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Anaerobic degradation of 3-hydroxybenzoate by a newly isolated nitrate-reducing bacterium

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1. SUMMARY

A Gram-negative nitrate-reducing bacterium, strain Asl-3, was isolated from activated sludge with nitrate and 3-hydroxybenzoate as sole source of carbon and energy. The new isolate was facultatively anaerobic, catalase- and oxidase-positive and polarly monotrichously flagellated. In addition to nitrate, nitrite, N_2O, and O_2 served as electron acceptors. Growth with 3-hydroxybenzoate and nitrate was biphasic: nitrate was completely reduced to nitrite before nitrite reduction to N_2 started. Benzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, protocatechuate or phenyl-acetate served as electron and carbon source under aerobic and anaerobic conditions. During growth with excess carbon source, poly-β-hydroxybutyrate was formed. These characteristics allow the affiliation of strain Asl-3 with the family Pseudomonadaceae. Analogous to the pathway of 4-hydroxybenzoate degradation in other bacteria, the initial step in anaerobic 3-hydroxybenzoate degradation by this organism was activation to 3-hydroxy-benzoyl-CoA in an ATP-consuming reaction. Cell extracts of 3-hydroxybenzoate-grown cells exhibited 3-hydroxybenzoyl-CoA synthetase activity of 190 nmol min^{-1} mg $protein^{-1}$ as well as benzoyl-CoA synthetase activity of 86 nmol min^{-1} mg $protein^{-1}$. A reductive dehydroxylation of 3-hydroxybenzoyl-CoA could not be demonstrated due to rapid hydrolysis of chemically synthesized 3-hydroxybenzoyl-CoA by cell extracts.

2. INTRODUCTION

Anaerobic degradation of all isomers of monohydroxybenzoate has been reported for various strains of bacteria [1]. However, only few anaerobic organisms are known to oxidize 3-hydroxybenzoate with either nitrate [2] or sulfate [3] as electron acceptor. Fermentative degradation of 3-hydroxybenzoate was demonstrated in defined syntrophic co-cultures with either *Desulfovibrio*

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vulgaris or *Methanospirillum hungatei* as hydrogen scavengers [4]. Nothing is known about the pathway of anaerobic degradation of 3-hydroxybenzoate, whereas aerobic metabolism has been studied extensively [5].

In this communication we report on isolation and characterization of a new 3-hydroxybenzoate-degrading, nitrate-reducing bacterium and demonstrate the initial activation of 3-hydroxybenzoate in cell extracts.

3. MATERIAL AND METHODS

3.1. Media and growth conditions

For anaerobic cultivation an anoxic, bicarbonate-buffered mineral medium was prepared as previously described [6], with the exception that sulfide was substituted by 2 mM Na_2SO_4 as sulfur source. Aromatic substrates were added from filter-sterilized stock solutions stored under N_2-atmosphere. Cultures were incubated statically in the dark at 30 °C.

For aerobic cultivation, a phosphate-buffered mineral medium prepared according to Weimer and Zeikus [7], supplemented with vitamin and trace element solutions [6], was used. Cultures were incubated at 30 °C on a rotary shaker (100 rpm).

3.2. Isolation and characterization

Pure cultures were obtained by repeated application of the agar-shake dilution method [8]. Growth was followed spectrophotometrically at 420 nm directly in the culture tubes or in 1-ml cuvettes. Gram-typing [9,10], flagella staining [11], oxidase and catalase tests [12] and PHB determination [13] were performed following standard procedures. Cytochromes were assayed in the soluble protein fraction and in membrane preparations obtained by ultracentrifugation (1 h at 150000 $\times g$) by redox difference spectroscopy [14]. For electron microscopy, glutaraldehyde-fixed cells were prepared according to Wolburg and Bolz [15].

3.3. Enzyme assays

Cell extracts (0.3 mg protein ml^{-1}) were prepared as previously described [16]. Acyl-CoA synthetase assays were performed by discontinuously measuring CoA ester formation by HPLC [17]. Assay mixtures (1 ml) contained 0.5 mM aromatic acid, 1 mM ATP, 0.5 mM CoA and 2.5 mM $MgCl_2$ in 50 mM sodium phosphate buffer (pH 7.2) and were incubated at room temperature [17].

3.4. Chemical analyses

Nitrate [18], nitrite [19], and ammonium [20] were determined as described. ATP, AMP, CoA, aromatic acids and their CoA esters were quantitated by HPLC analysis [17]. 3-Hydroxybenzoyl-CoA was synthesized, identified and quantified according to Schnell and Schink [17].

4. RESULTS AND DISCUSSION

4.1. Enrichment and isolation

Enrichment cultures in mineral salts medium containing 5 mM nitrate and either 2.5 mM benzoate, 2-hydroxybenzoate, 3-hydroxybenzoate, or 4-hydroxybenzoate, were inoculated with 10% activated sludge from the municipal sewage plant, Falmouth, MA. Turbidity developed within 10 days in all enrichment cultures, except for the enrichment with 2-hydroxybenzoate. Subcultures showed turbidity within less than 3 days. After several transfers, and subsequent agar dilution series, two dominating colony types appeared in all enrichments: lense-shaped, beige and white, diffuse colonies. From a beige colony of the 4-hydroxybenzoate enrichment, strain Asl-3 was isolated by two subsequent agar dilution series. Purity was checked by microscopy, and after growth in complex medium (AC medium, Difco, Detroit, MI).

4.2. Morphological and cytological characterization

Cells of strain Asl-3 were short motile rods of varying length (0.8 × 1–2.5 μm), containing light-refracting inclusions (Fig. 1A). In electron micrographs of ultra-thin sections, these inclusions appeared as large electron-permeable granules. As chemical analysis revealed, cells contained poly-β-hydroxybutyrate (PHB) up to 5% of their dry weight; these inclusions were probably

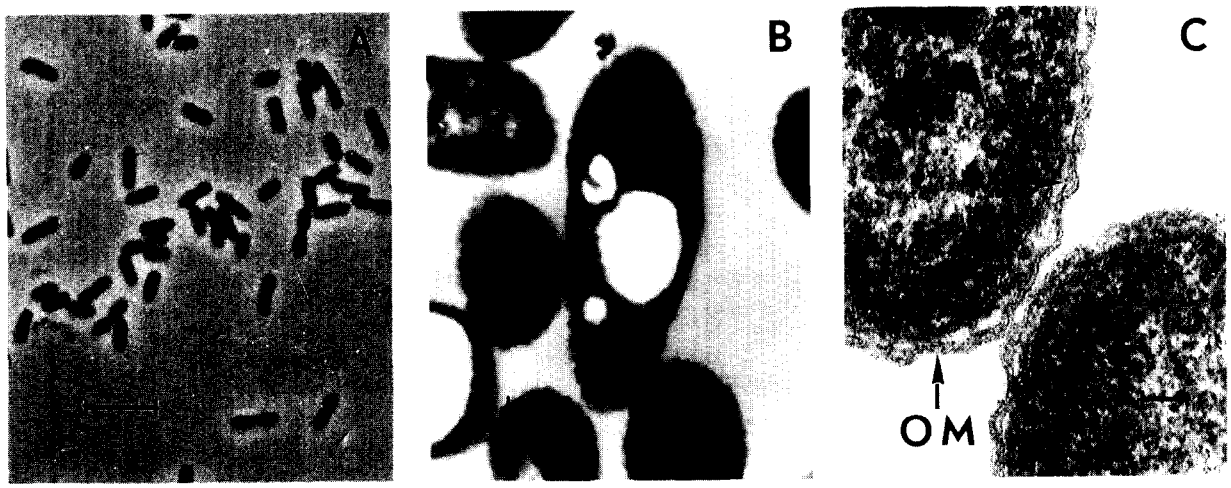

Fig. 1. **A.** Phase contrast photomicrograph of strain Asl-3. Bar equals 5 μm. **B.** Electron micrograph of ultra-thin section showing different types of granules. Bar equals 0.2 μm. **C.** Electron micrograph of ultra-thin section showing cell wall structure (OM, outer membrane; CM, cytoplasmic membrane; M, murein). Bar equals 50 nm.

PHB granules. In addition, ultra-thin sections showed smaller, electron-dense inclusions most likely representing polyphosphate granules (Fig. 1B). Spores were never observed. Strain Asl-3 possesses a Gram-negative cell wall structure (Fig. 1C), which was confirmed by results of Gram-staining and KOH test. Flagella staining revealed one polarly inserted flagellum. Strain Asl-3 was catalase- and oxidase-positive. The absorption bands of the soluble fraction at 420 nm, 523 nm, 553 nm, and of the membrane fraction at 425 nm, 522 nm and 553 nm of cells grown on 3-hydroxybenzoate and nitrate, indicated the presence of *b*- and *c*-type cytochromes.

Morphological and physiological characteristics (Gram-negative rod with one polarly inserted flagellum, catalase- and oxidase-positive, a strictly respiratory metabolism, and the ability to form PHB and to reduce nitrate) allow affiliation of strain Asl-3 with the family Pseudomonadaceae [21].

4.3. Physiological characterization

When strain Asl-3 was grown anaerobically with 2.5 mM 3-hydroxybenzoate and 5 mM nitrate, a biphasic growth curve was observed (Fig. 2). Nitrate was first completely reduced to nitrite, which was then further reduced to nitrogen. The respective growth rates were 0.08 h^{-1} and 0.02 h^{-1}. 3-Hydroxybenzoate was not completely oxidized because strain Asl-3 was grown under nitrate limitation to prevent nitrite intoxication. No ammonium formation was detected. In addition to nitrate, nitrite, N_2O and O_2 could serve as electron acceptors. No growth occurred in the absence of these electron acceptors. Strain Asl-3 grew at all temperatures between 16 °C and 37 °C, with a pH range of 6.6–9.2. Growth was optimal at 37 °C and pH 8.2–8.8. Phosphate concentrations up to 50 mM did not influence growth yields.

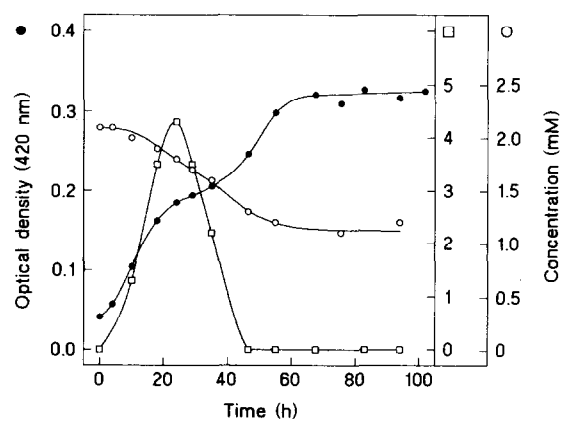

Fig. 2. Growth of strain Asl-3 with 3-hydroxybenzoate and 5 mM nitrate. Symbols, ●, cell density; ○, 3-hydroxybenzoate; □, nitrite.

Table 1

Molar growth yields (Y_s) and stoichiometry of 3-hydroxybenzoate (3-HB) degradation by strain Asl-3

Substrate degraded		Substrate assimilated		Products formed				Electron recovery	Y_s 3-HB	Y_s Nitrate
3-HB [a]	Nitrate [a]	Dry cell matter [b]	3-HB [c]	CO_2	NO_2^-	NH_4^+	N_2			
1.1 mM	4.3 mM	47 mg	0.28 mM	n.d. [d]	0 mM	0 mM	n.d.	96%	43 g/mol	11 g/mol

[a] Substrate consumed was calculated from the difference of substrate concentration at the beginning and the end of the experiment. Cultures were grown under nitrate limitation.

[b] Dry cell matter was calculated from culture turbidity using a conversion factor OD 1 at 420 nm = 167 mg dry matter l^{-1}, which was determined gravimetrically in 500-ml cultures.

[c] Substrate assimilation into cell matter was calculated after the following assimilation equation: $17\ C_7H_5O_3^- + 10\ H^+ + 54\ H_2O \rightarrow 28\langle C_4H_7O_3\rangle + 7\ HCO_3^-$; thus, 9.7 μmol 3-hydroxybenzoate was incorporated into 1 mg cell matter.

[d] n.d., not determined.

Growth yields and stoichiometry of 3-hydroxybenzoate degradation are documented in Table 1. The experimental results are in agreement with a complete oxidation of 3-hydroxybenzoate according to the following equation:

$$5\ C_7H_5O_3^- + 28\ NO_3^- + 6\ H_2O \rightarrow 35\ HCO_3^- + 14\ N_2 + 2\ H^+$$

$$\Delta G^{\circ\prime} = -3099\ \text{kJ/mol 3-hydroxybenzoate}$$

(calculated according to [22])

If strain Asl-3 was grown with 2.5 mM 3-hydroxybenzoate, Y_s for nitrate was constant between 0 and 5 mM nitrate. At higher concentrations, Y_s decreased even though 3-hydroxybenzoate was not limiting. Y_s for 3-hydroxybenzoate was similar to that reported for a physiologically related organism growing with phenol [23]. With strain Asl-3, Y_s for 3-hydroxybenzoate during nitrate reduction to nitrite is 30% higher than Y_s for 3-hydroxybenzoate during nitrite reduction to N_2. This phenomenon was also observed with a *Pseudomonas* strain growing with benzoate [24]. Thermodynamically, nitrite reduction to N_2 ($2\ NO_2^- + 3\ H_2 + 2\ H^+ \rightarrow N_2 + 4\ H_2O$; $\Delta G^{\circ\prime} = -265$ kJ/mol H_2) is more exergonic than nitrate reduction to nitrite ($NO_3^- + H_2 \rightarrow NO_2^- + H_2O$; $\Delta G^{\circ\prime} = -163$ kJ/mol H_2). Hence, it can be concluded that the thermodynamic efficiency of nitrate reduction is higher than that of nitrite reduction.

Strain Asl-3 anaerobically oxidizes a wide range of substrates (in the presence of nitrate), such as benzoate, mono- and dihydroxybenzoates, phenylacetate, short chain fatty acids, and dicarboxylic acids (see Table 2). All aromatic substrates degraded anaerobically were degraded aerobically

Table 2

Substrates tested for anaerobic degradation [a] by strain Asl-3 with 5 mM NO_3^-

Substrates degraded	Substrates not degraded
Benzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, protocatechuate, 2.5-dihydroxybenzoate [b], 3,5-dihydroxybenzoate [b], phenylacetate, mannose [b], lactose [b], fructose [b], arabinose [b] (all 2.5 mM).	2-Hydroxybenzoate, catechol, 2,6-dihydroxybenzoate, 2,4-dihydroxybenzoate, cyclohexanone, 1,3-cyclohexanedione, cyclohexane carboxylate, gallate, phloroglucinol, resorcinol, hydroquinone, phenol, anilin, *o*-cresol, *m*-cresol, *p*-cresol, syringate, vanillate, glucose, xylose, galactose (all 2.5 mM).
Propionate, butyrate, crotonate, pyruvate, lactate, malate, fumarate, succinate (all 5 mM).	Ethanol, glycolate (both 10 mM).

[a] With the exception of 3,5-dihydroxybenzoate, all substrates degraded anaerobically were degraded aerobically as well.

[b] Low turbidity as compared to controls after 4 weeks of incubation.

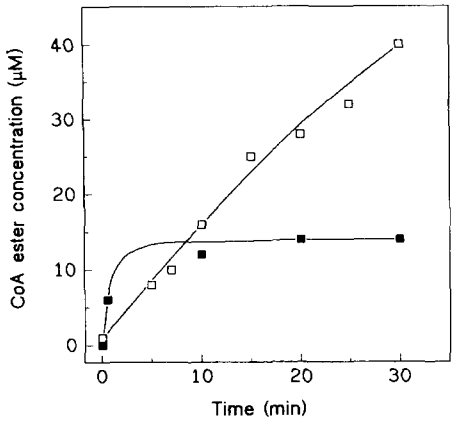

Fig. 3. Formation of benzoyl-CoA (□) or 3-hydroxbenzoyl-CoA (■) upon addition of benzoate or 3-hydroxybenzoate (both 0.5 mM). Assay mixtures contained 30 and 10 μg protein ml^{-1}, respectively.

as well, with the exception of 3,5-dihydroxybenzoate. At present we have no explanation for this phenomenon.

4.4. Acyl-CoA synthetase activities

Both benzoyl-CoA synthetase and 3-hydroxybenzoyl-CoA synthetase activities were present in cell extracts of 3-hydroxybenzoate-grown cells (86 and 190 nmol CoA ester min^{-1} mg $protein^{-1}$, respectively). These activities are sufficient to account for 3-hydroxybenzoate metabolism of growing cells (35 nmol min^{-1} mg $protein^{-1}$). In controls lacking ATP, CoA, the respective aromatic acid, or cell extract, no CoA esters were formed. Time courses of 3-hydroxybenzoate and benzoate activation are shown in Fig. 3. Only the benzoyl-CoA formation proceeded linearly over time, while 3-hydroxybenzoyl-CoA formation came to a standstill after accumulating 3-hydroxybenzoyl-CoA at high initial rates, even though no substrate of the assay was limiting. For this reason, only the rate of benzoyl-CoA formation was found to be linearly dependent on protein concentration (up to 120 μg protein). Besides the respective CoA esters, AMP was identified as another reaction product by HPLC. 2- and 4-hydroxybenzoate were not activated by cell extracts of 3-hydroxybenzoate-grown cells. As strain Asl-3 is capable of growing with 4-hydroxybenzoate, a different CoA ligase may be induced during growth on this substrate. The presumed subsequent step of anaerobic 3-hydroxybenzoate degradation, a reductive dehydroxylation of 3-hydroxybenzoyl-CoA to benzoyl-CoA, could not be demonstrated. Even though various electron acceptors were tested, no formation of benzoate or benzoyl-CoA was detected. However, the assay was severely hampered by rapid hydrolysis of 3-hydroxybenzoyl-CoA (219 nmol min^{-1} mg $protein^{-1}$) in the presence of cell extracts. The high thioesterase activity could also explain the non-linear kinetics of 3-hydroxybenzoyl-CoA formation in acyl-CoA synthetase assays.

There are reports indicating that 3-hydroxybenzoate may be degraded via the benzoate pathway [1,25]. Dense cell suspensions of a defined syntrophic co-culture incubated with 3-hydroxybenzoate accumulated benzoate in the supernatant when H_2 consumption by the syntrophic partner was inhibited [4]. However, with nitrate-reducing bacteria, we never observed any accumulation of aromatic intermediates if electron transfer to nitrate was limited (unpublished results). All 3-hydroxybenzoate-degrading organisms are able to use benzoate as substrate as well [3,4,23,24].

In this study, we were able to characterize the first step of anaerobic 3-hydroxybenzoate degradation as an activation of the aromatic acid by formation of a CoA ester. Further studies to elucidate the subsequent reactions involved in 3-hydroxybenzoyl-CoA metabolism are in progress.

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