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Azospirillum brasilense Locus Coding for Phosphoenolpyruvate:Fructose Phosphotransferase System and Global Regulation of Carbohydrate Metabolism

SUDIP CHATTOPADHYAY, AMIT MUKHERJEE,† AND SUDHAMOY GHOSH*
Department of Biochemistry, Bose Institute, Calcutta 700 054, India

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Mutants of *Azospirillum brasilense* unable to grow on fructose include ones affected only on fructose (Fru⁻) and others impaired on many or all carbohydrates through interference with induction of their specific pathways (Car⁻). Both types of mutants could be complemented by a cosmid in broad-host-range vector pLAFR1 containing a 27.5-kb genomic insert, Car⁻-complementing activity depending on a 2.2-kb fragment, and Fru⁻-complementing activity depending on an overlapping 9.6-kb fragment.

Azospirillum brasilense, like other strict aerobes, uses dicarboxylic acids in preference to carbohydrates, and a reverse diauxie occurs (19). This organism grows on a variety of dicarboxylic acids and carbohydrates; metabolism of the former seems to be constitutive, and that of the latter (e.g., fructose, galactose, gluconate, L-arabinose, and glycerol) seems to be inducible (6, 10, 12, 16, 20, 26, 28). Only fructose metabolism is known to use a phosphoenolpyruvate (PEP)-phosphotransferase system (7, 10), and there is no growth on glucose, lactose, mannose, sorbose, or sucrose (16, 26). As studied for succinate plus fructose, the reverse diauxie involves both transient and permanent apparent repression by succinate of enzymes I and II of the PEP: fructose phosphotransferase system and of fructose-1-P 6-kinase (1-PFK) (19).

The mechanism of reverse diauxie is unknown. In fermentative bacteria, certain elements, such as the *cya/crp* and *crr* gene products in *Escherichia coli*, affect a variety of inducible pathways, and the PEP-phosphotransferase system is intimately involved (1, 5, 9, 17, 21, 22). Cyclic AMP is not known to play a significant role in carbohydrate utilization by aerobes (24). Studies with aerobic *Pseudomonas* and *Rhizobium* spp. of transport and metabolism of carbohydrates and tricarboxylic acid cycle intermediates at the biochemical and genetic levels (11, 13, 15, 25, 27) so far have failed to reveal any such common elements that control the carbohydrate utilization pathways. In the present work we show that among mutants of *A. brasilense* selected as unable to grow on fructose but still using succinate, some are affected in growth on a variety of carbohydrates (i.e., Car⁻) through the impaired expression of inducible enzymes. Cloning shows that the function depends on a 2.2-kb fragment which overlaps with a 9.6-kb fragment presumably coding for an element(s) of the PEP:fructose phosphotransferase system.

A. brasilense RG (3), an efficient nitrogen-fixing wild-type strain, was treated with *N*-methyl-*N*-nitro-*N*′-nitrosoguanidine (18), and cultures were enriched for fructose-negative mutants by a 6-h treatment in FruMM with ampicillin (1 mg/ml) and cycloserine (100 μg/ml). After plating on minimal medium containing 0.4% fructose (FruMM) supplemented with 0.001% succinate, tiny colonies at 3 days were tested on FruMM and minimal medium containing 1% sodium succinate-6H_2O (SuccMM). Suc⁺ Fru⁻ mutants were screened on a variety of other carbon sources. Mutants affected only on fructose (type a), on all carbohydrates (i.e., Car⁻, type b), or on some others (type c) were obtained and tested for components of fructose metabolism (Table 1). A spontaneous revertant (CR17R) of Car⁻ strain CR17, obtained on FruMM, grew normally on all sugars and had abnormally high activities of the fructose enzymes (Table 1).

Activities of other inducible enzymes (as well as the fructose enzymes) were assessed in the wild type and revertant after growth on a variety of carbon sources (Table 2). The Car⁻ strain CR17 was tested for the same enzymes after 5 h of incubation following growth on succinate to allow time for induction: very low induction was observed for the fructose pathway, and none was observed for the other cognate inducible enzymes (Table 2). The induction pattern of carbohydrate catabolic enzymes in the wild type (Table 2) showed two important characteristics: (i) all carbohydrate-inducible enzymes tested remain unexpressed if the cells were grown on succinate, and (ii) a particular carbohydrate as an inducer can induce only a specific set of enzymes which was needed for its catabolism.

A gene bank of *A. brasilense* RG DNA in cosmid pLAFR1 (8) was obtained in *E. coli* S17.1. About 1,500 clones were screened, first in groups of 20 and then individually, for conjugation of Car⁺ into mutant CR17. This revealed cosmid pCG93, which, surprisingly, also repaired the growth defect of the Fru⁻ mutants F2 and F7 and of the type c mutants CR5 and CR10. Subcloning showed a 2.2-kb fragment to suffice for repair of the Car⁻ and type c mutants (possibly indicating different alterations in the same element), and an overlapping 9.6-kb fragment was needed for repair of the Fru⁻ strains (Fig. 1; Table 1). Enzyme assay of CR17 carrying pCG3-6 (the 2.2-kb fragment) showed restoration of near-normal fructose phosphorylation and galactose dehydrogenase activities, both, as in the wild type, being inducible.

Thus, the 2.2-kb fragment is likely to be the *carR* locus, and the element(s) affecting the fructose-phosphotransferase is likely to be closely linked to it. The role and nature of CarR are not known, other than perhaps being a positive

* Corresponding author.
† Present address: Department of Microbiology, The University of Kansas Medical Center, Kansas City, Kansas 66103.

TABLE 1. Characterization of carbohydrate mutants of *A. brasilense* RG by fructose-inducible enzyme activities, fructose uptake, and growth on carbohydrate minimal plates

A. brasilense strain	Type of mutant	Sp act[a] (nmol min^{-1} mg of protein^{-1}) of:			Fructose uptake[a] (nmol min^{-1} mg of cell protein^{-1})	Growth on[b]:					
		Enzyme I	Enzyme II	1-PFK		SuccMM	FruMM	AraMM	GalMM	GlyMM	GlnMM
RG	Wild type	70	15	240	15.3	++++	++++	+++	+++	++++	++++
F2	Type a	NA	NA	NA	NA	+++	±	+++	+++	++++	++++
F13	Type a	NA	NA	NA	0.2	++++	−	+++	+++	++++	++++
F7	Type a	9.5	NA	38	NA	++++	±	+++	+++	++++	+++
F8	Type a	4.2	NA	12.2	NA	++++	±	+++	+++	++++	++++
CR17	Type b	4.0	2.0	16	5.3	++	−	−	−	±	−
CR18	Type b	NA	NA	NA	NA	++	−	−	±	−	−
CR5	Type c	40	1.65	198	1.2	++++	−	±	±	++++	+
CR6	Type c	7.0	3.6	16.4	9.7	++++	−	+++	++	−	++
CR10	Type c	4.0	3.2	15.0	5.0	++++	−	+++	+	−	++
CR14	Type c	8.0	3.3	30	7.3	++++	−	+++	+++	±	++++
CR17R	Revertant of CR17	77	42	173	11	++++	++++	+++	+++	++++	++++
CR17(pCG3-3)	Complemented transconjugant	—	—	—	ND	++++	++++	+++	+++	++++	++++
CR17(pCG3-6)	Complemented transconjugant	—	—	—	ND	++++	++++	+++	+++	++++	++++

[a] Fructose uptake and fructose-inducible enzyme activities were measured in RG and CR17R after growth on FruMM and SuccMM, respectively, as described in reference 19. In the rest of the strains these activities were measured after the shift of exponentially growing cells (an optical density at 590 nm of about 1) from SuccMM to FruMM followed by shaking incubation at 32°C for 5 h. —, only enzymatic phosphorylation of fructose by the crude extracts was measured. Cells grown on SuccMM showed no activity; growth on FruMM showed 80% of the wild-type activity (7). NA (no activity), activity less than 0.2 nmol/min/mg of protein for fructose uptake and less than 0.5 nmol/min/mg of protein for enzyme I, enzyme II, and 1-PFK. ND, not determined.

[b] Minimal medium (MM) agar plates containing various carbon sources: sodium succinate-6H_2O (Succ), 1%; fructose (Fru), L-arabinose (Ara), galactose (Gal), glycerol (Gly), and gluconate (Gln), all 0.4%. Growth results are shown after incubation for 48 h at 32°C. In all cases growth was compared with that of the wild-type strain on SuccMM plates. Symbols: ++++, good growth; +++, fair growth; ++, poor growth; +, detectable growth; ±, faintly visible growth; −, no growth.

TABLE 2. Induced synthesis of carbohydrate-inducible enzymes in *A. brasilense* RG and its mutants

A. brasilense strain	Growth medium[a]	Sp act (nmol min^{-1} mg of protein^{-1}) of[b]:								
		Enzyme I	Enzyme II	1-PFK	ADH	GYK	α-GDH	GNK	ED pathway	GALD
RG	SuccMM	NA	NA	NA	NA	NA	NA	1.0	NA	NA
	FruMM	55	15	143	NA	NA	NA	1.0	NA	ND
	AraMM	NA	NA	NA	230	NA	ND	1.0	NA	ND
	GlyMM	1.0	1.0	1.0	1.9	42	49	1.0	NA	ND
	GlnMM	NA	NA	NA	NA	NA	ND	25	15	ND
	GalMM	ND	ND	ND	ND	ND	ND	ND	ND	177
CR17	MM	4.0	2.0	16	NA	NA	NA	1.0	NA	NA
CR17R	SuccMM	77	42	173	NA	NA	ND	1.0	NA	NA
	FruMM	210	28	559	NA	NA	NA	1.0	NA	ND
	AraMM	205	175	475	308	NA	NA	1.0	NA	ND
	GlyMM	260	174	736	NA	62	72	1.0	NA	ND
	GlnMM	210	215	662	NA	NA	NA	51	31	ND
	GalMM	ND	ND	ND	ND	ND	ND	ND	ND	104
CR17(pCG3-6)	GalMM	NA	NA	NA	ND	ND	ND	ND	ND	139
	FruMM	—	—	—	ND	ND	ND	ND	ND	NA

[a] Cultures of strains RG, CR17R, and CR17(pCG3-6) were grown exponentially in the indicated media for at least three generations, after which the cells were harvested and assayed for the enzymes. A culture of strain CR17 growing exponentially in SuccMM was shifted and incubated with shaking in (i) FruMM for 5 h for the assay of fructose-inducible enzymes (19), (ii) AraMM for 15 h for the assay of ADH (20), (iii) GlyMM for 19.5 h for the assay of α-GDH and GYK (28), (iv) GlnMM for 25 h for the assay of GNK and the ED pathway (2, 16), and (v) GalMM for 20 h for the assay of GALD. For the GALD assay, the procedure followed was the same as that in reference 20, except that galactose instead of L-arabinose was used as the substrate. Protein was measured by the method of either Lowry et. al. (14) or Bradford (4).

[b] ADH, L-arabinose dehydrogenase; α-GDH, L-α-glycerophosphate dehydrogenase; GYK, glycerol kinase; GNK, gluconokinase; ED, Entner-Doudoroff, GALD, galactose dehydrogenase; — and ND, see Table 1, footnote *a*, for definitions; NA (no activity), activity less than 0.5 nmol/min/mg of protein (activities between 0.5 and 1.5 nmol/min/mg are entered as 1.0).

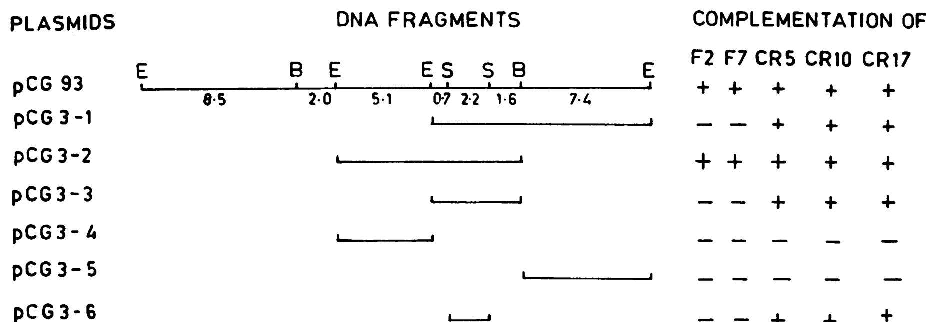

FIG. 1. Restriction map of 27.5-kb insert of pCG93 and ability of various restriction fragments to complement *A. brasilense* RG mutants. The restriction map (top line) was constructed by partial and complete digestion with *Eco*RI (E), *Bam*HI (B), and *Sal*I (S) and subcloning of the fragments (23) (all *Sal*I sites are not shown). Some of these subclones (pCG3-1 to pCG3-6), in multiple cloning sites of broad-host-range cosmid pLAFR3, are shown in the figure (bottom lines) to indicate their ability to complement the mutants (shown in the right side of each line).

regulatory element for expression of specific catabolic pathways. Analysis of the CR17R mutation should be informative. A possibly analogous factor to CarR, CRC (catabolite repression control), has been reported for *Pseudomonas aeruginosa* (15).

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