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Molecular Cloning and Sequencing of an Operon, *carRS* of *Azospirillum brasilense*, That Codes for a Novel Two-Component Regulatory System: Demonstration of a Positive Regulatory Role of *carR* for Global Control of Carbohydrate Catabolism

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A pleiotropic carbohydrate mutant, CR17, of *Azospirillum brasilense* RG (wild type) that assimilates C_4 dicarboxylates (succinate and malate) but not carbohydrate (fructose, arabinose, galactose, glycerol, and gluconate) as C sources for growth was used to identify the *car* (carbohydrate regulation) locus by complementation analysis. The 2.8-kb genomic fragment that complemented the Car$^-$ defect of CR17 and overlapped the *fru* operon (S. Chattopadhyay, A. Mukherjee, and S. Ghosh, J. Bacteriol. 175:3240–3243, 1993) has now been completely sequenced. The sequence contains an operon, *carRS*, coding for two proteins, CARR and CARS, having 236 and 352 amino acid residues, respectively. The 3′-flanking region of the *carRS* operon showed sequence homology with the 5′ terminus of the *fruB* gene of a related bacterium, *Rhodobacter capsulatus*. A complementation study with *carRS* deletion clones showed that only the *carR*$^+$ gene was required to complement the Car$^-$ defect of CR17, signifying that the carbohydrate pleiotropy was due to a lesion within this gene. Although the 2.8-kb DNA containing the *carRS* operon when introduced by conjugation into CR17 also complemented the Car$^-$ defect, the complemented transconjugant was unable to utilize succinate as a C source. The reason for this is not clear. A sequence analysis of the two protein products strongly suggests that the protein pair may constitute a novel two-component regulatory system for global expression of carbohydrate catabolic pathways in *A. brasilense*.

Gram-negative enterobacteria and strict aerobes differ remarkably in their regulation of carbohydrate catabolism. The preference of enterobacteria for the PTS (phosphoenolpyruvate:sugar phosphotransferase system) sugars as C sources for growth and their manifestation of the phenomena called diauxie and glucose effect are well known (for a review, see reference 16). On the other hand, aerobes prefer C_4 dicarboxylates (tricarboxylic acid cycle intermediates) rather than carbohydrates as C sources for growth, and a strong repression of inducible carbohydrate catabolic pathways in the presence of C_4 dicarboxylates that leads to the occurrence of reverse diauxie is observed (10, 18, 25, 26). The molecular basis of this reverse diauxie, i.e., the dominance of C_4 dicarboxylates (succinate and malate) over carbohydrates (glucose and fructose) as C sources for growth, in aerobic bacteria is not well understood.

Previous studies in several laboratories with *Azospirillum brasilense*, an N_2-fixing gram-negative aerobic bacterium, demonstrated that it utilizes succinate or malate in preference to carbohydrates (fructose, galactose, arabinose, gluconate, and glycerol) (11, 14, 18, 27). Fructose is the only sugar whose uptake and catabolism in this aerobic bacterium is mediated by the PTS (6, 11). Glucose is not assimilated by *A. brasilense* because of the absence of a transport system for the sugar (11). Studies from this laboratory showed previously that syntheses of fructose-inducible enzymes, enzyme I and enzyme II of the fructose PTS and 1-phosphofructokinase coded by the inducible *fru* operon, are subject to transient and permanent (catabolite) repression by succinate in *A. brasilense* (18). The bacterium also exhibited a succinate-fructose reverse diauxie (18).

To understand the regulatory mechanism for C-source utilization, biochemical and genetic investigation of the inducible carbohydrate catabolic enzymes and their repression by succinate was further carried out with wild-type *A. brasilense* RG and its Fru$^-$ and Car$^-$ (carbohydrate regulation) mutants (3). Previous studies from this laboratory (3, 18) demonstrated that (i) all carbohydrate (i.e., fructose, galactose, arabinose, glycerol, and gluconate)- inducible enzymes remain unexpressed when the bacterium is grown with succinate (or malate), (ii) a particular carbohydrate can induce, in the absence of succinate, only a specific set of enzymes needed for its transport and catabolism, and (iii) the bacterium possesses a global regulatory system that can switch off the syntheses of all inducible carbohydrate catabolic enzymes with the help of external succinate. Furthermore, complementation analysis of Fru$^-$ and Car$^-$ mutants with genomic clones in pLAFR3 (a broad-host-range cosmid vector) led to identification of a 2.2-kb *SalI-SalI* genomic fragment that contained the *carR* locus, which appeared to control positively the expression of all carbohydrate-inducible enzymes. The *fru* operon was found to be closely linked to the *carR* locus. We report here the identification and complete nucleotide sequencing of an operon *carRS* in the region of the *carR* locus coding for two

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
A. brasilense		
RG	Wild type, St^rAp^r(derived from sp81)	15
CR17	Car^- mutant (Fru^- Gal^- Gly^- Ara^- Gly^-) of RG	3
CR17R	Car^+ revertant of CR17	3
E. coli		
S17.1	C600::RP4-2 TC::Mu-KM::Tn7 *hsd*R *recA*	22
DH5α	F^- *rec*A φ80d *lacZ*ΔM15	BRL
Plasmids		
pLAFR1	IncP, Tc^r cosmid derived from pRK290	8
pLAFR3	Derived from pLAFR1, containing a multiple cloning site, Tc^r	23
pUC19	Ap^r cloning vector with a polylinker	31
pBluescript (KS^+)	Phagemid derived from pUC19	Stratagene
pCG93	*A. brasilense* genomic clone in pLAFR1 with 27.5-kb *Eco*RI-*Eco*RI insert	3
pCG3-6	pLAFR3 with 2.1-kb *Sal*I-*Sal*I fragment from pCG93	3
pCG3-3	pLAFR3 with 4.5-kb *Eco*RI-*Bam*HI fragment from pCG93	3
pCG3-7	pLAFR3 with 11.6-kb *Bam*HI-*Bam*HI fragment from pCG93	This study
pCG3-10	pLAFR3 with 3.8-kb *Sal*I-*Bam*HI fragment from the 4.5-kb insert of pCG3-3	This study
pCG3-11	pLAFR3 with 2.8-kb *Eco*RI-*Sal*I fragment from the insert of pCG3-3	This study
pCG3-16	pLAFR3 with 1,063-bp deletion fragment from the 2.1-kb insert of pCG3-6	This study

proteins, CARR and CARS. The deduced amino acid sequence data suggest that the two proteins may constitute a novel two-component regulatory system (1, 2, 19, 24) for global expression of carbohydrate catabolic pathways.

MATERIALS AND METHODS

Chemicals, enzymes, and reagents. Urea, acrylamide, bisacrylamide, Tris, glycerol, and sodium dodecyl sulfate were purchased from Bethesda Research Laboratories, Gaithersburg, Md. D-Galactose, L-arabinose, D-gluconic acid (K salt), D-fructose, isopropyl-β-D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal), low-melting-point agarose, and antibiotics were purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals and reagents used were of analytical grade and procured locally. All restriction enzymes, ligase, DNase, phosphatase, Klenow fragment, and DNA polymerase were supplied either by New England Biolabs, Beverly, Mass., or Boehringer Mannheim Biochemica, Mannheim, Germany. The DNA sequencing kit and nested deletion kit were obtained from U.S. Biochemical (Cleveland, Ohio) and Pharmacia (Uppsala, Sweden), respectively. [α-^{35}S] dATP was supplied by Amersham (Amersham, England).

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. *A. brasilense* RG is a wild-type strain that was isolated by us from a culture of *A. brasilense* sp81 (from the stock of N. R. Krieg, Blacksburg, Va.) and maintained by subculturing (15). *A. brasilense* and its mutants were grown in synthetic minimal medium (MM) containing 0.1% NH_4Cl and other salts, in addition to a C source, i.e., 1% sodium succinate · $6H_2O$ (SuccMM) or 0.4% other C source (fructose [FruMM], galactose, glycerol, arabinose, or gluconate), as described previously (3, 18). *A. brasilense* RG and its mutants were maintained on SuccMM or FruMM agar slants containing 1% peptone and 0.1% yeast extract in stoppered test tubes at 22 to 25°C and subcultured every 6 months. For routine work, these strains were maintained also in a cold room on SuccMM or FruMM agar plates with added 0.003% yeast extract and subcultured every 2 weeks. All media for growth of *A. brasilense* RG or its mutants routinely contained 50 μg of streptomycin per ml. Tetracycline, when necessary, was added at a concentration of 10 μg/ml. All *Escherichia coli* strains used in this work were maintained in 50% glycerol–0.01 M $MgSO_4$ at −20°C. *E. coli* strains were normally grown in LB (17) medium (with 1.6% agar for plates) and contained antibiotics, when necessary, at the following concentrations: streptomycin, 50 μg/ml; tetracycline, 20 μg/ml, and ampicillin, 50 μg/ml.

DNA procedures. Standard procedures were followed for small- and large-scale plasmid preparations, restriction enzyme digestion, ligation, agarose gel electrophoresis, elution of DNA from low-melting-point agarose, construction of recombinant plasmids, and transformation of *E. coli* (20). pCG3-10 and pCG3-11 were constructed by partial digestion of the 4.5-kb *Eco*RI-*Bam*HI DNA insert of pCG3-3 (3) with *Sal*I, isolation of 2.8- and 3.8-kb DNA fragments from low-melting-point agarose gel after electrophoresis, cloning in pUC19, and finally recloning of the DNA insert fragments in pLAFR3. pCG3-7 was constructed by the same strategy, using a 11.6-kb *Bam*HI-*Bam*HI DNA fragment from pCG93. Plasmid pCG3-16 was constructed from a 1,063-bp deletion fragment of the 2.1-kb *Sal*I-*Sal*I DNA (see below) and cloned in pLAFR3. S17.1 served as the donor for transfer of pCG plasmids into CR17 by conjugation.

Sequencing of the 2.8-kb DNA insert fragment of pCG3-11 by the nested deletion technique. The 2.8-kb DNA fragment was isolated from its clone in pUC19 by digestion with *Eco*RI and *Hin*dIII, purified by gel electrophoresis, and recloned in pBluescript (KS^+), using *E. coli* DH5α as the host. The recombinant pBluescript (KS^+) was cleaved from both sides of the insert with *Kpn*I-*Hin*dIII and *Sac*I-*Xba*I to obtain two linear products, and two sets of nested deletion clones were obtained by treatment of the linearized plasmids with exonuclease III and S1, following the protocol supplied with the nested deletion kit (Pharmacia). By using selected overlapping deletion clones, the 2.8-kb DNA was completely sequenced from both directions. Double-stranded DNA sequencing was performed by the chain termination method of Sanger et al. (21), using the Sequenase sequencing kit (U.S. Biochemical) as instructed by the manufacturer; 7-deaza-dGTP replaced dGTP to avoid compression, as *A. brasilense* DNA is known to be GC rich.

Nucleotide sequence analysis. Analysis of the nucleotide sequence was performed with a Microvax-II (VAX-VMS version V5.4), using the University of Wisconsin Genetics Computer Group sequence analysis software package, version 5.2 (5). Sequence comparisons were carried out with the GAP program of Genetics Computer Group package.

Nucleotide sequence accession numbers. The EMBL data library accession numbers for the 2,809-bp nucleotide sequence are X74935 and X70360.

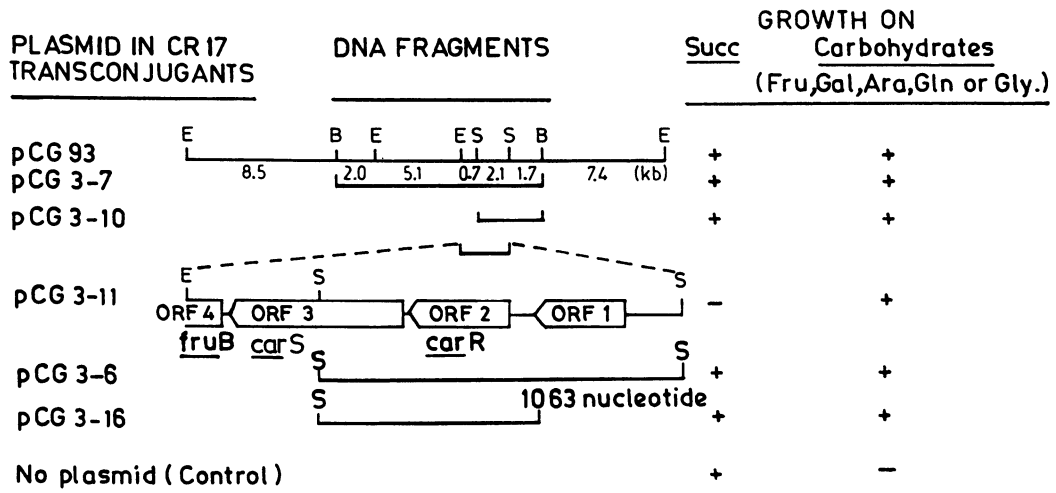

FIG. 1. Physical map of the region containing *car* and *fru* loci and Car$^-$-complementing activities of various DNA fragments. DNA inserts of pCG recombinant plasmids residing in CR17 are shown in the middle, and C sources on which these transconjugants can grow are shown on the right. The 2.8-kb *Eco*RI-*Sal*I fragment in pCG3-11 is shown in an expanded scale, and relative positions of various ORFs are indicated. The 2.1-kb *Sal*I-*Sal*I insert in pCG3-6 and the 1,063-bp deletion fragment in pCG3-16 are also shown in the expanded scale.

RESULTS

Physical map of the region containing *car* and *fru* loci of *A. brasilense*. The *car* and *fru* loci were identified previously within a 27.5-kb genomic fragment, and a close linkage between the two loci was inferred by subcloning of the 27.5-kb DNA and showing that it contained a DNA fragment with overlapping Car$^-$- and Fru$^-$-complementing activities (3). To more precisely identify the *car* and *fru* region, further complementation experiments were carried out (Fig. 1) with additional DNA fragments cloned in pLAFR3 for construction of transconjugants. It was previously shown that the 2.2-kb *Sal*I-*Sal*I fragment (the size is now revised to 2.1 kb) containing the Car$^-$-complementing activity was within the 9.6-kb *Eco*RI-*Bam*HI DNA that also complemented Fru$^-$ mutants. However, the 7.1-kb *Bam*HI-*Eco*RI fragment could not complement Fru$^-$ mutants (3). This finding implied that the 0.7-kb *Eco*RI-*Sal*I region flanking the 2.1-kb *Sal*I-*Sal*I DNA would be necessary for Fru$^+$ activity, and it could be a part of the bacterium's *fru* operon. The 2.8-kb *Eco*RI-*Sal*I and 3.8-kb *Sal*I-*Bam*HI fragments, like the 2.1-kb *Sal*I-*Sal*I fragment, showed the Car$^-$-complementing activity, and the transconjugants grew normally on all carbohydrates (Fig. 1). The transconjugant containing pCG3-11 (with the 2.8-kb *Eco*RI-*Sal*I insert), however, failed to grow on succinate (see below). To determine the physical linkage between *car* and *fru* and to characterize the *car* locus, the 2.8-kb DNA was completely sequenced.

Nucleotide sequence of the 2.8-kb EcoRI-SalI DNA fragment: identification of the *carRS* operon. The complete nucleotide sequence of the 2.8-kb insert in pCG3-11 is shown in Fig. 2. Computer analysis of the sequence revealed that there are three complete open reading frames (ORFs) present in the 2,809-bp-long DNA sequence (Fig. 2): one extending from positions 686 to 1066 (ORF1), the second extending from positions 1160 to 1570 (ORF2), and the third extending from positions 1595 to 2653 (ORF3). There is also an incomplete ORF (ORF4) which starts after ORF3 from position 2686 and is interrupted at position 2809 by an *Eco*RI site. All of these ORFs are present in the same strand and thus have the same orientation. ORF1 can code for a protein product of 126 amino acids, whereas ORF2 and ORF3 can code for protein products of 136 and 352 amino acids, respectively. ORF4 consists only of the N-terminal 41 amino acids coded for by a gene truncated by *Eco*RI. Potential ATG start codons, preceded by putative ribosome binding sites (Shine-Dalgarno [SD] sequence), are shown in ORF2, ORF3, and ORF4; stop codons are indicated in ORF1, ORF2, and ORF3 (Fig. 2). The SD sequence could not be identified for ORF1.

The G+C content of the entire sequence of 2,809 bp is 67%, consistent with the high G+C content of *A. brasilense*. The intermediate codon usage biases in the third positions of the ORFs are 83% G or C in ORF1, 87% in ORF3, and 84% in ORF4, consistent with these being coding regions. The coding regions of ORF1, ORF2, ORF3, and the N-terminal part of ORF4 have G+C contents of 71, 71, 72, and 67%, respectively, but the short sequence between ORF3 and ORF4 is remarkably A+T rich (60% A+T).

The genes coding for ORF2 and ORF3 have been designated *carR* and *carS*, respectively, as they appear to constitute an operon, *carRS*, from the nucleotide sequence analysis. The *carR* gene appears to contain a promoter with canonical σ^{-70} recognition sites (12) having −10 and −35 elements that resemble the *E. coli* promoter consensus sequence. It may be noted that *carR* has two possible −10 sequences (TATTCA at nucleotides 1124 to 1129 and 1140 to 1145) and two possible −35 sequences (TCGGCA at nucleotides 1095 to 1100 and TCGGCC at nucleotides 1113 to 1118); however, spacings between the −10 and −35 elements were found to be much longer, 23 and 21 bp, respectively, instead of the normal 17 bp as in *E. coli*. Also, an 11-bp repeat sequence that includes −10 element was observed in this region. The *carS* gene does not have its own promoter and is closely spaced in the sequence with an arrangement typical of a polycistronic bacterial operon. Interestingly, two SD sequences are present in upstream of ORF3, one overlapping the stop codon of ORF2 and the other overlapping the A of the start codon of ORF3, which is a very unusual position for an SD sequence. This might have significance in the regulation of expression of *carRS* products.

Demonstration of the ability of the *carR* gene to complement CR17. The 2.1-kb *Sal*I-*Sal*I DNA could rectify the pleiotropic Car$^-$ defect of CR17, and this led to a previous assumption that the regulatory gene *carR* was located within this region

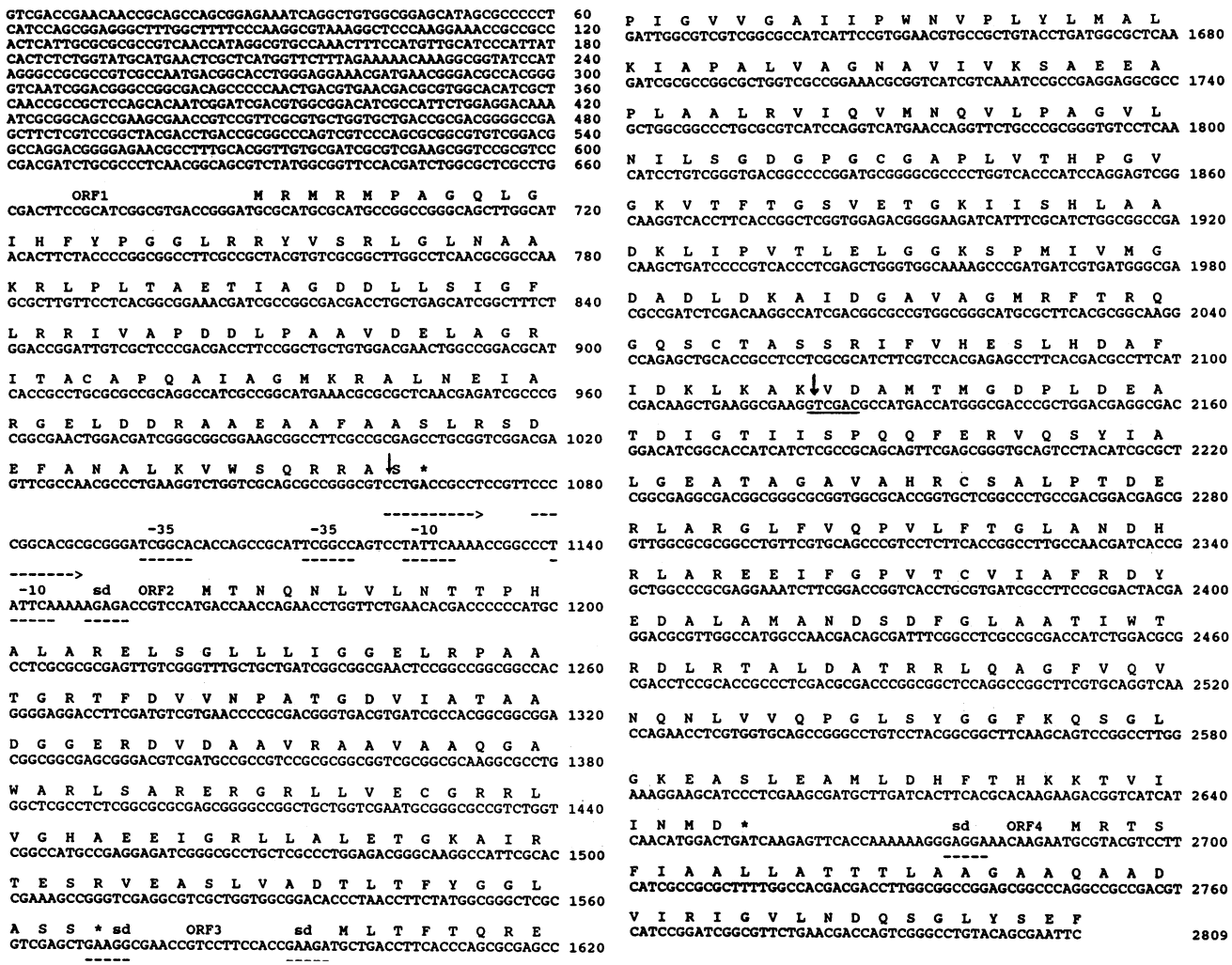

FIG. 2. Nucleotide sequence of the 2,809-bp fragment in pCG3-11 and the deduced amino acid sequence of the encoded proteins in different ORFs. SD sequences in upstream of ORF2, ORF3, and ORF4 are underlined. Duplication of −10 and −35 promoter elements of ORF2 is indicated by underlining. An 11-bp repeat sequence encompassing the −10 promoter element of ORF2 is shown by overlining. The 1,063-bp deletion fragment, cloned in pLAFR3 (pCG3-16), is shown by two vertical arrows. Asterisks indicate stop codons.

(3). It is clear from the 2,809-kb sequence and ORF analysis (Fig. 2) that there could be two possible candidates, ORF1 and ORF2, for the *carR* gene present within the 2.1-kb DNA. The partial *carS* gene within the 2.1-kb DNA fragment was not considered, as it contained a sequence coding only for the 177 N-terminal amino acid residues out of a total of 352 residues of CARS. To determine whether ORF1 or ORF2 or both are responsible for the rectification of the Car$^-$ defect, nested deletion fragments from the 2.1-kb *Sal*I-*Sal*I insert in pBluescript were isolated and cloned in pLAFR3 as described in Materials and Methods. Conjugation experiments with the deletion clones in pLAFR3 showed that the gene coding for ORF1 was not necessary for complementation of CR17. The minimum region from the *Sal*I site needed for Car$^-$ complementation activity was 1,063 bp, i.e., the insert in pCG3-16 which starts from position 2125 (*Sal*I site) and ends in position 1062 (Fig. 1 and 2). The only complete ORF present in the 1,063-bp insert of pCG3-16 is ORF2 encoded by the *carR* gene, the ORF1 region being completely deleted. Aberrant complementation by the partial *carS* gene in 1,063 bp through recombination is extremely unlikely, as all 50 transconjugants found in SuccMM-tetracycline plates after conjugation of CR17 with S17.1(pCG3-16) were Car$^+$.

Evidence for physical linkage of the *carRS* operon with the *fru* operon of *A. brasilense*. We have suggested previously that the 2,809-kb *Eco*RI-*Sal*I fragment might overlap the *fru* operon of *A. brasilense*, as complementation of Fru$^-$ mutants required the 0.7-kb *Sal*I-*Eco*RI region that flanks the 2.1-kb *Sal*I-*Sal*I DNA. Sequences of *fru* genes of *A. brasilense* are not known, but the complete sequence of the *fru* operon of a related bacterium, *Rhodobacter capsulatus*, has been published (28–30). We now find that the N-terminal 41-amino-acid sequence of ORF4 is very similar to the published N-terminal sequence of *fruB* coding for the enzyme II A domain of the *R. capsulatus* multiphosphoryl transfer protein (30). Figure 3 shows that the aligned sequences have identities of 32% at the amino acid level (out of 41) and 42% at the nucleotide level (out of 349). This result signifies a juxtaposition of *fruB* and *carRS* in the *A. brasilense* genome.

Growth of wild-type RG, mutant CR17, and their *car* and *fru* merodiploids. The pleiotropic carbohydrate mutant CR17 grows poorly on SucMM plates, yielding tiny colonies (3), and

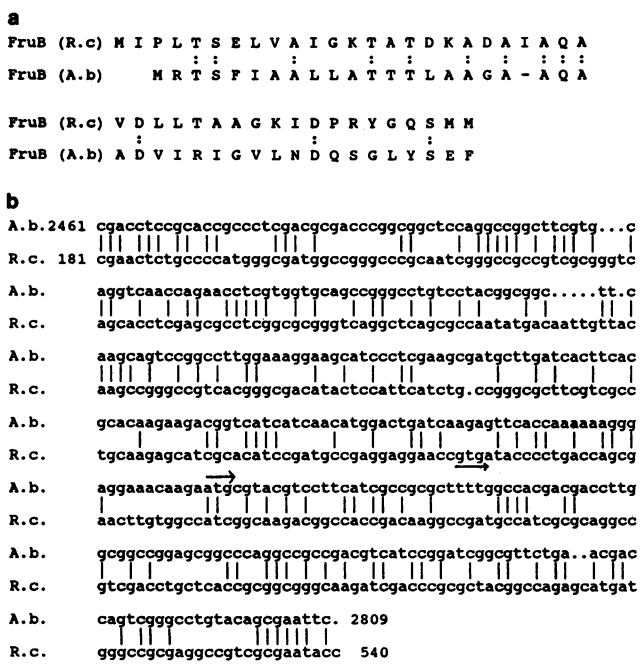

FIG. 3. Presence of an *R. capsulatus fruB*-like sequence in the ORF4 segment of *A. brasilense*. (a) Comparison of the derived N-terminal amino acid sequences of *fruB* of *R. capsulatus* and a *fruB*-like gene (ORF4) of *A. brasilense*. (b) Sequence homology of the 5′-terminal region of the *fruB* gene of *R. capsulatus* (R.c.) and the 3′-terminal flanking region of *A. brasilense* (A.b.) *carRS* at the nucleotide level. Arrows mark the translation initiation sites of *fruB* products.

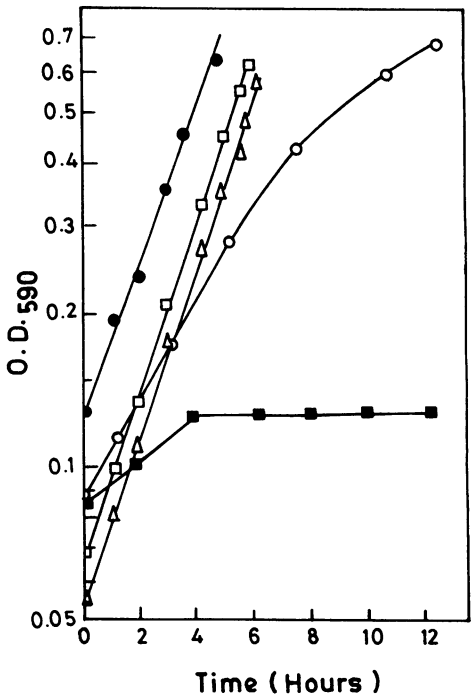

FIG. 4. Growth of *A. brasilense* RG (□), CR17 (○), CR17R (△), CR17(pCG3-6) (●), and CR17(pCG3-11) (■) in SuccMM. Inocula used were the cells of these strains growing in the early exponential phase in SuccMM. In the case of CR17(pCG3-11), cells were grown in FruMM and then shifted to SuccMM. $O.D._{590}$, optical density at 590 nm.

in SucMM liquid medium, its growth stops after a few generations (Fig. 4). Growth of CR17 in SucMM could be restored to that of the wild-type RG strain by a further mutation, as in CR17R (a revertant of CR17), that also restored the strain's ability to utilize all carbohydrates for growth (3). Construction of *carR*$^+$/*carR* merodiploids in CR17 (e.g., by using pCG3-6) also restored their ability to grow normally on succinate as well as carbohydrates (Fig. 1 and 4). However, although the wild-type *carRS* operon in *trans* could rectify the Car$^-$ defect of CR17, it uniquely created a problem for the merodiploid, CR17(pCG3-11), to grow in SuccMM (Fig. 1 and 4). The reason for the failure of the merodiploid to grow in SuccMM but not in any carbohydrate minimal media is not clear.

Sequence comparison of the products of *carRS* operon and the sensor-regulator class of proteins that constitute two-component regulatory systems in bacteria. The availability of the deduced amino acid sequences of the two protein products, CARR and CARS, of the *carRS* operon led us to investigate whether they could constitute a novel two-component regulatory system (1, 19) evolved for controlling global carbohydrate utilization in aerobic *A. brasilense*. Sequence analysis showed that only the CARS product contains two possible membrane-spanning segments at its N terminus (IGVVGAIIPWNVP LYLMALKI and VLPAGVLNILSGDGPGCGAPLV, from amino acid residues 10 to 30 and 62 to 83), as predicted from its hydropathy profile (13) by the method of Eisenberg et al. (7). CARR does not have any membrane-spanning sequence. Further evidence that CARS could be a sensor-like protein was found by looking for similarities to the conserved sequences in regions I, II, and III that exist in the C-terminal part of each of the sensor class proteins (such as Narx, PhoM, VirA, and DctB) (24). As shown in Fig. 5, when the C-terminal CARS sequence was divided into three such regions, the same conserved residues (i.e., residue H in region I, residue N in region II, and residues DXGXG and GXG in region III; shown by asterisks in Fig. 5a, except for one G replaced by A in region III) were found to be present. Whether CARR could be a protein of the response regulator class (24) was also investigated by comparing the N-terminal sequences of several proteins of this class (AlgR, PhoM2, OmpR, and ArcA) with that of CARR. As shown in Fig. 5b, the identity at the level of amino acid at the N-terminal region between CARR (approximately 100 residues out of a total of 136) and the four other regulatory proteins is about 20%. Therefore, there is a distinct possibility that CARR and CARS belong to a two-component regulatory system of *A. brasilense*.

DISCUSSION

This study was primarily carried out to identify, clone, and sequence the gene responsible for the pleiotropic carbohydrate utilization defect of mutant CR17. Complementation of CR17 by plasmid pCG3-16 carrying only the complete *carR*$^+$ gene of *A. brasilense* shows that the pleiotropic mutation is most likely due to a lesion in this gene. This result also indicates that the expression of carbohydrate catabolic pathways in *A. brasilense* is globally controlled by a positive regulatory system in which the *carR* gene product (CARR) is an essential factor.

The structure of the operon *carR* indicates that the CARR and CARS products are likely to be synthesized tandemly from a polycistronic message, which leads to the question of whether CARS also plays a role in this positive regulation. No experiments have been done in this study to obtain a direct answer to

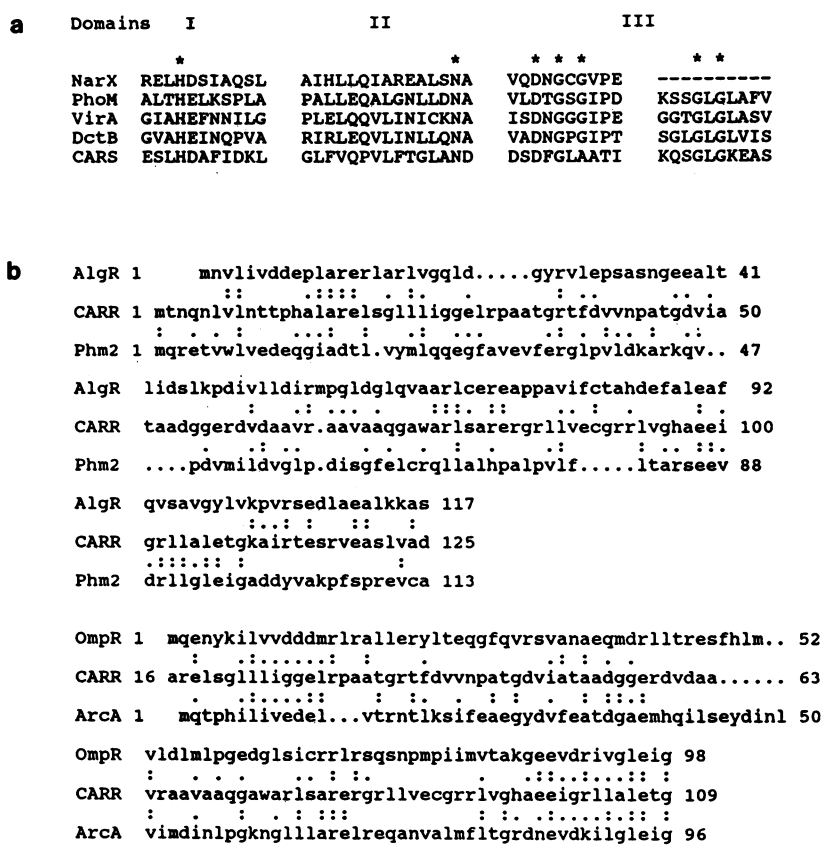

FIG. 5. Comparison of conserved primary structure motifs of some known two-component regulatory proteins with that of the CARR-CARS pair. (a) Homology of CARS in the three conserved domains (I, II, and III) with other known sensor proteins. The identical amino acids within these domains are shown by asterisks at the top. (b) Homology of CARR with different response regulators at N termini. Single and double dots indicate similar and identical amino acids, respectively.

this query. Nevertheless, it is worth noting that to elicit bacterial adaptive responses to external chemical stimuli, a minimum of two protein components are necessary, which are frequently the products of the same operon (1). It appears very likely that some kind of signal transduction mechanism for detecting external carbohydrates or C_4 dicarboxylates and transducing the signal to appropriate cellular response-regulatory systems exists in *A. brasilense*. Manifestation of the reverse diauxie and the succinate repression of all carbohydrate-inducible enzymes supports the existence of such a regulatory apparatus in *A. brasilense*. Taking advantage of the important observation that various two-component signal transduction proteins are identifiable by their characteristic primary structure motifs (24), we looked for such characteristic motifs in the CARR and CARS proteins. Analysis of the amino acid sequences convinced us that CARR as a response regulator and CARS as a sensor might constitute a novel bacterial two-component regulatory system. Similarity of primary structure motifs in the protein pairs of two-component regulatory systems is largely due to similar signalling strategies that depend on protein phosphorylation and dephosphorylation (2). The biochemistry of phosphorylation and dephosphorylation of CARR and CARS proteins remains to be investigated.

Although the expression of all carbohydrate-inducible operons is controlled positively by *carR*, the *carR* operon is found to be located immediately upstream of *fruB*. The significance of this locus relationship is unclear. Our previous observation that a spontaneous revertant of the pleiotropic carbohydrate mutant (CR17R) showed constitutive expression of the fructose-inducible enzymes, but not of other carbohydrate-inducible enzymes (3), makes sense with respect to the location of the *fru* operon in the 3′-flanking region of *carRS* in the chromosome.

A surprising observation that cannot be easily explained at this stage is the problem of growth of CR17 on succinate but not on carbohydrates in the presence of the 2.8-kb DNA (containing the wild-type *carS*, the 5′-terminal fragment of *fruB*, and ORF1) in *trans*. This gives an indication that processes of regulation of growth on succinate and carbohydrates are related by some common elements. This view is supported by the observation that *carR* mutant CR17 shows a partial defect of growth on SucMM, which completely disappears in the cases of *carR*/*carR*$^+$ merodiploids [one important exception being CR17(pCG3-11)]. Also, we observed previously (18) that the succinate uptake rate of *A. brasilense* increased by sixfold within 40 min (0.33 generation time) following addition of 1% sodium succinate · $6H_2O$ to an exponentially growing cell culture in FruMM. Therefore, external succinate is necessary to overcome the effect of fructose on lowering of the succinate uptake rate in the bacterium. This observation suggests that a mutual antagonistic relationship exists between succinate and fructose in their assimilation processes in *A. brasilense*, in which succinate dominates over carbohydrates. Another observation of importance has been that the problem of growth on succinate of CR17(pCG3-11) is not encountered in the wild-type RG(pCG3-11) transconju-

gant. This probably means that in certain genetic backgrounds, *carRS* can have an effect on succinate utilization (neglecting the possibility of any effect of ORF1 and a small 5′-terminal fragment of *fruB*).

An explanation for all of these observations becomes more difficult in view of the fact that a regulatory *fruR* gene has already been identified in enterobacteria (9), a mutation in which (*fruR*) caused constitutive expression of the *fruFKA* operon (encoding proteins needed for efficient utilization of fructose) while abolishing growth of the mutant on C_4 dicarboxylates (4). Although a *fru* operon possibly resembling the *E. coli fruFKA* exists in *A. brasilense*, the presence of a *fruR*-like gene has not yet been reported in an aerobic bacterium. This investigation indicates only some intimate relationship between the functions of the *carRS* products, expression of *fru* and other carbohydrate operons, and the succinate utilization system for the regulation C-source assimilation in *A. brasilense*. However, the molecular mechanism of this complex control system remains far from clear.

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