Malonate and Krebs Cycle Intermediates Utilization in the Presence of other Carbon Sources by *Rhizobium japonicum* and Soybean Bacteroids

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Free living cells of *Rhizobium japonicum 61-A-101* and bacteroids from *Glycine max* var. Mandarin infected with the same strain utilized malonate with a substrate saturation greater than 10^{-2} mol/l. At low concentrations of malonate (10^{-5} mol/l) the free living cells were significantly more active in utilizing malonate than bacteroids. In bacteroids two substrate saturation ranges were found, one between 3×10^{-4} mol/l and 10^{-3} mol/l, the other at more than 10^{-2} mol/l. Utilization of malonate was not affected by 10 to 100 times larger concentrations of either arabinose or xylose. 10^{-3} mol/l succinate inhibited the utilization of malonate (10^{-4} mol/l) completely in bacteroids, and by 90% in free living cells. Succinate utilization (10^{-4} mol/l) was reduced in those cells exposed to 100 times higher malonate concentration only by 20–30%. Utilization and incorporation of pyruvate and 2-oxoglutarate into bacteroids was also only slightly affected by 100 times larger malonate concentration. Citrate utilization by bacteroids however was reduced by more than 70%. The rate of endoxidation of malonate as sole carbon source ($^{14}CO_2$ production from [2-^{14}C] malonic acid)was about 1.5 μmol · h^{-1} · mg $protein^{-1}$ and about half the rate with *Pseudomonas putida* and 70% of the rate with *Pseudomonas fluorescens* under the same conditions (pH 6.0, 28 °C).

Introduction

Malonate is present in high concentrations in species of several plant families and especially in many legume species. Concentrations between 0.5 and 2 mg per gram of tissue fresh weight (corresponding to 7.5 to 30 mmol/l) malonate have been found in *e.g. Phaseolus, Medicago, Vicia, Astragalus, Trifolium, Lupinus, Lotus, Trigonella* [1]. In clover leaves up to 3% of the dry weight was found to be malonate, which represents more than 30% of the di- and tri-carboxylic acids [2]. In other organisms such as the leaves of *Phaseolus vulgaris*, malonate was less concentrated than malate and citrate but more concentrated than succinate [3]. Malonate concentration in soybean nodules as high as 10 mmol/l have been reported, which was twice that of malate and 20 fold the concentration of succinate [4]. Malonate concentration in the nodules have been found to be three times that found in infected root tissue [3, 4].

We have previously studied the effect of malonate relative to succinate and arabinose on the derepression of nitrogenase in pure cultures of *Rhizobium japonicum* [5]. 10 mmol/l malonate as sole carbon source and 50 mmol/l arabinose in surface cultures exposed to air gave no nitrogenase activity, whereas the combination of both gave the same peak activity as the combination of succinate and arabinose.

The high concentration of malonate in soybean nodules and the replacement of succinate by malonate left open the question of how malonate can be taken up, incorporated and endoxidized by soybean bacteroids and free living *Rhizobium japonicum* cells when affected by other carbon sources.

Materials and Methods

Organisms, media and growth

Rhizobium japonicum strain *61-A-101* was grown in liquid culture in the medium designated 20 E [6]. *Pseudomonas putida*, (received from Prof. Dr. G. Jagnow, Bundesforschungsanstalt für Landwirtschaft, Braunschweig, FRG) and *Pseudomonas fluorescens*, DSM 50415 (received from the Deutsche Sammlung für Mikroorganismen, Göttingen, FRG), were grown in Nutrient Broth as a batch culture at 28 °C. Cells from log-phase culture were harvested by centrifugation, washed twice and subsequently resuspended in a mineral medium at the following composition

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(mg/l): $MgSO_4 \cdot 7\,H_2O$: 123.25; $NH_4H_2PO_4$: 57.54; $Ca(NO_3)_2 \cdot 4\,H_2O$: 118.08; KNO_3: 50.55; H_3BO_3: 0.77; KCl: 1.86; $MnSO_4 \cdot H_2O$: 0.17; $ZnSO_4 \cdot 7\,H_2O$: 0.29; $CuSO_4 \cdot 5\,H_2O$: 0.006; $Na_2MoO_4 \cdot 2\,H_2O$: 0.06; $FeSO_4 \cdot 7\,H_2O$: 2.75; Titriplex III: 3.72; pH: 6 (½ concentrated Hoagland's medium) [7].

For the experiments described in Fig. 5 only, subsamples of 300 ml culture in salts of medium 20 E supplemented with 50 mmol/l arabinose and 10 mmol/l succinate were incubated with [^{14}C] arabinose for 2 h under either air or in microaerobic conditions (1% O_2, 99% N_2 in the gas phase). Nitrogenase activity under microaerobic conditions was tested directly, stopping the continuous gas supply, adding 10% acetylene and incubating for 1 h. Subsequently the cell suspension was returned to gas with air or 1% O_2 and 99% N_2 at the previous rate of 15 l/h. Growth was assessed in this experiment only by monitoring absorbance at 540 nm. Cell numbers were otherwise determined with a nephelometer (Corning, England). Nitrogenase activity was measured as described previously [5].

Isolation of bacteroids from nodules of Glycine max

Seedlings of *Glycine max* var. Mandarin were grown as previously described [8]. 4 to 5 weeks after infection, 500 mg of fresh nodules were washed twice with sterile water and squashed under anaerobic conditions in 2.5 ml in the mineral medium given above. The suspension was filtered through a 10 µm mesh size perlon gauze and the nodule debris re-extracted. The filtrate was centrifuged for 4 min at $350 \times g$ to remove contaminating cell walls and starch particles. Bacteroids were subsequently removed from the supernatant by centrifugation at $7400 \times g$ Resuspended bacteroids were washed in 2.5 ml of ½ concentrated Hoagland medium and cell number determined by plating on to medium 20 E containing 0.2 mol/l mannitol instead of 10 mmol/l.

Uptake, incorporation and utilization of carbon sources

[^{14}C]labelled substrates (Amersham Buchler, FRG) of the specific activities given in parenthesis were used:

[2-^{14}C]malonic acid, sodium salt: 2.06 GBq/mmol (55.8 mCi/mmol)
[U-^{14}C]pyruvic acid, sodium salt: 370 MBq/mmol (10 µCi/mmol)
2-Keto [5-^{14}C]glutaric acid, sodium salt: 747 MBq/mmol (20.2 mCi/mmol)
[1,4-^{14}C]succinic acid, sodium salt: 4.14 GBq/mmol (112 mCi/mmol)
[U-^{14}C]arabinose: 140 MBq/mmol (3.8 mCi/mmol).

To analyze *utilization* of the various carbon sources, cells were incubated for either 1 or 2 h. Cell samples were spun down by centrifugation at $35\,000 \times g$ and the radioactivity remaining in the supernatant determined. Blanks without cells were used as positive references. Fractions of the *substrate* which was *incorporated* into the cells was measured in the cell sediment after two washes with fresh medium. *Endoxidation* of the substrate in the short term (2 h) and long term (48 h) duration experiments was evaluated by a Warburg method in vessels containing 4 ml suspension with 0.4 ml KOH (5%) in the side arm. The vessels were sealed and incubated with constant shaking. 10 or 20 µl samples of the KOH were taken at appropriate time intervalls and the ^{14}C content determined.

Results

Fig. 1 shows the utilization of malonate by free living cells of *Rhizobium japonicum 61-A-101* compared with bacteroids from soybean nodules infected with the same effective strain. At low substrate concentrations (10^{-4} to 10^{-5} mol/l the free living cells

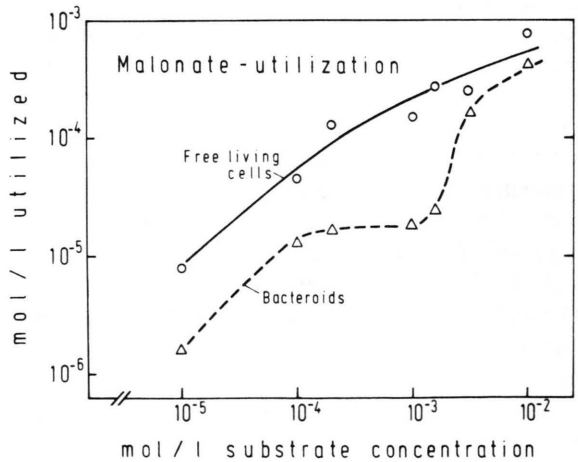

Fig. 1. Utilization of malonate as sole carbon source after a 2 h incubation period, by *Rhizobium japonicum 61-A-101*. Comparison of free living cells and isolated bacteroids under aerobic conditions at a biomass of 5×10^8 cells/ml (equivalent to about 50 nephelometer units).

utilized between 65 and 90% of the substrate, the bacteroids however only utilized 12 to 20% of the substrate during the 2 h incubation period. At high substrate concentrations (10^{-2} mol/l malonate) the difference between these two stages of *Rhizobium japonicum* was significantly decreased. The substrate utilization by free living cells was about 9%, in the bacteroids the figure was 6.3%. However, with the bacteroids only a first substrate saturation plateau between 5 times 10^{-3} and 10^{-3} mol/l malonate was observed. For comparison, the utilization of succinate as sole substrate with preparations of bacteroids and free living cells was determined with 1 mmol/l succinate: 22% of the succinate was utilized by the free living cells (compared to 19% of the malonate at 1 mmol/l) the bacteroids utilized 3.2% of the succinate (compared to 2.6% of the malonate) during the incubation period. The bacteroids used in these experiments were characterized by their viability on solid medium which contained 0.2 mol/l mannitol. Per gram of nodule fresh weight 3 to 8×10^{10} bacteroids were isolated. The viability varied between 22 and 78% with no significant difference in 4, 5 and 6 weeks old nodules (Table I). Malonate utilization was further compared between free living cells of *Rhizobium japonicum* and one strain, each of *Pseudomonas putida* and *Pseudomonas fluorescens.* The rate of endoxidation of malonate as sole carbon source ($^{14}CO_2$ production from [2-^{14}C]malonic acid was about 1.5 µmol · h^{-1} · mg protein^{-1} and about half the rate with *Pseudomonas putida* and 70% of the rate with *Pseudomonas fluorescens* under the same conditions (pH 6.0, 28 °C).

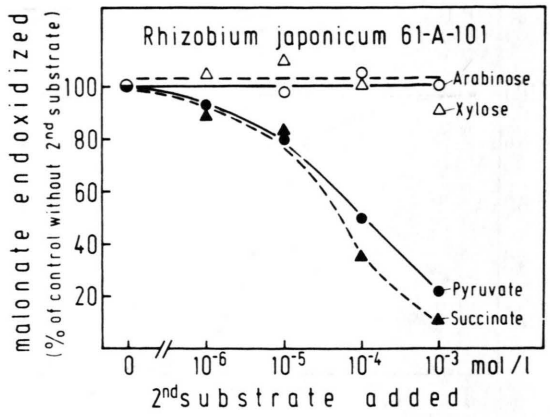

Fig. 2. Endoxidation of [2-^{14}C]malonate measured as $^{14}CO_2$ released by *Rhizobium japonicum 61-A-101* relative to concentrations of other carbon sources added (arabinose, xylose, pyruvate, succinate). The malonate concentration was 10^{-4} mol/l, the incubation period 2 h and a concentration of 5×10^8 cells/ml.

Utilization and endoxidation of malonate by *Rhizobium japonicum 61-A-101* was reduced to 10% by 10^{-3} mol/l succinate (Fig. 2). At the same concentration of succinate as malonate (10^{-4} mol/l) the reduction was about 70%. Pyruvate affected malonate utilization slightly less. Conversaly arabinose and xylose did not affect malonate utilization up to molar ratios of 10:1. The results with bacteroids are principally similar (Fig. 3). The effect of succinate, however, was even more pronounced.

Table I. Isolation of viable bacteroids from nodules of *Glycine max* var. Mandarin, infected with *Rhizobium japonicum 61-A-101.*

Nodule age after infection [weeks]	Isolation-Medium and conditions	Viable *Rhizobium japonicum* cells per gram nodule fresh weight	% Viable cells[a]
4	1/2 conc. Hoagland medium pH 6.0, anaerobic isolation	$5.7-8.5 \times 10^{10}$	43–60
5	1/2 conc. Hoagland medium pH 6.0, anaerobic isolation	$3.5-8.0 \times 10^{10}$	22–74
6	1/2 conc. Hoagland medium pH 6.0, anaerobic isolation	$3.2-4.5 \times 10^{10}$	56–78

[a] % of total cell number, as estimated by precalibrated nephelometer with 100 units as 10^9 cells · ml^{-1}.

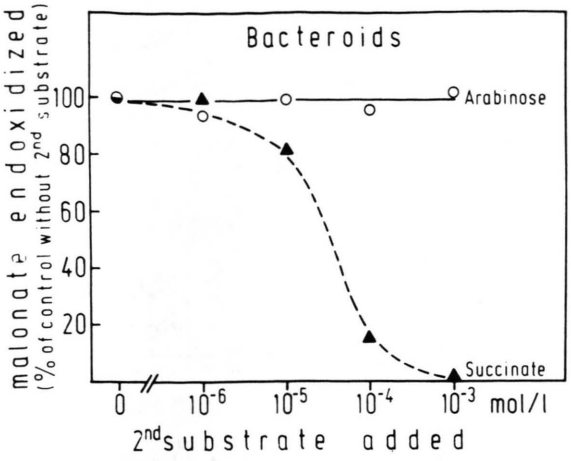

Fig. 3. Endoxidation of [2-^{14}C]malonate by bacteroids isolated from nodules of *Glycine max* as affected by increasing concentrations of succinate and arabinose. The malonate concentration was 10^{-4} mol/l the incubation period 4 h at a biomass of 5×10^8 cells/ml.

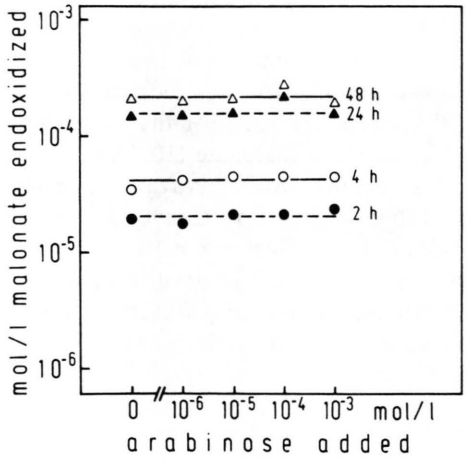

Fig. 4. Endoxidation of [2-^{14}C]malonate measured as $^{14}CO_2$ released by *Rhizobium japonicum 61-A-101* incubated for 2 to 48 h in the presence of various arabinose concentrations. The malonate concentration was 10^{-3} mol/l at a biomass of 5×10^8 cells/ml.

Malonate endoxidation was completely inhibited by 10^{-3} mol/l succinate and reduced by 85% at 10^{-4} mol/l. Arabinose again did not affect the utilization of the substrate malonate.

In the experiments described in Figs. 1 to 3, cells of *Rhizobium japonicum* and isolated bacteroids were incubated for one or 2 h. In previous publications we studied the effect of several carbon sources on derepression of nitrogenase activity in pure culture [5, 9]. In those studies cells were incubated for a much longer period. We therefore observed the utilization of malonate for 24 and 48 h in the presence of arabinose (Fig. 4). We found again no significant decrease or increase in malonate utilization by adding up to 1 mmol/l arabinose. The cells utilized about 2.2 µmol arabinose · h^{-1} · mg protein^{-1} under air (Fig. 5). This rate, however, depends very much on the stage of the growth curve where cells are harvested. Under microaerobic conditions (1% O_2 and 99% N_2 in the gas phase) the

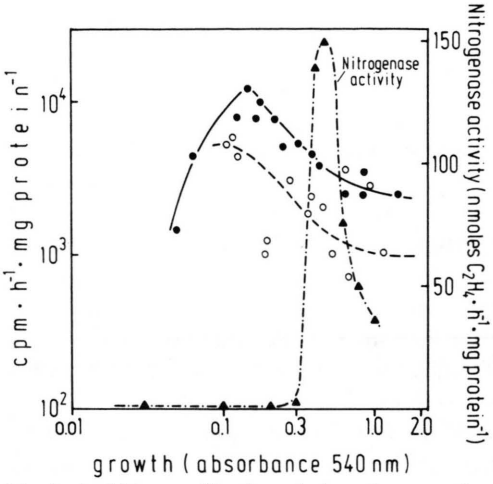

Fig. 5. Arabinose utilization during the growth of *Rhizobium japonicum 61-A-101* under aerobic (●) or microaerobic (○) conditions. 2.27 µmol arabinose correspond to 10^4 cpm. Nitrogenase activity only under microaerobic conditions.

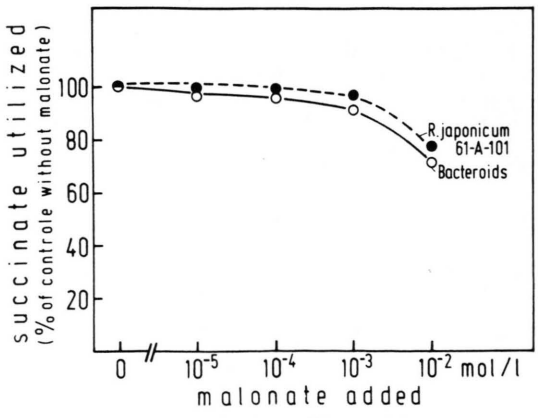

Fig. 6. Succinate utilization (10^{-4} mol/l) by *Rhizobium japonicum 61-A-101* and by bacteroids as affected by increasing malonate concentrations. The incubation period was 1 h, the initial succinate concentration was 10^{-4} mol/l at a biomass of 5×10^8 cells/ml.

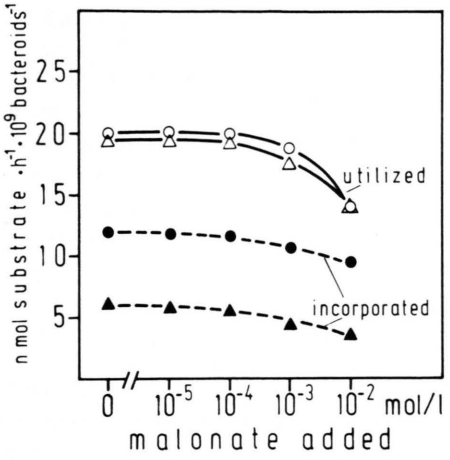

Fig. 7. Utilization (open symbols) and incorporation (closed symbols) of 2-oxoglutarate (△, ▲) and pyruvate (○, ●) within bacteroids as affected by increasing concentrations of malonate. The assay contained 5×10^8 cells/ml.

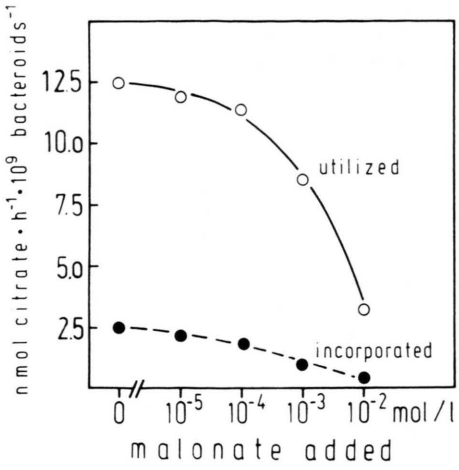

Fig. 8. Utilization and incorporation of citrate by bacteroids as affected by increasing concentrations of malonate. The biomass was 5×10^8 cells/ml.

utilization of arabinose varied more than under air (Fig. 5) and this may be significant. This utilization of arabinose alone is apparently not directly correlated with a maximum of nitrogenase activity (150 nmol C_2H_4 · mg protein^{-1} · h^{-1}) in pure culture.

A molar ratio malonate/succinate of 10 to 1 did not affect succinate utilization in free living cells nor in bacteroids (Fig. 6). A further increase by a factor of 10 of malonate reduced succinate utilization by 20 to 30%. This means that the molar ratio of malonate to succinate (20:1) found in nodules of soybeans [4] would not significantly affect continuous succinate utilization by bacteroids, assuming a homogenous distribution in infected and uninfected host cells. The rate of utilization of 2-oxoglutarate and pyruvate by viable bacteroids is also not affected by 1 mmol/l malonate and is reduced only about 25% by 10 mmol/l malonate (Fig. 7). Incorporation of these organic acids into bacteroids is also affected only shortly. However, independently of the malonate concentration, twice as much pyruvate as 2-oxoglutarate is incorporated into the bacteroids (Fig. 7).

Citrate utilization is different from that of other organic acids, being readily affected by 0.1 mmol/l malonate and reduced by about 80% by the addition of 1 mmol/l malonate (Fig. 8). Utilization of citrate occurs at about half the rate of utilization of 2-oxoglutarate and pyruvate under aerobic conditions by the bacteroids.

Discussion

The chemical constituents of the carbon cycle between bacteroids and host cells cytoplasm are, as yet, unidentified (see 10). Previous experiments using isolated bacteroids and free-living cells of rhizobia mainly relied on the use of respiration rates as affected by single carbon sources as criteria. This situation, however, is unlikely to occur *in vivo* within the bacteroid environment because several carbon sources may be present together in nodules. Pure cultures of *Rhizobium* may utilize simultaneously several carbon sources [11]. In the work of de Hollander and Stouthamer [11], the simultaneous consumption of combinations of mannitol, arabinose and gluconate or glucose and lactose were studied. The results were interpreted to mean that no catabolite repression mechanisms is present in the *Rhizobium trifolii* strain used. Unfortunately there is still no reliable system for assessing the derepression of nitrogenase in pure cultures with the so-called fast growing *Rhizobium* group which includes *Rhizobium trifolii* and *Rhizobium leguminosarum*. Therefore it has not been possibe to compare the interaction of several carbon sources between slow growing and fast growing rhizobia in the nitrogenase repressed and derepressed stages. The high concentrations of malonate in nodules of *Glycine max* found by Stumpf and Burris [4] is an average value for the whole nodules. However we have neither informa-

tion about possible malonate compartmentalization within the infection vacuoles, nor on transport rates of malonate through the peribacteroid membranes. Although malonate utilization by bacteroids at high malonate concentrations is almost as efficient as that of free-living cells (Fig. 1), we cannot conclude that malonate is *in vivo* one of the major substrates for the bacteroids. Assuming the same concentration of malonate (10.4 mmol/l) and succinate (0.46 mmol/l) in the infection vacuoles for succinate and malonate as found in the whole nodules by Stumpf and Burris [4] (a molar ratio of about 20:1), we can conclude from the data presented in Fig. 3 that malonate endoxidation must be almost completely inhibited in bacteroids by succinate at the same concentration as occurs in nodules. The result of this inhibition could be the accumulation of malonate within soybeans. Conversely the malonate accumulating in the soybean nodules (concentrations of 10 mmol/l) does not significantly reduce bacteroid utilization of succinate, 2-oxoglutarate or pyruvate (Fig. 6, 7). Bacteroids, however, have the capacity to endoxidize malonate without a lag period. Diurnal variations in the pools of other organic acids may perhaps change the situation, giving the bacteroids chance to utilize accumulated malonate as a reserve substrate. The two substrate saturation ranges which are found only in bacteroids, await a further explanation and should first be analyzed with other substrates.

Bacterial oxidation of malonate has been studied in *e.g. Azotobacter agilis* by Lineweaver [12], *Pseudomonas aeruginosa* by Gray [13] and *Enterobacter aerogenes* by Barron and Ghiretti [14]. In all cases anadapted cells showed a lag period of malonate oxidation, varying between 1 and 3 h. No such lag period was observed with anadapted cells of *Rhizobium japonicum 61-A-101* (Fig. 4). The pathway of malonate metabolism has been studied in *Pseudomonas fluorescens* [15, 16]. The proposed scheme of: malonate + ATP + CoA → Malonyl CoA → acetyl-CoA + Co_2 has been discussed in more detail by Webb [17]. In a forthcoming publication we will report on some differences between *Rhizobium japonicum* strains and other soil bacteria from the genus *Pseudomonas* and *Arthobacter* with respect to their efficiency in utilizing small concentrations of malonate and other carbon sources as well.

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