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A Novel Five-Subunit-Type 2-Oxoglutalate:Ferredoxin Oxidoreductases from *Hydrogenobacter thermophilus* TK-6

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A thermophilic, chemolithoautotrophic hydrogen-oxidizing bacterium, *Hydrogenobacter thermophilus* TK-6, fixes carbon dioxide via the reductive TCA cycle. 2-Oxoglutarate:ferredoxin oxidoreductase (OGOR) of this strain is one of the key enzymes of the pathway. OGOR of strain TK-6 has been reported to be a two-subunit-type OGOR and encoded by *korAB*. A gene cluster, *forDABGEF*, encoding another OGOR was found 148 bp upstream of *korAB* in the opposite orientation. Five of the *for* genes (*forDABGE*) were required for the expression of the active recombinant enzyme in *Escherichia coli*. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the purified enzyme showed five polypeptides corresponding to the *forDABGE* gene products, suggesting that the enzyme had a novel five-subunit structure. The recombinant enzyme had high substrate specificity toward 2-oxoglutarate as in the case of the gene products of *korAB*. Primer extension analysis showed that the *korA* and *forD* genes were transcribed from one and two transcriptional initiation sites, respectively. The results also suggested that both gene clusters were expressed in the cells of strain TK-6.

Key Words: **2-oxoglutarate:ferredoxin oxidoreductase; reductive tricarboxylic acid cycle; heterologous expression; *Hydrogenobacter thermophilus*.**

Hydrogenobacter thermophilus TK-6 is an aerobic, thermophilic, obligate autotrophic hydrogen oxidizing bacterium (1, 2). Phylogenetic analysis of 16S ribosomal RNA has shown that *Hydrogenobacter* is one of the members of the most deeply branching family in bacteria (3). In accordance with this, strain TK-6 has a unique sulfur-containing quinone called methionaquinone and a novel aminophospholipid (4). This bacterium also has a unique ability to fix carbon dioxide via the reductive tricarboxylic acid (RTCA) cycle, even under aerobic conditions (5). The RTCA cycle is a pathway for the assimilation of carbon dioxide by the reverse rotation of the TCA cycle, and is distributed mainly in anaerobic autotrophs. We have reported the isolation and characterization of one of the RTCA cycle enzymes, 2-oxoglutarate:ferredoxin oxidoreductase (OGOR), from strain TK-6 (6). OGOR is a member of the 2-oxoacid oxidoreductase (OR) family, which catalyzes the oxidative decarboxylation of 2-oxoacids to their acyl- or aryl-coenzyme A (CoA) derivatives (7). In the TCA cycle, OGOR catalyzes the oxidative decarboxylation of 2-oxoglutarate to succinyl-CoA, but in the RTCA cycle, the enzyme acts as the 2-oxoglutarate synthase and assimilates carbon dioxide by the reductive reaction. Strong reducing energy is required for the carboxylation reaction. Strain TK-6 uses hydrogen as the energy source, enabling the reaction to occur even under aerobic conditions (8, 9).

The OR-family enzymes carry iron-sulfur clusters and thiamine pyrophosphate (TPP). Low potential electron carriers such as ferredoxin are used as the electron acceptors or donors for the reaction. Most ORs from hyperthermophilic organisms consist of four different subunits ($\alpha\beta\gamma\delta$). Each subunit has a specific conserved motif. These subunits form a catalytic unit of mass ~120 kDa. In contrast, the catalytic unit of some mesophilic ORs consists of a single large subunit, which is composed of domains tentatively termed A-, B-, G-, and D-domains in the arrangement of A-G-D-B. The active enzymes are homodimers of 240 kDa. The A-, B-, G-, and D-domains are similar to the α-, β-, γ-, and δ-subunits of the four-subunit ORs, respectively (10). Two subunit ($\alpha\beta$)-type ORs have been found in organisms such as *Sulfolobus* and *Halobacterium* (11, 12). The α-subunit of the enzyme has a G + A domain structure, and the β-subunit has a single B domain structure. Archaeal indolepyruvate:ferredoxin oxidoreductases are also $\alpha\beta$-type ORs, but their α-subunit has an A + B + D domain structure, and their β-subunit has a single G domain structure. In this way,

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the diversity of the subunit structure of ORs can be attributed to the fusion patterns of the domains. ORs have evolved and diversified, probably by the rearrangement and fusion of the ancestral A-, B-, G-, and D-domains (12).

OGOR purified from strain TK-6 is a member of the $\alpha\beta$-type ORs similar to that from *Sulfolobus* (12). The α- and β-subunits are encoded by the *korAB* genes (13). Here, we refer to the OGOR enzyme derived from the *korAB* genes as Kor. The Kor enzyme has high substrate specificity toward 2-oxoglutarate, whereas the *Sulfolobus* enzyme has a broad specificity toward 2-oxoacids such as 2-oxoglutarate, 2-oxobutyrate, and pyruvate (12). In this work, we found another set of the genes, *forDABGEF*, encoding a novel multi-subunit OR upstream of *korAB* from strain TK-6. Here, we refer to the enzyme derived from the *for* genes as For. We succeeded in heterologous expression of For in *Escherichia coli* and analyzed its substrate specificity and subunit structure.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. *H. thermophilus* TK-6 (IAM 12695, DSM 6534) was cultivated in an inorganic medium at 70°C with CO_2 as a carbon source as described previously (14). *Escherichia coli* JM109 was used as a host for derivatives of pUC19 and pGEM-T Easy vector (Promega, Madison, WI). *E. coli* TH2 was used as a host for cloning using pKF3 (Takara, Kyoto, Japan). *E. coli* strains were grown in LB medium at 37°C. When necessary, 0.5 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the medium. Concentrations of antibiotics were as follows (μg/ml): ampicillin, 100; streptomycin, 50 and chloramphenicol, 12. For the microaerobic condition, a bottle (total volume: 390 ml) containing 350 ml of the medium was used. After the inoculation of 3.5 ml of aerobically grown culture, the bottle was sealed with a screw cap and cultivation was done statically.

Cloning and DNA sequencing. DNA manipulations were performed by standard methods (15). Restriction and modification enzymes were purchased from Toyobo (Osaka, Japan) or Takara. Southern and colony hybridization analyses were performed using Hybond-N nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) and a DIG DNA labelling and detection kit (Boehringer Mannheim, Mannheim, Germany).

Cloning of a 6.7-kb *Sac*I fragment containing the *korAB* genes for the anabolic OGOR was described previously (13). pYNA1 that carries the *Sac*I fragment is a derivative of Charomid 9-36 (Nippon Gene, Toyama, Japan). When a 1.6-kb *Sac*I-*Sph*I fragment from pYNA1 was used as a probe (probe 2), a 2.1-kb *Pst*I fragment of chromosomal DNA from strain TK-6 was hybridized. A gene library of *Pst*I fragments around the size of 2-kb was constructed with the enforcement cloning system pKF3 (Takara). One positive clone was identified out of 840 clones by colony hybridization with probe 2. The plasmid carrying the 2.1-kb *Pst*I fragment was designated as pYNA2. A 1.4-kb *Pst*I-*Sac*I fragment from pYNA2 (probe 3) was hybridized with a 1.4-kb *Hin*dIII fragment of chromosomal DNA from strain TK-6. The *Hin*dIII fragment was amplified using an LA PCR in vitro cloning kit (Takara) and cloned into pGEM-T Easy vector. The resultant plasmid was designated as pYNA3.

An ABI 377 DNA sequencer was used for sequence determination. A Big dye terminator cycle sequence kit (Applied Biosystems, Foster City, CA) was used for dideoxy chain-termination reactions. Synthetic oligonucleotides used as primers for sequence determination were prepared by Sawady Technology (Tokyo, Japan). The nucleotide sequence data determined in this work have been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence databases under the Accession No. AB054643.

Construction of plasmids. pYNA200 was constructed by ligation of an end-blunted 2.4-kb *Ssp*I-*Sac*I fragment from pYNA1, which carries *forDA* and a part of *forB*, with the *Hin*cII digest of pUC19 (Fig. 1). The direction of the genes was the same as that of the *lac* promoter of pUC19. pYNA201 was constructed by the insertion of a 1.2-kb *Sac*I-*Eco*RI fragment containing the remaining part of *forB* and *forG* in pYNA200. The fragment was prepared by PCR amplification from chromosomal DNA of strain TK-6 with synthetic oligonucleotides ogor25 (5′-TTCTCAGGCAGCAGTCC-3′) and ogor26 (5′-CTGTTTCGAATTCATCAGCCAC-3′, where the *Eco*RI site is underlined). pYNA202 was constructed by substitution of a 0.18-kb *Kpn*I-*Eco*RI fragment of pYNA201 with a 0.42-kb *Kpn*I-*Eco*RI fragment derived from a PCR fragment amplified from TK-6 DNA with ogor25 and ogor31 (5′-GCTCTTTTTGAATTCCTCCGCAAGG-3′, where the *Eco*RI site is underlined). pYNA203 was constructed in the same way by use of a 0.73-kb *Kpn*I-*Eco*RI fragment derived from a PCR fragment amplified with ogor25 and ogor35 (5′-CAGAAGAGAATTCTTGCCGCTTCC-3′, where the *Eco*RI site is underlined). pYNA204 was constructed by self-ligation of pYNA202 following digestion with *Nco*I. A 0.24-kb *Nco*I fragment within the *forD* gene on the plasmid was removed by this operation. pYNA208 was constructed as follows. A 0.73-kb *Kpn*I-*Eco*RI fragment from pYNA203 that carries *forEF* was subcloned into pUC19. The resultant plasmid was digested with *Bst*XI and self-ligated after the protruding 3′ ends were made blunt-ended by T4 DNA polymerase. A frame-shift mutation was introduced in the *forE* gene by this operation. The *Eco*RI-*Kpn*I fragment of pYNA202 was substituted with the fragment that carried the disrupted *forE* gene, resulting in pYNA208.

Preparation of cell-free extract and enzyme assay. Cell-free extract (CFE) was prepared from *E. coli* JM109 harboring pYNA201, 202, 203, 204 or 208 after cultivation under microaerobic conditions as described previously (13). Quantitative measurements of the enzymatic activity and substrate specificity of recombinant FOR were carried out according to the method described previously (13). The reaction was performed at 70°C under anaerobic conditions. The artificial electron acceptor methyl viologen was used for the assay. The activity was expressed as units per mg of protein, where one unit was defined as 1 μmol of methyl viologen reduced per min.

Purification of recombinant For and determination of N-terminal amino acid sequence. Recombinant For was purified from 5-1 culture of *E. coli* JM109 harboring pYNA203. CFE (30 ml) was prepared from the culture as described previously (13), but the condition of heat denaturation was changed (70°C for 10 min). The following procedures were carried out at room temperature under aerobic conditions. Proteins in the CFE were precipitated with ammonium sulfate (0 to 35%). The precipitated proteins were suspended in 2 ml of purification buffer (10 mM Tris-HCl, pH 8.0; 0.01% Triton X-100, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM sodium dithionite). Ammonium sulfate was removed by passing through a PD-10 column (Pharmacia, Uppsara, Sweden). The same buffer was used as an eluant. The solution (2 ml) was applied to a HiLoad Q Sepharose High Performance column (2.6 by 10 cm; Pharmacia) preequilibrated with the purification buffer. The OGOR activity came out at a NaCl concentration of 0.39 to 0.40 M after being eluted with a liner gradient of 0 to 1 M NaCl at 5 ml/min. The total gradient volume was 1 l. The active fraction was stored under an argon atmosphere at 4°C. Polyacrylamide gel electrophoresis (PAGE) was carried out in the presence of sodium dodecyl sulfate (SDS) with an 18% gel. The N-terminal amino acid sequence of the peptide that migrated in the SDS-PAGE gel was determined by using the Procise cLC protein sequencer system (Applied Biosystems) after transferring the pep-

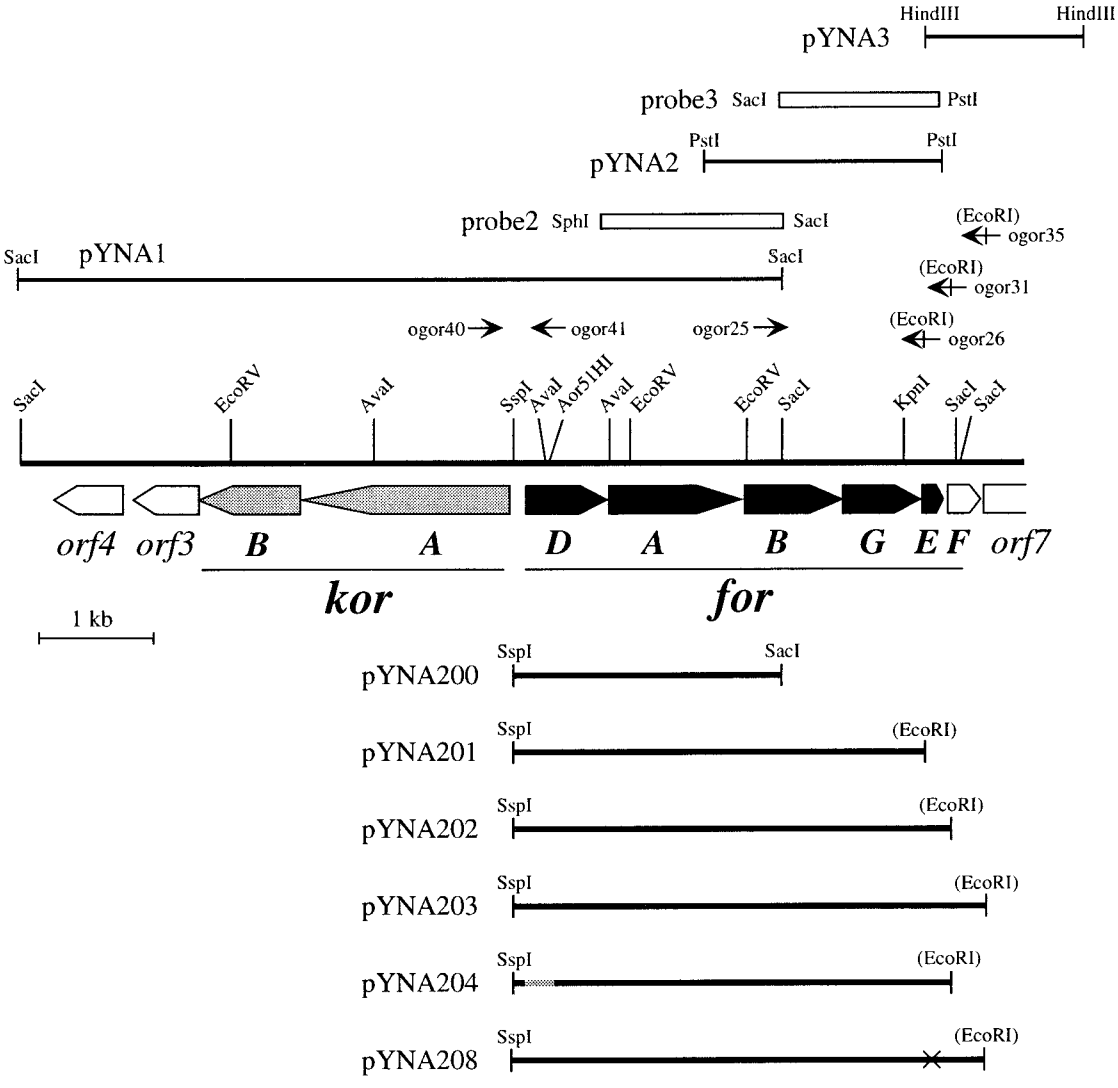

FIG. 1. Physical map of the two OGOR genes from *H. thermophilus* TK-6. The arrow boxes indicate the size and direction of the genes. The subunits of For are shown in black. The subunits of Kor are shown in gray. The fragments labeled pYNA1, pYNA2, and pYNA3 were cloned from chromosomal DNA of strain TK-6. The fragments labeled probe 2 and probe 3 were used as probes for cloning the fragments of pYNA2 and pYNA3, respectively. Small arrows indicate the synthetic oligonucleotides used for PCR amplification and primer extension analyses. Lower bars labeled pYNA200–208 show the fragments used for heterologous expression of the For genes in *E. coli*. The *Eco*RI sites shown in parentheses were generated by PCR with synthetic oligonucleotides. The region missing in pYNA204 is shown in gray. A frame-shift mutation in pYNA208 is indicated by ×.

tide onto a Sequi-Blot PVDF membrane (0.2 μm; Bio-Rad, Hercules, CA).

RNA extraction and primer extension analysis. RNA was isolated from TK-6 cells at mid-log phase (OD_{660} of 0.6 to 0.8) by using ISOGEN (Nippon Gene) according to the instructions of the manufacturer, and was treated with RNase-free DNase (Nippon Gene). The primer extension reaction was performed with a primer extension kit (Promega). The oligonucleotide primers used for the reaction were ogor40 (5′-CACATAATAACCAGCCCGTGCTGC-3′) and ogor41 (5′-AGCCTTACCCGGAGGTGGTAGCTC-3′), which were complementary to the mRNAs of *korA* and *forD*, respectively. The primers were labeled with [γ-^{32}P]ATP (Amersham Pharmacia Biotech) by T4 polynucleotide kinase. The labeled primer and RNA were annealed at 70°C for 10 min. Extension was carried out with AMV reverse transcriptase at 42°C for 30 min. The primer extension products were compared on an 8% polyacrylamide/6M urea gel with the products of sequence reactions made with the same primers.

RESULTS

Arrangement of the Genes Encoding a Novel 2-Oxoacid Oxidoreductase

Sequence analyses of the upstream region of *korAB* on pYNA1 (13) suggested the existence of a set of genes encoding another putative multi-subunit OR in the opposite orientation (Fig. 1). We cloned a 2.1-kb *Pst*I fragment (pYNA2) and a 1.5-kb *Hin*dIII fragment (pYNA3) from chromosomal DNA of strain TK-6 for the

TABLE 1

Specific Activity of OGOR from Recombinant *E. coli* Strains

Plasmid	Gene carried	Specific activity (U/mg)[a]
pUC19		0.03 ± 0.01
pYNA201	*forDABG*	0.04 ± 0.03
pYNA202	*forDABGE*	0.31 ± 0.05
pYNA203	*forDABGEF*	0.47 ± 0.05
pYNA204	*forABGE*	0.04 ± 0.03
pYNA208	*forDABGF*	0.02 ± 0.00

[a] The values are means ± standard deviation from three independent experiments.

purpose of obtaining the whole gene cluster. Six complete genes designated as *forDABGEF* and a partial open reading frame (*orf7*) were identified in this region. *korA* and *forD* were separated by 147-bp. *forABG* encode proteins consisting of 389, 288 and 227 amino acid residues, respectively. These proteins are similar to the α-, β-, and γ-subunits of the four-subunit ($\alpha\beta\gamma\delta$-type) ORs, respectively. ForA carries a YPITP motif, which is highly conserved in the A-domain of ORs but whose role has not yet been identified (12). ForB carries a B-domain-specific motif (GXGC//GC//GDG//C), which is required for the binding of a [4Fe-4S] cluster and TPP. ForG carries a G-domain-specific GXXG motif, which is a putative CoA-binding site. *forD* encodes a protein of 237 amino acid residues. ForD is 63% identical with PorD, the δ-subunit of pyruvate:ferredoxin oxidoreductase (POR) from strain TK-6 (16, DDBJ accession no. AB042412). POR is also a member of the OR-family enzymes and assimilate carbon dioxide in the RTCA cycle (16). ForD is not similar to the δ-subunit of the other $\alpha\beta\gamma\delta$-type ORs. ForD and PorD of strain TK-6 do not carry the D-domain-specific ferredoxin-like motif (CXXCXXCXXXC), which is required for the binding of [4Fe-4S] clusters. The ferredoxin-like motif was found in the translated sequence of *forE*. ForE was similar to the δ-subunit of the $\alpha\beta\gamma\delta$-type ORs, although the size of ForE (74 amino acids) was much smaller than the sizes of the other δ-subunits. *forF* encodes a small protein consisting of 97 amino acid residues. No protein that has overall similarity with ForF was found in the protein sequence databases, but a TFASTA search of DNA databases revealed two putative open reading frames starting from GTG triplets on the genome of *Aquifex aeolicus* (17). One of the open reading frames is located downstream of *forA2B2G2-fdx2* (aq_1166, 1167, 1168, and 1171a), which correspond respectively to *forABGE* of strain TK-6. The putative open reading frame encodes a protein of 110 amino acids that is 46% identical with ForF. The other open reading frame is located between *murE* (aq_1747) and *tyrS* (aq_1751). It encodes a protein of 102 amino acids that is 33% identical with ForF. *orf7* was similar to *cobW* (aq_147) of *A. aeolicus* encoding a putative cobalamin synthesis-related protein (17). All of the *forDABGEF* and *orf7* genes are probably transcribed as an operon because the genes are located close to one another or overlap, and no terminator-like sequence was found between the genes.

Heterologous Expression of the for Genes in E. coli

The pUC19-derivative plasmids designated as pYNA201, 202, 203, 204 and 208 (Fig. 1) were constructed for the expression of the recombinant For enzyme in *E. coli* JM109. The plasmids carried the fragments of *forDABG* (pYA201), *forDABGE* (pYNA202), *forDABGEF* (pYNA203), *forABGE* (pYNA204) and *forDABGF* (pYNA208), respectively. *E. coli* JM109 was transformed with the plasmids and cultivated under the microaerobic condition in the presence of IPTG. CFE of the *E. coli* culture was used for the enzyme assay. The activity was determined by the reduction of methyl viologen under anaerobic conditions. 2-Oxoglutarate was used as a substrate for the reaction. The active enzyme was produced in *E. coli* cells when pYNA202 or pYNA203 was used, but not when pYNA201, 204 or 208 was used, suggesting that *forD* and *forE* genes were necessary for the expression of the active enzyme (Table 1). The *forABG* genes are likely to be necessary for the activity because they respectively carry the conserved motifs specific for the A-, B-, and G-domains of ORs. The activity in the CFE of JM109 (pYNA203) was slightly higher than that in JM109 (pYNA202), suggesting that ForF had some effect on the activity of the For enzyme.

The recombinant For was purified from the CFE of *E. coli* JM109 harboring pYNA203 (Table 2). Five bands were detected on the SDS-PAGE gel of the purified For (Fig. 2). The sizes of the four large bands corresponded to the calculated molecular weights of the gene products of *forA*, *forB*, *forD*, and *forG*, respectively in order of size. The smallest band was weaker

TABLE 2

Purification of the Recombinant For from *E. coli*

Step	Protein (mg)	Activity (U)	Sp. Act. (U/mg)	Yield (%)	Purification (fold)
Cell-free extract	55.8	89.7	1.61	100	1
Ammonium sulfate	6.8	17.8	2.62	20	1.6
Q-sepharose	0.2	0.67	3.34	0.7	2.1

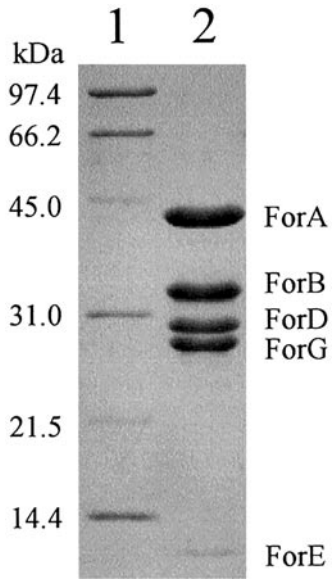

FIG. 2. SDS–PAGE of the purified For enzyme. Lane 1, low-molecular-weight standard proteins (Bio-Rad). Lane 2, 10 μg of For purified from *E. coli* JM109 carrying pYNA203. The gel was subjected to coomassie brilliant blue staining after electrophoresis.

than the other bands. It was not certain whether the band was derived from *forE* or *forF;* therefore, the N-terminal sequence of the polypeptide in the band was sequenced. The nine N-terminal amino acid residues were completely identical with those of the translated sequence of *forE* (data not shown), indicating that the band was derived from the *forE* gene product. These results indicated that For is composed of five subunits, i.e., ForA, ForB, ForG, ForD, and ForE.

Substrate Specificity of For

The substrate specificity of For was determined by using the purified enzyme. Eight 2-oxoacids (2-oxoglutarate, 2-oxobutyrate, 2-oxoisocaproate, 2-oxoisovalerate, oxalacetate, oxomalonate, pyruvate, and phosphoenolpyruvate) were used as substrates for the enzyme assay. As in the case of Kor (6), For showed an extremely high specificity toward 2-oxoglutarate (Table 3). The specificity of the purified For was almost the same as that of the CFE prepared from JM109 (pYNA202) (data not shown).

Transcriptional Start Points of the kor and for Genes

Transcriptional initiation sites of the *kor* and *for* genes were determined by primer extension analysis (Fig. 3). The mRNA used for the template was prepared from the cells of strain TK-6 autotrophically grown under aerobic conditions. Primer extension products were detected when both primers complementary to *korA* and *forD* were used. The result indicated that both *kor* and *for* genes were transcribed in the strain TK-6 cells under the growth conditions. The transcriptional start site of the *kor* genes was found 15 bp upstream from the initiation codon of *korA* (Figs. 3A and 3C). Two adjacent transcriptional start points of the *for* genes were detected 23 bp and 24 bp upstream from the initiation codon of *forD* (Figs. 3B and 3C). A putative −10 sequence was found, but the −35 sequence was not in the *kor* promoter region. On the contrary, a typical −35 sequence was found, but the −10 sequence was not in the *for* promoter region (Fig. 3C).

DISCUSSION

OGOR is one of the key enzymes of the RTCA cycle and catalyzes the reductive carboxylation of succinyl-CoA to give 2-oxoglutarate and CoA in the autotrophically growing strain TK-6 cells (5, 6). In this work, we found a set of the genes (*forDABGE*) encoding the second OGOR. The For enzyme, the gene product of *forDABGE*, is similar to the four-subunit ORs. ForA, ForB, ForG, and ForE corresponded to the α-, β-, γ-, and δ-subunits of the four-subunit-type enzymes, respectively. The results of the heterologous expression of For and the SDS-PAGE of the purified enzyme indicated that For is not a member of the well known four-subunit-type ORs but is a novel five-subunit-type enzyme. One of the subunits, ForD, is not similar to any components of the reported ORs except PorD, the δ-subunit of POR from strain TK-6 (DDBJ accession no. AB042412). Yoon *et al.* reported that POR of strain TK-6 is a four-subunit enzyme (16). The subunits were encoded by *porDABG* genes, whose gene products are 64%, 50%, 46%, and 57% identical with the *forDABG* gene products, respectively. Interestingly, an open reading frame encoding a protein, which is 70% identical with ForE, is located upstream of *porD*. This open reading frame also carries the D-domain-specific ferredoxin-like motif for the binding of [4Fe-4S] clusters. The [4Fe-4S] clusters of the D-domain are expected to be the site of electron transfer with the phys-

TABLE 3

Substrate Specificity of Purified For

Substrate	Relative activity (%)[a]
2-Oxoglutarate	100
2-Oxobutyrate	0.1
2-Oxoisocaproate	0.8
2-Oxoisovalerate	0.4
Oxalacetate	0.0
Oxomalonate	0.4
Pyruvate	1.9
Phosphoenolpyruvate	0.3

[a] Values are the percentage of each activity when the specific activity for 2-oxoglutarate is defined as 100%.

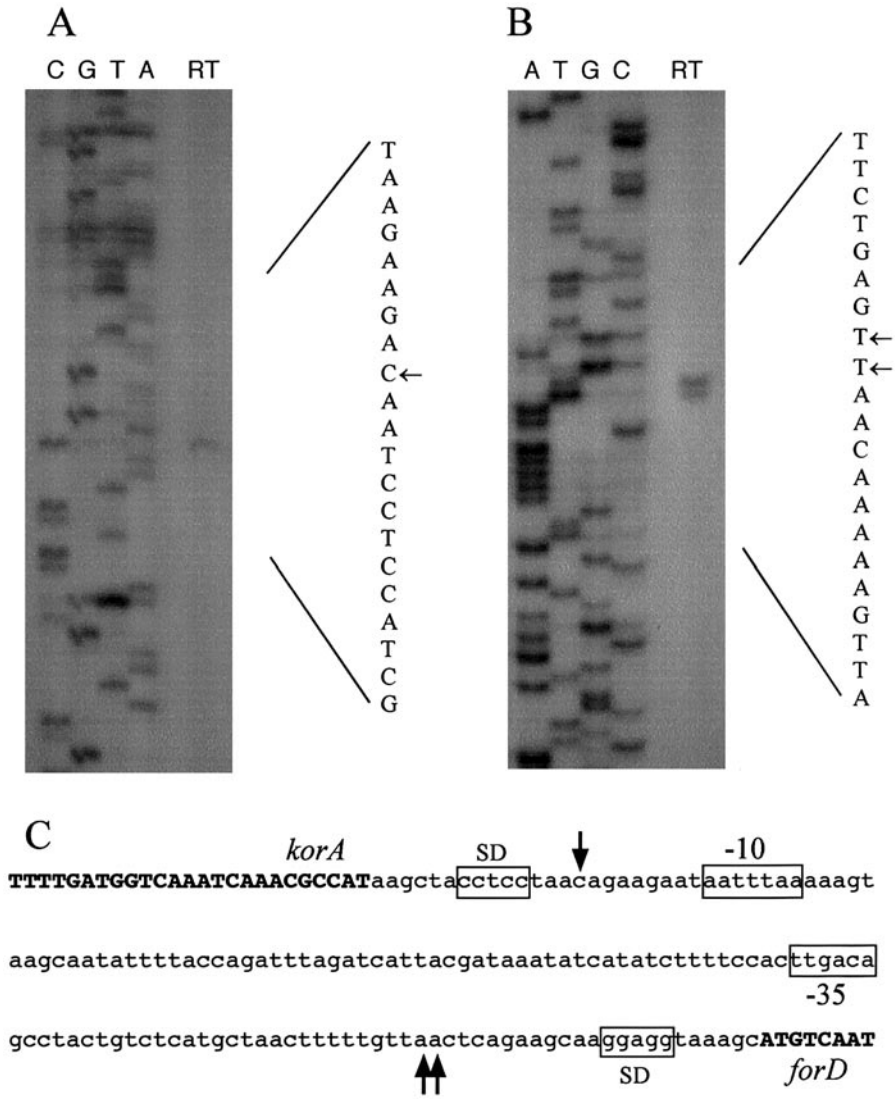

FIG. 3. Mapping of the transcriptional start sites of *korA* (A) and *forD* (B) by primer extension analyses. Lane RT contains the primer extension products from RNA isolated from autotrophically growing strain TK-6 cells. Sequencing ladders generated with the same primers are shown. The arrows indicate the start sites of transcription. (C) Nucleotide sequence of the *korA–forD* intergenetic promoter region. The ribosome-binding sites (SD) and putative −10 and −35 sequences are indicated by boxes. Coding regions of *korA* and *forD* are indicated by capital letters.

iological electron acceptor/donor, ferredoxin. Because the D-domain motif was not found in the other *por* gene products, the *forE*-like open reading frame might also be involved in the enzymatic activity of POR. The band of ForE that appeared on SDS-PAGE was weaker than those of the other subunits (Fig. 2). That relative weakness was probably the reason that the ForE-like fifth subunit in POR was not detected. It is not certain whether the molar ratio of ForE is lower than that of the other subunits or if ForE ran out during purification. We think the latter is the case, because the yield of the activity was very low (Table 2). ForE is smaller than the other δ-subunits of the four-subunit ORs because of the lack of the N-terminal extension that might act as an anchor to the enzyme complex, which may be the reason for the detachment.

The *for* genes is located 147 bp upstream of the *korAB* genes encoding another OGOR. The Kor enzyme, the gene product of *korAB*, is an $\alpha\beta$-type heterodimer enzyme. The *kor* and *for* genes were divergently transcribed from each other. Such a structure suggested the existence of some regulatory system that regulates a ratio of the *kor* and *for* gene products. Because no typical regulatory motif was found in this region and no transcriptional regulatory system of *Hydrogenobacter* has been reported so far, we cannot speculate on the regulatory mechanism at present.

Both Kor and For enzymes had high substrate specificity toward 2-oxoglutarate for oxidative reactions. When we purified OGOR from strain TK-6, the Kor enzyme was obtained, but the For enzyme was not (6). However, the For enzyme might also be expressed in the strain because primer extension products were detected when a primer complementary to *forD* was used. We do not know why strain TK-6 has two different types of OGOR at present. Studies on the kinetic properties and regulation of the two enzymes would be necessary to determine this and these experiments are now under way. We compared the enzymatic activity of the oxidative decarboxylation reaction by using methyl viologen as an artificial electron acceptor, because quantitative analysis of the reductive carboxylation reaction is difficult in the absence of a physiological electron donor at present. The development of an analytical method for the carboxylation reaction might be necessary to determine the physiological roles of the two OGORs in strain TK-6.

Interestingly, *A. aeolicus,* which is phylogenetically very close to *Hydrogenobacter,* does not have genes corresponding to *korAB.* The genome sequence of *A. aeolicus* showed that the bacterium has two gene clusters encoding putative ORs. They are similar to the *for* gene cluster encoding OGOR and the *por* gene cluster encoding POR of strain TK-6, respectively (17). *A. aeolicus* also fixes carbon dioxide by the RTCA cycle, suggesting that Kor is not necessary for the RTCA cycle in the presence of For. Because *Hydrogenobacter* grows much faster than *Aquifex,* carrying two OGOR enzymes might have some advantageous effect on the growth of *Hydrogenobacter.*

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