Metabolism of pyruvate and glucose by intact cells of *Helicobacter pylori* studied by $^{13}$C NMR spectroscopy

Peter A. Chalk, Andrew D. Roberts and W. Michael Blows

Author for correspondence: Peter A. Chalk. Tel: +44 81 966 2497. Fax: +44 81 423 5579. e-mail: PAC0367@GGR.CO.UK

The metabolic routes of substrate catabolism by intact cells of *H. pylori* have been investigated by $^{13}$C NMR. Real time analyses of metabolic transformations under anaerobic conditions have been obtained with dense cell suspensions incubated with $^{13}$C-labelled pyruvate and glucose. In addition, time point studies have been carried out with cells incubated under aerobic conditions. Anaerobically, pyruvate was rapidly metabolized to lactate, ethanol and acetate. In addition, alanine was produced in significant quantities by cells provided with a nitrogen source and the metabolic incorporation of nitrogen from urea was demonstrated. Under aerobic conditions acetate was the major oxidation product from pyruvate; no evidence was obtained for tricarboxylic acid cycle activity. Glucose was metabolized more slowly than pyruvate. Anaerobically, two major products were observed and identified as sorbitol and gluconate by gas chromatography/mass spectrometry. Evidence was obtained for the oxidation of glucose to acetate under aerobic conditions. The fate of the $^{13}$C label with glucose substrates labelled in different positions showed that this oxidation takes place via the Entner-Doudoroff pathway.

**Keywords:** *Helicobacter pylori*, NMR, glucose metabolism, pyruvate metabolism, urease

**INTRODUCTION**

*Helicobacter pylori* is a Gram-negative, microaerophilic, flagellate, curved or spiral bacterium that colonizes the mucous layer of the human gastric epithelium. There is now a wealth of evidence linking *H. pylori* infection with gastritis and peptic ulcer disease (Goodwin & Worsley, 1993; Rathbone & Heatley, 1989; Rauws & Tytgat, 1989). In addition, recent evidence suggests that past infection with *H. pylori* is strongly associated with increased risk of gastric carcinoma and may be a causative factor in the pathogenesis of this condition (Parsonnet *et al.*, 1991; Nomura *et al.*, 1991).

*H. pylori* grows slowly on culture in *vitro*, and is generally grown on rich undefined media containing blood products in a controlled gas atmosphere (Goodwin & Armstrong, 1990; Shahamat *et al.*, 1991). Despite the importance of this pathogen its preferred growth substrates either in *vitro* or in *vivo* are largely unknown and its key metabolic processes poorly understood. A greater knowledge of *H. pylori* metabolism might aid the design of novel drugs for the treatment of infections and promote an understanding of how the bacterium is adapted to survive in a specialized ecological niche. It has been proposed that *H. pylori* obtains its carbon and energy requirements from nutrients in the blood, rather than from the stomach lumen (Mendz & Hazell, 1993). An interesting characteristic is the production of copious amounts of urease by the bacterium and this enzyme has been studied extensively (Ferrero & Labigne, 1993). In addition, other enzyme activities have been reported such as cytochrome oxidase, catalase, $\gamma$-glutamyl transpeptidase, alkaline phosphatase and DNAase (Megraud, 1989). Glucose metabolism by *H. pylori* has been a matter of some debate. It has been reported that sugars are not major substrates for the organism (McNulty & Dent, 1987). However, glucose metabolism has since been detected and the enzymes of the pentose phosphate pathway demonstrated (Mendz & Hazell, 1991). Recently, evidence has been presented for a specific glucose kinase (Mendz & Hazell, 1993), and for the metabolism of glucose to lactate (Mendz *et al.*, 1993).

$^{13}$C Nuclear magnetic resonance (NMR) is a powerful technique for metabolic studies since it allows the simultaneous analysis of label distribution by divergent metabolic routes (Jeffrey *et al.*, 1991; Lundberg *et al.*, 1991).
1990). Studies of biosynthetic pathways can be carried out by feeding cultures on 13C labelled substrates and determining the labelling patterns of extracted cell constituents (Eikel et al., 1983; Houwen et al., 1991). However, this technique requires that growth is obtained utilizing only specific carbon sources. This restriction does not apply to catabolic processes, however, which can be studied by following the metabolism of 13C-labelled substrates by suspensions of intact cells. The distribution of 13C label among different end-products allows one to study flux position in metabolism being at the branch point of metabolic pathways involved.

We have used 13C NMR to study the catabolic capabilities of H. pylori. Pyruvate metabolism is a good starting point for such investigations since pyruvate occupies a key position in metabolism being at the branch point of several potential metabolic routes. These vary between different organisms and may be affected by growth conditions and particularly oxygen availability (Schlegel, 1986). Therefore, we investigated the utilization of pyruvate by H. pylori under different conditions of oxygenation. We have also analysed glucose catabolism, utilizing glucose substrates with 13C label at different carbon atoms to discriminate between the potential metabolic pathways involved.

**METHODS**

**Bacterial strains, media and growth conditions.** H. pylori type strain NCTC 11637 and a clinical isolate, strain 8091 (isolated in February 1988 from a duodenal ulcer patient at Wexham Park Hospital, Slough, UK) were maintained by subculturing twice-weekly on chocolate Columbia agar (supplemented with 10 μg ml⁻¹ each of amphotericin B, polymyxin B and vancomycin) in an atmosphere of 12% CO₂, 5% O₂, 5% H₂, balance N₂ (by vol.). Broth cultures were prepared in 10 ml of brain heart infusion media (Oxoid) supplemented with 5% (v/v) foetal calf serum and antibiotics in 25 cm² vented tissue culture flasks (Costar) and were used after overnight incubation. For NMR experiments cells were obtained by seeding agar plates with 100 μl of 24 h broth cultures and harvesting after 2 d incubation when growth had become confluent. All experiments were performed using both strains of organism. The results shown are for strain NCTC 11637 unless stated otherwise.

Where indicated, a urease-negative isogenic strain of NCTC 11637 and a clinical isolate, strain 8091 (isolated in February 1988 from a duodenal ulcer patient at Wexham Park Hospital, Slough, UK) were used for such investigations since pyruvate occupies a key position in metabolism being at the branch point of several potential metabolic routes. These vary between different organisms and may be affected by growth conditions and particularly oxygen availability (Schlegel, 1986). Therefore, we investigated the utilization of pyruvate by H. pylori under different conditions of oxygenation. We have also analysed glucose catabolism, utilizing glucose substrates with 13C label at different carbon atoms to discriminate between the potential metabolic pathways involved.

**Labelling studies.** [1-13C]Pyruvate, [3-13C]glucose and [5-13C]glucose were obtained from Cambridge Isotope Laboratories. [2-13C]Pyruvate was from MSD Isotopes. [1-13C]Glucose was from Euriso-top Groupe CED. [2-13C]Glucose, sodium [13C]formate and [1,3,15N]urea were purchased from Sigma/Aldrich.

Cells were resuspended in brain heart infusion media (BHI) or phosphate-buffered saline (NaCl, 136 mM; KCl, 2 mM; Na₂HPO₄, 8 mM; KH₂PO₄, 1.5 mM; pH 7.2) (PBS) and harvested by centrifugation at 3500 g for 20 min at 4 °C. They were washed twice, and pellets were resuspended at approximately 50% wet weight in BHI or PBS. Labelled compounds were dissolved in D₂O and added to the samples to give a final concentration of 50 mM substrate and ~ 5% D₂O. 13C NMR spectra were obtained at 100 MHz on a Varian VX400 spectrometer equipped with a 5 mm broad band probe and operating in the Fourier transform mode. A repetition time of 1 s and 35° pulses were used. Proton decoupled carbon spectra were acquired for 256 scans and integrals used to quantify metabolic products. Chemical shifts were referred to tetramethylsilane. They are reported in parts per million (p.p.m.) and are a measure of the position of an NMR resonance which is dependent on the structure of a particular molecule. For anaerobic studies either cell suspensions were incubated directly, under nitrogen, at 37 °C, in the NMR spectrometer, or 1 ml cultures were sealed in 1·8 ml, 10 mm diameter, NUNC screw-capped vials, and were incubated statically at 37 °C, allowing cell respiration to generate anaerobic conditions. After incubation the vials were frozen on cardice, without opening, and stored until NMR analysis. Aerobic incubations were carried out with 1 ml cultures incubated in six-well tissue culture trays (Costar) at 37 °C in an atmosphere of 20% O₂, 10% CO₂, balance N₂ (by vol.) with gentle agitation. Following incubation 1 ml samples were frozen until NMR analysis.

Metabolic products from pyruvate were identified by comparison of chemical shift and structural data with that expected for putative products and confirmed by spiking cell supernatants with authentic standards.

**Gas chromatography and mass spectrometry.** Glucose metabolites were identified from NMR data and by analysing supernatants of cell suspensions by gas chromatography (GC) and mass spectrometry (MS) on a VG analytical TRIO-1 machine. Cell suspensions were centrifuged at 13000 g for 10 min and 0·1 ml portions of supernatant fractions taken to dryness under nitrogen at 75 °C. Silyl derivatives were prepared by adding 0·5 ml of pyridine/N-trimethylsilylimidazole (2:3, v/v) to the residue and heating for 30 min at 75 °C. Separations were carried out on a Quadrex 25 m x 0·25 mm diameter fused silica capillary column, coated with a 25 μm film of OV-1701 silicone. Splitless injections of 0·1 to 1 μl samples were used with an injection temperature of 250 °C. A temperature programme of 135 °C for 2 min followed by a rise of 5 Centigrade degrees min⁻¹ to 250°C was used. Helium carrier gas total flow was 50 ml min⁻¹ with 5 p.s.i. head pressure and 1·5 ml min⁻¹ septum purge. Product identities were confirmed by comparison of spectra with standard spectra from the National Bureau of Standards mass spectral library (Washington DC, USA) and against retention time and spectra obtained with authentic standards.

**RESULTS**

**Pyruvate metabolism**

[2-13C]Pyruvate (chemical shift 202 p.p.m.) proved particularly useful for studies of pyruvate metabolism due to the large differences in chemical shift of its products. The major product of anaerobic pyruvate utilization by H. pylori cells suspended in growth medium was lactate (66 p.p.m.). In addition, acetate (179 p.p.m.) and alanine (48 p.p.m.) were significant products and ethanol (55 p.p.m.) was produced to a lesser extent. A minor contaminant of the labelled pyruvate was observed in these samples (91·4 p.p.m.) that was metabolized at a similar rate to pyruvate. This NMR signal has been
Glucose and pyruvate metabolism in *H. pylori*

**Fig. 1.** Production of labelled metabolites during [2-13C]pyruvate catabolism by cells suspended anaerobically in PBS. Cells were incubated with 50 mM [2-13C]pyruvate in the NMR spectrometer under nitrogen as described in Methods. Products were quantified at time points using NMR integrals. ●, Pyruvate; ○, impurity; ■, lactate; □, ethanol; ●, acetate; ○, alanine.

**Table 1.** Effect of urea and glutamate on the distribution of products from [2-13C]pyruvate after 2 h anaerobic incubation

<table>
<thead>
<tr>
<th>Product</th>
<th>No additions to incubation buffer</th>
<th>% Urea</th>
<th>% Glutamate</th>
<th>% Urea and glutamate</th>
<th>% Glutamate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>46</td>
<td>42</td>
<td>27</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Lactate</td>
<td>45</td>
<td>20</td>
<td>26</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>4</td>
<td>tr.</td>
<td>4</td>
<td>tr.</td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>5</td>
<td>38</td>
<td>43</td>
<td>57</td>
<td></td>
</tr>
</tbody>
</table>

*Fig. 2.** Doublet signal due to [13C]alanine after anaerobic [2-13C]pyruvate metabolism by cells suspended in PBS containing [15N]urea. *H. pylori* NCTC 11637 cells were incubated in PBS with 50 mM [2-13C]pyruvate and 50 mM [15N]urea for 2 h at 37°C in sealed tubes and products analysed by NMR as described in Methods.

20% of the pyruvate was converted to alanine whereas cells resuspended in PBS produced minimal alanine (Fig. 1).

In view of the potent urease activity of *H. pylori* we investigated the potential of urea to act as a nitrogen source in this system. Cells resuspended in PBS containing 50 mM urea converted typically about 40% of [2-13C]pyruvate into alanine; glutamate likewise was able to provide a nitrogen source (Table 1). Further evidence for the metabolic incorporation of the nitrogen of urea was obtained using [15N]urea. 15N alone gives too weak an NMR signal for use as a metabolic label but if incorporated adjacent to a 13C in a molecule its coupling to the 13C signal can be demonstrated. When *H. pylori* cells were incubated with [2-13C]pyruvate plus 50 mM [15N]urea, the resultant 13C signal due to alanine was a doublet, separated by 6 Hz, indicative of 15N,13C coupling (Fig. 2). From the results shown in Table 1, it appears that under the conditions of this experiment about 17% of the urea nitrogen was scavenged into alanine. The ability of urea to promote alanine synthesis was dependent on urease activity and was not observed in a urease-negative mutant (results not shown).

When *H. pylori* cells were incubated aerobically in PBS containing [2-13C]pyruvate numerous products were detected by NMR at incubation times of less than about 20 min. In addition to resonances corresponding to pyruvate, acetate, lactate, ethanol and alanine, spectra showed unidentified products with chemical shifts of 199, 73-9, 61.7, 51.2 and 49.5 p.p.m. However, at incubation times greater than 80 min the only product detectable was acetate which was not metabolized further even after 48 h. There was no evidence for the operation of the TCA cycle aerobically, which would generate [13C]CO₂ from [2-13C]pyruvate and cause a substantial decrease in [13C]acetate.

A feature of these experiments was that the production of alanine was dependent on the provision of a nitrogen source. Thus, when cells were resuspended in BHI about documented previously in aqueous solutions of sodium pyruvate and assigned to the hydrated form of pyruvate (Margolis & Coxon, 1986). More than 80% of the added pyruvate was metabolized within 1 h (Fig. 1); however, further metabolism of the products formed was not detected even after extended incubations of up to 120 h and products were readily detectable in supernatant fractions after centrifugation of cell suspensions. Results for both strains of organism were similar.
Investigation of the fate of the 1-carbon of pyruvate (169.5 p.p.m.) anaerobically (Fig. 3) showed the production of lactate (182 p.p.m.) and alanine (175 p.p.m.); all the remaining labelled carbon appeared as bicarbonate (160 p.p.m.) and CO₂ (124 p.p.m.). As with the [2-13C] material a minor contaminant (178 p.p.m.) of the [1-13C]pyruvate was observed in these samples, most likely due to carboxyl carbon of the hydrate of pyruvate (Margolis & Coxon, 1986). No production of ["C]formate (170 p.p.m.) was seen and, in separate experiments, we observed no utilization of [13C]formate. In contrast, in control experiments conducted with E. coli, formate was initially detected as the predominant product from [1-13C]pyruvate and was also avidly metabolized (results not shown). The results for both strains of H. pylori were similar.

**Glucose metabolism**

The metabolism of glucose by cells suspended in growth medium was relatively slow compared to that of pyruvate and under the experimental conditions incubations of around 18 h were required to detect the formation of metabolic products. Even so, typically only about 40% of the added glucose (50 mM) was utilized and this fraction could not be increased by extending the incubation period.

In initial experiments, we investigated [1-13C]glucose metabolism anaerobically by NMR. Two products were seen (see Fig. 4); the major component containing, most likely, a 13C-labelled primary alcohol at 62.6 p.p.m. and a minor product probably containing a 13C-labelled carboxylic acid at 175.8 p.p.m. These metabolites were identified as sorbitol and gluconate respectively by analysing supernatant fractions of cell suspensions using GC/MS. No other products were detected, even when cell suspensions were incubated aerobically.

The metabolism of glucose was investigated further by comparing the disposition of label from [1-13C], [2-13C], [3-13C] and [5-13C]glucose. In all cases sorbitol and gluconate were seen as the major products when cells were incubated anaerobically in growth medium. [2-13C] and [5-13C]glucose were less satisfactory as substrates for these studies because they gave rise to 13C-labelled sorbitol and gluconate, products with NMR signals less readily distinguishable from those of the α- and β-glucose starting materials. However, when cells were incubated aerobically with [2-13C], [3-13C] and [5-13C]glucose evidence was obtained for the further oxidation of glucose (Fig. 5). In addition to sorbitol and gluconate, [2-13C] and [5-13C]glucose both produced small peaks at 179 p.p.m.
Fig. 5. Comparison of aerobic [1-13C], [2-13C], [3-13C] and [5-13C]glucose disposition by *H. pylori*. Cells were incubated for 24 h at 37 °C aerobically in growth medium supplemented with 50 mM [1-13C]glucose (a), [2-13C]glucose (b), [3-13C]glucose (c) or [5-13C]glucose (d) and products analysed by NMR as described in Methods.
which we ascribe to the carboxylic acid carbon of acetate. [3-13C]Glucose likewise gave rise to 13C-labelled acetate, but with a chemical shift of 20.8 p.p.m. (Fig. 5) since this label is incorporated into methyl carbon. Results for both strains of H. pylori were similar.

DISCUSSION

The metabolites of glucose and pyruvate detected during this study probably represent the end-products of H. pylori metabolism since they were present in supernatant fractions of centrifuged cell suspensions. Also the metabolism was presumably due to intracellular transformation of substrates since we detected no such metabolic activity in culture supernatants. 13C NMR is a relatively insensitive technique, which necessitated the use of dense cell suspensions. Nevertheless, short-lived metabolites and compounds at low concentration may not have been detected. Intracellular compounds might also have been missed because of masking by cellular constituents. Similarly, the NMR integrals depicted in Figs 1, 3 and 4 and in Table 1 may not be strictly related to concentrations of products.

However, the results shown here demonstrate that the main oxidation product of pyruvate metabolism by H. pylori is acetate whereas anaerobically the reduction products of lactate and ethanol are also produced. In addition, alanine is produced when cells are provided with a nitrogen source.

The results also clearly show that urea can act as a nitrogen source for H. pylori. Urease activity has been reported to be important for the survival of H. pylori in vivo (Ferrero & Labigne, 1993; Hazell & Mendz, 1993) and a number of possible functions have been suggested for the enzyme. For example, it has been proposed that urease creates an alkaline micro-environment that helps neutralize gastric acidity and that urease may have a direct role in modulating the physiology of the gastric epithelium (Ferrero & Labigne, 1993). The results shown here provide direct evidence that as with other bacteria (Mobley & Hausinger, 1989; Collins & D’Orazio, 1993) urease activity in H. pylori may also be important for the provision of a nitrogen source. Possibly, the alanine production we observed was from pyruvate via the activities of transaminase and glutamate dehydrogenase which synthesizes glutamate from 2-oxoglutaric acid and NH4+. Glutamate dehydrogenase activity has been reported in H. pylori (Hazell & Mendz, 1993) and in the studies reported here glutamate itself was able to act as a nitrogen donor. It is possible that this process in some circumstances may cause the suicidal destruction of H. pylori in vitro; Neithercut et al. (1991) and Greig et al. (1991) reported that urea is toxic to H. pylori under conditions of fixed pH, if citrate is available to the organism. They suggested that this effect may be due, in part, to an uncontrolled diversion of carbon flux caused by transaminase activity in the presence of excess intracellular ammonia (derived from urea by the action of urease). Our results support this hypothesis. Ugurbil et al. (1978) similarly showed the production of alanine as an end-product by E. coli cells metabolizing [1-13C]glucose and in addition showed label incorporation into the amino acids valine and glutamate.

In this study, we have seen no evidence for the accumulation of succinate as an anaerobic end-product from pyruvate in H. pylori suggesting that pyruvate carboxylase is not a major route for disposing of pyruvate, at least under the conditions of these experiments. However, the possibility remains that succinate may be produced by H. pylori as a metabolic end-product from other substrates. In facultative anaerobes pyruvate is often metabolized anaerobically to acetate (and ethanol) via pyruvate-formate lyase activity, resulting in the production of formate from the 1-carbon of acetate (Abbe et al., 1982; Knappe, 1990). Anaerobically, we observed no production of formate from 13Cpyruvate presumably indicating that acetate formation by H. pylori does not involve pyruvate-formate lyase. Further studies are needed to define what enzymes are involved.

In these studies we have observed no evidence for the operation of a tricarboxylic acid (TCA) cycle. Acetate arising from pyruvate appeared not to be further metabolized; however, since we are unable to precisely relate NMR integrals to concentrations and since CO2, the expected TCA cycle product, would be lost to the atmosphere under aerobic conditions we cannot rule out that some TCA cycle activity is present. Clearly, if present, the rate of TCA cycle activity must be less than that of acetate production from acetyl-CoA, the likely source of acetate we observed in these experiments. It would be of interest to investigate the metabolic fate of potential TCA cycle intermediates in H. pylori.

The low rate and extent of glucose metabolism, compared to that of pyruvate, suggests that glucose is not a major energy source for H. pylori. In contrast to the extended incubations required for glucose metabolism by H. pylori Ugurbil et al. (1978), using similar conditions obtained the complete utilization of 50 mM glucose in 10 min by cell suspensions of E. coli. Sorbitol is a reduction product of glucose and presumably its synthesis during anaerobiosis relates to the maintenance of the cell redox state. Gluconate may arise from the breakdown of 6-phosphogluconate, a key intermediate in the pentose phosphate and Entner–Doudoroff pathways. From the results with pyruvate one might expect acetate to be the principal oxidation product of glucose. The catabolism of glucose could occur by three routes: glycolysis, pentose phosphate pathway or the Entner–Doudoroff pathway. These three pathways may all lead to the generation of acetate but the fates of the glucose carbons are different (Schlegel, 1986) (Table 2). Therefore by investigating the labelling patterns of the end-products of metabolism it is possible to deduce the metabolic pathways involved.

In this study, we observed that glucose is indeed oxidized to acetate by H. pylori, albeit in small amounts. The metabolism of glucose to lactate has been reported by Mendz et al. (1993) perhaps suggesting limited oxygen
Table 2. Fate of glucose carbon on metabolism to acetate and CO₂ via three potential metabolic routes

<table>
<thead>
<tr>
<th>Fate of carbon</th>
<th>Glycolysis</th>
<th>Entner–Doudoroff</th>
<th>Pentose phosphate pathway*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate, CH₃ group</td>
<td>1, 6</td>
<td>3, 6</td>
<td>2, 6</td>
</tr>
<tr>
<td>Acetate, COOH group</td>
<td>2, 5</td>
<td>2, 5</td>
<td>3, 5</td>
</tr>
<tr>
<td>CO₂</td>
<td>3, 4</td>
<td>1, 4</td>
<td>1, 2, 3, 4</td>
</tr>
</tbody>
</table>

* Other labelling possibilities exist.

availability in their experiments. In our study, the labelling patterns obtained with differently labelled glucose substrates suggest that oxidation takes place via the Entner–Doudoroff pathway. Glycolysis does not appear to be active since [1-13C]glucose does not give rise to acetate labelled in the methyl carbon. The oxidation of glucose to acetate via either the Entner–Doudoroff or pentose phosphate pathways results in the 1-carbon of glucose giving rise to CO₂. 13CO₂ formation was not observed during aerobic incubations of [1-13C]glucose; however, this is not surprising as the small amount of label would be dissipated as gaseous CO₂ during the course of the incubations. The enzymes of the pentose phosphate pathway have been demonstrated in H. pylori (Mendz & Hazell, 1991) and may participate in the metabolism of glucose to lactate (Mendz et al., 1993). We have seen no evidence for the oxidation of glucose by the pentose phosphate pathway (Table 2), perhaps suggesting that it has a predominantly biosynthetic role.

Clearly, the metabolic profiles that we have seen in H. pylori in this study reflect the physiological adaptations of the organism in the particular growth conditions we have employed. It would be interesting to extend this study by investigating the effect of growth conditions on the metabolic profiles obtained.

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REFERENCES


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