Plant and Soil 120, 195–201 (1989).
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PLSO 8184

Some properties of carbohydrate and C_4-dicarboxylic acid utilization negative mutants of *Rhizobium leguminosarum* biovar *phaseoli* strain P121

P. J. LAFONTAINE, CAROLE LAFRENIÈRE[1] and H. ANTOUN
Département des Sols, Faculté des Sciences de l'Agriculture et de l'Alimentation, Université Laval, Québec, Canada G1K 7P4. [1] Present address: Station de recherche, Agriculture Canada, Kapuskasing, Ontario, Canada P5N 2X9

Received 20 March 1989. Revised July 1989

Key words: french bean, oxygen consumption, *Phaseolus vulgaris, Rhizobium leguminosarum* biovar *phaseoli*, symbiotic effectiveness

Abstract

After NTG treatment of the very effective wild type strain P121 of *Rhizobium leguminosarum* biovar *phaseoli*, mutants defective in the utilization of sugars or organic acids were obtained. All the mutants nodulated the cultivar Goldie of *Phaseolus vulgaris*. The arabinose, fructose, glucose and pyruvate utilization mutants formed nodules similar in shape and size to the nodules formed by the wild type strain. These mutants exhibited an acetylene reduction activity significantly lower than the activity observed with the wild type strain. All the C_4-dicarboxylic acid utilization mutants, formed ineffective nodules that did not show a significant acetylene reduction activity. The C_4-dicarboxylic acids uptake system is apparently inducible in the free-living bacteria of strain P121. When P121 cells were grown on glucose in the presence of 2.5 m*M* malate, the rate of glucose-dependent O_2 consumption significantly decreased suggesting the presence of a catabolite repression-like phenomenon. Isolated bacteroids of strain P121, under the experimental conditions used, were able to oxidize succinate, fumarate or malate but did not oxidize pyruvate, glucose, fructose or sucrose.

Introduction

In the Rhizobium-legumes symbiosis, the exchange of metabolites between plant and bacteroids play a crucial role in the establishment of an effective nitrogen fixing system. Although the exact nature of the carbon source supplied by the plant to the bacteria is not known, bacteroids are believed to receive primarily dicarboxylic acids (Emerich *et al.*, 1988). In fact, a functional C_4-dicarboxylic acid transport system is essential for N_2 fixation to occur in pea nodules (Finan *et al.*, 1983), and the capacity to utilize sugars is apparently not essential for bacteroid development or the establishment of effective N_2 fixation in pea (Glenn *et al.*, 1984). However, sucrose and glucose supported acetylene reduction by bacteroids extracted from French bean, soybean and pea root nodules in the presence of low O_2 concentrations (Trinchant *et al.*, 1981).

The present study shows that in *Rhizobium leguminosarum* biovar *phaseoli* the ability to utilize dicarboxylates is essential for the occurrence of effective N_2 fixation in French bean nodules, and that the ability to utilize some other sugars is also apparently important in this beneficial process, as indicated by the significant alterations observed in sugar utilization mutants.

Material and methods

Bacteria and growth conditions

Rhizobium leguminosarum biovar *phaseoli* strain P121, is a wild type strain very effective on *Phaseolus vulgaris* cv. Goldie (Lalande *et al.*, 1986). Bac-

teria were grown as previously described (Lafrenière *et al.*, 1987) on BM_1 or BM_2 media supplemented with 0.5% sugars or 10 m*M* organic acids.

Isolation of mutant and revertant strains

Mutagenesis of strain P121 was carried out by the treatment of the log phase cells grown on BM_1-mannitol, with 200 μg/mL N-Methyl-N′-nitro-N-nitrosoguanidine (NTG) for 30 min at 25°C. The treated cells (20–40% survival) were washed twice with phosphate buffer saline (PBS; 3 m*M* phosphate buffer in 0.7% NaCl, pH 6.8), suspended in BM_1-mannitol broth and incubated for 24 h at 28°C on a rotary shaker (160 rev.min^{-1}), to allow segregation and expression of mutations. For enrichment, the cells were washed twice in PBS and resuspended in BM_2 supplemented with the appropriate carbon source and 500 μg mL^{-1} carbenicillin were added. After incubation at 28°C for 24 h on a rotary shaker, the enrichment cycle was repeated. Enriched cultures were washed and plated for single colonies on BM_2 medium modified (Lafrenière *et al.*, 1987) by using 0.6 g L^{-1} proteose peptone (Difco) as a nitrogen source and by adding filter sterilized 2,3,5-triphenyl tetrazolium chloride (TTC, Sigma) to a final concentration of 25 mg L^{-1}. On this medium wild type colonies have a good growth and reduce TTC (pink colonies), while mutants show poor growth and TTC is not reduced (white colonies). Revertants were selected by plating a dense washed culture on BM_2-succinate plates. Mutants and revertants were preserved at −80°C in yeast extract mannitol broth containing 10% glycerol.

Plant growth conditions

French bean (*Phaseolus vulgaris* cv. Goldie) plants were grown in an autoclaved hydroponic growth system as previously described (Lafontaine *et al.*, 1989).

Acetylene reduction activity

The acetylene reduction activity was measured 22 days after plants inoculation by incubating for 30 min, the 2 detached root systems obtained from each hydroponic jar, in a 150 mL glass bottle, capped with Subba Seal (William Freeman Co., Barnsley, England), and containing 10% acetylene. The ethylene produced was measured with a Perkin Elmer Sigma 3B dual FID gas chromatograph equipped with a 1 m stainless steel column packed with porapack R (80–100 mesh) and operated at 45°C. The injector and detector temperatures were maintained at 55°C and 125°C respectively.

Bacteroids isolation

Phaseolus vulgaris cv. Goldie nodules were removed from the roots and gently crushed in a solution of 0.15 *M* NaCl and 50 m*M* KH_2PO_4, pH 7.6 at 4°C. This crude homogenate was filtered through 6 layers of cheesecloth, and 1 mL was layered on top of a 70% Percoll solution (Reibach *et al.*, 1981) and centrifuged at 40, 360 × *g* for 40 min at 4°C, in an IEC 33° angle head rotor (IEC model B-20 refrigerated centrifuge). The cytosol fraction, on top of the Percoll gradient and the bacteroids fraction near the bottom of the centrifuge tube were collected with Pasteur pipettes. Percoll was removed by diluting the bacteroid fraction 1:5 (v/v) with 0.15 *M* NaCl plus 50 m*M* KH_2PO_4 pH 7.6 and centrifuging 10 min at 12000 × *g*. The bacteroid pellet was resuspended in BM_1 without carbon source.

Measurement of O_2 consumption

Substrates dependent O_2 consumption by free-living bacteria and bacteroids was measured by using a biological oxygen monitor (Yellow Spring Instrument, Ohio) as previously described (Lafrenière *et al.*, 1987).

Protein determination

For protein determination cells were washed in PBS and digested at 90°C in 1 *M* NaOH for 10 min and the protein content was determined by the

Folin phenol method (Lowry *et al.*, 1952) using bovine serum albumin as a standard.

Results and discussion

Isolation and growth properties of carbohydrate and organic acid-utilization mutants

Strain P121 is a very effective strain of *R. leguminosarum* biovar *phaseoli*, isolated from a Quebec soil (Lalande *et al.*, 1986). This strain grows on sugars and organic acids commonly used by rhizobia. After NTG mutagenesis and carbenicillin enrichment, the carbohydrate (arabinose, P121A18; glucose, P121DH; fructose, P121FH1 and FH2) mutants isolated, were able to use organic acids and other carbohydrates as sole carbon source (Table 1). Pyruvate was the only carbon source not used by mutant P121P22. All C_4-dicarboxylic acid mutants (P121S4, P121S9 and P121S21) did not use succinate, fumarate and malate as sole carbon source, but they were able to grow on glucose and arabinose indicating that they have a functional tricarboxylic acid cycle (Bolton *et al.*, 1986; Duncan and Fraenkel, 1979). Thus, the failure of a mutant to utilize succinate, fumarate and malate was taken as an indication of a defect in the C_4-dicarboxylic acid transport system, recognized to be common for the three organic acids (Finan *et al.*, 1981). Strain P121F16 is a double mutant defective in the utilization of dicarboxylic acids and fructose, but able to grow on glucose and arabinose (Table 1). Strains P121S21R10 and P121S21R14 are revertants of the mutant strain P121S21 selected on succinate. These revertants grew on all carbon sources used by the wild type strain P121.

Symbiotic properties of mutant and revertant strains

All mutant and revertant strains nodulated French bean (*Phaseolus vulgaris* cv. Goldie). The carbohydrate utilization mutants P121FH1, P121FH2 and P121DH, the pyruvate mutant P121P22 and the revertant strains P121S21R10 and P121S21R14, formed effective pink nodules similar in shape, size and number to those formed by the wild type strain P121. The arabinose utilization mutant P121A18 also formed effective nodules but in lesser number. The C_4-dicarboxylic acid utilization mutants P121S4, P121S9 and P121S21 and the double mutant P121F16 nodulated French bean, but the nodules were ineffective, small and white to greenish.

Table 1. Growth of *R. leguminosarum* biovar *phaseoli* P121 and its mutants and revertants on some carbon sources

Strain	Carbon source						
	Pyruvate	Succinate	Fumarate	Malate	Glucose	Fructose	Arabinose
Wild type							
P121	+	+	+	+	+	+	+
Mutants							
Carbohydrates							
P121A18	+	+	+	+	+	+	−
P121DH	+	+	+	+	−	+	+
P121FH1	+	+	+	+	+	−	+
P121FH2	+	+	+	+	+	−	+
Organic acids							
P121F16	+	−	−	−	+	−	+
P121P22	−	+	+	+	+	+	+
P121S4	+	−	−	−	+	+	+
P121S9	+	−	−	−	+	+	+
P121S21	+	−	−	−	+	+	+
Revertants							
P121S21R10	+	+	+	+	+	+	+
P121S21R14	+	+	+	+	+	+	+

All the carbohydrate utilization mutants retained their ability to fix dinitrogen as indicated by the presence of an acetylene reduction activity (Table 2). This suggests as previously reported for the *R. leguminosarum* biovar *viceae* (Glenn *et al.*, 1984) that the capacity to utilize some sugars is apparently not essential for bacteroid development or the establishment of effective N_2 fixation. However, the nitrogenase activity detected in French bean plants nodulated with the carbohydrate utilization mutants was always significantly lower than that observed in plants nodulated with the wild type strain P121 (Table 2). The lowest nitrogenase activity observed with the arabinose utilization mutant P121A18 (32% of P121 activity) could be attributed in part, to the low number of nodules formed. The acetylene reduction activities observed with the other mutants ranged from 47 to 78% that of the wild type strain P121, with the pyruvate (P121P22) and the glucose (P121DH) utilization mutants respectively. As the nodulation of these mutants was similar to the nodulation of the wild type effective strain, this might indicate that the capacity to utilize glucose, fructose or pyruvate by *R. leguminosarum* biovar *phaseoli* bacteroids, is in part essential for an optimum nitrogenase activity. In fact, sucrose and glucose supported acetylene reduction by bacteroids extracted from French bean root nodules in the presence of low O_2 concentrations but not under O_2 tensions usually able to support acetylene reduction with succinate (Trinchant *et al.*, 1981).

All the mutants that are altered in the utilization of succinate, fumarate and malate, including the double mutant P121F16, have lost completely their ability to fix N_2. The acetylene reduction activities observed with these mutants are not statistically different from the activity observed with the uninoculated plants (Table 2). The results shown, corroborate previous observations (Arwas *et al.*, 1985; Finan *et al.*, 1983) indicating that utilization of exogenous dicarboxylates (*i.e.* possession of a functional C_4-dicarboxylic acid transport system) is essential for N_2 fixation to occur in nodules. However, all carbohydrate mutants had altered nitrogenase activity and one revertant (P121S21R10) had a restored nitrogenase activity which is also significantly lower than that of the

Table 2. Nodulation and acetylene reduction activity (μmoles C_2H_4 h^{-1} $plant^{-1}$) of *Phaseolus vulgaris* cv. Goldie inoculated with *R. leguminosarum* biovar *phaseoli* P121 and its mutant and revertant strains

Strain	Nodulation	Acetylene reduction activity
Wild type		
P121	+	6.44 a[a]
Mutants		
Carbohydrates		
P121A18	+	2.06 d
P121FH1	+	3.72 cd
P121FH2	+	4.72 bcd
P121DH	+	5.04 b
Organic acids		
P121F16	+	0.12 e
P121P22	+	3.05 cd
P121S4	+	0.89 e
P121S9	+	0.29 e
P121521	+	0.10 e
Revertants		
P121S21R10	+	4.69 bc
P121S21R14	+	5.22 ab
Uninoculated	–	0.09 e

[a] Means followed by the same letter are not significantly different ($P \leqslant 0.05$) according to the Waller Duncan's multiple range test. Means are from 3 replicates.

effective wild type strain (Table 2). This suggests that *R. leguminosarum* biovar *phaseoli* bacteroids are using more than one carbon source, to establish an effective nitrogenase activity in *Phaseolus vulgaris* nodules. The ability of the double mutant P121F16 to nodulate French bean also suggests that *R. leguminosarum* biovar *phaseoli* can use carbon sources other than C_4-dicarboxylic acids or sugars to fuel the nodulation process. Similar observations were previously reported with mutants of *R. leguminosarum* biovar *viceae* (Arwas *et al.*, 1986).

Oxidation of substrates by R. leguminosarum biovar phaseoli P121 and its C_4-dicarboxylic acid utilization mutant P121S21

Because of the importance of the role played by dicarboxylates in the symbiotic nitrogen fixation by French bean nodules, we have compared the oxidation of malate and glucose by the effective wild type strain P121 and its ineffective C_4-dicarboxylic acid utilization mutant strain P121S21.

R. leguminosarum biovar *phaseoli* P121 cells grown on glucose were not able to oxidize readily pyruvate, succinate, malate and fumarate (Table 3). However, when grown on malate P121 was capable of oxidizing the three organic acids succinate, malate and fumarate but pyruvate was not oxidized, and the glucose and fructose-dependent O_2 consumptions significantly decreased. These observations show that as previously reported for *Rhizobium leguminosarum* biovar *viceae* (Finan *et al.*, 1981; Glenn *et al.*, 1980), *R. leguminosarum* biovar *phaseoli* P121 possesses a C_4-dicarboxylic acid uptake system which is inducible and mediates the uptake of succinate, malate and fumarate. The absence or very low pyruvate, glucose and fructose-dependent O_2 consumptions suggests, that strain P121 has an inducible oxidation system for the catabolism of these carbon sources, or the presence of a catabolite repression-like phenomenon. The systems for the catabolism of pyruvate, glucose and fructose are constitutive in a strain of *Rhizobium leguminosarum* biovar *viceae* (Glenn and Dilworth, 1981). Regardless of the carbon source used in the cell culture medium, strain P121S21 was not able to oxidize succinate, malate, fumarate or pyruvate but it oxidized glucose and fructose (Table 3).

No significant organic acid-dependent O_2 consumption was observed with P121 cells grown on glucose. The presence of a catabolite repression-like phenomenon (Lafrenière *et al.*, 1987) was investigated by growing strain P121 in BM_2 medium containing glucose and an increasing concentration of malate. The addition of 2.5 m*M* or more malate significantly decreased the rate of glucose-dependent O_2 consumption (Table 4), indicating the presence of a catabolite repression-like phenomenon in strain P121. In fact, such a repression is absent in the C_4-dicarboxylic acid utilization mutant P121S21 (Table 3). The presence of the lowest concentration of malate used, was also necessary for the occurrence of a significant malate-dependent O_2 consumption (Table 4), which confirm the presence of an inducible C_4-dicarboxylic acid uptake system in strain P121.

Oxidation of substrates by bacteroids of strain P121 and its C_4-dicarboxylic acid utilization mutant P121S21

The isolated bacteroids of strain P121, exhibited a significant substrate-dependent O_2 consumption

Table 3. Substrate-dependent O_2 consumption by free-living cells of *R. leguminosarum* biovar *phaseoli* P121 and its C_4-dicarboxylic acid utilization mutant P121S21

Carbon source in growth medium	Strain	Rate of O_2 consumption in nmoles O_2 min^{-1} (mg protein)$^{-1}$ in the presence of:					
		Pyruvate (10 m*M*)	Succinate (10 m*M*)	Fumarate (10 m*M*)	Malate (10 m*M*)	Glucose (0.5%)	Fructose (0.5%)
Malate (10 m*M*)	P121	11.99	375.15	238.62	295.20	23.78	39.69
	P121S21[a]	0.00	1.85	20.30	0.00	125.54	115.41
Glucose (0.5%)	P121	9.43	5.33	7.79	11.77	180.81	211.56
	P121S21	6.97	4.51	0.00	11.89	135.72	194.34

[a] Mutant was grown on BM_2 with 0.5% glucose and transferred on BM_2 with 10 m*M* malate for 15 h prior to O_2 measurements.

Table 4. Substrate-dependent O_2 consumption by free-living cells of *R. leguminosarum* biovar *phaseoli* P121 grown on BM_2 medium containing 0.5% glucose and supplemented with increasing concentrations of malate

Concentration of malate (m*M*)	Rate of O_2 consumption in nmoles O_2 min^{-1} (mg protein)$^{-1}$ in the presence of:	
	Glucose (0.5%)	Malate (10 m*M*)
0.0	137.60	14.76
2.5	32.80	136.65
5.0	23.78	144.74
10.0	12.71	137.60

Table 5. Substrate-dependent O_2 consumption by isolated bacteroids of *R. leguminosarum* biovar *phaseoli* P121 and its C_4-dicarboxylic acid utilization mutant P121S21

Substrate	Concentration	Rate of O_2 consumption in nmoles O_2 min^{-1} (mg protein)$^{-1}$	
		P121	P121S21
Pyruvate	10 m*M*	0.9	0.5
6-P-Gluconate	5 m*M*	3.3	1.8
Succinate	10 m*M*	68.9	1.3
Fumarate	10 m*M*	32.6	0.6
Malate	10 m*M*	47.8	0.9
Glucose	0.5%	1.4	0.8
Fructose	0.5%	0.8	0.9
Sucrose	0.5%	0.3	0.7
Cytosol		52.8	1.1

only with the organic acids succinate, malate and fumarate (Table 5). The bacteroids of the ineffective C_4-dicarboxylic acid utilization mutant P121S21 did not oxidize any of the carbon sources tested. The respective plant cytosol fractions, stimulated the respiration of P121 bacteroids but had not any effect on P121S21 bacteroids. Our results are similar to other observations made with bacteroids of a strain of *R. leguminosarum* biovar *viceae* (Glenn and Dilworth, 1981) and a strain of cowpea *Rhizobium* (Saroso *et al.*, 1984).

The results presented here indicate that C_4-dicarboxylic acid utilization by bacteroids is essential for the establishment of an effective *Rhizobium leguminosarum* bv. *phaseoli* — *Phaseolus vulgaris* symbiosis. This supports the previous hypothesis (Ronson *et al.*, 1981) which states that C_4-dicarboxylic acids are the main source of energy and reducing power for N_2-fixing bacteroids. In *Phaseolus vulgaris* nodules, a high concentration of malate can be detected (Lafontaine *et al.*, 1989; Streeter, 1987). Moreover, in ineffective nodules induced by strain P121S21, the concentration of malate was tenfold higher than in effective P121-induced nodules (Lafontaine *et al.*, 1989). Thus, the possibility of the existence of a catabolic repression-like phenomenon mediated by malate and blocking the catabolism of glucose in bacteroids should be investigated. Reports on the support of acetylene reduction by glucose in French bean under low O_2 concentrations (Trinchant *et al.*, 1981) and the significant alterations of the nitrogenase activity in some sugar utilization negative mutants, are points in favor of the repression hypothesis. More studies on the transport of metabolites across the peribacteroid membrane under different conditions, and an exhaustive biochemical characterization of the carbohydrate mutants obtained in this study, are essential to elucidate the exact nature of the carbon source supplied to the nitrogen fixing bacteroids.

Acknowledgments

This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada (NSERC). Cost of the present paper is supported by the Conseil de recherches et services agricoles du Québec.

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