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Radiorespirometry evidence for the discrimination between ^{13}C-enriched glucose and unlabelled glucose molecules by *Paracoccus denitrificans*

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Paracoccus denitrificans was grown on either unlabelled glucose, [1-^{13}C]glucose or [6-^{13}C]glucose as the sole carbon source for growth. The cells were then incubated with a range of ^{14}C-glucose substrates to compare the $^{14}CO_2$-evolution rates between cells grown on the glucose and the ^{13}C-labelled glucose. Cells grown on ^{13}C-glucose had significantly faster rates of $^{14}CO_2$-evolution than those grown on unlabelled glucose. The % yields of $^{14}CO_2$, per [1-^{14}C]-, [6-^{14}C]- and [U-^{14}C]glucose supplied were also substantially greater than those measured for cells grown on unlabelled glucose. The data indicated that growth of *Paracoccus* on ^{13}C-enriched glucose substrates resulted in cells with notably different ^{14}C-glucose oxidation metabolism compared to that observed in cells grown on unlabelled glucose.

Radiorespiration; Isotope discrimination; Stable isotope; *Paracoccus denitrificans*

1. INTRODUCTION

Paracoccus denitrificans has been grown on [1-^{13}C]glucose and [6-^{13}C]glucose to investigate the flux of carbon in vivo during glucose oxidation [1-4]. The distribution of ^{13}C-labelling and the %-enrichments confirmed the activities of the Entner-Doudoroff and pentose phosphate pathways during growth on the ^{13}C-labelled substrates. In addition the data also indicated that a third glucose oxidation pathway was active in *Paracoccus* [2]. This pathway decarboxylates the hexose unit in the C-6 position and is named the 'G_6' pathway. The enzymes of the glucuronic acid cycle are thought to be utilized for this decarboxylation sequence. The ^{13}C-enrichments of the metabolites of *Paracoccus* grown on [1-^{13}C]glucose and [6-^{13}C]glucose suggested that this organism can discriminate between these isotopes when they are supplied as the sole carbon and energy sources for growth.

The observed 'isotope discrimination' could be explained by an initial detection of the different isotope distribution by the bacterium, leading to a differential reading out of the bacterial genome and the consequent induction of different enzymes in response to the presence of [6-^{13}C]glucose or [1-^{13}C]glucose. An alternative mechanism might be a change in the activation of enzyme proteins in response to C-1 or C-6 labelled substrates [2]. This induction/activation represents a potentially large amplification above the normal 'chemical' isotope response, which would be expected to be small.

The aim of the work described in this paper was to further investigate the ability of *Paracoccus* to discriminate between the different isotope analogues of glucose. Cells were grown on [1-^{13}C]glucose, [6-^{13}C]glucose or unenriched glucose as the sole carbon source. The cultures were then harvested and replicate samples were suspended in dilute (0.5 mM) solutions of specifically labelled [^{14}C]glucose. The patterns of $^{14}CO_2$ production were determined at 10 min intervals for the next 2 h. The distribution of ^{13}C-label in some key amino acids from the cells grown on ^{13}C-glucose substrates was also determined by gas chromatography-mass spectrometry, in order to confirm previous data [2].

2. MATERIALS AND METHODS

2.1. *Bacterial strain and culture conditions*

Paracoccus denitrificans (N.C.I.B. 8944) was grown in a cooled Gallenkamp orbital shaking incubator (120 rpm) at 28°C. The basic medium was prepared as described previously [1] and contained 500 mg/l of either unlabelled glucose, [1-^{13}C]glucose (92 at.%) or [6-^{13}C]glucose (92 at.%) as described in the text. All cultures (50 ml) were started with inoculum from a single stock culture and were grown in 100 ml Erlenmeyer flasks.

2.2. *Preparation of material for radiorespirometry*

After 15 h of growth, the absorbances (550 nm) of the cultures were measured in a Phillips UV/VIS Spectrophotometer to determine the growth yield. The average growth yield (± 95% confidence limits) of the four cultures grown on unlabelled glucose was 31.4 ± 5.6 mg. The bacterial yields from the cells grown on [1-^{13}C]glucose and

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[6-^{13}C]glucose were 27.0 and 27.1 mg, respectively. These values indicate that the cultures had reached similar stages of growth at harvest. The cells were then harvested by centrifugation at 20,000 × *g* for 15 min at 4°C in an MSE Europa 24 refrigerated centrifuge. The cells were resuspended and homogenised in a calculated volume of 50 mM potassium phosphate buffer (pH 6.8) with 9 mM ND_4Cl at 4°C, to give a final approximate cell concentration equivalent to 2 mg dried cell mass/ml.

2.3. *Radiorespirometry*

The initial incubation mixture (3 ml) contained 50 mM potassium phosphate buffer (pH 6.8) with 9 mM ND_4Cl, 4 mg carbonic anhydrase (EC 4.2.1.1), and 50 μg unlabelled glucose. The reaction mixtures were supplied with 0.2 μCi of either [1-^{14}C]glucose, [2-^{14}C]glucose, [3,4-^{14}C]glucose, [6-^{14}C]glucose or [U-^{14}C]glucose in 50 ml Erlenmeyer flasks. The experiments were initiated by adding 1 ml of the cell suspension and incubating at 28°C for 2 h in a shaking waterbath (100 rpm). Carbonic anhydrase was added to facilitate release of CO_2 from the solution at pH 6.8. The flasks were sealed by rubber stoppers which each supported a 200 μl glass well extending into the flask. The glass wells contained 200 μl of fresh 10% (w/v) KOH, and the stopper plus glass well assembly could be rapidly and easily exchanged for a new assembly containing fresh KOH. This sampling of KOH was performed at 10 min intervals for each incubation over the 2 h period.

Alkali was quantitatively removed from the glass wells and made up to 2 ml with water in a plastic vial. Then 2 ml 'optiphase safe' scintillation cocktail (LKB Instruments Ltd., Croydon, U.K.) were added and the sample stored in the dark for 48 h before being counted by liquid scintillation spectrometry (Pack and Tri-Carb 2000 Liquid Scintillation Analyser). Control experiments indicated that the non-biological release of ^{14}C was negligible.

2.4. *Preparation of cell material for gc-ms*

Cells were harvested, hydrolysed and fractionated as described previously [2], and trimethylsilyl (TMS) derivatives of extracts were prepared as described by Dunstan et al. [1].

2.5. *Gc-ms*

The derivatised samples were separated and analysed in a Finnigan 1020 automated gc-ms system (incorporating a Data General Nova 3 computer) and the run conditions were as described previously [1]. The ^{13}C-enrichments were calculated as described by Dunstan [5].

2.6. *Chemicals*

[1-^{13}C]glucose (92 at.%) was synthesised as described by Isbell et al. [6] except that the gluconolactone was reduced to glucose using

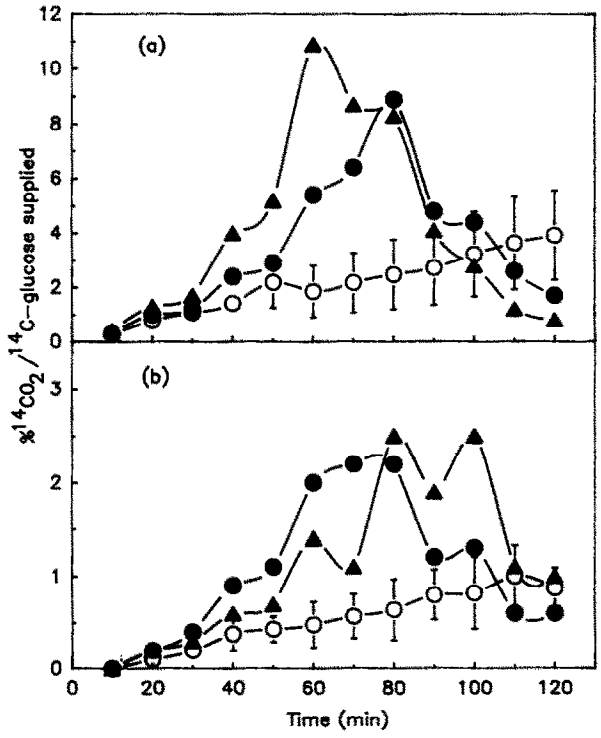

Fig. 1. The $^{14}CO_2$ evolution from (a) [1-^{14}C]glucose and (b) [6-^{14}C]glucose, by cells grown on unlabelled glucose (○), [1-^{13}C]glucose (●) and [6-^{13}C]glucose (▲). The cells were washed and incubated with either [1-^{14}C]glucose or [6-^{14}C]glucose, and the $^{14}CO_2$ evolved was collected at 10 min intervals as described in the text. The $^{14}CO_2$ produced during each 10 min interval was calculated as the % yield of the [^{14}C]glucose supplied. The $^{14}CO_2$ yields from cells grown on unlabelled glucose are the mean values ± 95% confidence limits from 6 separate incubations.

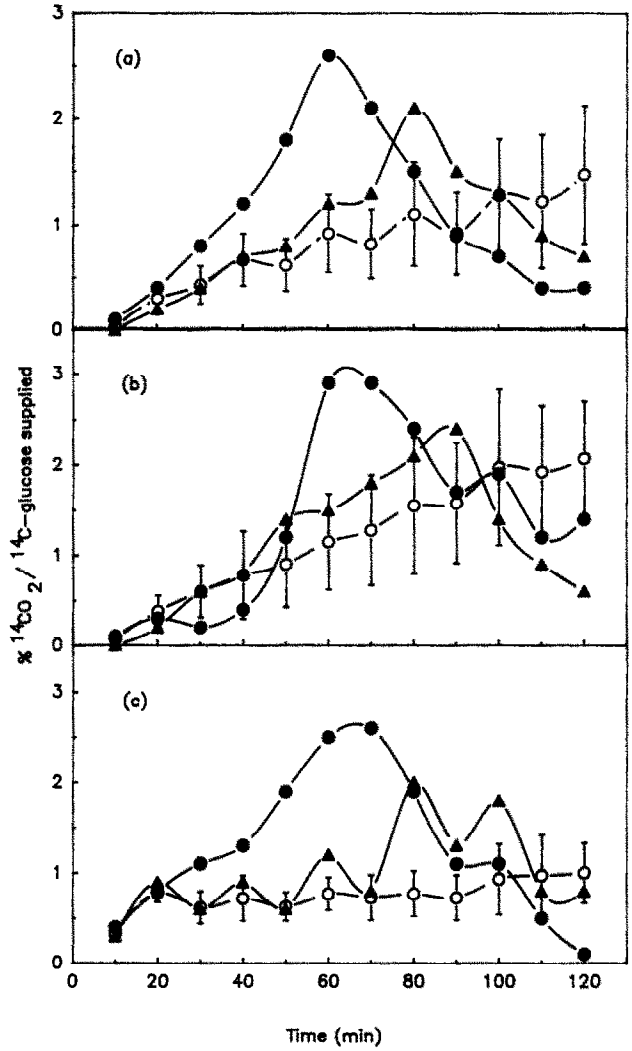

Fig. 2. The $^{14}CO_2$ evolution from (a) [2-^{14}C]glucose, (b) [3,4-^{14}C]glucose and (c) [U-^{14}C]glucose, by cells grown on unlabelled glucose (○), [1-^{13}C]glucose (●) and [6-^{13}C]glucose (▲). The cells were washed and incubated with the respective ^{14}C-glucose substrates, and the $^{14}CO_2$ evolved was collected at 10 min intervals as described in the text. The $^{14}CO_2$ produced during each 10 min interval were calculated as % yields of the [^{14}C]glucose supplied. The $^{14}CO_2$ % yields from cells grown on unlabelled glucose are the mean values ± 95% confidence limits from 6 separate incubations.

Table I

The % $^{14}CO_2$ produced per [^{14}C]glucose supplied, by *Paracoccus* after 120 min incubation with a range of [^{14}C]glucose substrates

	% $^{14}CO_2$ produced / ^{14}C-glucose supplied			
	Cells grown on unlabelled glucose		Cells grown on ^{13}C-glucose	
Substrate	Mean	95% Confidence range	[1-^{13}C]glucose	[6-^{13}C]glucose
[1-^{14}C]glucose	25.2	13.8–36.6	49.4	42.0
[2-^{14}C]glucose	9.8	5.8–13.8	12.9	11.1
[3,4-^{14}C]glucose	14.3	8.3–20.3	16.6	13.7
[6-^{14}C]glucose	6.3	4.3–8.3	12.7	13.3
[U-^{14}C]glucose	9.0	6.8–11.2	15.3	12.0

diborane [7]. [6-^{13}C]glucose (92 at.%) was synthesised as described by Schaffer and Isbell [8] and purified by chromatography on Dowex 50wx8 (200–400 mesh, Ba^{2+}) prior to crystallisation [9]. The ^{14}C-glucose substrates were obtained from Amersham, UK. Other chemicals were obtained from either Sigma Chemical Company or from British Drug Houses Ltd.

3. RESULTS

3.1. *Radiorespirometry*

The $^{14}CO_2$ yields from metabolism of ^{14}C-glucose by cells grown on unlabelled glucose were measured during 6 separate incubation experiments as described in Materials and Methods. The levels of $^{14}CO_2$ produced during each 10 min interval were calculated as percentage yields (i.e. $^{14}CO_2$ produced per [^{14}C]glucose supplied). These data were then used to calculate the average percentage yields (± 95% confidence limits) of $^{14}CO_2$ produced at each sequential 10 min interval. The $^{14}CO_2$ evolutions from the ^{14}C-labelled glucose substrates by cells grown on [1-^{13}C]glucose and [6-^{13}C]glucose were each determined simultaneously with one of the cultures grown on unlabelled glucose as a control.

During the first 80 min of the incubations, the rates of increase in $^{14}CO_2$ evolution from [1-^{14}C]glucose by the cells grown on ^{13}C-labelled substrates were substantially greater than those measured for cells grown on unlabelled glucose (Fig. 1a). After this time, the yields of $^{14}CO_2$ from the cells grown on ^{13}C-labelled glucose decreased rapidly. The yields of $^{14}CO_2$ from the cells grown on unlabelled glucose continued to increase at a slower rate throughout the incubation.

The rates of increase in $^{14}CO_2$ evolution from [6-^{14}C]glucose by cells grown on ^{13}C-labelled glucose were also greater than those measured from cells grown on unlabelled glucose during the first 90–100 min (Fig. 1b). The yields decreased after this time, whilst the $^{14}CO_2$ yields from the cells grown on unlabelled glucose continued to increase until after 110 min.

Similar trends in $^{14}CO_2$ evolution by cells grown on [^{13}C]glucose were also observed during incubations with [2-^{14}C]-, [3,4-^{14}C]- and [U-^{14}C]glucose (Fig. 2). The increases in rates of $^{14}CO_2$ evolution were most pronounced in the cells grown on [1-^{13}C]glucose.

The yields of $^{14}CO_2$ produced after 120 min incubation, per ^{14}C-glucose supplied (calculated as % yields), were determined for each ^{14}C-glucose substrate (Table I). The % yields from [1-^{14}C]-, [6-^{14}C]- and [U-^{14}C]glucose produced by cells grown on ^{13}C-glucose were substantially greater than the upper 95% confidence levels calculated from the cells grown on unlabelled glucose. The % yields produced from [2-^{14}C]- and [3,4-^{14}C]glucose were however, within the 95% confidence limits.

Table II

The ^{13}C-enrichments of the amino acids observed in the hydrolysate fraction of cells grown on ^{13}C-labelled glucose substrates

Glucose substrate	Compound	Ion fragment	Relative frequencies (composition %)		
			m+1	m+2	m+3
[1-^{13}C]glucose	Alanine	$[M-15]^+$	34.0 ± 1.0		
	Valine	$[M-15]^+$	30.3 ± 2.5		
	Leucine	$[M-15]^+$	1.8 ± 0.5		
	Isoleucine	$[M-15]^+$	3.3 ± 1.1		
[6-^{13}C]glucose	Alanine	$[M-117]^+$	24.6 ± 2.1		
	Valine	$[M-117]^+$	37.5 ± 0.2	8.3 ± 0.3	
	Leucine	$[M-117]^+$	38.7 ± 0.8	16.2 ± 0.8	2.8 ± 0
	Isoleucine	$[M-117]^+$	38.3 ± 0.1	15.7 ± 0.1	2.2 ± 0

The relative frequencies (composition %) of the amino acid fragments (indicated) that contained 1 label (m+1), 2 labels (m+2), and 3 labels (m+3), were calculated from the mass spectra of the amino acids derived from cells grown on the ^{13}C-labelled substrates; 4 scans were analysed for each fragment.

Table III

The relative fluxes of glucose carbon through the Entner-Doudoroff, pentose phosphate and G_6 pathways during growth on [1-^{13}C]glucose and [6-^{13}C]glucose

Pathway	Growth on [1-^{13}C]glucose	Growth on [6-^{13}C]glucose
Entner-Doudoroff	66 % } 95 %	} 48 %
Pentose phosphate	29 % }	
G_6	5 %	52 %

The relative fluxes were calculated from the ^{13}C-enrichments of alanine, valine, leucine and isoleucine as described by Dunstan et al. [2].

3.2. *^{13}C-labelling*

Samples of the cells (equivalent to 5 mg bacterial dry wt) grown on unlabelled and ^{13}C-labelled glucose (i.e. those cells used for the radiorespirometry experiments) were prepared for analysis by GC-MS as described in Materials and Methods. The ^{13}C-enrichments measured in the hydrolysis products alanine, valine, leucine and isoleucine are shown in Table II. The balances of fluxes of glucose carbon through the Entner-Doudoroff, pentose phosphate and G_6 pathways were calculated for *Paracoccus* during growth on [1-^{13}C]glucose and during growth on [6-^{13}C]glucose, using the equations described by Dunstan et al. [2]. The balance of fluxes for cells grown on [1-^{13}C]glucose was different from that calculated for cells grown on [6-^{13}C]glucose: half of the [6-^{13}C]glucose supplied was metabolised via the G_6 pathway, whereas the same pathway metabolised only 1/20 of the [1-^{13}C]glucose (see Table III). These results were very similar to those obtained in previous growth experiments using ^{13}C-labelled glucose substrates as the sole carbon sources for growth [2].

4. DISCUSSION

Growth of *Paracoccus* on ^{13}C-enriched glucose substrates resulted in cells with notably different ^{14}C-glucose oxidation metabolism compared to that in cells grown on unlabelled glucose. This suggests that *Paracoccus* adapted its metabolism during growth on [1-^{13}C]glucose and [6-^{13}C]glucose and supports the thesis that *Paracoccus* can discriminate between ^{13}C-labelled glucose and unlabelled glucose molecules when these are supplied as the sole carbon and energy sources for growth [2]. The results are also consistent with the hypothesis that the discrimination involves an initial detection of the different isotope distributions leading to a differential reading out of the bacterial genome, and the consequent induction of different enzymes in response to the ^{13}C-glucose substrates [2].

The observed responses by cells growing on [1-^{13}C]glucose were notably different to those by cells growing on [6-^{13}C]glucose. These responses by cells to growth on the ^{13}C-labelled substrates might involve a differential increase in synthesis of active glucose decarboxylation enzymes in relation to the Entner-Doudoroff enzyme sequence. This response would be consistent with the ^{13}C-enrichments measured in these cells after growth and with those measurements from previous experiments [2], which indicate that the balance of fluxes of [6-^{13}C]glucose through the glucose oxidation pathways is different from that during growth on [1-^{13}C]glucose and unlabelled glucose.

It was concluded that *Paracoccus* can discriminate between [6-^{13}C]glucose, [1-^{13}C]glucose and unlabelled glucose when they are supplied as the sole carbon and energy sources for growth. Consequently, the metabolism of [1-^{13}C]glucose is notably different to the metabolism of [6-^{13}C]glucose and of unlabelled glucose.

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