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^{13}C-NMR study of glucose and pyruvate catabolism in four acetogenic species isolated from the human colon

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Abstract

Glucose fermentation by four acetogenic species (two *Clostridium* strains, one *Streptococcus* strain and *Ruminococcus hydrogenotrophicus)* isolated from the human colon was of a mixed-acid type, whereas pyruvate metabolism was characterised by homoacetogenesis. Acetate formation from [1-^{13}C] and [2-^{13}C]glucose was consistent with the formation of acetyl-SCoA from pyruvate generated by the Embden-Meyerhof-Parnas pathway. Labelling of lactate and ethanol demonstrated that these metabolites were formed by reduction of pyruvate and acetyl-SCoA, respectively. In contrast, the reductive pathway of acetate formation was the preferential means of re-oxidising cofactors formed during [1-^{13}C]pyruvate catabolism.

Keywords: Human colon; Acetogenic bacteria; NMR spectroscopy; Catabolism; [^{13}C]Glucose; [^{13}C]Pyruvate

1. Introduction

Reductive acetogenesis is a metabolic process corresponding to the reduction of two moles of CO_2 to form one mole of acetate. Most acetogenic bacteria isolated from environmental and digestive anaerobic ecosystems are able to undertake both autotrophic and heterotrophic acetogenesis [1,2]. In the latter, one hexose equivalent produces two acetates by oxidative decarboxylation of pyruvate, and a third is formed by reduction of the previously released CO_2. In the autotrophic process, two exogenous CO_2 are reduced by four molecular H_2 to form one acetate [1,3]. In humans, reductive acetogenesis has been demonstrated to be an important pathway for H_2 disposal in the colon of subjects harbouring low numbers of methanogens [4–6]. Recently, H_2/CO_2-utilising acetogenic strains, belonging to several different bacterial genera, including *Clostridium*, *Streptococcus* and *Ruminococcus*, were obtained in our laboratory from non-methane-producing faecal samples [7]. A new species, *Ruminococcus hydrogenotrophicus*, was identified in these studies [8]. All these bacteria were able to use H_2/CO_2 as their sole energy source to produce acetate according to the stoichiometric equation of reductive acetogenesis. These colonic acetogens were also able to utilise a broad range of organic substrates, but glucose and fructose fermentation was of a mixed-acid type, rather than homoacetogenesis [7]. The aim of the present work was to elucidate the pathways involved in heterotrophic metabolism in four of these colonic aceto-

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gens using NMR spectroscopy. The oxidative pathways of acetate, lactate and alcohol formation were investigated using [1-^{13}C] and [2-^{13}C]glucose as precursors, and the reductive pathway of acetate formation was studied using [1-^{13}C]pyruvate as precursor of $^{13}CO_2$.

2. Materials and methods

2.1. Source of strains

Four H_2/CO_2-utilising acetogenic strains, isolated in our laboratory [7], were selected for this study: strain M5a3 (DSM 10510) and strain F5a15 corresponded to two different species of the genus *Clostridium*, strain S5a2 belonged to the genus *Streptococcus*, and strain S5a33 (DSM 10507) was identified as *Ruminococcus hydrogenotrophicus*, a new species [8].

2.2. Culture methods and growth media

The anaerobic techniques employed were those previously described by Hungate [9] using 100% CO_2 gas. H_2/CO_2-utilising acetogens were grown in semi-synthetic AC-21 medium modified in our laboratory [7,8] in which yeast extract was omitted and replaced by a vitamin solution (5 ml l^{-1}) containing (mg l^{-1} of distilled water): biotin, 0.33; choline chloride, 30.0; folic acid, 0.15; inositol, 140.0; nicotinic acid, 59.8; *p*-aminobenzoic acid, 76.3; pantothenic acid, 27.4; pyridoxine, 4.3; riboflavin, 11.6; thiamine, 53.0; thymidine 21.7. Samples of the medium (5 ml) were placed in capped serum tubes (18×150 mm).

2.3. Glucose and pyruvate fermentation in acetogenic isolates

The acetogenic bacteria were grown on modified AC-21 medium containing glucose (11 mM) or pyruvate (10 mM) as sole carbon sources. Inocula consisted of 0.3 ml of a 24 h culture grown on glucose or a 48 h culture grown on pyruvate. The end products of glucose and pyruvate fermentation were determined after 25 h incubation at 37°C by capillary gas chromatography, after conversion to tertiary butyldimethylsilyl derivatives [10]. The ethanol, glucose and pyruvate remaining after incubation were measured enzymically (Boehringer Mannheim). Each experiment was done in triplicate.

2.4. NMR studies of [1-^{13}C]glucose, [2-^{13}C]glucose, and [1-^{13}C]pyruvate metabolism in bacterial cell suspensions

All manipulations were done under anaerobic conditions using 100% N_2 gas. Cell suspensions were prepared from bacterial cultures grown in 1 litre flasks containing 250 ml of modified AC-21 medium, with either 2 g l^{-1} glucose or pyruvate as carbon source. The inocula consisted of 5 ml of late exponential phase cultures (15 h for cultures grown on glucose or 48 h for cultures grown on pyruvate). When bacteria grown in flasks at 37°C reached late exponential phase (20–24 h for glucose, 48 h for pyruvate), cells were harvested by centrifugation (8800×*g*, 30 min, 4°C). Bacterial pellets were suspended in 2.5 ml incubation medium (pH 6.8) containing (g l^{-1}): KH_2PO_4, 15.0; K_2HPO_4, 15.0; NH_4Cl, 0.25; KCl, 0.5; $CaCl_2{\cdot}2H_2O$, 0.15; $MgCl_2$. 6 H_2O, 0.6; K_2SO_4, 0.1; tryptone, 2.0. In addition, 1 ml trace element solution [11], 0.1 ml of Na_2WO_4+Na_2SeO_3 (0.1 mM each), 5 ml vitamin solution [12], 1 ml resazurin solution (0.1% w/v) and 20 ml cysteine HCl+Na sulfide (1.25% each) were added per litre of the basal medium. Bacterial suspensions (2 ml) were added to capped serum tubes containing 3 ml of the incubation medium. Bacterial protein concentrations, assessed by the method of Bradford [13], were approximately 1–2 mg ml^{-1}. [1-^{13}C]-Glucose, [2-^{13}C]glucose, or [1-^{13}C]pyruvate (99% enrichment; Isotec, France) was added to bacterial suspensions giving a concentration of 20 mM. Triplicate incubations of cells were performed at 37°C for 5 h with [^{13}C]glucose and for 24 h with [^{13}C]pyruvate. The suspensions were then harvested by centrifugation (6000×*g*, 20 min, 4°C). Residual glucose and pyruvate were assessed enzymically and short chain fatty acids were analysed by gas chromatography as described above (see Section 2.3).

^{13}C-Labelled metabolites in supernatant fluids were measured by NMR spectroscopy. The pH of the supernatant fluids was adjusted to 8.0 and 2 ml samples were prepared by adding 0.3 ml ^2H_2O, con-

taining dioxane (frequency and intensity reference) at a final concentration equivalent to 5 mM ^{13}C. 300 μl of relaxing reagent, comprising chromium sodium diethylenetriaminepentaacetate (CrDTPA) in $^{2}H_2O$, was also added to a final concentration of 2 mM CrDTPA. NMR spectra were recorded on a Bruker AM-300 spectrophotometer at a frequency of 75.45 MHz, as described previously [5].

3. Results

3.1. End products of glucose and pyruvate fermentation

Glucose fermentation by the four acetogenic species led to production of acetate, formate, ethanol and, except for *Clostridium* strain F5a15, lactate. While acetate was the main end product of glucose fermentation in *Clostridium* strains and *R. hydrogenotrophicus*, lactate was the major metabolite formed by *Streptococcus* sp. In contrast, pyruvate was metabolised principally to acetate (70–100% of total fermentation products) in all the acetogenic species. Whereas the amount of lactate formed from glucose was 71% and 17% respectively from the *Streptococcus* and *Ruminococcus* species, lactate production was only 17% and 4% respectively from pyruvate. In contrast, *R. hydrogenotrophicus* produced slightly more ethanol (3–4%) from pyruvate than from glucose.

3.2. Metabolism of [1-^{13}C]glucose and [2-^{13}C]glucose in resting cells of acetogenic bacteria

Metabolites produced from [1-^{13}C] and [2-^{13}C]glucose by resting cells of the different acetogenic species were similar to those observed with actively growing bacteria. Metabolism of [1-^{13}C] and [2-^{13}C]glucose in the *Clostridium* strains and *R. hydrogenotrophicus* yielded 60–70% acetate, which was mono-labelled on its methyl or carboxyl group, depending on the position of the glucose label (Table 1). The ratio of labelled acetate to total acetate reached 30–40% for all strains. Resting cells of the *Streptococcus*, however, mainly produced lactate (60–70%), labelled either on its methyl group from fermentation of [1-^{13}C]glucose or on its alcohol group from [2-^{13}C]glucose utilisation (Table 1). La-

Table 1
Labelled end products formed from [1-^{13}C]glucose, [2-^{13}C]glucose, or [1-^{13}C]pyruvate fermentation by resting cells of four human acetogenic bacteria, incubated at 37°C under 100% N_2 for 5 h with [^{13}C]glucose and for 20 h with [^{13}C]pyruvate

		Labelled end products (% of total ^{13}C metabolites)[a]									^{13}C recovery (%)[b]
		Formate	Acetate			Lactate			Ethanol		
		[1-^{13}C]	[1-^{13}C]	[2-^{13}C]	[1,2-^{13}C]	[1-^{13}C]	[2-^{13}C]	[3-^{13}C]	[1-^{13}C]	[2-^{13}C]	
Clostridium strain F5a15	[1-^{13}C]G[c]	–[d]	–	69.0	–	–	–	–	–	31.0	45.8
	[2-^{13}C]G	–	70.6	–	–	–	–	–	29.5	–	42.5
	[1-^{13}C]P	–	68.9	–	31.2	–	–	–	–	–	47.7
Clostridium strain M5a3	[1-^{13}C]G	–	–	68.5	–	–	–	4.2	–	27.3	54.0
	[2-^{13}C]G	–	67.3	–	–	–	9.1	–	23.6	–	62.7
	[1-^{13}C]P	3.8	58.0	–	36.8	–	–	–	–	–	49.5
Ruminococcus hydrogenotrophicus strain S5a33	[1-^{13}C]G	–	–	58.0	–	–	–	25.8	–	11.0	56.9
	[2-^{13}C]G	–	56.8	–	–	–	24.2	–	9.7	–	47.8
	[1-^{13}C]P	–	69.0	–	31.0	0.0	–	–	–	25.8	
Streptococcus strain S5a2	[1-^{13}C]G	3.0	–	12.8	–	–	–	65.3	–	18.7	59.0
	[2-^{13}C]G	4.1	10.7	–	–	–	69.1	–	16.1	–	59.7
	[1-^{13}C]P	11.0	40.4	–	22.6	24.8	–	–	–	–	38.3

[a] Labelled end products expressed as a percentage of the total ^{13}C metabolites measured.
[b] The percentage of C recovery does not take into account cell carbon or CO_2.
[c] [1-^{13}C]G: [1-^{13}C]glucose; [2-^{13}C]G: [2-^{13}C]glucose; [1-^{13}C]P: [1-^{13}C]pyruvate.
[d] Not detected. Results are mean values of three determinations.

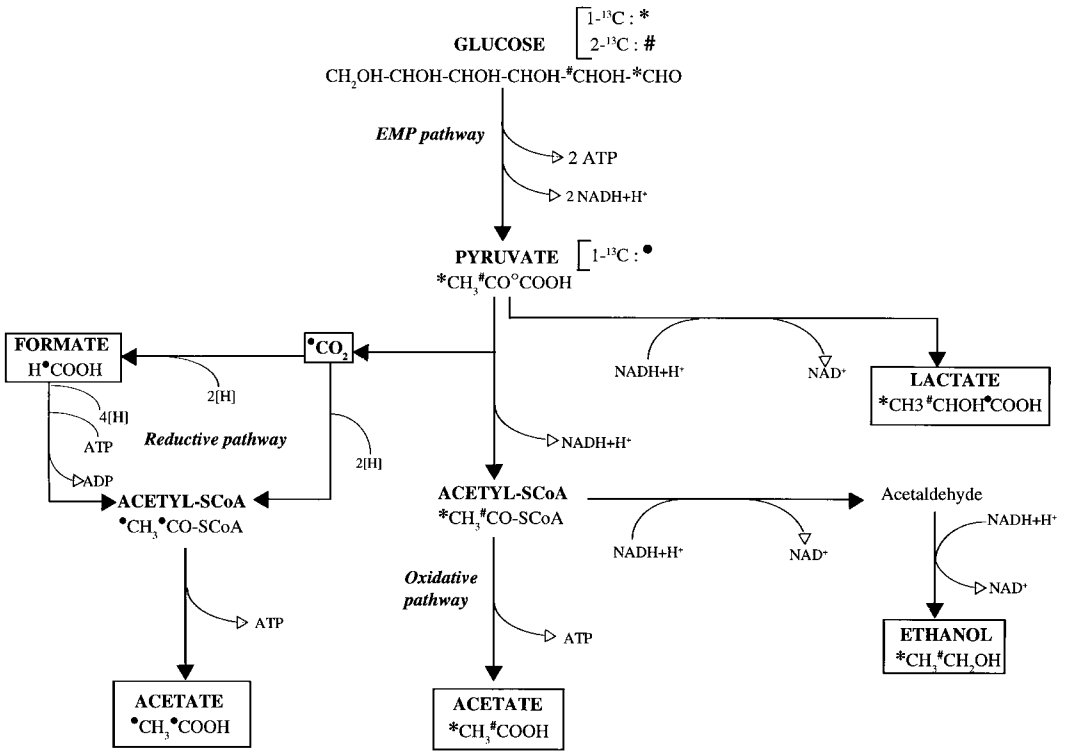

Fig. 1. Scheme showing the overall pathways involved in [1-^{13}C], [2-^{13}C]glucose and [1-^{13}C]pyruvate catabolism in acetogenic bacteria from the human colon. Distribution of ^{13}C label represented by: (*) from [1-^{13}C]glucose, (#) from [2-^{13}C]glucose, (●) from [1-^{13}C]-pyruvate. EMP pathway: Embden-Meyerhof-Parnas pathway.

belled lactate represented 40–45% of the total amount produced. Similar lactate labelling was found with resting cells of *Clostridium* strain M5a3 and *R. hydrogenotrophicus* strain S5a33. Ethanol formed by all of the acetogens was labelled on its methyl group from [1-^{13}C]glucose metabolism, or on its alcohol group from [2-^{13}C]glucose (Table 1). Trace amounts of labelled formate were also detected during [1-^{13}C] and [2-^{13}C]glucose utilisation by resting cells of *Streptococcus* sp.

3.3. Metabolism of [1-^{13}C]pyruvate in resting cells of acetogenic bacteria

Acetate was the sole metabolite formed by the clostridia and *R. hydrogenotrophicus* resting cells from [1-^{13}C]pyruvate (Table 1). Labelled acetate represented 30–34% of the total amount of acetate produced. The percentage of acetate labelled on both its methyl and carboxyl groups ranged from 20 to 31%, depending on the strain. Small amounts of labelled formate were also formed by *Clostridium* strain M5a3. The *Streptococcus* also produced labelled acetate as the main end product of [1-^{13}C]-pyruvate metabolism. However, ^{13}C labelling was also found in lactate and formate (Table 1). The percentage of labelled acetate to total acetate was close to 30%, whereas labelled lactate represented 66% of the total lactate produced.

4. Discussion

As shown previously [7], glucose fermentation by the four colonic acetogens was of a mixed-acid type. Labelling of acetate by [1-^{13}C] and [2-^{13}C]glucose was consistent with formation of acetyl-SCoA from pyruvate generated by the Embden-Meyerhof-Parnas (EMP) pathway (Fig. 1). The large amounts of unlabelled acetate formed from [^{13}C]glucose fermenta-

tion could be assumed to derive in part from CO_2 reduction by the Wood-Ljungdahl pathway. These acetogenic strains were previously shown to use this metabolic process for energy conservation during autotrophic growth from H_2/CO_2 [7], and it is likely that acetate synthesised in this reductive pathway cannot be labelled using [1-^{13}C] and [2-^{13}C]-glucose precursors.

Lactate was also formed from glucose by most of these colonic acetogens. Labelling of this metabolite by [1-^{13}C] and [2-^{13}C]glucose was consistent with the reduction of pyruvate by lactate dehydrogenase (Fig. 1). In the case of *Streptococcus* S5a2, lactate formation from glucose was mainly used to re-oxidise reduced electron carriers produced during glycolysis. Ethanol was also synthesised by all test species as an alternative means of regenerating reduced cofactors formed in the EMP pathway. Ethanol seemed to be formed by reduction of acetyl-SCoA formed by the EMP pathway, as demonstrated by its labelling from [1-^{13}C] and [2-^{13}C]glucose (Fig. 1).

In contrast to glucose, homoacetogenesis was the main characteristic of pyruvate breakdown in all four species. All test strains produced mainly labelled acetate from [1-^{13}C]pyruvate, attesting to their ability to reduce $^{13}CO_2$ released during acetyl-SCoA formation (Fig. 1). The percentage of acetate labelled was close to 30–33% which would be expected from the stoichiometric equation of homoacetogenesis. Furthermore, this result is comparable with the previous findings of Miller and Wolin [6], that one third of the acetate formed from [^{14}C]glucose fermentation by a human faecal suspension derived from CO_2 reduction. Energy considerations can explain the homoacetate fermentation of pyruvate observed in colonic acetogens, especially the clostridia. Since the reductive pathway of acetate formation is coupled to energy conservation, it is likely that hydrogen equivalents obtained from pyruvate are preferentially used to reduce CO_2 to acetate, instead of being used for ethanol or lactate formation. Accumulation of [^{13}C]formate, observed in some of these bacterial incubations, might be due to its rapid production rate, as compared to its utilisation as a precursor of acetate in the reductive pathway (Fig. 1).

As previously observed [14], low ^{13}C recoveries were obtained in all bacterial incubations. Since NMR measurements were only done on supernatant fluids, ^{13}C-compounds present inside the cells were not detected, which may account for loss of part of the label. Furthermore, CO_2 production by acetogenic strains, which could not be measured by NMR, could also explain the loss of label. Exchange reaction between [1-^{13}C]pyruvate and unlabelled CO_2 may have occurred as previously reported [15]. This could be responsible for the high percentage of [1-^{13}C]acetate formed from [1-^{13}C]pyruvate catabolism.

In this investigation, we have demonstrated the different fermentative capacities in human colonic acetogens, which depended on the species and substrate considered. Only a few studies have dealt with the fermentative characteristics of acetogenic bacteria from other ecosystems, whereas their ability to use one-carbon or aromatic compounds as electron sinks has been widely documented [1,16]. Furthermore, our results are consistent with the recent report of Misoph and Drake [17] showing utilisation of alternative fermentation pathways for conservation of energy and growth in the acetogen *Peptostreptococcus productus* U1, isolated from a sewage digester. Because of the great nutritional versatility of colonic acetogens, together with their abilities to grow both autotrophically and heterotrophically [7], further investigations are needed to determine how the metabolism of H_2/CO_2 is modulated by organic compounds in these species.

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