SHORT COMMUNICATION

Oxidation of Substrates by Isolated Bacteroids and Free-living Cells of *Rhizobium leguminosarum* 3841

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Free-living cells of *Rhizobium leguminosarum* 3841 have inducible oxidation systems for the catabolism of histidine, malonate, *p*-hydroxybenzoate, glycerol, mannitol and sorbitol. In addition to *p*-hydroxybenzoate, strain 3841 is able to use a variety of other aromatic substrates as sole carbon source. Cultured free-living bacteria of this strain have constitutive systems for the catabolism of acetate, pyruvate, succinate, fumarate, malate, glucose, fructose, sucrose, lactose, ribose and arabinose. However, isolated pea bacteroids of the same strain are unable to oxidize monosaccharides or disaccharides although they can oxidize the organic acids succinate, fumarate, malate and pyruvate.

INTRODUCTION

The physiology of the free-living and bacteroid forms of legume root nodule bacteria (rhizobia) is still poorly understood, though it has obvious relevance to the survival of the organisms in the soil and their participation in the development of N₂-fixing nodules with a leguminous plant partner. In addition, the changes from the free-living to the bacteroid form must be a reflection of changes in physiology. An understanding of the physiology of the free-living form should provide a base-line from which significant and specific events in bacteroid development may be recognized. It may then be possible to establish a temporal sequence, not only of morphological events (Vincent, 1980) but also of biochemical events.

The supply of carbon to the bacteroid by the plant is clearly a vital event in symbiosis and is believed (Hardy, 1977; Pate, 1977) to be a major factor limiting N₂ fixation. Although the exact nature of the carbon source(s) has been unclear, recent work with succinate uptake mutants of *Rhizobium trifolii* (Ronson *et al.*, 1981) suggests that an exogenous supply of C₄ dicarboxylic acids is essential for N₂ fixation, since such mutants are unable to fix N₂ (Fix⁻). However, these mutants undergo apparently normal bacteroid development and form nodules (Nod⁺) (Ronson *et al.*, 1981). This suggests that carbon sources other than C₄ dicarboxylic acids may be used in bacteroid development. These conclusions are supported by work with succinate-resistant (*sucII*) mutants of *R. leguminosarum* (Glenn & Brewin, 1981). The *sucII* mutants are unable to grow on low concentrations of succinate, show decreased rates of uptake of succinate and are able to nodulate (Nod⁺) but are unable to fix N₂ (Fix⁻); revertants able to grow on succinate also regain the ability to fix N₂.

The experiments by Ronson *et al.* (1981) and Glenn & Brewin (1981) suggest that the bacteroid can utilize different carbon sources. However, little is known about the regulation of carbon metabolism in rhizobia. We have, therefore, investigated how growth on specific carbon compounds affects the ability of *R. leguminosarum* 3841 to utilize other carbon substrates.

METHODS

Organism. *Rhizobium leguminosarum* 3841 is a Str^R derivative of strain 300 (Johnston & Beringer, 1975) and has been described previously (Glenn *et al.*, 1980).

Nodulation and preparation of bacteroids. Pea plants (*Pisum sativum* cv. Greenfeast) were nodulated by *R. leguminosarum* 3841 and bacteroids were prepared as described by Glenn *et al.* (1980); they were used immediately after isolation.

Media. Bacteria were grown in batch culture at 28 °C on the minimal medium of Brown & Dilworth (1975) with NH₄⁺ (3.8 mM) as nitrogen source and a carbon source at 10 mM, and buffered with 40 mM-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), pH 7.2.

Measurement of O₂ consumption. Bacteria were harvested at an $A_{600}^{1\text{cm}}$ of 0.6–0.8 (about 0.6 mg dry wt ml⁻¹), washed twice with prewarmed buffered salts solution and resuspended to the same cell density in fresh buffered salts solution. Substrate-dependent O₂ consumption was measured polarographically at 25 °C using Hansatech oxygen electrodes (Hansatech, Norfolk, U.K.). Results are expressed as nmol O₂ min⁻¹ (mg protein)⁻¹.

Protein determination. Protein was determined by the Lowry method using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Growth of free-living bacteria. *Rhizobium leguminosarum* 3841 was able to utilize a range of single carbon sources for growth. Care was required with pH control since there was substantial acid production from the metabolism of sugars such as glucose, and alkali production arising from succinate utilization. The addition of increasing concentrations of HEPES, pH 7.2, up to 40 mM, increased growth on a wide range of carbon sources. The mean generation times with 40 mM-HEPES varied from 3.5 h on glucose to 10 h on *p*-hydroxybenzoate; other substrates gave intermediate values, e.g. succinate 4.1 h, malonate 5.5 h, histidine 7.5 h. The capacity of exponentially growing cells grown on a single carbon source to oxidize a range of other carbon compounds was then investigated.

Inducible systems in free-living bacteria. Histidine-, malonate- or *p*-hydroxybenzoate-dependent O₂ consumption was high in cells grown on these particular substrates (Table 1). However, cells grown on a wide range of other carbon substrates showed no O₂ consumption dependent on the presence of histidine, malonate or *p*-hydroxybenzoate. This indicates that these systems are inducible in strain 3841.

The cleavage of the aromatic ring of substrates like *p*-hydroxybenzoate was demonstrated by measuring the decrease in A_{255} . Cells grown on *p*-hydroxybenzoate oxidized *p*-hydroxybenzoate [240 nmol O₂ min⁻¹ (mg protein)⁻¹] and had an immediate capacity to oxidize 3,4-dihydroxybenzoate [145 nmol O₂ min⁻¹ (mg protein)⁻¹] and 3-methoxy-4-hydroxybenzoate [66 nmol O₂ min⁻¹ (mg protein)⁻¹]. However, such cells failed to oxidize 2,4-dihydroxybenzoate [3 nmol O₂ min⁻¹ (mg protein)⁻¹] or 3,5-dihydroxybenzoate, *m*-hydroxybenzoate, benzoate, or catechol (no O₂ uptake). This suggests that *p*-hydroxybenzoate is degraded by *R. leguminosarum* 3841 via the protocatechuate pathway.

In addition to *p*-hydroxybenzoate, strain 3841 grew, at varying rates, on catechol, 2,4-dihydroxybenzoate, 3,4-dihydroxybenzoate, 3,5-dihydroxybenzoate, 3,4,5-trihydroxybenzoate, 3-methoxy-4-hydroxybenzoate, 3-methoxycinnamate, *m*-hydroxybenzoate, 3-methoxy-4-hydroxyphenylpropanoate, phenol, salicylate and L-tyrosine. Some of these aromatic compounds are likely to originate from the breakdown of soil humic acids, and the capacity to utilize such substrates may be of significance in the survival of rhizobia in soil. The genes coding for enzymes involved in the catabolism of many aromatic substrates are plasmid-borne in the related genus *Pseudomonas* (Chakrabarty, 1976), and it is obviously of interest to examine rhizobia for plasmid-coded catabolic systems. Such functions could prove to be useful genetic markers for rhizobial plasmids.

Glycerol-, mannitol- or sorbitol-dependent O₂ consumption was maximally expressed when *R. leguminosarum* 3841 was grown in the presence of the inducing substrate. Cells grown on mannitol were capable of oxidizing mannitol and sorbitol and vice versa, indicating that these polyols induced the same enzyme system or separate enzyme systems which utilized both

Table 1. Substrate-dependent O₂ consumption by free-living cells and pea bacteroids of *R. leguminosarum* 3841

O ₂ consumption* by free-living cells, grown on:																			
Substrate		O ₂ consumption* by free-living cells, grown on:																	
		Malonate	Histidine	<i>p</i> -Hydroxybenzoate	Glycerol	Mannitol	Sorbitol	Acetate	Succinate	Fumarate	Malate	Pyruvate	Glucose	Fructose	Sucrose	Lactose	Ribose	Arabinose	O ₂ consumption* by isolated bacteroids
Inducible	Malonate	143.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Histidine	0	78.2	ND	ND	0	ND	ND	0	ND	ND	ND	0	ND	ND	0	ND	0	0
	<i>p</i> -Hydroxybenzoate	0	ND	243.2	ND	0	ND	0	0	ND	ND	0	0	ND	0	0	0	0	0
	Glycerol	25.6	ND	34.0	149.1	19.8	17.8	27.0	0	0	0	20.0	22.8	10.0	18.0	21.9	19.6	15.8	1.2
	Mannitol	11.2	ND	15.0	15.0	123.5	122.6	20.2	0	0	0	10.0	14.0	10.0	14.0	5.0	16.0	11.7	ND
Constitutive	Sorbitol	32.0	ND	19.6	28.8	122.6	111.3	27.0	0	0	0	8.4	10.6	15.0	22.5	20.5	29.3	20.1	ND
	Acetate	70.1	ND	22.5	30.8	89.0	60.1	107.8	43.6	31.2	42.0	21.8	41.9	58.0	41.0	71.2	54.0	50.4	ND
	Succinate	78.5	57.0	31.7	41.6	124.0	79.0	50.4	220.5	193.5	148.1	78.8	58.0	67.0	30.4	59.9	49.8	103.1	55.3
	Fumarate	71.7	ND	43.2	31.5	91.5	75.0	37.0	114.3	180.5	76.5	53.0	45.0	35.0	31.0	63.5	40.2	56.0	47.7
	Malate	69.9	ND	44.2	27.7	97.5	74.0	40.4	135.5	146.5	191.6	71.9	42.0	54.0	25.2	32.2	41.2	55.5	36.6
	Pyruvate	135.5	ND	88.6	59.0	56.2	64.3	180.1	79.0	76.0	107.0	164.6	141.0	125.2	70.0	68.9	103.9	72.4	18.7
	Glucose	108.2	103.0	86.8	86.0	112.0	117.0	90.5	90.0	73.2	77.0	103.8	168.0	144.0	120.0	137.0	91.0	86.8	0
	Fructose	77.5	ND	ND	42.3	182.0	150.0	70.7	50.0	72.0	40.0	85.7	90.0	136.0	60.0	59.2	87.4	61.9	0.8
	Sucrose	107.0	56.0	83.7	72.0	119.0	99.0	82.3	45.0	67.5	42.0	105.6	116.0	129.0	127.5	110.0	92.0	95.5	0.4
	Lactose	79.5	ND	ND	59.0	84.0	57.6	75.8	43.0	36.5	39.0	73.9	96.5	70.4	58.0	91.0	45.0	93.9	0.2
	Ribose	75.8	ND	ND	53.0	42.0	49.0	63.7	46.7	42.4	48.0	43.0	119.3	99.4	41.0	64.5	65.2	62.7	0.6
	Arabinose	95.2	ND	101.7	86.0	75.0	93.4	66.4	127.0	96.0	107.0	95.3	150.2	113.0	72.0	136.0	90.8	161.9	ND

ND, Not determined.

* All O₂ consumption rates are expressed in nmol O₂ min⁻¹ (mg protein)⁻¹.

substrates (Table 1). Primrose & Ronson (1980) have reported similar cross-induction of polyol dehydrogenases in *R. trifolii*. In contrast to the oxidative systems for histidine, malonate or *p*-hydroxybenzoate, cells of 3841 grown on a range of carbon sources showed some glycerol-, mannitol- or sorbitol-dependent O₂ consumption, indicating a much higher basal level of these particular enzyme systems compared with those for histidine, malonate or *p*-hydroxybenzoate oxidation. It is interesting to note, however, that cells grown on succinate, fumarate or malate showed little or no glycerol-, mannitol- or sorbitol-dependent O₂ consumption (Table 1).

Constitutive oxidation systems in free-living bacteria. Acetate-, succinate, fumarate- or malate-dependent O₂ consumption was observed in some measure in cells grown on all the carbon sources tested (Table 1). However, in some cases the O₂ consumption was much lower than that of bacteria grown on the homologous substrate (e.g. for succinate oxidation, O₂ consumption by sucrose-grown cells was about 15% of that of succinate-grown cells). Pyruvate- and sugar-dependent O₂ consumption was considerable in cells grown on any of the carbon sources. These results suggest that the oxidative systems for the metabolism of organic acids and of sugars are constitutive in *R. leguminosarum* 3841.

Oxidation of substrates by isolated bacteroids. Freshly isolated bacteroids of *R. leguminosarum* 3841 displayed succinate-, fumarate-, malate- and pyruvate-dependent O₂ consumption (Table 1). This indicated that the enzyme systems for the catabolism of these organic acids were present in the isolated bacteroids. By contrast, isolated bacteroids showed little or no glucose-, fructose-, sucrose-, lactose- or ribose-dependent O₂ consumption (Table 1). This suggests that bacteroids, unlike free-living cells, lack the ability to metabolize sugars. Separate experiments on the uptake of radioactive solutes have shown that isolated bacteroids are able to take up [¹⁴C]succinate, [¹⁴C]leucine and [¹⁴C]phenylalanine, but are unable to take up [¹⁴C]glucose, [¹⁴C]sucrose or [¹⁴C]lactose (Hudman & Glenn, 1980; Glenn *et al.*, 1980; Dilworth & Glenn, 1981).

These results imply that cells of strain 3841 contain a number of enzymes for which there is no apparent immediate use. Of the 17 carbon substrates examined in this study only six (histidine, malonate, *p*-hydroxybenzoate, glycerol, mannitol and sorbitol) had inducible oxidation systems. Even in this group it is worth noting that cells grown on most of the carbon sources displayed some, albeit small, glycerol-, mannitol- or sorbitol-dependent O₂ consumption. Implicit in this is that given a mixture of carbon substrates the cells should have some capacity to catabolize many of them at some rate, unless there are catabolite repression or other inhibitory effects which are not seen when cells are grown on a single carbon substrate.

It has been shown previously that free-living cells of *R. leguminosarum* have constitutive uptake systems for glucose (Hudman & Glenn, 1980) and succinate (Glenn *et al.*, 1980), whereas the bacteroid has only the latter. This study has extended those observations to other sugars and organic acids. The free-living form of strain 3841 has constitutive uptake systems for monosaccharides and disaccharides but these appear to be absent in the bacteroid form. One possible explanation for this difference is that there is a specific change in the regulation of sugar metabolism genes such that constitutive systems in the free-living form are repressed in the bacteroid. A simpler, and perhaps more likely, explanation is that the sugar catabolic systems remain constitutive in the bacteroid form but that some essential components of sugar transport, for example, sugar-binding proteins, are lost on bacteroid isolation. This view is consistent with the observations of Dilworth & Glenn (1981) that lysozyme/EDTA treatment (Glenn & Dilworth, 1979) of free-living cells of *R. leguminosarum* markedly decreased the uptake of [¹⁴C]glucose, [¹⁴C]sucrose and [¹⁴C]lactose, but had no effect on the uptake of [¹⁴C]leucine, [¹⁴C]succinate or [¹⁴C]phenylalanine. Sugar-binding activity has been detected in periplasmic proteins isolated from free-living rhizobia and in pea nodule supernatants (Dilworth & Glenn, 1981).

Further studies on the physiology of free-living and bacteroid forms of strain 3841 and other strains, including the use of mutants, should help to clarify the carbon metabolism of the two forms.

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