The metabolism of [2-13C]acetate by Pseudomonas M27 (Icl−) and Pseudomonas MA (Icl+) was studied in vivo using 13C-NMR spectroscopy. The flux of 13C-label into bicarbonate, glutamate and citrate was observed in both organisms. In addition 13C-labelled α,α-trehalose was synthesized as a major metabolite by Pseudomonas M27 but not by Pseudomonas MA. The presence of this disaccharide in cell extracts of Pseudomonas AM1 (Icl−) grown with [13C]methanol was also observed. The data from analysis of the trehalose multiplet signal observed in the spectra of Pseudomonas M27 cell extracts were consistent with the absence of the glyoxylate cycle in this methylotroph.

INTRODUCTION

Recent advances in 13C high resolution Fourier transform NMR spectroscopy have made it possible to study metabolism and its regulation in intact cells and isolated perfused organs (Shulman et al., 1979; London, 1988). Using this technique, studies on the metabolism of glucose, formaldehyde and methanol in bacteria (Ugurbil et al., 1978; Hunter et al., 1984; Cornish et al., 1984) and of glucose and acetate in yeast (den Hollander et al., 1979, 1986; Dickinson et al., 1983) have been described. It provides an extremely useful method for calculating the fluxes of metabolites via different pathways as well as measuring the kinetics of individual reactions. den Hollander et al. (1986) studied aerobic and anaerobic glycolysis in the yeast Saccharomyces cerevisiae and the flow of glucose via different pathways was estimated. Barton et al. (1982) examined the mobilization of trehalose in yeast spores and Walker et al. (1982) and Walker & London (1987) were able to determine the pathways involved in the biosynthesis of glutamate by a Microbacterium sp. and by Brevibacterium flavum.

In the present study we describe the utilization of [2-13C]acetate by intact cells of the methylotrophs Pseudomonas M27 (Icl−) and Pseudomonas MA (Icl+). We show significant synthesis of trehalose in both Pseudomonas M27 grown on acetate and Pseudomonas AM1 grown on methanol. These facultative methylotrophs use the serine pathway for assimilation of C1 compounds. An important step of this pathway is the regeneration of glyoxylate from acetyl-CoA. In Pseudomonas MA this conversion is achieved via a series of tricarboxylic acid cycle enzymes and isocitrate lyase. The latter enzyme is also essential for growth of this bacterium on substrates which require the operation of the glyoxylate cycle. In Pseudomonas M27 and AM1 this enzyme cannot be detected and the route for the regeneration of glyoxylate from acetyl-CoA is not clearly understood. By examining the distribution of 13C label in trehalose, we have been able to determine the flux of the carbon via gluconeogenesis and to establish whether or not the glyoxylate cycle is present.

Abbreviations: RF, radio frequency; TSS, sodium trimethylsilylpropanesulphonate.
METHODS

Organisms and growth. Bacterial strains used in this study were the methylotrophs Pseudomonas AM1 (NCIMB 9133), Pseudomonas M27 (NCIMB 9686) and Pseudomonas MA (NCIMB 11590). They were obtained from the National Collection of Industrial and Marine Bacteria, Torry Research Station, Aberdeen, UK.

Liquid media were prepared by addition of a single carbon and energy source to a basal inorganic salts solution containing (g l\textsuperscript{-1}): KH\textsubscript{2}PO\textsubscript{4}, 5; (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 1; MgSO\textsubscript{4}.7H\textsubscript{2}O, 0.05; FeSO\textsubscript{4}.7H\textsubscript{2}O, 0.005. The pH of all the media was adjusted to 7.0 with 5 m\textsubscript{NaOH}. The concentrations of acetate and methanol were 20 mM and 125 mM, respectively. Methanol media were supplemented by the addition of a trace element solution (0.2 ml l\textsuperscript{-1}). This contained (% w/v): EDTA, 5; ZnSO\textsubscript{4}.7H\textsubscript{2}O, 2.2; CaCl\textsubscript{2}.2H\textsubscript{2}O, 0.55; MnCl\textsubscript{2}.5H\textsubscript{2}O, 0.50; (NH\textsubscript{4})\textsubscript{6}Mo\textsubscript{7}O\textsubscript{24}.4H\textsubscript{2}O, 0.11; CuSO\textsubscript{4}.5H\textsubscript{2}O, 0.16; CoCl\textsubscript{2}.6H\textsubscript{2}O, 0.16.

Cultures were incubated at 30 °C in an orbital shaker (150 r.p.m.). Growth was measured as the optical density at 540 nm using a Cecil CE 272 spectrophotometer.

Preparation of perchloric acid extracts. Starter cultures of Pseudomonas M27 and Pseudomonas MA were grown in acetate minimal media. Cells were harvested at mid-exponential phase by centrifugation (7500 g, 15 min, 4 °C), and washed once in 15 mM-potassium phosphate buffer (pH 7). Sufficient quantity of the washed cells was added to 250 ml minimal salts medium to give 0.7 mg (dry wt equivalent) cells ml\textsuperscript{-1}. After temperature equilibration at 30 °C, [2-\textsuperscript{13}C]acetate was added at 20 mM final concentration, and the cultures were reincubated. At intervals samples (50 mg dry wt equivalent) of culture were harvested (3000 g, 10 min, 4 °C) and the cell pellet was immediately fixed in 10% (w/v) perchloric acid for 1 h at 0 °C. After further centrifugation, the supernatant was removed and neutralized with 10 M-KOH (stored on ice). The precipitate of potassium perchlorate formed was then removed by centrifugation; the supernatant was freeze-dried and reconstituted in 0.5 ml 15 mM-potassium phosphate buffer containing 20% (v/v) D\textsubscript{2}O.

NMR spectroscopy. [13C]-NMR spectra were obtained at 90.55 MHz on a Bruker WM360 spectrometer, performing in the Fourier transform mode. Sodium trimethylsilylpropanesulphonate (TSS) was used as an external standard. Chemical shifts of all other signals were referred to those of TSS (0 p.p.m.). All test samples contained 20% (v/v) D\textsubscript{2}O to provide the field frequency lock signal.

Assignment of the chemical shifts was based on the comparison with the spectra of standard compounds obtained under similar experimental conditions. Assignments for trehalose and glutamate were further confirmed by spiking the sample with known standards.

In vitro [13C]-NMR spectra were obtained using 0.5 ml samples of perchloric acid extracts in 5 mm NMR tubes. Broad-band [1H]-decoupled spectra were produced at 30 °C by acquisition of around 1500 transients with 32 K data points over 22700 Hz spectral width using 42° pulse angles with a relaxation delay time of 3 s.

For in vivo [13C]-NMR spectroscopy, cells of Pseudomonas M27 and Pseudomonas MA were cultured separately in 500 ml volumes in 1 litre flasks at 30 °C (300 r.p.m.) and harvested aseptically by centrifugation (7000 g, 10 min, 4 °C). The cells were washed once in cold, sterile 15 mM-phosphate buffer and resuspended in basal salts medium lacking a carbon source to give a cell concentration of 6-7 mg dry wt ml\textsuperscript{-1}. Samples (3-6 ml) were transferred to a 10 mm NMR tube fitted with a drilled Teflon insert carrying three Portex tubes to allow aeration and sample injection. Pure O\textsubscript{2} was bubbled continuously at the rate of 20 ml min\textsuperscript{-1} via the lower tube placed below the level of the radiofrequency (RF) coil and at 130 ml min\textsuperscript{-1} through the upper bubbler placed above the RF coil (to reduce its effect on the lock signal and the peak resolution) and also above the suspension.

The experiment was started by injection of 0.4 ml 1.5 M-[2-13C]sodium acetate. Broad-band [1H]-decoupled spectra were acquired in non-spinning 10 mm tubes using 16 K data points over a spectral width of 22700 Hz with a pulse angle of 37° and a delay time of 0.8 s. High-level irradiation (5 W) was used to ensure decoupling during the acquisition period (0-36 s), and a lower power level (0.1 W) was used during the delay periods to maintain nuclear Overhauser enhancement while minimizing dielectric heating. The temperature remained at 30–35 °C during the experiment. Spectra were accumulated into blocks of 2000 and transformed after application of a 5 Hz line-broadening. At the end of each run the cell density and the pH of the culture were measured. The latter was found to have shifted-up by less than 0.4 units.

Chemicals. [2-13C]sodium acetate and [13C]methanol (both 90 atom%) were obtained from Amersham; all other chemicals obtained were of the purest grade commercially available.

RESULTS

Fig. 1 shows a sequence of time-dependent in vivo [13C]-NMR spectra obtained as a result of [2-13C]acetate metabolism by Pseudomonas M27. The decrease in acetate C2 intensity was coupled to a corresponding increase in peak intensities of a number of metabolites, namely trehalose, glutamate and citrate. The evolution of CO\textsubscript{2} as a result of oxidative respiration is indicated by the continuous increase in intensity of the bicarbonate resonance (Fig. 2).
Fig. 1. Time-dependent 90.5 MHz $^{13}$C-NMR spectra of Pseudomonas M27 growing on sodium [2-$^{13}$C]acetate (150 mM) at 30 °C. The time indicated for each spectrum represents the middle of the accumulation period (39 min). At the end of the experiment the cell concentration in the NMR tube had increased from $5.2 \times 10^{10}$ to $7.1 \times 10^{10}$ cells ml$^{-1}$. Abbreviations: Act, acetate; Tre, trehalose; Glu, glutamate; Cit, citrate; Me, methyl.
Apart from CO₂ the earliest signal to appear was at 22.2 p.p.m. This shift is typical of a methyl group in a small metabolite such as ethanol. However, comparison of chemical shift with authentic samples run under identical conditions did not show good agreement for ethanol, or for lactate, alanine, β-methylaspartate, aspartate or mesaconate. As this signal was absent from the perchlorate extracts, further attempts to identify its origin were not possible.

The glutamate pool became labelled after 40 min, label appearing in the C2, C3 and C4 atoms. Though not well-resolved, the resonance of the above peak intensities showed 13C–13C coupling. The carboxyl carbons of glutamate would not be expected to be observed due to the short relaxation interval used. The only carboxyl group resonance present was that of the C1 atom of the added acetate and was in a doublet form.

After 2 h incubation peaks appeared with increasing intensities corresponding to the carbon atoms of trehalose. The resonances of C1 and C6 indicated 13C–13C coupling. The results of in vivo [2-13C]acetate metabolism in Pseudomonas MA are shown in Figs 3 and 4. The initial rise in acetate C2 resonance was probably due to inadequate mixing of the injected substrate at the start of the experiment. The subsequent reduction of the C2 acetate peak, the evolution of CO₂ and the synthesis of glutamate and citrate were again observed. However, the major difference compared with Pseudomonas M27 was the conspicuous absence of the trehalose peaks in the region 63–100 p.p.m. The intensity of the unknown possible methyl group was reduced and did not appear until after 3 h incubation. Higher intensities of the glutamate peaks suggest a larger pool size of this metabolite in Pseudomonas MA.

The observed increases in the cell densities (Figs 1 and 3) of both methylotrophs indicate that during the course of the in vivo experiments the bacterial cells were actively growing.

To improve the resolution of the 13C–13C coupled signals of the labelled intermediates, 13C-NMR spectra of perchlorate extracts of Pseudomonas M27 and Pseudomonas MA grown on acetate and Pseudomonas AM1 grown on methanol were obtained (Fig. 5). The trehalose C3 resonances, hidden by the broadened trehalose C5 peaks in the in vivo spectra, were now clearly separated. Trehalose was observed in both of the Icl⁻ facultative methylotrophs, but not in the Icl⁺ Pseudomonas MA. Since no trehalose signals were observed in the lyophilized samples of culture supernatants of M27 (data not shown) trehalose was present only intracellularly.
Fig. 3. Time-dependent 90.5 MHz $^{13}$C-NMR spectra of *Pseudomonas* MA growing on sodium [2-$^{13}$C]acetate (150 mM). During the experiment the cell density in the NMR tube increased from $4.9 \times 10^{10}$ to $7.2 \times 10^{10}$ cells ml$^{-1}$. For abbreviations refer to Fig. 1.
Fig. 4. Time-course of the labelling pattern of various metabolites in *Pseudomonas* MA observed by *in vivo* $^{13}$C-NMR. The data were obtained from results shown in Fig. 3. The intensities shown are Act-C2 (▼), bicarbonate (○) and the unidentified Me group (□).

Table 1. *Comparison of the relative $^{13}$C-enrichment of the carbon atoms of trehalose observed in perchloric acid extracts of Pseudomonas M27*

$^{13}$C-Enrichment values are from the integrated intensities of the multiplet signals and have been corrected for the differences in the relaxation times of the individual carbon atoms. Chemical shift values indicated are those for the centre lines of the multiplets.

<table>
<thead>
<tr>
<th>Carbon atom of trehalose</th>
<th>Chemical shift (p.p.m.)</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>95.82</td>
<td>35.80</td>
</tr>
<tr>
<td>C2</td>
<td>73.72</td>
<td>33.86</td>
</tr>
<tr>
<td>C3</td>
<td>75.23</td>
<td>24.15</td>
</tr>
<tr>
<td>C4</td>
<td>72.37</td>
<td>24.43</td>
</tr>
<tr>
<td>C5</td>
<td>74.78</td>
<td>37.58</td>
</tr>
<tr>
<td>C6</td>
<td>63.21</td>
<td>37.00</td>
</tr>
</tbody>
</table>

Growth of bacteria on acetate would necessitate the anaplerotic replenishment of the TCA cycle (Kornberg, 1966). In most bacteria this is achieved via the glyoxylate cycle, but in Icl$^-$ methylotrophs the nature of the anaplerotic sequence is still unclear (Anthony, 1982).

$^{13}$C-NMR allows the determination of the presence or absence of the glyoxylate cycle by analysis of the labelling pattern of oxaloacetate, either directly or indirectly. The incorporation of [2-$^{13}$C]acetate via the TCA cycle will first label equally the C2 and C3 positions of the oxaloacetate; the next turn of the cycle will introduce label into the C1 and C4 positions. Incorporation of the label via the glyoxylate cycle would label positions C2 and C3 only. The details of such analysis of enrichment patterns due to label scrambling via these two routes have previously been explained in detail (Dickinson *et al.*, 1983; Walsh & Koshland, 1984; London, 1988).

The labelling pattern of the synthesized trehalose, presumably via gluconeogenesis, in acetate-grown *Pseudomonas* M27 indirectly reflects the labelling of oxaloacetate, where C1, C2, C3 and C6, C5, C4 of trehalose originate from C3, C2 and C1 of oxaloacetate, respectively. Therefore trehalose derived from oxaloacetate via glyoxylate cycle activity only would not be labelled at C3 and C4. Operation of the tricarboxylic acid cycle would label the C3 and C4 positions of trehalose at 50% intensity of the other sites.
The results in Table 1 indicate the relative intensities of different carbon atoms of trehalose in a spectrum of *Pseudomonas* M27 cell extract prepared after 4 h incubation. Allowing for the differences in the relaxation times of the individual carbon atoms of trehalose, the results show that the intensities of the $^{13}$C label in positions C1, C2, C5 and C6 were almost equal. The intensities of C3 and C4 of trehalose were 68 ± 4% and 66 ± 4%, respectively of the levels of the other four carbon atoms. These values rule out the operation of the glyoxylate cycle. This is not surprising since *Pseudomonas* M27 is known to lack the key enzyme isocitrate lyase (Dunstan *et al.*, 1972). The values of greater than 50% suggest that an alternative anaplerotic pathway exists.
which preferentially labels C1 of oxaloacetate. Consideration of an alternative proposed pathway (Kortstee, 1980) does not explain this discrepancy.

**DISCUSSION**

Using high resolution $^{13}$C-NMR we were able to observe *in vivo* the metabolism of [2-$^{13}$C]acetate in *Pseudomonas* M27 (Icl') and *Pseudomonas* MA (Icl'). We also examined *in vitro* the metabolism of [2-$^{13}$C]acetate by the above organisms and the metabolism of [${^{13}$C}]methanol by the Icl$^{-}$ *Pseudomonas* AM1. It was possible to observe the synthesis of a number of metabolic intermediates and the evolution of CO$_2$. Significant levels of trehalose were synthesized in the two Icl$^{-}$ methylotrophs, but not in *Pseudomonas* MA. $^{13}$C-NMR spectra of perchlorate cell-extracts confirmed the trehalose peak assignments and also showed the detailed labelling pattern in glutamate.

In $^{13}$C-NMR studies trehalose has been detected during glucose metabolism in *Propionibacter shermanii* (Burton *et al.*, 1980) and *Microbacterium ammoniaphilum* (Walker *et al.*, 1982). The only methylotroph where trehalose synthesis has been implicated (during a $^{14}$C tracer study) is *Pseudomonas* AM1 growing on methanol or succinate (Salem *et al.*, 1972). Trehalose is an important storage compound in the vegetative cells and spores of fungi (Elbein, 1974; Panek, 1963). It is present in large quantities in spores of the yeast *Saccharomyces cerevisiae* where it serves as an endogenous energy supply for germination (Thevelein, 1984). Trehalose has an osmoregulatory role in *Escherichia coli* cells grown under osmotic stress (Larsen *et al.*, 1987). Its presence was also observed in osmotically stressed cells of cyanobacteria (Mackay *et al.*, 1984; Reed *et al.*, 1986). Whether the accumulated trehalose in the methylotrophs studied has an osmoregulatory role cannot be confirmed since the cells were not intentionally under osmotic stress. It is possible that trehalose may act as a storage carbohydrate, although poly-3-hydroxybutyrate is the major storage polymer in these pink facultative methylotrophs. Using the C2 peak of acetate as the internal standard the final concentration of trehalose in the NMR tube was calculated to be 1 mM, implying that about 10% of the acetate utilized was converted to trehalose in *Pseudomonas* M27. The accuracy of such quantification is limited due to differences in the relaxation times of the C2 atoms of acetate and trehalose.

The absence of isocitrate lyase in Icl$^{-}$ methylotrophs is well-documented (Anthony, 1982; Hou, 1984). However, the possibility that the lack of detection was due to some peculiar property of the enzyme, making it undetectable by standard assay methods, rather than its complete absence could not be ruled out. The multiplet analysis of the $^{13}$C-labelling pattern in the trehalose indicates that there is no evidence for the glyoxylate cycle in *Pseudomonas* M27.

The unidentified signal at 22.2 p.p.m. is clearly of interest, particularly since it is present in *Pseudomonas* M27 to a much greater extent than in *Pseudomonas* MA. Further work will be necessary to try to establish the origin of this signal and to investigate its significance for metabolism. The absence of this signal from the perchlorate cell-extracts suggests that this labelled metabolite may be either acid labile or volatile.

As expected, C5 of glutamate was not enriched in [2-$^{13}$C]acetate-grown *Pseudomonas* M27 or *Pseudomonas* MA since this carbon atom is derived from the unlabelled C1 of acetate. This suggests that in these organisms a possible scrambling of the label from C2 to C1 of acetyl-CoA (Walker *et al.*, 1982) does not exist. In *Pseudomonas* AM1 grown on methanol, however, significant label was incorporated into the C5 position of glutamate, since all the carbon atoms of this amino acid were synthesized from the single carbon of [${^{13}$C}]methanol.

In the spectra of extracts of *Pseudomonas* AM1 grown on methanol no intermediates specific for the serine pathway were detected. This reflects their small pool size. Similar lack of detection was implicated by Cornish *et al.* (1984) during *in vivo* metabolism of [${^{13}$C}]methanol in the methylotroph *Methylosinus trichosporium* OB3b. Such insensitivity to low concentration of metabolites is a major disadvantage of $^{13}$C-NMR since the serine pathway intermediates were detected in $^{14}$C-tracer studies (Large *et al.*, 1961; Salem *et al.*, 1972).

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