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# Anaerobic degradation of sorbic acid by sulfate-reducing and fermenting bacteria: pentanone-2 and isopentanone-2 as byproducts

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*Key words:* anaerobic degradation, β-oxidation, decarboxylation, ketones, sorbic acid

**Abstract**

Strictly anaerobic bacteria were enriched and isolated from freshwater sediment sources in the presence and absence of sulfate with sorbic acid as sole source of carbon and energy. Strain WoSo1, a Gram-negative vibrioid sulfate-reducing bacterium which was assigned to the species *Desulfoarculus* (formerly *Desulfovibrio*) *baarsii* oxidized sorbic acid completely to $CO_2$ with concomitant stoichiometric reduction of sulfate to sulfide. This strain also oxidized a wide variety of fatty acids and other organic compounds. A Gram-negative rod-shaped fermenting bacterium, strain AmSo1, fermented sorbic acid stoichiometrically to about equal amounts of acetate and butyrate. At concentrations higher than 10 mM, sorbic acid fermentation led to the production of pentanone-2 and isopentanone-2 (3-methyl-2-butanone) as byproducts. Strain AmSo1 fermented also crotonate and 3-hydroxybutyrate to acetate and butyrate, and hexoses to acetate, ethanol, hydrogen, and formate. The guanine-plus-cytosine content of the DNA was 41.8 ± 1.0 mol%. Sorbic acid at concentrations higher than 5 mM inhibited growth of this strain while strain WoSo1 tolerated sorbic acid up to 10 mM concentration.

## Introduction

Sorbic acid (hexa-2,4-dienoic acid) is present in many fruits, e.g. in rowanberries (*Sorbus aucuparia*) and strawberries (Bayer & Walter 1988). Synthetical sorbic acid is used as antibacterial and antifungal food additive in cheese, yoghurt, and conserved meat. Enterobacteriaceae, staphylococci and pseudomonads are inhibited by sorbic acid at concentrations of 50–100 μg/ml (Russell & Gould 1988). Like benzoic acid, sorbic acid affects the proton motive force and accelerates proton movement from the medium into the cytoplasm, as studies with *Escherichia coli* revealed (Salmond et al. 1984; Eklund 1985). Other effects were described for *Clostridium sporogenes,* which can basically be attributed to protonophoric activity as well: sorbic acid inhibited uptake of essential amino acids, decreased the rate of protein synthesis, altered the patterns of phosphorylated nucleotides (GTP and ppGpp) and caused drastic morphological changes at 50–200 mM concentrations (Ronning & Frank 1987, 1989). In *Bacillus subtilis,* uptake of serine or *a*-amino-isobutyric acid is inhibited by sorbic acid (Freese et al. 1973), whereas yeasts are inhibited in phosphate uptake (Borst-Pauwels & Jager 1969). Some enzymes of bacterial carbohydrate metabolism like enolase and lactic dehydrogenase are directly inhibited by sorbic acid; perhaps essential sulfhydryl groups of these enzymes are masked or

oxidized by the conjugated double bonds of sorbic acid (Fulgraff 1989). Certain other bacteria such as lactic acid bacteria appear to be unaffected by sorbic acid.

Except for its toxicity, sorbic acid does not pose any basic problems to microbial degradation, especially in the presence of oxygen or nitrate as electron acceptor but no detailed studies on sorbic acid degradation have been published so far. In the present communication we report on degradation of sorbic acid by strictly anaerobic sulfate-reducing and fermenting bacteria.

## Materials and methods

### *Organisms and cultivation*

Enrichment cultures were inoculated with black sediment samples from two polluted freshwater creeks near Konstanz, Germany (Wollmatinger Graben) and near Tübingen, Germany (Ammerkanal), as well as with a marine sediment sample taken from Rio della Pergola, a canal in the city of Venice, Italy. *Methanospirillum hungatei* strain M1h was obtained from Prof. Dr. F. Widdel, München, Germany.

All procedures for cultivation were essentially as described by Widdel & Pfennig (1981). The mineral medium for enrichment, isolation, and cultivation contained 30 mM sodium bicarbonate buffer, 1 mM sodium sulfide as reducing agent, the trace element solution SL 10 (Widdel et al. 1983) and a 7 vitamin solution (Widdel & Pfennig 1981). Freshwater medium contained per liter 0.5 g NaCl and 0.4 g $MgCl_2 \times 6H_2O$, saltwater medium 20.0 g and 3.0 g, respectively. The pH was 7.1–7.3, and the incubation temperature 30° C. For isolation of pure cultures, the agar shake dilution method was applied (Pfennig 1978). Gram staining was carried out after Magee et al. (1975).

### *Enzyme measurements*

Carbon monoxide dehydrogenase (Diekert & Thauer 1978) and 2-oxoglutarate synthase (Brandis-Heep et al. 1983) were measured with methyl viologen as electron acceptor under strictly anoxic conditions with cell-free extracts prepared by French pressure treatment of fresh cell material (for details see Schnell & Schink 1991). Protein was quantified after Bradford (1976).

### *Fermentation product analysis*

Fatty acids and ketones were identified and quantified with a Vega 6000 gas chromatograph (Carlo Erba Strumentazione, Milano, Italy) equipped with a 2 m × 2 mm glass column packed with 60/80 Carbopak C/0.3% Carbowax 20 M/0.1% $H_3PO_4$ (Supelco Inc., Bellefonte, PA, USA), injector and detector temperature 200° C, temperature program 80–160° C, carrier gas nitrogen at 40 ml/min (modified after Schink & Pfennig 1982). Samples were acidified with formic acid (0.5 M final concentration) prior to injection.

### *HPLC-Analysis of sorbic acid*

Samples (100 µl) were preserved by injection into 1 ml glass vials containing 400 µl of 0.1 M $H_3PO_4$, and stored at − 20° C prior to analysis. Samples were analyzed with a System Gold high-pressure liquid chromatograph (Beckman Instruments, München, Germany) on an Ultrasphere-ODS column (4.6 × 150 mm), using a 50% 100 mM ammonium phosphate 50% methanol sovent system (pH 2.6). Samples (20 µl) were injected with a Spark Promis II autosampler (Beckman Instruments, München, Germany) and eluted at a flow rate of 1 ml/min. Sorbic acid was detected in a variable-wavelength detector (Beckman type 166) at 263 nm. Data were analyzed by a computer program and quantified by comparison with external standards.

### *Identification of pentanones by mass spectrometry*

Fermentation products in 10 ml culture supernatant of strain AmSo1 were purified and concentrat-

ed by solid phase extraction using C-18 Bond Elut column (ict Handels GmbH, Frankfurt). The columns were conditioned with 2 × 0.5 ml methanol and 2 × 0.5 ml $H_2O$ and eluted with 2 × 0.5 ml methanol. Analysis was carried out on a gas chromatography mass spectrometry system (Hewlett Packard HP 5890/5971 A, electron ionization at 70 eV) equipped with a HP 1 column (dimethyl polysiloxane), 25 m × 0.2 mm × 0.33 μm in size, column pressure 50 kPa. The temperature of the injector was 270° C, the MS interface was kept at 280° C and the ion source at 170° C.

*Analysis of DNA*

The guanine-plus-cytosine content of the DNA was determined by the thermal denaturation method according to DeLey (1970) after extraction as described by Marmur (1961).

*Chemicals*

All chemicals used were of analytical grade quality and obtained from Fluka, Neu Ulm, Merck, Darmstadt, and Sigma, München, Germany. $H_2$, $N_2$ and $N_2/CO_2$ gas mixtures were obtained from Messer Griesheim, Ludwigshafen and CO from Linde, Unterschleißheim, Germany.

## Results

*Enrichment, isolation and morphology of sorbate-degrading anaerobes*

Enrichment cultures with black anoxic mud from a ditch near Konstanz, Germany, in freshwater mineral medium containing 2 mM sorbic acid and 20 mM sulfate showed sulfide production within three weeks. After repeated transfers in the same medium, motile vibrioid bacteria dominated. These bacteria were isolated in two subsequent agar dilution series with 2 mM sorbic acid and 20 mM sulfate. Yellowish-brown, irregularly shaped colonies developed within four weeks and consisted of the motile vibrios. After subjection to a further agar dilution series, strain WoSo1 was isolated.

In enrichment cultures with saltwater medium inoculated with sediment of a canal in Venice, Italy, with 2 mM sorbic acid and 20 mM sulfate, a diverse bacterial population developed rather slowly. These cultures were not characterized any further. Enrichment cultures with sulfate-free freshwater medium inoculated with sediment from a polluted freshwater creek near Tübingen exhibited methane formation within 3 weeks of incubation. After two subsequent transfers, the predominant bacterial type, strain AmSo1, was isolated by agar shake dilutions.

Cells of strain WoSo1 were curved rods, 2–4 μm long and 0.5 μm in diameter (Fig. 1a). Cells were motile and rotated around the long cell axis both in young and old cultures. They stained Gram-negative; spores could not be detected.

Cells of strain AmSo1 were short straight rods, 0.6 × 1.0–1.5 μm in size, after growth with sorbic acid (Fig. 1b). Glucose-grown cells were wider (0.8 μm) and up to 5 μm long (Fig. 1c). Mutual checks were made by agar shake dilutions with both substrates to make sure that sorbate-grown and glucose-grown cells were bacteriologically identical. Cells stained Gram-negative and did not form spores.

*Growth physiology and stoichiometry of substrate degradation by strain WoSo1*

Strain WoSo1 grew only in freshwater medium. No growth occurred in brackish water medium or saltwater medium. The pH range of growth was pH 6.8 to 7.6, with an optimum at pH 7.2. No growth was observed below 20° C or above 35° C; best growth occurred at 28° C.

Sorbic acid was oxidized completely to $CO_2$ with concomitant reduction of sulfate to sulfide. No inhibition of growth (initial rates) was observed up to 10 mM sorbic acid. Substrate-dependent growth and sulfide formation are shown in Fig. 2. In Table 1, the stoichiometry of sorbic acid oxidation by strain WoSo1 is documented. The molar ratio of

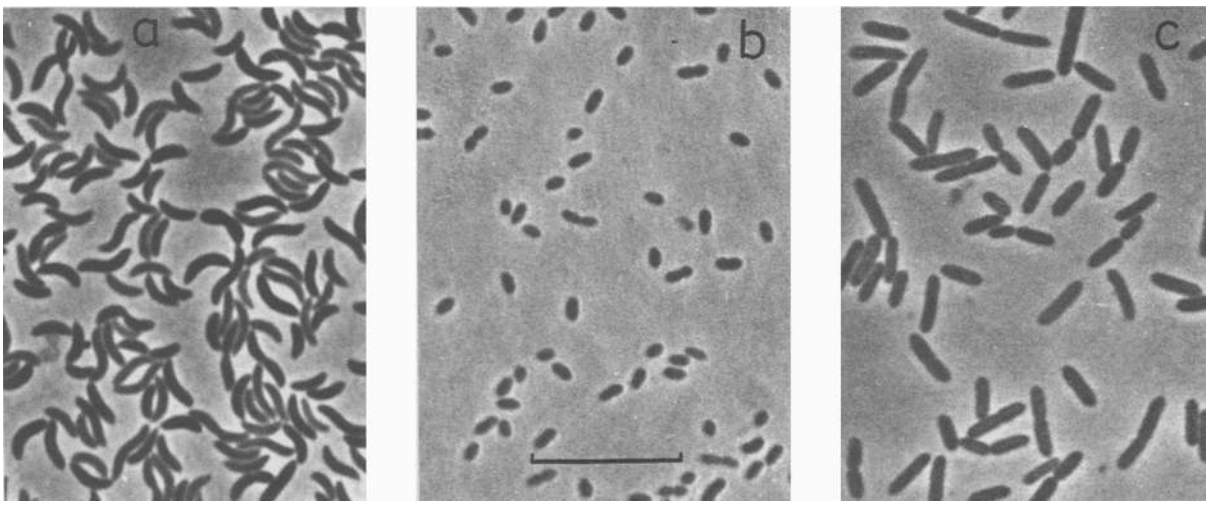


*Fig. 1.* Phase-contrast photomicrograph of the new isolates. (a) Strain WoSo1, grown with 2 mM sorbic acid and 20 mM sulfate. (b) Strain AmSo1, grown with 5 mM sorbate. (c) Strain AmSo1, grown with 2 mM glucose. Bar equals 10 $\mu$m for all panels.

sorbic acid dissimilated over sulfide formed was 1 : 3.5. The growth yield determined was 39.7 g per mol sorbic acid, and the doubling time 55 h ($\mu$ = 0.013 $h^{-1}$) for growth with sorbic acid. Strain WoSo1 grew also with fatty acids with chain lengths of $C_1$–$C_8$ and with the unsaturated fatty acids crotonate and trans-2-hexanoate. Further substrates supporting growth were pyruvate, lactate, and aliphatic alcohols. Dicarboxylic acids were not degraded (Table 1).

The pathway of acetate oxidation by strain

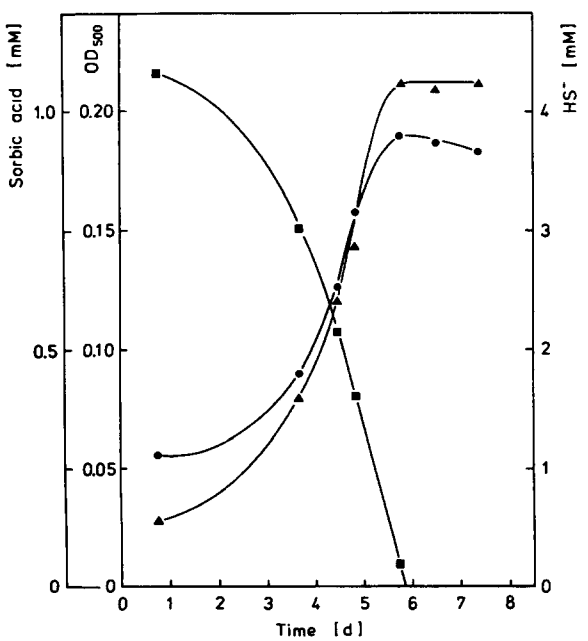


*Fig. 2.* Time course of sorbic acid degradation by strain WoSo1. (■) sorbic acid, (●) optical density, (▲) sulfide.

*Table 1.* Stoichiometry of sorbic acid oxidation by strain WoSo1.

| | |
|---|---|
| Sorbic acid degraded [mmol] | 1.08 |
| Sulfide produced [mmol] | 3.68 |
| Net final $OD_{500}$ | 0.135 |
| Dry matter formed [mg] | 29.2 |
| Sorbic acid assimilated[a] [mmol] | 0.17 |
| Sorbic acid dissimilated [mmol] | 0.91 |

[a] Substrate assimilated for cell matter synthesis was calculated by the following equation:

17 $C_6H_8O_2$ + 10 $CO_2$ + 30 $H_2O$ → 18 <$C_4H_7O_3$>

5.95 mmol sorbic acid is assimilated into 1 g of dry cell matter.

Other substrates supporting growth (mM concentrations in brackets): Formate (7), acetate (10), propionate (10), butyrate (5), valerate (5), caproate (4), heptanoate (1.5), caprylate (1), lactate (10), pyruvate (10), crotonate (5), trans-2-hexenoate (2), n-propanol (5), n-butanol (5).

Substrates not supporting growth: Maleinate (3), succinate (5), fumarate (5), malate (5), glutarate (3), adipate (2), pimelate (1), acrylate (5), ethanol (10), 1,3-propanediol (5).

WoSo1 was studied by measurement of key enzymes in cell-free extracts. Carbon monoxide dehydrogenase was found at high activity (11.5 μmol CO per min and mg protein); 2-oxoglutarate synthase activity could not be detected with methyl viologen as electron acceptor.

### *Growth physiology and stoichiometry of substrate degradation by strain AmSo1*

Strain AmSo1 was strictly anaerobic, catalase and oxidase negative, and did not contain cytochromes as visualized in redox difference spectra of crude cell extracts. The guanine-plus-cytosine content of the DNA was 41.8 ± 1.0 mol%.

Sorbate was fermented to about equal amounts of acetate and butyrate, and a small amount of a further product which was later identified as pentanone-2 (Fig. 3). The doubling time was 33 h ($\mu$ = 0.021 $h^{-1}$) during growth with sorbic acid. Other substrates supporting growth were 3-hydroxybutyrate and crotonate which were fermented to acetate and butyrate, with traces of acetone (Table 2). Fructose and glucose were fermented to acetate, ethanol, formate, and traces of hydrogen.

Two unknown byproducts of sorbic acid degra-

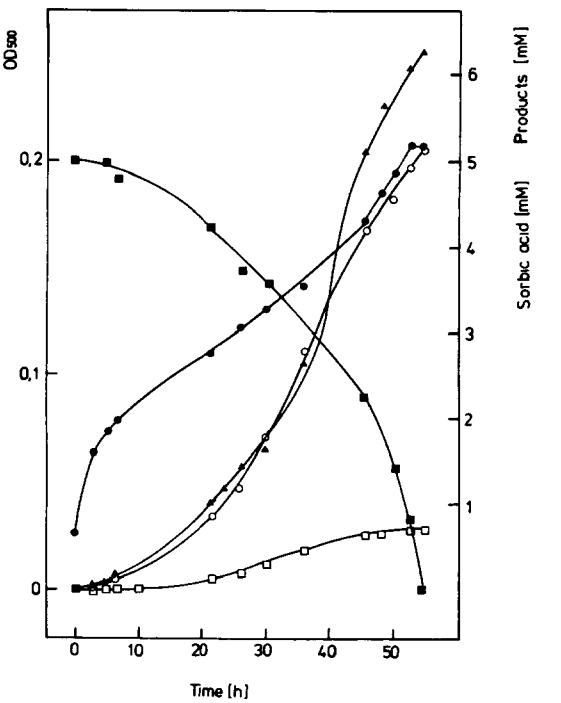


*Fig. 3.* Time course of sorbic acid degradation by strain AmSo1. (■) sorbic acid, (●) optical density, (▲) acetic acid, (○) butyric acid, (□) pentanone-2.

*Table 2.* Growth and stoichiometry of substrate degradation by strain AmSo1.

| Substrate | Concentration (mM) | net $OD_{500}$ | Cell[a] matter formed (mg/ml) | Substrate assimil. (mM)[b] | Products formed (mM) | | | | $H_2$ | Formate | Electron balance (%) | Growth yield (g/mol) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Acetate | Butyrate | Pentanone | Ethanol | | | | |
| Sorbate | 4 | 0.117 | 30.7 | 0.18 | 4.34 | 3.38 | 0.02 | – | – | – | 96.2 | 8.6 |
| Sorbate | 5 | 0.156 | 41.0 | 0.24 | 4.8 | 4.2 | 0.04 | – | – | – | 92.5 | 8.7 |
| 3-Hydroxybutyrate | 10 | 0.194 | 51.0 | 0.46 | 12.85 | 3.14 | –[c] | 0.91 | 0.17 | – | 98.3 | 5.4 |
| Crotonate | 5 | 0.134 | 35.3 | 0.32 | 5.28 | 1.57 | –[c] | 0.21 | 0.07 | – | 90.4 | 7.7 |
| Glucose | 5 | 0.450 | 118.5 | 1.07 | 2.61 | – | – | 4.97 | 0.16 | 6.9 | 100.3 | 20.5 |
| Fructose | 5 | 0.390 | 102.7 | 0.93 | 3.11 | – | – | 4.41 | 0.14 | 6.6 | 94.7 | 17.8 |

Growth with acetoin was possible as well, however, growth was only very low and substrate degradation was incomplete.

[a] Cell dry matter formed was calculated via the experimentally determined conversion factor 0.1 $OD_{500}$ = 26.3 mg cell mass per liter.

[b] Substrate assimilation into cell material was calculated after the equation 17 $C_6H_8O_2$ + 10 $CO_2$ + 30 $H_2O$ → 28 [$C_4H_7O_3$].

[c] Small amounts of acetone (< 0.07 mM) were found as byproduct.

Substrates not supporting growth (mM concentrations in brackets): Methanol (10), ethanol (10), propanol (10), ethylene glycol (10), glycerol (10), 2,3-butanediol (10), formate (20), methoxyacetate (10), lactate (10), acrylate (2), malate (10), betaine (10), choline (10), arabinose (5), xylose (5), phloroglucinol (5), pyrogallol (5), $H_2/CO_2$ (80%/20%).

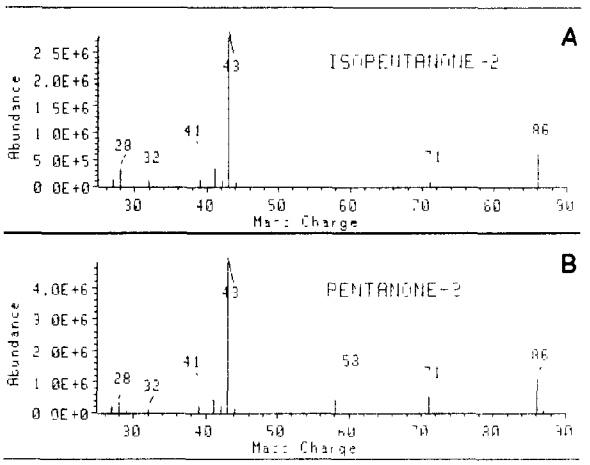


*Fig. 4.* Mass spectrometric fragmentation patterns of by-products of sorbate fermentation by strain AmSo1. (A) Isopentanone-2 (3-methyl-2-butanone), (B) pentanone-2.

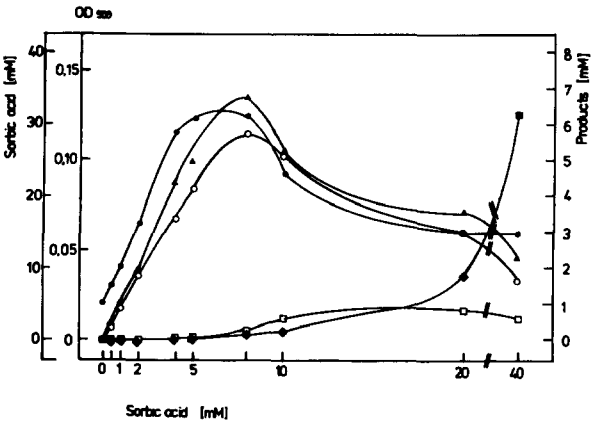


*Fig. 5.* Growth and product formation pattern by strain AmSo1 with increasing amounts of sorbate provided. (■) Sorbic acid, (●) optical density, (▲) acetic acid, (○) butyric acid, (□) pentanone-2.

dation which showed up as a small and a large peak ($t_r$ at 1.06 min and 1.38 min) in gas chromatographic fatty acid analysis were identified by gas chromatography mass spectrometry. The fragmentation patterns (Fig. 4) were compatible with isopentanone-2 and pentanone-2, respectively. Isopentanone-2 made up less than 20% of total pentanone formed.

Strain AmSo1 exhibited substrate-dependent growth and product formation up to about 5 mM sorbate; at higher concentrations, growth and substrate conversion were inhibited, and pentanone-2 accumulated up to 0.8 mM concentration (Fig. 5). In mixed cultures of strain AmSo1 with *Methanospirillum hungatei,* 10 mM butyrate was not oxidized during more than 2 months of incubation.

## Discussion

### *Anaerobic degradation of sorbate*

In the present publication, degradation of sorbate by strictly anaerobic bacteria was studied in detail for the first time. Two different types of anaerobes were found to degrade this substrate: a sulfate-reducing bacterium oxidized it completely to $CO_2$, according to the equation:

$$2\ C_6H_7O_2^- + 7\ SO_4^{2-} + 9\ H^+ \rightarrow 12\ CO_2 + 7\ HS^- + 8\ H_2O,$$

and a fermenting bacterium converted sorbate mainly to about equal amounts of acetate and butyrate:

$$C_6H_7O_2^- + 2\ H_2O \rightarrow C_2H_3O_3^- + C_4H_7O_2^- + H^+.$$

Both bacteria isolated tolerated this bacteriostatic agent only up to concentrations around 10 mM (= 1.12 g/l) without significant impairment of energy metabolism; higher concentrations caused significant decreases in cell yields. Obviously, sorbate is a rather potent agent also against these sorbate-degrading bacteria.

### *Biochemistry of sorbate degradation*

The double bonds in the sorbate molecule are localized in a pattern well suited for degradation through classical β-oxidation. After binding to coenzyme A, hydration and further oxidation to acetyl-CoA units should not pose any basic difficulties. However, it cannot be ruled out at present that hydration occurs with the non-activated acid, and that the β-hydroxy acid is activated afterwards. The

sulfate-reducing bacterium oxidizes the three acetyl residues to $CO_2$, as most acetate-oxidizing sulfate reducers do (Widdel 1988), through the noncyclic carbon monoxide pathway (Schauder et al. 1986) as presence of this enzyme and lack of 2-oxoglutarate synthase activity indicate.

With the fermenting bacterium AmSo1, the initial reactions are probably the same as discussed above, and the unsaturated acid derivative is subsequently dismutated to acetate and butyrate (Fig. 6), analogous to crotonate dismutation by *Ilyobacter polytropus* (Stieb & Schink 1984) or *Ilyobacter delafieldii* (Janssen & Harfoot 1990), or to crotonate or sorbate dismutation by *Syntrophomonas wolfei* (Beaty & McInerney 1990). An unexpected finding was the formation of pentanone-2 and isopentanone-2 (3-methyl-2-butanone) as byproducts, especially at enhanced substrate concentrations. Formation of pentanone-2 can be easily explained through a sidepass of sorbate dismutation (Fig. 6): Reduction of the intermediate 3-oxy-4-hexenoic acid and subsequent decarboxylation leads directly to this product:

$$C_6H_7O_2^- + H^+ + H_2O \rightarrow C_5H_{10}O + CO_2$$
$$\Delta G^{\circ\prime} \approx -75 \text{ kJ} \cdot \text{mol}^{-1}$$

This transformation is probably not coupled to energy conservation, and can be understood as a side reaction occurring especially when the cellular energy metabolism is partly uncoupled, e.g. by sorbate at enhanced concentrations.

Formation of small amounts of isopentanone-2 together with pentanone-2 can be understood as an isomerization of the butyryl constituent which would be released by thiolytic cleavage of 3-oxohexanoyl-CoA. Butyrate-isobutyrate and reverse isomerisation have been observed in anaerobic enrichment cultures from sludge samples (Tholozan et al. 1988) and a pure culture of a recently isolated strict anaerobe (Matthies & Schink 1991). The reaction is catalyzed by a coenzyme $B_{12}$-dependent acyl-CoA mutase enzyme (Matthies & Schink 1991). Excretion of significant amounts of isopentanone together with *n*-pentanone indicates that the various activated fatty acid residues involved in

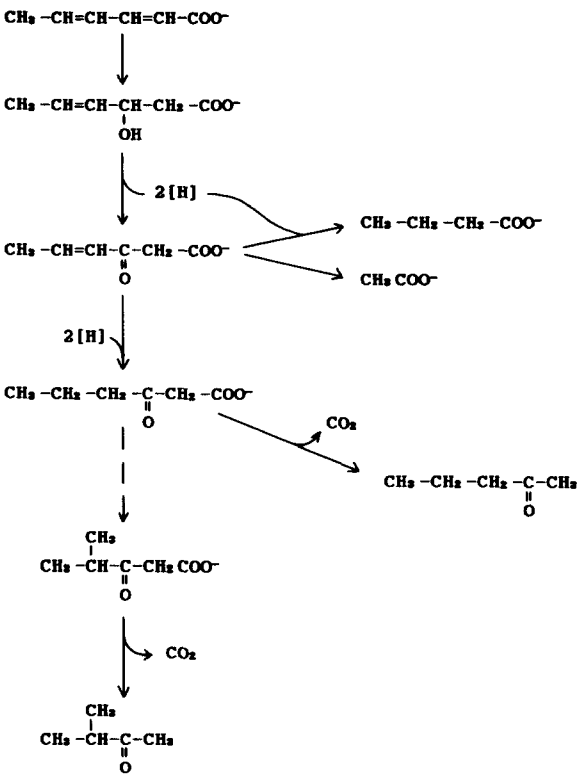


*Fig. 6.* Hypothetical pathway of sorbate degradation by strain AmSo1. All conversions of acid residues occur probably with the respective coenzyme A derivatives.

sorbate degradation exchange actively, probably via CoA transferases.

### *Taxonomy*

On the basis of its morphology and the pathway of acetate oxidation, strain WoSo1 can be affiliated with *Desulfoarculus* (formerly *Desulfovibrio) baarsii* (Widdel & Bak 1991). Also the capacity to utilize numerous fatty acids agrees with this affiliation. However, strain WoSo1 degraded also alcohols, lactate and puryvate, and differs with this from *D. baarsii.* The type strain of *D. baarsii* was tested for growth with sorbic acid, too, and good growth was observed. Therefore, strain WoSo1 should be affiliated with *D. baarsii.*

Strain AmSo1 is less easy to affiliate. As a Gram-negative strict anaerobe, it belongs to the family

Bacteroidaceae. Physiologically, it resembles most *Ilyobacter polytropus* and *I. delafieldii,* however, the guanine-plus-cytosine content of these species (29–32%) are too low to assign the new isolate to this genus. Strain AmSo1 differs from the also sorbate-dismutating *Syntrophomonas wolfei* (Beaty & McInerney 1990) by its inability to oxidize saturated fatty acids in syntrophic cooperation with methanogens. Final taxonomic assignment of this strain will be postponed until more data on its relationship to members of other taxa are at hand.

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