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^{13}C-NMR Study of propionate metabolism by sludges from bioreactors treating sulfate and sulfide rich wastewater

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Abstract

Applications of nuclear magnetic resonance (NMR) to study a variety of physiological and biochemical aspects of bacteria with a role in the sulfur cycle are reviewed. Then, a case-study of high resolution ^{13}C-NMR spectroscopy on sludges from bioreactors used for treating sulfate and sulfide rich wastewaters is presented. ^{13}C-NMR was used to study the effect of sulfate and butyrate on propionate conversion by mesophilic anaerobic (methanogenic and sulfate reducing) granular sludge and microaerobic (sulfide oxidizing) flocculant sludge. In the presence of sulfate, propionate was degraded via the randomising pathway in all sludge types investigated. This was evidenced by scrambling of [3-^{13}C]propionate into [2-^{13}C]propionate and the formation of acetate equally labeled in the C_1 and C_2 position. In the absence of sulfate, [3-^{13}C]propionate scrambled to a lesser extend without being degraded further. Anaerobic sludges converted [2,3-^{13}C]propionate partly into the higher fatty acid 2-methyl[2,3-^{13}C]butyrate during the simultaneous degradation of [2,3-^{13}C]propionate and butyrate. [4,5-^{13}C]valerate was also formed in the methanogenic sludges. Up to 10% of the propionate present was converted via these alternative degradation routes. Labeled butyrate was not detected in the incubations, suggesting that reductive carboxylation of propionate does not occur in the sludges.

Introduction

NMR studies of bacteria involved in the sulfur cycle

NMR techniques offer a range of unique tools to study the functioning of waste treatment systems (Lens & Hemminga 1998). They include the determination of bioconversion and transport processes in wastewater, sludge, solid waste and soils. Also for sulfur cycle-dependent bioconversions, NMR techniques have been applied. Tables 1, 2 illustrate the spectrum of applications where NMR has been applied to resolve specific aspects of the metabolism, physiology and biochemistry of sulfur cycle bacteria.

One of the most attractive aspects of the application of NMR to study cellular physiology is the possibility to perform non-destructive measurements. Different aspects of metabolic processes can be studied by NMR, which can involve the detection of different nuclides (Table 1). ^{31}P NMR has been used to monitor the energetic status (ATP/ADP/AMP content) of bacterial cells. Other applications of ^{31}P NMR include measurement of enzyme kinetics *in vivo* and the determination of the intracellular pH and thus the transmembrane proton gradient. Substrate consumption, product formation and the metabolic fate of individual carbon atoms can be followed by ^{13}C NMR (Table 1). ^{1}H NMR can be used to confirm the structure of intermediates and products during a bioconversion (Table 1). The application of *in vivo* NMR to study the metabolism of pure cultures of sulfate reducing bacteria has been reviewed in detail by

Table 1. Applications of high resolution NMR spectroscopy to study the metabolism of sulfur cycle bacteria

Application	Organism used	Technique	Reference
SULFATE REDUCING BACTERIA			
Aerobic metabolism of polyglucose	*Desulfovibrio gigas*	^{31}P NMR	Santos et al. 1993
Metabolism of carbon reserves	*Desulfovibrio gigas*	^{13}C NMR	Fareleira et al. 1997
Ethanol fermentation	*Desulfobulbus propionicus*	^{13}C NMR	Stams et al. 1984
Propionate metabolism	*Desulfobulbus propionicus*	^{13}C NMR	Houwen et al. 1991
Propionate metabolism	Sulfate reducing granular sludge	^{13}C NMR	Omil et al. 1997
Butyrate isomerisation	*Desulforhabdus amnigenus*	^{1}H and ^{13}C NMR	Oude Elferink et al. 1996
Furfural fermentation	*Desulfovibrio furfuralis*	^{1}H NMR	Schoberth et al. 1993
Selenate and selenite metabolism	*Desulfovibrio desulfuricans*	^{13}C NMR	Tomei et al. 1995
SULFUR REDUCING BACTERIA			
Autotrophic CO_2 fixation	*Thermoproteus neutrophilus*	^{13}C NMR	Schaefer et al. 1989; Strauss et al. 1992
SULFIDE OXIDIZING BACTERIA			
Acetate metabolism	*Chromatium vinosum*	^{13}C and ^{31}P NMR	Nicolay et al. 1983
Acetate metabolism	*Thiobacillus versutus*	^{13}C NMR	Claassen et al. 1986
OTHER BACTERIA			
Monomethyl sulfate biodegradation	*Hyphomicrobium* sp. *Agrobacterium* sp.	^{13}C NMR	Higgings et al. 1996

Santos et al. (1994). An interesting example illustrating the potentials of NMR is the study of the aerobic metabolism of the "strict" anaerobe *Desulfovibrio gigas* using *in vivo* high resolution NMR (Santos et al. 1994).

^{1}H high resolution, especially 2 dimensional, NMR and ^{1}H relaxation measurements are powerful techniques to study the composition and architecture of proteins and enzyme systems. These NMR techniques have been extensively applied to study the electron carriers of many sulfate reducing and sulfide oxidizing bacteria, including their cytochromes, ferredoxins and flavodoxins (Table 2). Using NMR, the structure and functioning of both the iron-sulfur cluster moieties and the associated proteins of these electron carriers have been elucidated.

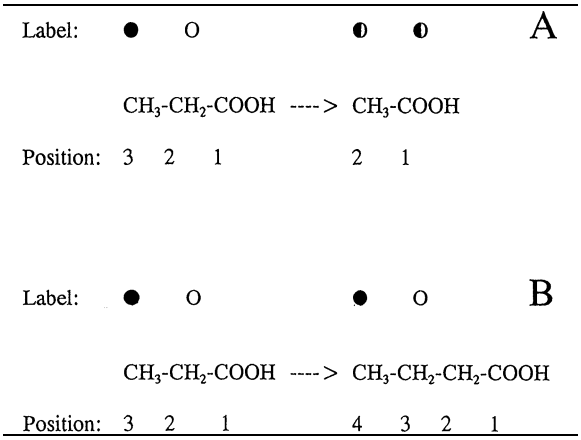

Figure 1. Schematic representation of the fate of the labeling position during propionate conversion via the randomising pathway (A) and via reductive carboxylation (B).

Propionate conversion under anaerobic conditions

In methanogenic environments, oxidation of propionate is thermodynamically difficult (Schink 1997). The $\Delta G^{0'}$ value for propionate oxidation to acetate under standard conditions amounts to +76.1 kJ/mol (Thauer et al. 1977). Usually, acetate is formed via the succinate pathway, which randomises the label in both the acetate (Figure 1A) and the propionate molecule (Houwen et al. 1991). Propionate oxidation can become exergonic due to interspecies transfer of reducing equivalents from acetogenic to methanogenic bacteria, either as molecular hydrogen or as formate (Dong et al. 1994). Besides the syntrophic oxidation of propionate to the methanogenic substrates H_2, formate and acetate, reduced end products such as higher (C_4-C_7) volatile fatty acids (VFA) and alcohols can be formed from propionate as well (Smith & McCarty 1989). In general, these side reactions are associated with suboptimal reactor performance (Smith & Mc-

Table 2. NMR applications to study sulfur cycle dependent bioconversions

Application	Organism used	Technique	Reference
PHYSIOLOGY			
Energy status	*Desulfovibrio* sp.	^{31}P high resolution NMR spectroscopy	Santos et al. 1991
Study of intracellular inclusions	*Desulfovibrio gigas*	^{31}P high resolution NMR spectroscopy	Hensgens et al. 1996
Determination of the transmembrane proton gradient	*Desulfovibrio desulfuricans*	^{31}P high resolution NMR spectroscopy	Kroder et al. 1991
Determination of the intracellular pH	*Desulfovibrio gigas*	^{31}P high resolution NMR spectroscopy	Santos et al. 1994
ENZYME SYSTEMS			
Sulfite reductase	*Desulfovibrio* sp.	NOE measurements*	Matthew et al. 1995
Sulfite reductase	*Escherichia coli*	Relaxation and NOE measurements	Kaufman et al. 1993
Sulfhydrylase	*Salmonella typhimurium*	^{1}H high resolution NMR spectroscopy	Tai et al. 1993
ELECTRON CARRIERS			
Cytochrome c-5	*Desulfovibrio vulgaris*	2 dimensional exchange NMR	Turner et al. 1996
Cytochrome c-553	*Desulfovibrio vulgaris*	NMR spectroscopy	Blackledge et al. 1996
Ferrocytochrome c-550	*Thiobacillus versutus*	^{1}H, ^{13}C, ^{15}N high resolution NMR and ^{15}N relaxation studies	Ubbink et al. 1996
Cu(I) rusticyanin	*Thiobacillus ferrooxidans*	^{1}H, ^{13}C, ^{15}N high resolution NMR	Botuyan et al. 1996
Primary electron acceptor of green sulfur bacteria	*Prosthecochloris* sp. *Chlorobium* sp.	^{1}H high resolution NMR spectroscopy	van de Meent et al. 1992
STRESS METABOLITES			
Osmoregulator halophilic purple sulphur bacterium	*Ectothiorhodospira marismortui*	NMR mass spectrometry	Galinski & Oren 1991
Osmoregulator	Purple and green sulphur bacteria	^{13}C high resolution NMR spectroscopy	Welsh & Herbert 1993
MASS TRANSPORT PROCESSES			
Diffusional properties	Sulfate reducing granular sludge	Pulsed field gradient ^{1}H NMR	Lens et al. 1997
Flow properties	Sulfate reducing granular sludge	Pulsed field gradient ^{1}H NMR	Lens et al. 1997

*: NOE = Nuclear Overhauser Effect.

Carty 1989). Little is known about the biochemistry of these conversions and their *in situ* role in organic matter degradation in bioreactors.

In this paper, ^{13}C NMR was used to study possible alternative propionate conversion routes in sludges present in bioreactors treating sulfate and sulfide rich wastewater. Special attention was given to the effect of sulfate and butyrate on the propionate degradation pathway. The use of ^{13}C-labeled propionate offered the possibility to study the incorporation of ^{13}C-label into intermediates and end products derived from propionate.

Materials and methods

Source of biomass

Sulfide-oxidizing sludge was harvested from an expanded bed reactor with a spatially separated aeration unit operating at a sulfide loading rate of 3 g HS^-/l.day (Janssen et al. 1997). The reactor operated under heterotrophic conditions (acetate:propionate mixture in a ratio of 1:1 on chemical oxygen demand (COD) basis, 0.6 g COD/l) at 22 °C. Sulfate-reducing granular sludge was sampled anaerobically from a mesophilic (30 °C) granular sludge reactor (Omil et al. 1996) treating an acetate:propionate:butyrate mixture [1:1:1 on COD basis, 12 g COD/l] at 4.8 g COD/l.day with an excess of sulfate (influent COD/SO_4^{2-} 0.5).

The effect of the wastewater composition on the propionate-degrading properties of methanogenic granular sludge was studied in mesophilic (30 °C) fed batch reactors operated for 3 months at a volumetric loading rate of 4.0 g COD per litre reactor per day. One batch reactor was fed with a mixture of 10% glucose and 90% volatile fatty acids (acetate:propionate:butyrate mixture in a ratio of 5:3:2 on COD basis). The feed of two other batch reactors

was supplemented with 35 μM and 35 mM Na_2SO_4, respectively. In a fourth batch reactor, glucose was omitted from the basic feed.

Degradation studies and nuclear magnetic resonance spectroscopy

The fate of ^{13}C-labeled compounds was studied in batch experiments at 35 ($\pm$ 2) °C using about 50 mg volatile suspended solids as described by Lens et al. (1996). Degradation of the substrates (10 mM initial concentration) was studied both in the absence and the presence of 30 mM sulfate. At regular time intervals, the supernatant was sampled, centrifuged for 10 min at 13000 g and stored at -20 °C until analysis.

The ^{13}C proton-decoupled NMR spectra of the supernatants were recorded on a Bruker spectrometer (AMX-300) as described previously (Houwen et al. 1991). The ^{13}C chemical shifts were referenced to the carbon-2 of propionate (31.6 ppm). Presented spectra are representative for the results of replicate experiments.

Analytical methods

Volatile fatty acids (VFA) and methane were measured by high-performance liquid chromatography and gas chromatography, respectively (Lens et al. 1996).

Chemicals

^{13}C-labeled compounds (> 99 atom% ^{13}C) were obtained from Isotec Inc. (Pixie Corporatie B.V., Tjuchem, The Netherlands).

Results

Effect of sulfate on propionate metabolism

The sulfide oxidizing sludge was cultivated at dissolved oxygen concentrations lower than 0.1 mg/l and it contained an active sulfate-reducing population (Janssen et al. 1997). In the presence of sulfate, the sludge converted [3-^{13}C]propionate to [2-^{13}C] propionate (Table 3) and to acetate which was equally labeled in the C_2 (Figure 2) as well as the C_1 position. Labeled intermediates of the propionate degradation route were not observed. Also during incubation of the sludge with [3-^{13}C]propionate at an excess of unlabeled acetate (10 mM) no intermediates were detected.

Table 3. Effect of sulfate on the degree of scrambling of sodium [3-^{13}C]propionate by sulfate reducing and sulfide oxidizing sludge after, respectively, 24h and 44h incubation

Sludge type[a]	Propionate degraded (%)	Degree of scrambling (%)
SULFATE REDUCING GRANULAR SLUDGE		
3-Propionate + sulfate	94.7	13.3
3-Propionate	N.D.	7.7
SULFIDE OXIDIZING SLUDGE		
3-Propionate + sulfate	61.2	32.0
3-Propionate	22.9	5.9

[a] Incubations were performed with 10 mM VFA and 30 mM sulfate.
N.D. Not determined.

When sulfate reducing granular sludge was incubated with [3-^{13}C]propionate as the sole substrate in the presence of sulfate, [2-^{13}C]propionate was formed during propionate degradation (Table 3), and both [1-^{13}C]acetate and [2-^{13}C]acetate accumulated in the medium. After 24 hours of incubation, the sulfate reducing granular sludge had converted 13.3% of the [3-^{13}C]propionate to [2-^{13}C]propionate (Table 3). Also all methanogenic granular sludges converted [3-^{13}C]propionate to [2-^{13}C]propionate, [1-^{13}C]acetate and [2-^{13}C]acetate in the presence of sulfate (data not shown).

In the absence of sulfate, little scrambling occurred in the sludges investigated, as evidenced by the small amount of [2-^{13}C]propionate formed from [3-^{13}C]propionate (Table 3). No (labeled) intermediates of propionate conversion and no acetate could be detected in these incubations in the absence of sulfate (data not shown).

Effect of butyrate on propionate metabolism

No labeled butyrate was formed out of [3-^{13}C] propionate when methanogenic granular sludge was incubated with equimolar quantities of [3-^{13}C]propionate and butyrate, neither in the presence nor in the absence of sulfate. In order to identify intermediates of propionate conversion, sludge was incubated with equimolar quantities of [2,3-^{13}C]propionate and butyrate. [2,3-^{13}C]propionate has typical doublet structures for the 2- and 3-carbon resonance positions, separated by 34 Hz due to spin-spin coupling between the two labeled carbon atoms. This typical doublet structure can facilitate the detection and assignment of peaks in a NMR spectrum. Besides the degradation product [1,2-

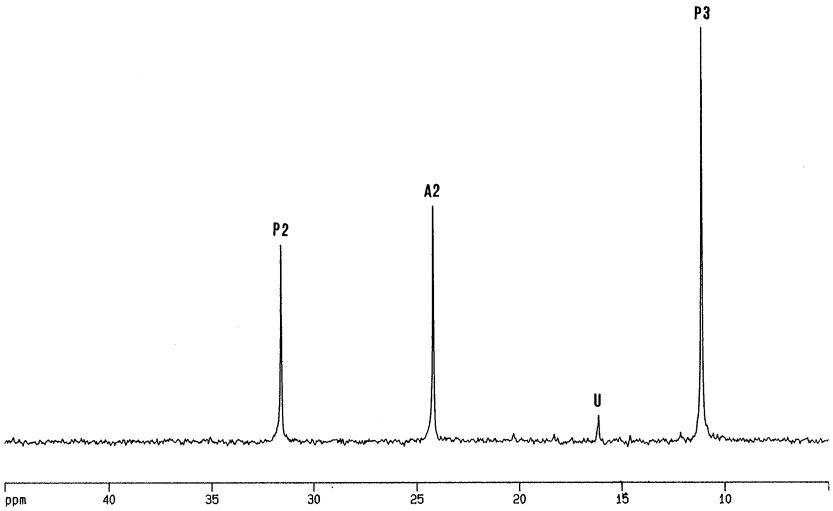

Figure 2. High resolution ^{1}H-decoupled ^{13}C NMR spectrum of medium sample from [3-^{13}C]propionate degradation by the sulfide oxidizing sludge recorded after 44h incubation. A, acetate; P, propionate; U, unidentified peak. The numbers following the one-letter abbreviations give the positions of the carbon in the molecule.

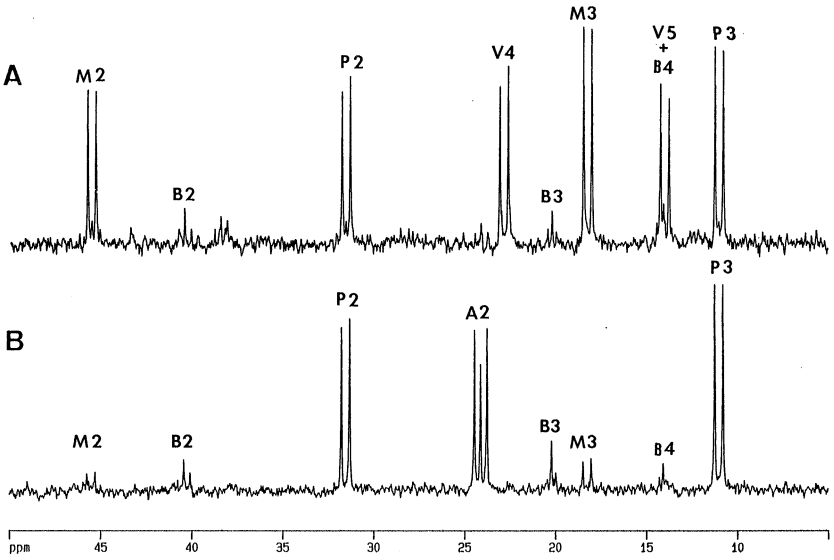

Figure 3. High resolution ^{1}H-decoupled ^{13}C NMR spectrum of medium samples from [2,3-^{13}C]propionate degradation by the methanogenic (A) and sulfate reducing (B) sludge in the presence of butyrate, recorded after 24h incubation. A, acetate; B, butyrate; M, methyl-butyrate; P, propionate; V, valerate. The numbers following the one-letter abbreviations give the positions of the carbon in the molecule.

^{13}C]acetate, label of [2,3-^{13}C]propionate appeared to be incorporated in [4,5-^{13}C]valerate and 2-methyl[2,3-^{13}C]-butyrate, both in the presence (Figure 3A) and the absence (data not shown) of sulfate.

Differences in feed composition (omission of glucose, presence of sulfate) of the methanogenic granular sludge did not alter the observed propionate conversion routes. In the presence of sulfate, [3-^{13}C]propionate scrambled faster. This corroborated with the higher metabolic activity of this sludge (data not shown). Independently of the feed composition, all methanogenic sludges produced [4,5-^{13}C]valerate and 2-methyl[2,3-^{13}C]-butyrate during the concomitant degradation of [2,3-^{13}C]propionate and butyrate. However, no labeled butyrate was formed.

The presence of butyrate diminished the degree of scrambling of [3-^{13}C]propionate (5.9% after 24 hours of incubation) into [2-^{13}C]propionate by the sulfate

reducing granular sludge. Both in the presence or absence of sulfate, labeled butyrate was not produced from [3-^{13}C]propionate and butyrate. Incubating the sulfate reducing granular sludge with equimolar quantities (10 mM) of [2,3-^{13}C]propionate and butyrate yielded double labeled acetate and 2-methyl[2,3-^{13}C]-butyrate, both in the presence (Figure 3B) or absence (data not shown) of sulfate.

Discussion

Propionate metabolism by sludges treating sulfate and sulfide rich wastewater

This ^{13}C-NMR study showed that propionate was converted to acetate via the randomising pathway in all the sludge types investigated. This was evidenced by scrambling of [3-^{13}C]propionate to [2-^{13}C]propionate (Figure 2) and acetate equally labeled in the C_1 and C_2 position. In this pathway, studied in propionate-oxidizing cultures of *Desulfobulbus propionicus* (Stams et al. 1984; Kremer & Hansen 1988) and in methanogenic enrichment cultures (Houwen et al. 1991), propionate is first carboxylated by a transcarboxylase reaction, and then degraded through the methylmalonyl-CoA pathway which involves succinate and fumarate as intermediates. The presence of the symmetrical 4-carbon compounds in the degradation pathway explains the exchange of label between the methyl and methylene group of propionate. The randomisation of the label is an energy-independent process, thus randomisation may also be performed by sulfate-reducing bacteria in the absence of sulfate (Table 3). Alternatively, randomisation in the absence of sulfate can be due to the activity of syntrophic associations, which use the same randomising pathway (Houwen et al. 1991).

Effect of butyrate on propionate metabolism

[2,3-^{13}C]propionate was converted into [4,5-^{13}C]valerate and 2-methyl[2,3-^{13}C]butyrate in the presence of butyrate (Figure 3). These two higher VFA have been observed during the concomitant degradation of propionate and butyrate in methanogenic sludges (Wu et al. 1993) and defined tricultures (Wu et al. 1994). Previously, the same labelling pattern of 2-methyl-butyrate and valerate as found in this study was observed in a methanogenic flocculant sludge (Lens et al. 1996). It is unlikely that the synthesis of these higher fatty acids is accomplished by the respiration of sulfate reducing bacteria as their formation occurred both in the presence and absence of sulfate.

The conversion of propionate to butyrate, i.e. by reductive carboxylation (Tholozan et al. 1988), was not observed in the anaerobic sludges investigated. Reductive carboxylation, in contrast to the randomising pathway, preserves the carbon skeleton of the propionate molecule, i.e. [3-^{13}C]propionate is converted into [4-^{13}C]butyrate, and [2,3-^{13}C]propionate to [3,4-^{13}C]butyrate (Figure 1B). This labeling pattern of butyrate was not detected in any of the spectra recorded. Surprisingly, the propionate skeleton was incorporated into the valerate molecule similar to its incorporation into butyrate during reductive carboxylation, i.e. [2,3-^{13}C]propionate was converted into [4,5-^{13}C]valerate (Figure 3). Hence, a more general reaction mechanism might be involved. The reductive carboxylation of propionyl-CoA to oxobutyrate with the ultimate formation of butyrate (Tholozan et al. 1988) resembles the reductive carboxylation of acetyl-CoA to pyruvate. The latter reaction mechanism is omnipresent in *eubacteria* (Gottschalk 1985). More research with enrichment or pure cultures is required for the elucidation of the exact biochemical pathways of the interactive degradation of propionate and butyrate.

Acknowledgments

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