 30-kb segment (Fig. 5) and also cannot utilize CO or H_2 (24). Mutant OM5-24 carries a 15-kb deletion in pHCG3 and can utilize CO but not H_2 (24). It has the *cox* and *cbb* genes but lacks the 2.2-kb *Eco*RV fragment carrying *hoxS*, the 3′ region (309 bp) of *hoxV*, and the 5′ region (632 bp) of *hoxL* (Fig. 2 and 5). Since the 11-kb *Eco*RV fragment upstream of the 2.2-kb *Eco*RV fragment (Fig. 2) is present in mutant OM5-24, the 15-kb deletion extends downstream from *hoxV* (Fig. 5). These considerations show that the 30-kb DNA segment of pHCG3 (Fig. 5) assembles the essential genes involved in CO and H_2 chemolithoautotrophy of *O. carboxidovorans.*

We have reported here for the first time the arrangement and sequences of *hox* genes in an aerobic CO-oxidizing bacterium. Up to now, only two sequences of Mo-containing CO dehydrogenases (*cox* and *cut*) have been published (41, 38). The construction of evolutionary trees would provide further clues, e.g., a possible convergent or divergent evolution of carboxidotrophy and hydrogenotrophy in aerobic CO-oxidizing bacteria. It would thus be of considerable interest to analyze a greater number of *cox* and *hox* sequences from species of taxonomically distant carboxidotrophic bacteria.

ACKNOWLEDGMENTS

We thank Elisabeth Keese for expert technical assistance.

This work was financially supported by the Deutsche Forschungsgemeinschaft (Bonn, Germany) and the Fonds der Chemischen Industrie (Frankfurt am Main, Germany).

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