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Cloning, Sequencing, and Oxygen Regulation of the *Rhodobacter capsulatus* α-Ketoglutarate Dehydrogenase Operon

F. P. DASTOOR,[1] M. E. FORREST,[2] AND J. T. BEATTY[1,3]*

Departments of Microbiology and Immunology[1] and Medical Genetics,[3] University of British Columbia, Vancouver, British Columbia V6T 1Z3, and Department of Biology, Okanagan University College, Kelowna, British Columbia V1V 1V7,[2] Canada

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The *Rhodobacter capsulatus sucA*, *sucB*, and *lpd* genes, which encode the α-ketoglutarate dehydrogenase (E1o), the dihydrolipoamide succinyltransferase (E2o), and the dihydrolipoamide dehydrogenase (E3) components of the α-ketoglutarate dehydrogenase complex (KGD), respectively, were cloned, sequenced, and used for regulatory analyses. The KGD enzymatic activity was greater in cells grown under aerobic, respiratory growth conditions than under anaerobic, photosynthetic conditions. Similarly, the *sucA* gene was transcribed differentially, leading to a greater accumulation of *sucA* mRNAs under respiratory growth conditions than under photosynthetic conditions, although differential rates of mRNA decay could also contribute to the different amounts of *sucA* mRNAs under these two growth conditions. The *sucA* promoter was located about 4 kb upstream of the 5′ end of the *sucA* gene, and transcripts greater than 9.5 kb hybridized to a *sucA* probe, suggesting the presence of an operon that produces a polycistronic mRNA. Thus, these genes seem to be expressed as an unstable primary transcript, and we speculate that posttranscriptional processes control the stoichiometry of KGD proteins.

Rhodobacter capsulatus is a purple nonsulfur phototrophic bacterium capable of growth by a number of different metabolic processes, including aerobic respiration and anaerobic photosynthesis (21, 22). During aerobic respiratory growth, *R. capsulatus* uses the citric acid cycle (CAC) to produce reducing equivalents for ATP synthesis by oxidative phosphorylation (4, 12). When the oxygen tension in the growth environment drops below a critical concentration, cells of *R. capsulatus* respond by modification of metabolic pathways and synthesis of differentiated membrane structures for photosynthetic energy transduction (14). Although the photosynthetic physiology has been much studied, little is known of the regulation of aerobic metabolism in *R. capsulatus.*

The KGD enzyme complex catalyzes the oxidative decarboxylation of α-ketoglutarate to succinyl coenzyme A (succinyl-CoA), which links the tricarboxylic acid branch (conversion of oxaloacetate plus acetyl-CoA to α-ketoglutarate) to the dicarboxylic acid branch (conversion of succinyl-CoA to oxaloacetate) of the CAC. It has been reported that the activities of KGD and other enzymes involved in respiration, although present, are lower in cultures of *R. capsulatus* grown under anaerobic photosynthetic growth conditions than under aerobic conditions (4, 12). These changes in KGD and other respiratory enzyme activities suggest that although the CAC functions under photosynthetic growth conditions, its activity is reduced because it is required only for the generation of biosynthetic precursors.

The KGD enzyme complex has been purified from several eukaryotes and prokaryotes and is invariably composed of three proteins: α-ketoglutarate dehydrogenase (E1o), dihydrolipoyl succinyltransferase (E2o), and dihydrolipoamide dehydrogenase (E3). These three proteins form a large complex with a molecular weight in excess of 10^6 and a normalized protein stoichiometry of 1E1o:2E2o:1E3 (for summaries of α-keto acid dehydrogenase complexes, see reference 29). Oxygen regulation of KGD enzymatic activity appears to be important for the modulation of CAC activity in organisms (such as *R. capsulatus* and *Escherichia coli*) that are capable of aerobic and anaerobic growth, and the KGD activity generally reflects overall CAC activity and respiration (4, 12, 35, 38).

The *E. coli* genes *sucA*, *sucB*, and *lpd* (which encode the E1o, E2o, and E3 proteins, respectively) have been cloned and sequenced, and their structures, as well as aspects of their regulation of expression, have been elucidated in a series of elegant publications by Guest and colleagues (25). The *sucAB* operon gives rise to a dicistronic mRNA, although some transcripts also encode adjacent CAC genes, and a *sucAB* promoter is located within 250 bases of the 5′ end of the *sucA* gene (37). Two transcripts initiated from the *sucAB* promoter were detected. One is long enough to comprise the *sucAB* genes (*ca.* 4.4 kb). The second is longer and, in addition to the *sucAB* genes, encodes the downstream *sucC* and *sucD* genes (which encode the β and α subunits of succinyl-CoA synthetase, respectively [37, 38]). The *lpd* gene is part of the *aceEFlpd* (pyruvate dehydrogenase) operon, which is not closely linked to the *sucAB* locus (25).

As part of a study of the oxygen regulation of respiratory enzyme genes in *R. capsulatus*, we have initially focused on the KGD complex. In this paper, we present the *R. capsulatus* E1o, E2o, and E3 protein sequences as predicted from the *sucA*, *sucB*, and *lpd* gene sequences and show that the transcriptional organization of these genes is different from that in other species. We also present data which show that transcription initiation of the *sucA* gene (and presumably of *sucB* and *lpd*) is influenced by the levels of oxygen in the culture medium and that this difference in transcription corresponds to steady-state *sucA* mRNA levels and the activity of the KGD enzyme complex. This oxygen regulation of *sucA* is independent of the previously described RegB-RegA sensor kinase/response regulator system (2, 18).

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of British Columbia, 300-6174 University Blvd., Vancouver, British Columbia, V6T 1Z3, Canada. Phone: (604) 822-6896. Fax: (604) 822-6041. E-mail: jbeatty@unixg.ubc.ca.

MATERIALS AND METHODS

Bacterial strains and plasmids. The following *R. capsulatus* strains have been described: the wild-type strain B10 (40); the phage-free, rifampin-resistant derivative of B10, SB1003 (36); the KGD$^-$ mutant KGD11 (5); the *regA* derivative of SB1003, MS01 (33); and the *regB* derivative of SB1003, JS9 (18, 27). The *E. coli* strains used for cloning and subcloning and as plasmid donors in conjugations were C600 $r^- m^+$ (7), JM83 (43), SM10 (34), and MC1061 (9). Strain HB101(pRK2013) (13) was used as the helper strain in some conjugations, and DH5αF′ (Gibco BRL) was the host when single-stranded DNA was produced for sequencing. Plasmids pTZ18U and pTZ19U (24), pRK415 (19), pXCA601 (1), and pLAFR1 (16) were used.

Growth conditions. *E. coli* strains were routinely grown in Luria-Bertani medium (31). All *R. capsulatus* strains were grown on minimal RCV or complex YPS medium (4, 40). Media used for the growth of *R. capsulatus* KGD11 were deoxygenated by storage in BBL GasPak jars (Becton Dickinson) for at least 1 day before use.

The conditions for high aeration and photosynthetic growth of *R. capsulatus* strains were essentially as described previously (42), except that the cultures were started from low-oxygen precultures (20).

Antibiotics were added when appropriate to the following concentrations: ampicillin, 200 μg/ml (*E. coli*); kanamycin, 10 μg/ml (*E. coli* and *R. capsulatus*) or 70 μg/ml (during preparation of single-stranded DNA for sequencing); tetracycline, 10 μg/ml (*E. coli*) or 0.5 μg/ml (*R. capsulatus*). Succinate at 5 mM was used as a growth supplement for strain KGD11. The *E. coli* and *R. capsulatus* strains were grown at 37 and 34°C, respectively, and culture densities were measured with a Klett-Summerson photometer fitted with a no. 66 (red) filter.

Transformation and conjugation. *E. coli* cells were made competent by the $CaCl_2$ method and transformed as described previously (23). Plasmid DNA was introduced into *R. capsulatus* strains by either triparental (13) or biparental (34) conjugations with *E. coli* donors; recipient cells were purified by streaking onto RCV agar plates supplemented with the appropriate antibiotics. The conjugative transfer of pLAFR1 from *E. coli* into *R. capsulatus* occurs at a frequency of 10^{-1} to 10^{-2}.

Differentiation between complementation in *trans* and recombination. Conjugation was allowed to occur overnight under anaerobic photosynthetic conditions in BBL GasPak jars where the cell suspension was spotted onto 13-mm-diameter, 0.45-μm-pore-size Millipore filters on RCV agar plates supplemented with 0.1% glucose (for the fermentative growth of *E. coli*) and succinate. The filters carrying the conjugated cells were used to inoculate 21-ml screw-cap tubes filled with deoxygenated RCV medium supplemented with tetracycline. After tetracycline-resistant KGD11 exconjugants had arisen, dilutions were spread on duplicate plates of RCV medium containing tetracycline. One plate was incubated under aerobic conditions to select for the KGD$^+$ phenotype, and the second plate was incubated photosynthetically to allow both KGD$^-$ and KGD$^+$ cells to grow. The colonies that arose were counted, and the number of colonies on the aerobically incubated plate was compared to the number on the photosynthetically incubated plate. If the number of aerobically competent (KGD$^+$) cells was similar to the number arising on photosynthetic plates, *trans* complementation was assumed. Where the number of colonies on aerobic plates was much smaller than that on photosynthetically incubated plates (KGD$^+$ and KGD$^-$; typically ≤1%) and this number increased over a 5-day incubation period (usually to ~20%), the plasmid being tested was credited with allowing recombination to take place. If few of the cells spread formed colonies (~10^{-6}, due to reversion of the KGD11 mutation) under aerobic conditions, it was assumed that neither recombination nor complementation was possible with the plasmid being tested.

DNA sequence analysis. Nested deletions were created with the New England Biolabs Exo-Size deletion kit. The S1-S2 DNA fragment (see Fig. 1) was subcloned into pTZ19U in both orientations, so that unique 5′ and 3′ restriction enzyme sites existed adjacent to the universal sequencing primer site, and these sites were used to initiate deletion fragments. Gaps in sequences were filled by use of oligonucleotide primers complementary to sequences obtained with the universal primer or by restriction enzyme digestion to eliminate sequenced portions of individual clones of interest.

Computer analysis of the sequence data was performed with the Geneworks 2.45 software package (IntelliGenetics, Inc.) as well as BLAST analysis through the National Center for Biotechnology Information (WWW URL, http://www.ncbi.nlm.nih.gov), and the free energy of formation of RNA stem-loop structures was calculated by the method of Zuker and Turner (WWW URL, http://www.ibc.wustl.edu/~zuker/rna/form1.cgi).

RNA isolation, blotting, and hybridization. High-oxygen and anaerobic photosynthetic cultures of *R. capsulatus* B10 were grown to late logarithmic phase (ca. 8×10^8 CFU/ml), the cells were pelleted by centrifugation through an ice slurry, and total RNA was extracted with 65°C water-saturated unbuffered phenol as previously described (39). In some experiments, a guanidine isothiocyanate method was used for RNA isolation (11).

Samples containing 20 μg of RNA were ethanol precipitated, resuspended in 10 μl of RNA resuspension buffer, denatured, and subjected to formaldehyde gel electrophoresis as described previously (30). The gels were rinsed for 5 min in distilled H_2O, photographed on a UV light transilluminator to determine the position and quality of the various RNA species with respect to the molecular size markers (0.24- to 9.5-kb RNA ladder), and then equilibrated in two changes of 0.5× Tris-borate-EDTA (TBE) buffer (31) for 25 min at room temperature.

The RNA was transferred to Bio-Trans nylon membranes (prewetted in 0.5× TBE buffer) by electroblotting in 0.5× TBE buffer at 20 V for about 16 h followed by 80 V for 2 h. The buffer was cooled with a water circulation coil during the transfer. The membrane was soaked in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) (31), the membranes and gels were photographed on a UV transilluminator to confirm efficient transfer, and the RNA was fixed to the membrane by being baked in a vacuum oven at 80°C, followed by a 10-min UV cross-linking step. The membranes were prehybridized for a minimum of 2 h at 42°C in 20 ml of 5× SSPE–0.3% sodium dodecyl sulfate–50% formamide–100 μg of sheared salmon sperm DNA per ml. The prehybridization solution was replaced with 3 ml of fresh solution, to which was added approximately 50 ng of alkali-denatured ^{32}P-labelled *sucA*-specific DNA probe (labelled by the random oligonucleotide primer method [15]).

Hybridization was carried out overnight at 42°C, after which the membranes were washed three times for 7 min each in 200 ml of 2× SSPE–0.1% sodium dodecyl sulfate at 45°C and then used to expose X-ray film at −80°C with an intensifying screen.

Densitometer scans of RNA blot autoradiograms. The intensities of the signals detected on RNA blot autoradiograms were determined by scanning defined areas (0.2 to 10 kb) over each lane with a Molecular Dynamics densitometer. The data were obtained with the ImageQuant V.3.0 Fast Scan software package with integration over the entire exposed area of the lane.

RNA pulse-labelling experiments. High-oxygen and photosynthetic cultures (20 ml) of *R. capsulatus* B10 were grown to ca. 7.4×10^8 CFU/ml. The cultures were pulsed with 1.2 mCi of [5,6-^{3}H]uridine (37.6 mCi/mmol, 1.0 mCi/ml) for 2 min. Following the pulse, the cells were pelleted and total RNA was isolated as described above. The specific incorporation of labelled uridine into RNA was determined by precipitation of 0.6 to 1.5 μg of RNA in ice-cold 10% trichloroacetic acid (TCA), collection of the precipitate on fiberglass filters (no. 25; Schleicher & Schuell), and counting of radioactive decay in a toluene-based scintillation fluid. The amounts of radioactivity were normalized to RNA concentrations, which were determined by measurement of absorption at 260 nm. The specific activities of the RNA preparations from aerobically grown cultures ranged from 6.2×10^4 to 2.2×10^5 cpm μg of RNA^{-1}, and those from photosynthetically grown cultures ranged from 2.9×10^4 to 5.7×10^4 cpm μg of RNA^{-1}.

Single-stranded phage DNA containing the *Eco*RV-*Bam*HI segment of the *sucA* gene (see Results and Discussion), either complementary (in pTZ18U) or noncomplementary (in pTZ19U) to the *sucA* transcript, was spotted in 0.5-, 1.0-, and 2.0-μg amounts onto 0.45-μm-pore-size nitrocellulose filters. The filters were allowed to air dry before being baked in a vacuum oven at 80°C for 2 h. Each set of complementary- and noncomplementary-strand DNA-spotted filters was placed into screw-cap vials (19 by 65 mm) and prehybridized for 2 h in 5 ml of prehybridization solution as described above (yeast tRNA was substituted for sheared salmon sperm DNA). The prehybridization solution was replaced with 2.1 ml of fresh solution to which was added the entire pulse-labelled RNA preparation (24 to 59 μg of total cellular RNA). Hybridization took place overnight at 42°C, the filters were washed as described above and air dried under a 150-W flood lamp for 6 h, and radioactive decay events were counted over a 10-min period. The counts resulting from hybridization to the noncomplementary probe were subtracted from the values obtained with the complementary probe to yield net values of radioactive *sucA*-specific RNA. Portions of 50 μl (before addition of the probe) and 10 μl (after hybridization) of the hybridization liquid were withdrawn, and the RNA in these samples was TCA precipitated, collected, and counted as a control to ensure that the RNA was not degraded during hybridization (extensive degradation would have significantly reduced the number of TCA-precipitable counts, which was not observed).

Enzyme assays. For KGD assays, *R. capsulatus* cultures (20 ml) were grown and cells were harvested as described for RNA isolation (omitting the ice slurry). Cell pellets were resuspended in 1.0 ml of 50 mM KPO_4 buffer (pH 7.0) and disrupted by sonication. Sonicated cells were centrifuged for 1 min at 15 600 × *g* to remove intact cells and large membrane particles, and the supernatant fluids were used for KGD enzyme assays as described previously (5). Enzyme activities were normalized to the protein contents of the extracts as determined by a modified Lowry method (28) with bovine serum albumin as a standard, and units were expressed as the net number of (αKG-dependent) nanomoles of NADH produced per minute per milligram of protein.

Cells used for β-galactosidase assays were resuspended in 1 ml of Z buffer (26) and disrupted by sonication, and extracts were cleared of cell debris as described above. The β-galactosidase activities in cleared cell extracts were assayed by continuous measurement of the rate of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) cleavage as previously described (41). β-Galactosidase activities were normalized to the total protein content in the extracts as described above, and units were expressed as nanomoles of ONPG cleaved per minute per milligram of protein.

Other methods. The *R. capsulatus* B10 gene library was prepared by *Eco*RI partial digestion of chromosomal DNA which was size fractionated by agarose gel electrophoresis to obtain fragments of 15 to 25 kb, ligated with the cosmid vector pLAFR1, packaged in vitro, and used to infect *E. coli* MC1061.

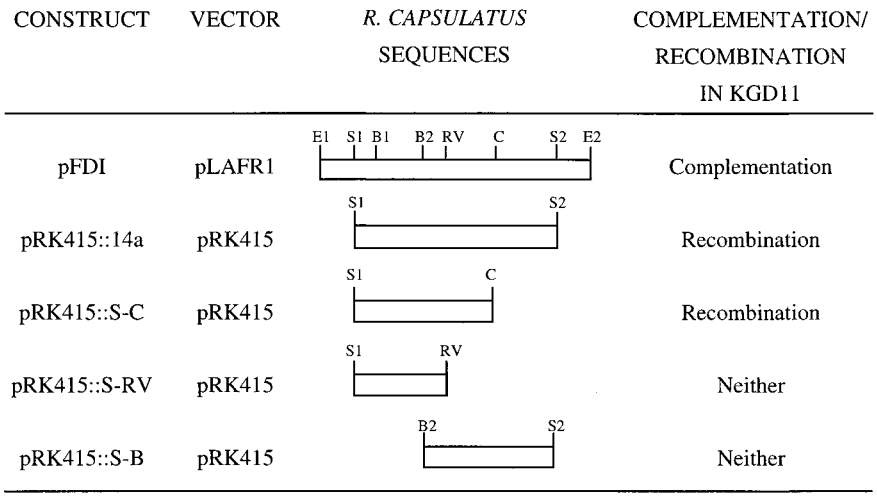

FIG. 1. Diagrammatic representation of the *R. capsulatus* DNA segments used in complementation/recombination assays with the KGD$^-$ mutant strain KGD11. Additional restriction sites used in subcloning experiments are also indicated. Restriction sites are represented as follows: E1 and E2, *Eco*RI; S1 and S2, *Sst*I; B1 and B2, *Bam*HI; RV, *Eco*RV; C, *Cla*I.

Routine molecular biology techniques were carried out as described previously (31).

Nucleotide sequence accession number. The *R. capsulatus sucAB lpd* sequence has been deposited in GenBank and assigned accession no. U84577.

RESULTS AND DISCUSSION

Cloning and sequencing of the *R. capsulatus sucA* gene and flanking sequences. The *R. capsulatus* B10 gene library was mobilized into the KGD$^-$ strain KGD11, and a selection for aerobic growth of this mutant strain (which is incapable of aerobic growth on CAC intermediates) was used to identify cosmids that complemented the KGD$^-$ phenotype. Two different cosmid clones were found to complement in *trans* the KGD$^-$ phenotype in KGD11. An *Eco*RI digestion of both cosmids produced a pattern suggesting that they contained overlapping segments of DNA and differed in the amount of flanking sequences. The smaller cosmid, containing a 15-kb insert of *R. capsulatus* DNA, was chosen for further studies and designated pFDI (Fig. 1). Complete *Eco*RI digestion of pFDI generated six fragments, none of which individually complemented the KGD$^-$ phenotype after subcloning in pRK415 and conjugation into strain KGD11. However, it was observed that a 12-kb *Sst*I fragment (in plasmid pRK415::14a) and an 8-kb *Sst*I-*Cla*I fragment (in pRK415::S-C) rescued the KGD$^-$ phenotype at a much lower frequency than did the original pFDI plasmid (Fig. 1). Furthermore, the number of KGD11 (pRK415::14a) and KGD11(pRK415::S-C) colonies that arose on aerobically incubated plates increased to approximately 20% of the number of colonies that grew photosynthetically over a 5-day incubation period (the reversion frequency of KGD11 in these experiments was 1.9×10^{-6}).

These results led us to conclude that plasmids pRK415::14a and pRK415::S-C contain the wild-type sequence and sufficient flanking DNA to allow replacement by homologous recombination of the mutation in KGD11. However, the lack of *trans* complementation implied that parts of the gene mutated in KGD11 and/or parts of its regulatory sequences were missing on the subcloned fragments.

Since plasmid pRK415::S-C, but not pRK415::S-RV or pRK415::S-B, restored aerobic growth to KGD11 through recombination (Fig. 1), we suspected that the ca. 0.9-kb region between, or a sequence very close to the *Bam*HI (B2) and *Eco*RV (RV) sites contained the mutation that caused the KGD11 phenotype. Sequencing of the B2-RV region revealed a nucleotide sequence with homology to previously sequenced *sucA* genes. Thus, the KGD11 strain probably has a mutation of the *sucA* gene, which accounts for the loss of KGD activity.

The DNA regions flanking the B2-RV restriction sites were sequenced and found to contain a 989-codon open reading frame with 43% amino acid sequence identity to the *sucA*-encoded E1o protein of *E. coli*. The 2,970-bp *R. capsulatus sucA* gene is preceded by a putative ribosome binding site (AGGA) 7 nucleotides upstream of the proposed translation start site, but there are no sequences compellingly indicative of a promoter in the 50 nucleotides 5′ of the ATG codon (although the elements of an *R. capsulatus* promoter are not well understood), and it ends with a TGA codon (see GenBank deposition U84577). In addition to the overall 43% amino acid sequence identity between the *R. capsulatus* and *E. coli* E1o proteins, the *R. capsulatus* sequence contains regions corresponding to sites of phosphorylation found in the mammalian pyruvate dehydrogenase enzyme (underlined amino acids 431 to 444 and 483 to 495 in the *R. capsulatus* sequence) (Fig. 2A) (29), as well as a region predicted to contain the binding domain for a thiamine pyrophosphate cofactor (underlined amino acids 402 to 430 in Fig. 2A) (17).

Sequencing of the *sucA* 3′-flanking region revealed the 1,238-bp *sucB* gene, which is separated from the *sucA* stop codon by 1 nucleotide and is preceded by a putative ribosome binding site (GGAGG) within the last two *sucA* codons (see GenBank deposition U84577). The predicted *sucB* gene product shares 50% amino acid sequence identity with the *E. coli* E2o protein (Fig. 2B). The predicted *R. capsulatus* E2o protein contains a conserved lysine residue (Lys_{42}) believed to be the binding site for the lipoic acid moiety, as well as conserved histidine (His_{383}) and aspartic acid (Asp_{387}) residues believed to be in the active site (8).

Sequencing of the *sucB* 3′-flanking region revealed a third open reading frame, *lpd*, which is 1,364 nucleotides long, starts 128 nucleotides downstream of *sucB*, and encodes a protein that shares 38% amino acid sequence identity with the *E. coli* E3 protein (Fig. 2C). The *R. capsulatus* E3 protein contains the

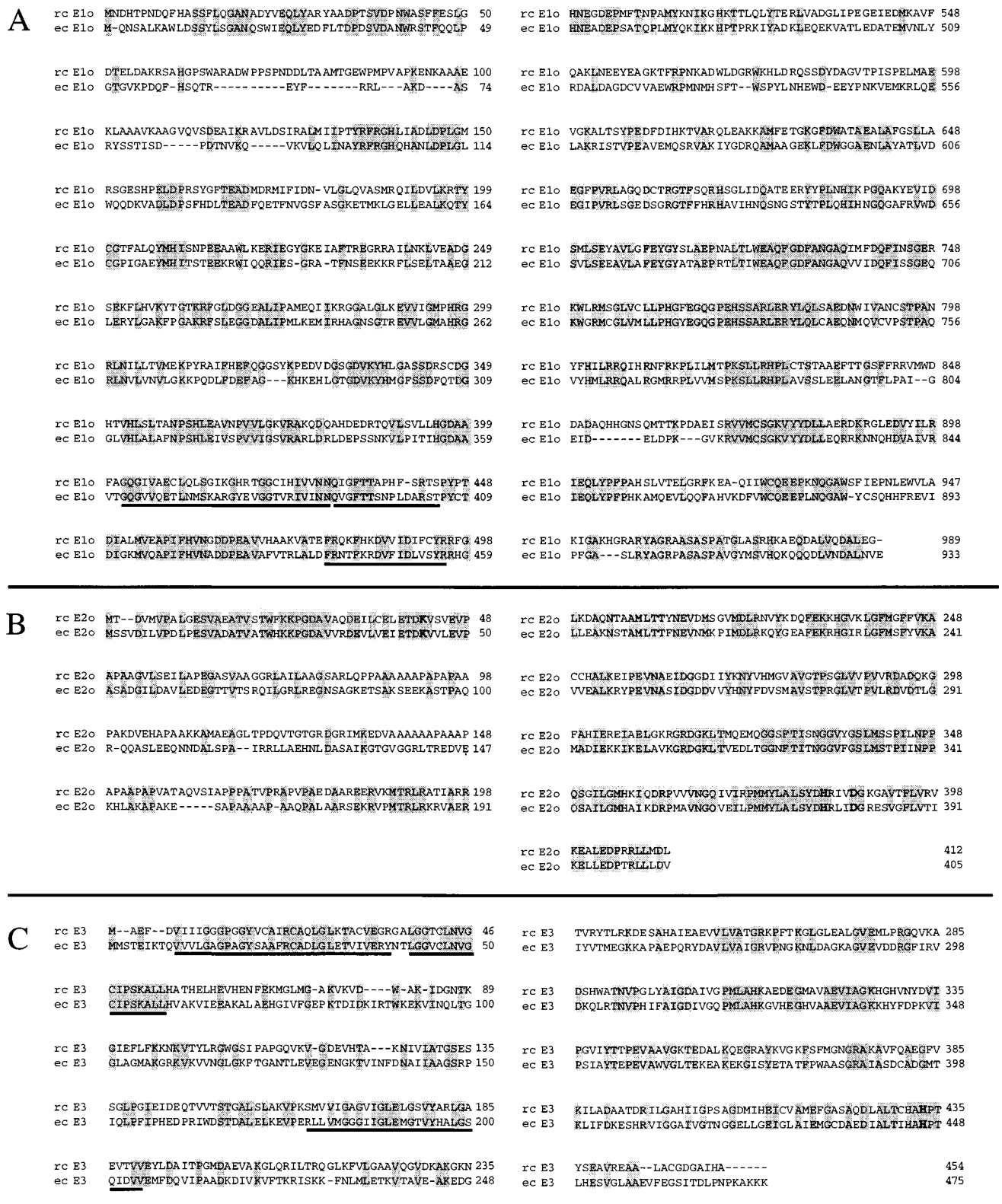

FIG. 2. Amino acid sequence alignments between the predicted *R. capsulatus* (rc) proteins E1o (A), E2o (B), and E3 (C) and the corresponding *E. coli* (ec) homologs. Underlined and boldface sequences are described in the text. Identical amino acids are indicated by shading, and gaps are indicated by hyphens.

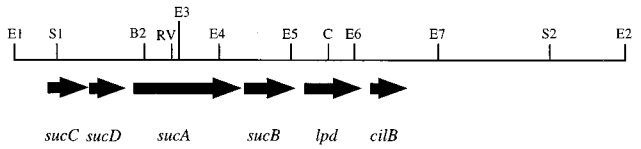

FIG. 3. Organization of *sucC*, *sucD*, *sucA*, *sucB*, *lpd*, and *cilB* genes in relation to the fragments used for complementation/recombination and other assays. Restriction sites are designated as for Fig. 1. Arrows indicate the approximate sizes of genes and the direction of transcription.

ADP-binding site of flavin adenine dinucleotide (underlined amino acids 5 to 35), the catalytic disulfide bridge (underlined amino acids 38 to 54), the ADP-binding site of NAD^+ (underlined amino acids 163 to 191), and the catalytic histidine (His_{433}) (6).

The *sucB* structural gene is followed by two mutually exclusive potential mRNA stem-loop structures at bases 4310 to 4345 and bases 4314 to 4341 with predicted free energies of formation of −21.5 and −20.8 kcal/mol, respectively (see GenBank deposition U84577). Similarly, two mutually exclusive potential mRNA stem-loop structures are located downstream of *lpd* at bases 4310 to 4345 and bases 4314 to 4341 and are predicted to have free energies of formation of −24.5 and −23.8 kcal/mol, respectively (see GenBank deposition U84577).

The close physical association of *sucA* and *sucB* strongly suggests that the transcription and perhaps even the translation of these two genes is coupled. Whether the stem-loop structures predicted at the end of *sucB* and *lpd* occur in vivo and have a function in transcription termination, mRNA stability or processing remains to be tested.

The preliminary DNA sequence (to be reported elsewhere) of the *sucA* 5′ region revealed the presence of open reading frames with sequence homology to the *sucC* and *sucD* genes, and a sequence homologous to *cilB* (citrate lyase β subunit) genes was found to begin 137 nucleotides 3′ of *lpd*. The genetic organization of this region of the *R. capsulatus* chromosome is summarized in Fig. 3.

The observation that aerobic growth of KGD11 (pRK415::14a) occurred only after recombination between the wild-type sequence on pRK415::14a and the chromosomal mutated sequence whereas pFDI was able to complement the mutation in *trans* suggested that one of the regions bordered by the *Eco*RI and *Sst*I sites (designated E1-S1 and E2-S2 in Fig. 1) is important for expression of the *sucA* gene. The orientation and close proximity of the *sucA*, *sucB*, and *lpd* genes (Fig. 3), the lack of sequences indicative of a promoter immediately upstream of *sucA*, and the location and polarity of *sucC* and *sucD*, taken together, suggested that the E1-S1 region contains a *cis*-active element, such as a promoter, necessary for expression of these genes. This putative promoter would be at least 4 kb upstream of the start of the *sucA* gene. This organization is different from *E. coli*, in which a promoter of the *sucAB* operon is located relatively close to the start of the *sucA* gene and *sucC* and *sucD* are located downstream (25, 37, 38). Southern blots of *R. capsulatus* chromosomal DNA probed with DNA fragments from pFDI indicated that the arrangement of the *sucA* flanking regions found in pFDI is the same as in the chromosome and is not due to a cloning artifact (data not shown).

The complementation/recombination and DNA sequence data, taken together with the transcriptional data (see below), suggest that these genes are encoded by a primary transcript that includes the *sucC*, *sucD*, *sucA*, *sucB*, and *lpd* (and perhaps *cilB*) genes for oxygen regulation of CAC enzymes in *R. capsulatus.*

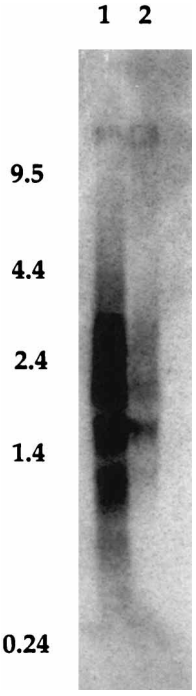

FIG. 4. Autoradiogram of a blot hybridization of *R. capsulatus* B10 RNA. Lanes: 1, 20 μg of RNA isolated from aerobically grown cultures; 2, 20 μg of RNA from photosynthetically (anaerobically) grown cultures. The faint areas of hybridization seen above and below the ca. 1.5-kb region are due to interference from rRNA. Molecular lengths of RNA size standards are given in kilobases on the left. The probe used was the radioactively labelled *Bam*HI-*Eco*RV fragment (Fig. 3).

Oxygen regulation of KGD activity and *sucA* transcripts. We found that under the growth conditions used in this study, the KGD enzyme specific activity in cell extracts of B10 cultures grown with high aeration averaged 57 ± 19 U, compared to 5 ± 0.8 U in extracts of photosynthetically grown anaerobic cultures, a difference of ca. 10 fold. This oxygen control of KGD activity could result from modulation of transcription initiation of the *sucAB lpd* genes. However, since these genes encode separate components of a multienzyme complex and potential E1o phosphorylation sites were revealed from the sequence analyses, there was the possibility for regulation of KGD activity at several loci through one or more of the various transcriptional and posttranscriptional mechanisms that have been documented in prokaryotes.

To test for oxygen regulation of KGD activity by modulation of the steady-state levels of *sucA* gene transcripts, RNA blots were done with RNA isolated from B10 cultures grown either with high aeration or under anaerobic, photosynthetic conditions. The B2-RV fragment within *sucA* (Fig. 3) was used as a probe in RNA blots for *sucA* mRNAs, and an autoradiogram from these experiments is shown in Fig. 4. It can be seen that RNA preparations from aerobic and photosynthetic cultures contained molecules complementary to the probe that ranged in length from about 0.5 to greater than 9.5 kb, although the most intense signal was obtained from species of 1 to 3 kb. Different methods for RNA purification yielded essentially the same results, although these methods gave discrete bands with other relatively stable *R. capsulatus* mRNAs (1, 10, 11, 33, 44). Therefore, we interpret the results shown in Fig. 4 as being suggestive that *sucA* mRNA is synthesized as a large (>9.5-kb) primary transcript that is very unstable. This interpretation is

TABLE 1. Hybridization of pulse-labelled total cellular RNA to a *sucA* mRNA-specific probe to evaluate the frequency of initiation of transcription of the *sucA* gene under high-oxygen respiratory and anaerobic photosynthetic growth conditions

Growth conditions	Amt (μg) of probe used	Hybridization[a]		Net cpm/μg of probe[b]	% of *sucA* RNA/μg of probe[c] (10^{-4})
		A	B		
High oxygen	0.5	370	180	380	
	1.0	651	190	462	
	2.0	1,103	225	439	
Net avg					81 (8.1)
Photosynthetic	0.5	126	119	14	
	1.0	125	113	12	
	2.0	155	140	7.4	
Net avg					4.3 (1.3)

[a] Hybridization of ^{3}H-labelled RNA to the *sucA* probe; A, specific hybridization to the complementary pTZ18U probe; B, nonspecific hybridization to the noncomplementary pTZ19U probe.
[b] Determined by subtracting nonspecific hybridization from specific hybridization and normalizing the result to 1.0 μg of probe.
[c] Results expressed as a percentage of total TCA-precipitable counts in the hybridization mixture. Values within parentheses are standard deviations. The ratio of high-oxygen to photosynthetic *sucA* mRNA detected was 19.

consistent with the finding that a *sucA* promoter seemed to be more than 4 kb upstream of the 5′ end of the *sucA* gene (see above) and also implies that there is *sucA* transcription readthrough into the *sucB*, *lpd*, and perhaps *cilB* sequences downstream of the *sucA* gene. It is noteworthy that very large, unstable primary transcripts of oxygen-regulated photosynthesis gene "superoperons" exist in *R. capsulatus* (3).

Densitometry of autoradiograms of three independent RNA blots similar to that in Fig. 4 showed that there were on average seven times more *sucA*-encoding mRNA in cells from cultures grown with high aeration than in photosynthetic cultures. The steady-state levels of *sucA* mRNA observed in high-oxygen and photosynthetically grown cells closely reflect the relative KGD specific activities measured (see above), indicating that the different KGD enzyme activities measured were due to different amounts of the enzyme complex, which corresponded to differences in the amounts of *sucA* mRNA.

The differences in steady-state levels of *sucA* mRNA noted above could have resulted from differential transcription initiation or from different rates of RNA decay under the two growth conditions. To assess the dependence of *sucA* mRNA levels on differential transcription initiation, as opposed to different mRNA degradation rates, the relative amounts of radioactively labelled *sucA* mRNA (as defined by molecules complementary to the B2-RV *sucA* probe [Fig. 3]) in high-oxygen and anaerobic photosynthetic cultures of *R. capsulatus* B10 were compared following a pulse with [^{3}H]uridine. The results are shown in Table 1. It can be seen that *sucA* mRNA comprised 81×10^{-4}% of labelled RNA in aerobically grown cultures compared to 4.3×10^{-4}% in photosynthetically grown cultures. That is, approximately 19 times more *sucA* mRNA was synthesized during the pulse in aerobically grown cultures than in photosynthetically grown cultures. The two cultures had the same growth rates and yielded similar amounts of total RNA, and so it seems that a major determinant of the steady-state level of *sucA* mRNA is the frequency with which transcription of the *sucA* gene initiates. Since there appeared to be a sevenfold difference in steady-state *sucA* mRNA levels in RNA blot experiments and a 19-fold difference in the levels in pulse-label experiments, it would seem that *sucA* mRNAs have different rates of decay under the two conditions of growth.

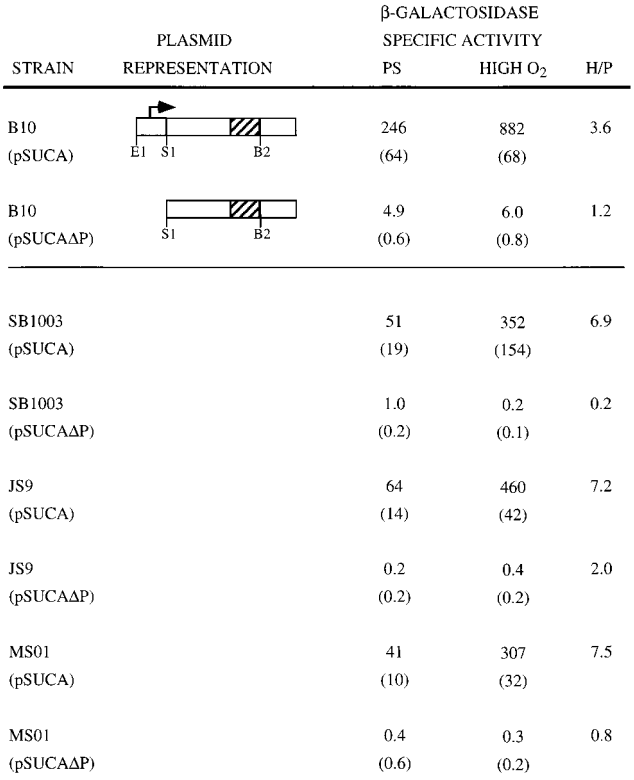

FIG. 5. β-Galactosidase specific activities in extracts of cells containing *sucA′*::*′lacZ* fusions. B10 is the parent of KGD11; SB1003 is the parent of *reg* mutant strains; JS9 is the *regB* mutant strain derived from SB1003; MS01 is the *regA* mutant strain of SB1003. Fusion constructs are as represented, where the hatched box represents the *sucA′* gene of *R. capsulatus* followed by the *′lacZ* allele. The approximate location of the putative *sucA* promoter is indicated by the arrow. Restriction sites are represented as follows: E1, *Eco*RI; S1, *Sst*I; B2, *Bam*HI. β-Galactosidase specific activity is expressed as nanomoles of ONPG cleaved per minute per milligram of protein; the values in parentheses are standard deviations for three independent assays. PS, photosynthetic cultures; high O_2, aerobic cultures. H/P, ratio of aerobic to photosynthetic β-galactosidase specific activities.

Although this possibility is worthy of additional investigation, we interpret the [^{3}H]uridine pulse results as only a semiquantitative index of *sucA* induction, mainly because the net values of radioactive *sucA* mRNA obtained from photosynthetically grown cultures were so low. To further evaluate this oxygen regulation, we measured the degree of oxygen induction of *sucA′*::*′lacZ* gene fusions as an independent method for assessment of *sucA* gene transcription initiation.

Oxygen control of expression of *sucA′*::*′lacZ* gene fusions. Two translationally in-frame gene fusions between the *sucA′* gene and the *′lacZ* allele of pXCA601 were engineered (Fig. 5). The plasmid pSUCA contains 4 kb of *R. capsulatus* DNA upstream of the B2 fusion site, including the sequences located between the E1 and S1 restriction sites that were required for complementation in *trans* of the KGD$^-$ phenotype of strain KGD11 (Fig. 1). Plasmid pSUCAΔP contains the same *sucA′*::*′lacZ* fusion joint as pSUCA, but the region between the E1 and S1 sites (which was presumed to contain the *sucA* promoter) is absent. These two plasmids were conjugated into strain B10, and the specific activities of β-galactosidase in extracts of cells grown with high aeration or photosynthetically were determined. The results obtained (Fig. 5) show that relatively large amounts of β-galactosidase were produced when the DNA region between the E1 and S1 sites was present. In contrast, ca. 2% of these

activities was produced from the plasmid from which these sequences are absent. These findings are consistent with the KGD11 complementation results, and the simplest interpretation is that a promoter of the *sucA* gene is located between the E1 and S1 sites.

The B10(pSUCA) β-galactosidase specific activities increased with increased culture aeration, and the ratio of average high-oxygen to anaerobic photosynthetic specific activities obtained was 3.6. This indicates that the activity of the *sucA* promoter is regulated by O_2, in support of the conclusions drawn from the RNA studies. However, the magnitude of oxygen regulation was not as great (see above). This could be due to several reasons, including plasmid copy number effects or different stabilities of the native *sucA* and *sucA′::′lacZ* gene fusion mRNAs.

The *regB* and *regA* genes recently discovered in *R. capsulatus* are believed to encode proteins of a "two-component" sensor kinase/response regulator system required for anaerobic activation of the *puf*, *puh*, and *puc* promoters of photosynthesis gene operons (2, 18, 27). Since the *sucA* gene promoter is also modulated by O_2 availability (although inversely, relative to the photosynthesis genes controlled by the RegB/RegA system), it was of interest to evaluate the effects of *regA* and *regB* mutations on expression of the *sucA* gene. Therefore, the β-galactosidase activities in extracts of the *R. capsulatus* parental strain SB1003 and the mutant MS01 (*regA*) or JS9 (*regB*) containing either of the *sucA′::′lacZ* fusion plasmids (pSUCA or pSUCAΔP) were measured under the same conditions of oxygen availability used in the experiments with the wild-type strain B10. The results, shown in Fig. 5, indicate that transcription of the *sucA* gene is not affected by RegA and RegB, since the β-galactosidase activities in both mutant strains were equivalent to that in the parental wild-type strain SB1003 under the conditions tested. Strain SB1003 yielded lower absolute β-galactosidase activities and a greater H/P ratio than did B10, which we attribute to strain differences. Therefore, oxygen-dependent modulation of *sucA* transcription initiation is controlled by a process independent of the RegB/RegA sensor kinase/response regulator system.

Concluding remarks. This paper reports the cloning and sequencing of the *R. capsulatus sucA*, *sucB*, and *lpd* genes, which encode the E1o, E2o, and E3 components of the KGD complex, respectively, and the use of the cloned *sucA* gene to evaluate mechanisms operative in oxygen control of KGD activity. The DNA sequence of the *sucA* 3′-flanking region revealed the close linkage and order of the *sucAB lpd* genes. The gross organization of the *sucA* gene with respect to the position of its promoter (and/or essential *cis*-active control sequences) in *R. capsulatus* appears to be different from that in other bacteria, because these sequences in *R. capsulatus* are located at least 4 kb upstream of the 5′ end of the *sucA* structural gene. Preliminary data reveal the presence of *sucC* and *sucD* genes between *sucAB lpd* and the promoter, as well as the location of the downstream *cilB* gene (Fig. 3). The DNA sequence, RNA blot, and *lacZ* fusion data indicate that the *sucA* gene is expressed as part of a large (>9.5-kb) primary transcript which is rapidly degraded to give smaller segments. Our results indicate that regulation of transcription initiation is the primary mechanism for modulation of KGD activity and perhaps of the overall activity of the CAC in response to the environmental signal of O_2 concentration. There could also be different rates of decay of mRNA segments under aerobic respiratory relative to anaerobic photosynthetic growth conditions, although we did not directly address this possibility. However, the different stoichiometries of E1o, E2o, and E3 in the KGD complex (29) invite speculation that in *R. capsulatus* this stoichiometry is achieved by differential decay rates of segments of a primary transcript that contains *sucA*, *sucB*, and *lpd*.

The *R. capsulatus* RegB/RegA sensor kinase/response regulator system does not regulate transcription initiation at the *sucA* promoter, which indicates that oxygen control of photosynthesis and CAC genes is carried out by separate, independent systems. Our cloning of these CAC genes and location of *cis*-active oxygen control sequences open the door to more detailed genetic and biochemical analyses of the processes required for the regulation of respiratory metabolic pathways in response to changes in O_2 concentration.

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