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ANAEROBIC–NITRATE, SYMBIOTIC AND AEROBIC GROWTH OF *RHIZOBIUM JAPONICUM*: EFFECTS ON CYTOCHROME P450, OTHER HAEMOPROTEINS, NITRATE AND NITRITE REDUCTASES

R. M. DANIEL* AND C. A. APPLEBY

Division of Plant Industry, C.S.I.R.O., Canberra (Australia)

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SUMMARY

1. *Rhizobium japonicum* strain 505 (Wisconsin) was grown symbiotically in soybean root nodules, in liquid culture under air or 2% O_2, also aerobically and anaerobically with nitrate (6 mM).

2. Anaerobic–nitrate growth induces a haemoprotein pattern similar to but not identical with that of N_2-fixing symbiotic bacteria (bacteroids). In particular, the induced cytochrome P450 has different properties.

3. Whole bacteroids and anaerobic–nitrate cells showed high levels of nitrate reductase activity, respectively 1490 and 1240 μmoles$\cdot h^{-1} \cdot g^{-1}$ protein; nitrate reductase activity in air-grown or air–nitrate-grown cells was low, respectively 330 and 190 μmoles$\cdot h^{-1} \cdot g^{-1}$ protein. Nitrite reductase activity was detected only in anaerobic–nitrate-grown cells.

4. It is concluded that nitrate respiration is significant in anaerobic–nitrate-grown cells, but not in air–nitrate cells or bacteroids. The characteristic haemoprotein pattern of bacteroids seems dependent on near-anaerobiosis rather than the presence of nitrate.

INTRODUCTION

The observations of Appleby[1,2] and others[3] have shown considerable difference between the haemoprotein and respiratory enzyme patterns of air-grown free-living cells of *Rhizobium japonicum*, and the N_2-fixing 'bacteroid' form of this organism grown symbiotically in soybean root nodules. O_2 tension within the root nodule is very low[4], but so far it has not been shown whether the bacteroid haemoprotein pattern simply reflects this low-O_2 environment.

We now report the anaerobic or near-anaerobic growth of *R. japonicum* in the presence of nitrate as terminal electron acceptor, and show that these cells acquire some bacteroid characteristics. However, we were unable to obtain any

* Present address: Department of Cell Biology, University of Glasgow, Glasgow, W.2., Scotland.

evidence that nitrate respiration is a significant feature of bacteroid energy conservation *in vivo*. A preliminary account of this work has been presented elsewhere[5].

MATERIALS AND METHODS

Preparation of free-living bacteria

R. japonicum strain 505 (Wisconsin) was grown in a medium consisting of K_2HPO_4, 0.05 %; $MgSO_4 \cdot 7H_2O$, 0.02 %; crude NaCl, 0.01 %; Oxoid dry yeast extract, 0.06 %; mannitol, 1.0 %. The medium was adjusted to pH 7.0 before autoclaving.

The cells were grown at 25 °C in 15-l batches in 20-l bottles sparged with air–N_2 mixtures or air at 200 $l \cdot h^{-1}$. Studies with submerged O_2 electrodes (Titron-Instruments, Sandringham, Victoria, Australia) showed that the O_2 demand of *R. japonicum* is sufficiently low that the medium remains saturated with air ($\simeq$ 200 μM O_2) throughout growth under this condition. The cells were harvested in late logarithmic phase after 10 days.

For growth in the presence of nitrate, separately autoclaved 1 M KNO_3 was added to the sterile medium to give 6 mM final concentration. For anaerobic growth the medium was sparged with high purity N_2 (Commonwealth Industrial Gases, Sydney) containing $<$ 10 ppm O_2, or the inoculum put into full, rubber stoppered 19-l, thick-walled polypropylene bottles, and the cells harvested after 6 days at 25 °C.

Preparation of bacteroids

These were isolated and purified[1] from N_2-fixing soybean root nodules (cv. Lincoln) inoculated with *R. japonicum* strain 505 (Wisconsin), and grown as described elsewhere[6].

Determination of nitrate and nitrite

Nitrate was determined by the method of Szekely[7] as described by Lam[8] and nitrite determined as described by Nicholas and Nason[9].

Determination of nitrate and nitrite reductases

Nitrate reductase was measured by estimating both the rate of disappearance of nitrate and, in the absence of nitrite reductase, the rate of appearance of nitrite. Nitrite reductase was measured by estimating the rate of disappearance of nitrite.

The assay mixture for determination of nitrate reductase consisted of 800 μg chloramphenicol, 1 mmole KH_2PO_4–Na_2HPO_4 (pH 6.8), 100 μmoles sodium succinate, 100 μmoles glucose, 20–200 μmoles KNO_3 and a suitable quantity of whole cells (2–20 mg protein) in a final volume of 10 ml. The reactions were run under argon in 10-ml conical flasks, and 0.2-ml samples taken through a rubber ampoule cap (Subaseal) at appropriate intervals. The reaction in these 0.2-ml samples was stopped by the addition of 3 ml of ethanol and 0.2 ml 1 M zinc acetate, and the supernatant assayed for nitrite after centrifuging for 10 min at 3000 $\times$ g; alternatively the reaction was stopped with 12 % trichloroacetic acid (1.8 ml) and the supernatant assayed for nitrate after centrifuging for 10 min at 3000 $\times$ g.

For the nitrite reductase assay various concentrations of KNO_2 (100 μM–1

mM) were substituted for KNO_3. Values given for nitrite reductase are probably minimal; although the enzyme appeared to have a very low affinity for nitrite and the rate of reaction increased with increasing concentrations of nitrite, at high concentrations nitrite may be inhibitory[10]. However, rates of nitrate and nitrite reductase activity remained constant for the period of the longest assay (3 h) despite the presence of up to 650 μM nitrite (the usual concentration).

Estimation of nitrate and nitrite in root nodules and plant nutrient solution

Attempts were made to extract nitrite or nitrate present in root nodules or in bacteroids by homogenizing in a Waring blendor with an equal volume of 0.1 M KH_2PO_4–Na_2HPO_4 (pH 6.8) at 2 °C, or water at 90 °C, or with 12 % trichloroacetic acid. The lower limit of detection by these methods is 2 nmoles nitrite·g^{-1} wet wt for nodule breis or bacteroids and 10 nmoles nitrate·g^{-1}. No nitrite or nitrate could be detected in nodules or bacteroids, nor in the concentrated plant nutrient solution of McKnight[11], where the detection limit was 10 nmoles·l^{-1} for nitrite and 25 nmoles·l^{-1} for nitrate.

Molecular weight determination of soluble haemoproteins

The 144000 × *g* supernatant from an appropriate quantity of ruptured bacteria (28 g wet wt of anaerobic–nitrate cells or bacteroids, or 150 g wet wt of air-

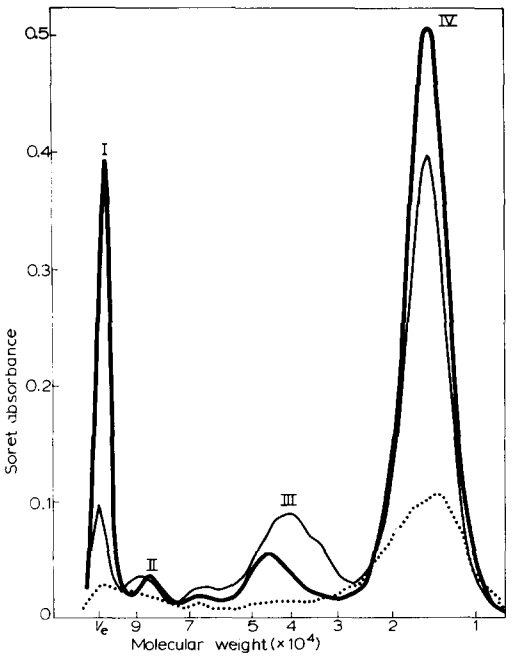

Fig. 1. Elution profiles, from Sephadex G-100 columns, of soluble haemoproteins from equal wet weights (28 g) of bacteroids (———), anaerobic–nitrate cells (▬▬) and air-grown cells (·····) of *R. japonicum*. Details are given in Materials and Methods. Although the extract from a larger amount (150 g wet wt) of air-grown cells was actually applied to the column, the observed absorbances were recalculated and plotted to allow direct comparison with the other profiles. The profile from air–nitrate cells, not shown, generally resembled the anaerobic–nitrate profile.

grown cells, suspended in 3 vol. of 0.1 M KH_2PO_4–Na_2HPO_4, pH 6.8) was concentrated to < 15 ml by pressure filtration over a Diaflo PM 10 membrane (Amicon Corporation, Lexington, Mass.) and applied to an 80 cm × 5 cm column of Sephadex G-100 (Pharmacia, Uppsala) pre-equilibrated and eluted with 0.1 M KH_2PO_4 – Na_2HPO_4 (pH 6.8) at 4 °C. Optical spectra of the fractions (16 ml) were recorded in the Soret region (450–350 nm) and absorption at the Soret maximum plotted against elution volume (Fig. 1). The column exclusion volume (V_e) and mol. wt calibrations marked on the abscissa of Fig. 1 were determined with the aid of Blue Dextran (Pharmacia) and crystalline proteins with molecular weights between 160 000 and 12 400.

Other procedures

Cell rupture and fractionation, protein determination, haemoprotein identification and quantitative spectrophotometry were carried out as described by Appleby[1,2]. CO equilibria were determined as described by Daniel[12] and Appleby[1].

RESULTS AND DISCUSSION

Achievement of anaerobic growth

When 14 l volumes of sterile medium were inoculated with 1 l of air-grown culture no measurable growth was achieved by sparging with pure N_2 or air–N_2 mixtures containing < 1% O_2, during incubation at 25 °C. From a wide range of added organic or inorganic electron acceptors, including acetate, fumarate, formate, pyruvate, cupric and ferric salts, sulphate and nitrate, only nitrate (6 mM) supported measurable growth under pure N_2 or in filled, stoppered bottles (Materials and Methods). The yield was ≃ 4 g wet wt pink cells per 15 l, compared with ≃ 16 g of air-grown cells per 15 l.

Cytochrome pattern in whole cells

Whereas Table I shows that the cytochrome $a + a_3$ content of *R. japonicum* was almost unaffected by air–nitrate or 2% O_2 growth, neither bacteroids (*cf.*

TABLE I

EFFECT OF GROWTH CONDITIONS ON HAEMOPROTEINS IN *R. japonicum* WHOLE CELLS

Concentrations of cytochromes $a + a_3$, b, c were determined from dithionite-reduced *minus* oxidized spectra[1,2] and of cytochrome a_3 from CO difference spectra[2]. The total amount of CO-reactive cytochromes b and c, which may include cytochrome c-552 (ref. 1), cytochrome c-554, cytochrome o and *Rhizobium* haemoglobin[2], was approximately estimated by assuming $\Delta\varepsilon_{mM} = 18$ for peak *minus* trough (approx. 550–570 nm) in CO difference spectra. All concentrations are expressed as nmoles·g^{-1} cell protein, but the phenomenon of multiple internal reflection (*cf.* ref. 1) may have caused 50% enhancement of the true values.

Growth conditions	*Cytochrome* $a + a_3$	*Cytochrome* a_3	*Cytochrome* b	*Cytochrome* c	*CO-reactive cytochromes* b *and* c
Air	90	55	190	250	60
Air–nitrate	50	40	280	600	80
2% O_2	60	< 50	270	450	120
Anaerobic–nitrate	0	0	190	780	230
Bacteroid	0	0	430	870	270

ref. 1) nor anaerobic–nitrate cells contained any trace of this terminal oxidase (*cf.* ref. 2). This simple result, and the further observation (Table I) that the CO-reactive cytochromes *b* and *c* increase during anaerobic–nitrate growth, might suggest that anaerobiosis, rather than nitrate is a determinant of bacteroid cytochrome pattern. However, air–nitrate growth caused some increase toward the bacteroid level of total cytochromes *c* and *b* (Table I), so this simple conclusion might not be valid.

Soluble haemoproteins

The soluble CO-reactive cytochrome P450 is supposedly characteristic of bacteroids[1,2], but its spectrum may be obscured by the dominating maxima and minima of major cytochrome components in the CO difference spectra of whole *Rhizobia* or crude extracts. Consequently, to assist the search for cytochrome P450 and other minor haemoprotein constituents in anaerobic–nitrate and other *Rhizobium* cells, we chromatographed the crude extracts on Sephadex G-100 (Materials and Methods). The elution profiles (Fig. 1) revealed four major regions of haemoprotein absorption. Band I, eluted in the exclusion volume, contained an unresolved mixture of high mol. wt or particle-bound cytochromes. Band II, mol. wt $\simeq$ 85 000, contained a new CO-reactive cytochrome with α peak (reduced pigment) at 554 nm, and tentatively identified as *Rhizobium* cytochrome *c*-554. Its properties will be described elsewhere. Band III, mol. wt $\simeq$ 45 000 contained cytochrome P450 (refs 1, 13 and 14) and Band IV, molecular wt $\simeq$ 15 000, contained *Rhizobium cytochrome* *c*-550, with smaller amounts of the CO-reactive cytochrome *c*-552 (ref. 1) sometimes present near mol. wt 13 000, and *Rhizobium* haemoglobin, sometimes appearing near mol. wt 30 000. The area under each peak in Fig. 1 is approximately proportional to the amount of oxidized or reduced cytochrome present, but Table II shows quantitative measurements made on the pooled fractions.

Cytochrome *c*-554 does not vary significantly with growth condition. The small

TABLE II

EFFECT OF GROWTH CONDITIONS ON THE SOLUBLE HAEMOPROTEINS OF *R. japonicum*

Cytochromes *c*-554 and *c*-550 were determined from reduced *minus* oxidised spectra (*cf.* ref. 1) and cytochromes *c*-552, P450 and *Rhizobium* haemoglobin from CO difference spectra[1,2] on the pooled fractions of each component as isolated from Sephadex G-100 columns (Fig. 1, also Materials and Methods). All pigment concentrations are expressed as nmoles·g^{-1} of original whole-cell protein. The values in parentheses for newly-described cytochrome *c*-554 are very approximate.

Haemoprotein	*Mol. wt*	*Band*	*Growth condition:*			
			Bacteroid	*Anaerobic–nitrate*	*Air–nitrate*	*Air*
Cytochrome *c*-554	$\simeq$ 85 000	II	(4.6)	(3.4)	(3.6)	(2.0)
Cytochrome P450	$\simeq$ 45 000	III	50	17	3.6	5.8
Rhizobium haemoglobin*	$\simeq$ 30 000	III–IV	0	0	++	++
Cytochrome *c*-550	$\simeq$ 15 000	IV	267	264	77	81
Cytochrome *c*-552	$\simeq$ 13 000	IV	28	23	14	2.1

* It is difficult to make a quantitative estimation of *Rhizobium* haemoglobin. The actual content in air or air–nitrate cells may lie between 2 and 20 nmoles·g^{-1} of whole-cell protein.

amount of cytochrome P450, shown here for the first time (*contra* ref. 2) to be present in air-grown *Rhizobia*, is not increased by air–nitrate growth but is increased toward the bacteroid level by anaerobic–nitrate growth (Table II). Similarly, the absence of *Rhizobium* haemoglobin and a high content of soluble cytochrome *c*-550 seems correlated with low O_2 tension rather than the presence of nitrate (Table II), so again the simple conclusion may be that near-anaerobiosis, not nitrate, is a determinant of bacteroid cytochrome pattern. But again this simple conclusion might not be justified, as our measurement of CO affinity (Materials and Methods) for bacteroid P450, showed $K_i \simeq 0.25$ μM, and K_i for P450 from anaerobic–nitrate cells to be < 0.08 μM CO. Elsewhere[14], we show that these cytochromes P450 have different reactivities and may indeed be distinct chemical species.

Nitrate and nitrite metabolism by R. japonicum

Fig. 2 shows the steady disappearance of nitrate, and transitory appearance of nitrite during anaerobic–nitrate growth of *R. japonicum*, and Table III describes the presence of both nitrate and nitrite reductases in anaerobic–nitrate cells. We

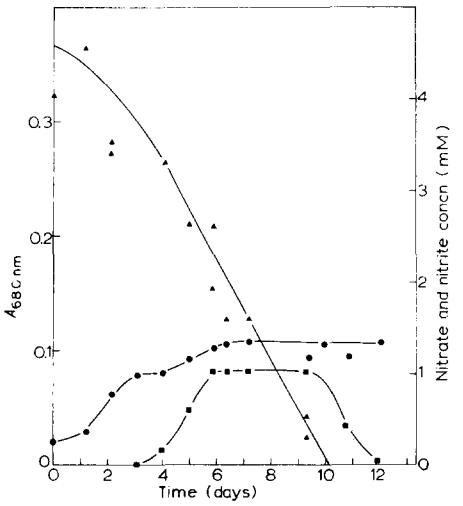

Fig. 2. Nitrate and nitrite levels in the culture medium during anaerobic–nitrate growth of *R. japonicum*. General conditions for growth at 25 °C and nitrate (▲) and nitrite (■) analysis are given in Materials and Methods. Bacterial growth (●) was monitored by measuring absorbance at 680 nm in 10-mm cuvettes in a Unican SP600 spectrophotometer.

TABLE III

NITRATE/NITRITE REDUCTASE ACTIVITY IN WHOLE CELLS OF *R. japonicum* GROWN UNDER DIFFERENT CONDITIONS

Activities are in μmoles·h^{-1}·g^{-1} of whole-cell protein, and were determined as described in Materials and Methods.

Enzyme	*Bacteroid*	*Anaerobic–nitrate*	*Air–nitrate*	*Air*
Nitrate reductase	1490	1240	190	330
Nitrite reductase	0	>290	0	0

therefore assume that dissimilatory nitrate respiration is a feature of anaerobic–nitrate growth of *R. japonicum*. As described in Materials and Methods, no trace of nitrate or nitrite was detected in legume nodules or plant nutrient solution, and Table III shows the absence of nitrite reductase activity from bacteroids. Table III does show that isolated bacteroids exhibit high nitrate reductase activity, but its physiological significance is unknown, as nitrite might be expected to accumulate in the absence of a corresponding nitrite reductase.

Table III also shows that the addition of nitrate to air-grown cultures of *R. japonicum* induces neither nitrate nor nitrite reductase activity and R. M. Daniel (unpublished experiments) has found no disappearance of nitrate, nor any appearance of nitrite in the culture medium during a 12-day air–nitrate growth experiment.

CONCLUSIONS

A significant feature of this work is the demonstration that nitrate can substitute for O_2 as electron acceptor, and so allow anaerobic ($<$ 10 ppm gaseous O_2 or $<$ 13 nM dissolved O_2) growth of *R. japonicum*. Murphy and Elkan[15] previously reported nitrate respiration by several *Rhizobium* strains, but O_2 diffusion through their unshaken cultures (exposed to air) could have allowed significant additional O_2 respiration. This growth of free-living *R. japonicum* at an O_2 tension equal or less than the tension within soybean nodules[4,6] may be a useful tool in the search for those components of *Rhizobium* nitrogenase not subject to host genetic control, nor requiring the whole nodule environment for their appearance.

While assuming that nitrate respiration is responsible for energy conservation during anaerobic growth of free-living *R. japonicum*, it has not been established that the nitrate reductase found in isolated bacteroids (Table III) is capable of providing useful energy. The fact that there is no detectable anaerobic N_2 fixation by soybean nodules (Burris *et al.*[16], Bergersen[17]) and the fact that we could detect no nitrate or nitrite in nodules or plant nutrient solutions makes it unlikely that nitrate respiration or any other anaerobic pathway for ATP production is of major physiological significance in bacteroids.

We do not yet know whether the increased amount of cytochrome P450 and of certain other cytochromes (Tables I and II), observed in both anaerobic–nitrate cells and bacteroids, indicates the appearance of an oxidase pathway able to function efficiently at the extremely low O_2 tension of the bacteroid environment[4,6].

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