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A strictly anaerobic nitrate-reducing bacterium growing with resorcinol and other aromatic compounds

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Abstract. With resorcinol as sole source of energy and organic carbon, two stains of gram-negative, nitrate-reducing bacteria were isolated under strictly anaerobic conditions. Strain LuBRes1 was facultatively anaerobic and catalase- and superoxide dismutase-positive. This strain was affiliated with *Alcaligenes denitrificans* on the basis of substrate utilization spectrum and peritrichous flagellation. Strain LuFRes1 could grow only under anaerobic conditions with oxidized nitrogen compounds as electron acceptor. Cells were catalase-negative but superoxide dismutase-positive. Since this strain was apparently an obligate nitrate reducer, it could not be grouped with any existing genus. Resorcinol was completely oxidized to CO_2 by both strains. Neither an enzyme activity reducing or hydrolyzing the resorcinol molecule, nor an acyl-CoA-synthetase activating resorcylic acids or benzoate was detected in cell-free extracts of cells grown with resorcinol. In dense cell suspensions, both strains produced a compound which was identified as 5-oxo-2-hexenoic acid by mass spectrometric analysis. This would indicate a direct, hydrolytic cleavage of the resorcinol nucleus without initial reduction.

Key words: Obligate nitrate reducer – Resorcinol hydrolysis – Aromatic compounds – *Alcaligenes denitrificans* – Anaerobic degradation

As recently reviewed (Evans and Fuchs 1988; Tschech 1989; Schink et al. 1991) aromatic compounds are degraded anaerobically through three key intermediates. Most aromatic compounds are degraded via benzoyl-CoA as the last aromatic intermediate before ring reduction, and several reactions modifying the aromatic nucleus or its substituents such as carboxylation (Tschech and Fuchs 1989), reductive dehydroxylation (Glöckler et al. 1989) or oxidation (Rudolphi et al. 1991) are known. Phloroglucinol and resorcinol, two aromatic compounds with hydroxy groups in *meta*-position to each other, are degraded without CoA activation because their mesomeric π-electron system is destabilized by a tautomeric equilibrium. Reduction of phloroglucinol to dihydrophloroglucinol was shown with a *Coprococcus* strain (Patel et al. 1981), with *Eubacterium oxidoreducens* (Krumholz et al. 1987) and *Pelobacter acidigallici* (Samain et al. 1986), and a methyl viologen-dependent resorcinol reductase activity was found in a *Clostridium* sp. (Tschech and Schink 1985; Kluge et al. 1990). Also a preliminary description of a nitrate reducer degrading resorcinol was presented recently (Kluge et al. 1990).

In this study we report on nitrate-dependent degradation of resorcinol which appears to take a pathway different from that described for the *Clostridium* strain.

Material and methods

Sources of organisms

Strain LuBRes1 and strain LuFRes1 were isolated from samples of activated sludge and of digested anaerobic sludge of the municipal sewage plant in Tübingen-Lustnau, FRG. *Propionibacterium freudenreichii* subsp. *shermanii* (DSM 20270) and *Escherichia coli* (DSM 498) were obtained from the Deutsche Sammlung für Mikroorganismen, Braunschweig, FRG.

Media and growth conditions

All methods for enrichment and cultivation were essentially the same as described in an earlier paper (Widdel and Pfennig 1981). The bicarbonate-buffered mineral medium contained 1 mM Na_2SO_4 as sulfur source, vitamin solution (Widdel and Pfennig 1981), selenite-tungstate solution (Tschech and Pfennig 1984) and the trace element solution SL 10 (Widdel et al. 1983), but no reducing agent. It was dispensed anoxically into test tubes or infusion bottles sealed with butyl rubber septa. Substrates and electron acceptors were added from sterile stock solutions. Growth experiments were carried out at 28 °C. Growth was followed by measuring turbidity in an Eppendorf 1101 M photometer at 578 nm (light path 1 cm).

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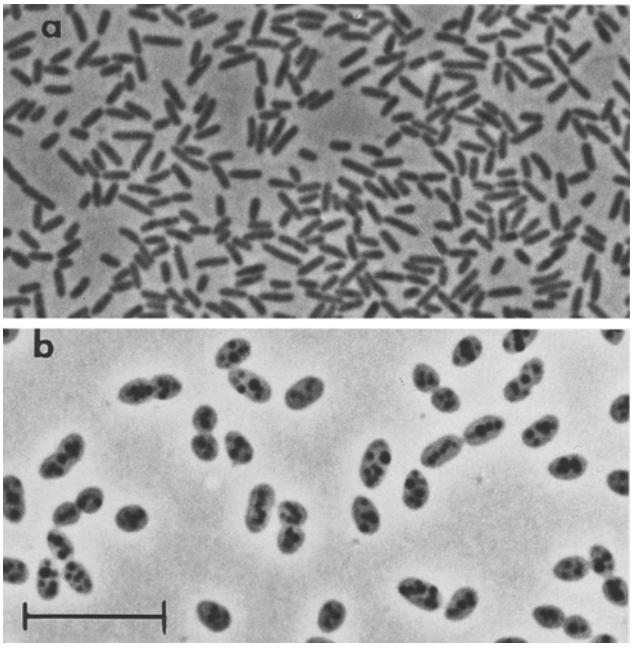
a
b

up to 1% of the dry matter. Cells were motile by four peritrichously inserted flagella. The guanine-plus-cytosine content of the DNA was 69.4 ± 0.4 mol%.

Cells of strain LuFRes1 were short, oval rods, 2.7–3.3 × 1.5 μm in size (Fig. 1), forming round inclusions in all growth phases. Poly-β-hydroxybutyrate accumulated to 14% of cell dry matter. Cells stained Gram-negative and were motile by a single polar flagellum. The DNA base ratio was 66.5 ± 0.5 mol%.

Physiology

Both strains grew at temperatures ranging between 10 °C and 37 °C with an optimum at 30 °C. The pH-range was 6.0 to 8.5 with an optimum between 7.2 and 7.6. Growth was not inhibited by phosphate at concentrations up to 50 mM. Substrates utilized are listed in Table 1. Both strains grew with citric acid cycle intermediates and with short-chain fatty acids or alcohols. Resorcinol and β-resorcylate were the only aromatic substrates used by strain LuBRes1, whereas strain LuFRes1 oxidized many others, especially hydroxybenzoates and other compounds which are presumably degraded through benzoyl-CoA.

Strain LuBRes1 was facultatively anaerobic and grew under anaerobic conditions only with nitrate, nitrite or dinitrogen oxide as electron acceptor. The doubling time with 2 mM resorcinol was 6.2 h ($\mu = 0.11\ h^{-1}$) and only small amounts of nitrite were accumulated during growth.

Table 1. Substrates tested for growth under denitrifying conditions with strain LuBRes1 and strain LuFRes1. The chosen concentrations [mM] are given in brackets

Substrate	LuBRes1	LuFRes1
Acetate (5)	+	+
Propionate (5)	+	+
Butyrate (5)	+	+
Valerate (5)	+	+
Ethanol (10)	+	+
Propanol (10)	+	+
Lactate (10)	+	+
Pyruvate (10)	+	+
D, L-Malate (5)	+	−
Fumarate (5)	+	+
Succinate (5)	+	+
Adipate (5)	+	−
Cyclohexane carboxylate (1)	−	+
Phenol (1)	−	+
Resorcinol (1)	+	+
Benzoate (1)	−	+
3-Hydroxybenzoate (1)	−	+
4-Hydroxybenzoate (1)	−	+
2,4-Dihydroxybenzoate (1)	±	±
2,6-Dihydroxybenzoate (1)	−	±
Phenylacetate (1)	−	+
p-Cresol (1)	−	+
Phenylalanine (1)	−	+
Tyrosine (1)	−	+

No growth within six weeks was found with the following substrates: D(+)-xylose, L(−)-arabinose, D(+)-glucose, D(−)-fructose, formate, 5-oxo-caproate, pimelate, catechol, hydroquinone, 2-hydroxybenzoate, *o*-cresol, *m*-cresol

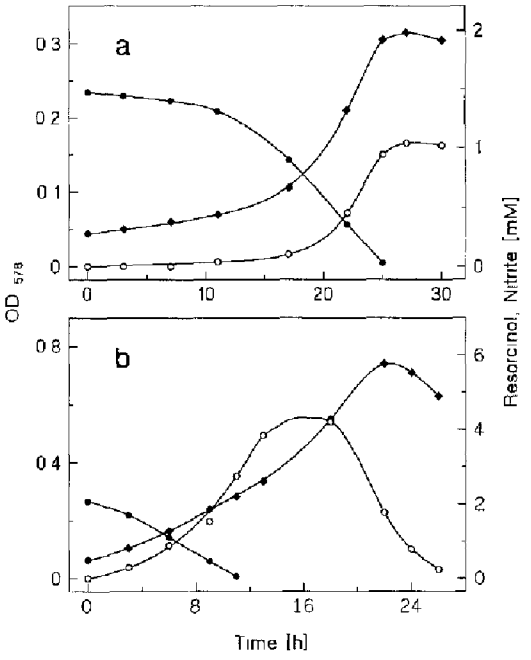

Fig. 2a, b. Time course of growth, resorcinol degradation and nitrite formation. **a** Strain LuBRes1; **b** strain LuFRes1. OD_{578}: optical density at 578 nm. *Symbols:* ◆ cell density; ● resorcinol; ○ nitrite

Table 2. Electron recovery after growth with resorcinol and nitrate

Strain	LuBRes1	LuFRes1
Resorcinol consumed [mmol]	1.48	2.09
Cell material formed [mg]	100.1	145.6
Nitrate consumed [mmol]	5.49	5.98
Nitrite formed [mmol]	1.10	0.24
Electron recovery [%]	89.8	98.2
Growth yield [g/mol]	67.6	69.7

Amounts of resorcinol consumed were calculated after the following reaction equations:

$$5\ C_6H_6O_2 + 26\ NO_3^- + 2\ H_2O \rightarrow 30\ HCO_3^- + 13\ N_2 + 4\ H^+$$

$$20\ C_6H_6O_2 + 44\ H_2O + 2\ NO_3^- + 2\ H^+ \rightarrow 30\ \langle C_4H_7O_3\rangle + N_2$$

Strain LuFRes1 did not grow aerobically, not even at low oxygen pressure in oxygen gradient cultures. Thiosulfate, sulfite, or sulfur were not reduced. No growth was detected either if $K_3[Fe(CN)_6]$, $Fe(OH)_3$, trimethylamine N-oxide, dimethylsulfoxide or fumarate (each 5 mM) was supplied as electron acceptor and ethanol (10 mM) as source of carbon and energy. The doubling time with resorcinol was 4.8 to 7.4 h ($\mu = 0.09$–$0.14\ h^{-1}$). Nitrate was reduced quantitatively to nitrite before the latter was further reduced to nitrogen. Growth time courses for both strains are shown in Fig. 2, electron recovery after growth with resorcinol in Table 2. In contrast to strain LuBRes1, no catalase activity was detected in cells of strain LuFRes1. Superoxide dismutase activity was found with both strains.

Resorcinol transformation in cell suspensions

In dense cell suspensions ($OD_{578} = 2.0$) of strain LuBRes1 and strain LuFRes1, resorcinol degradation was

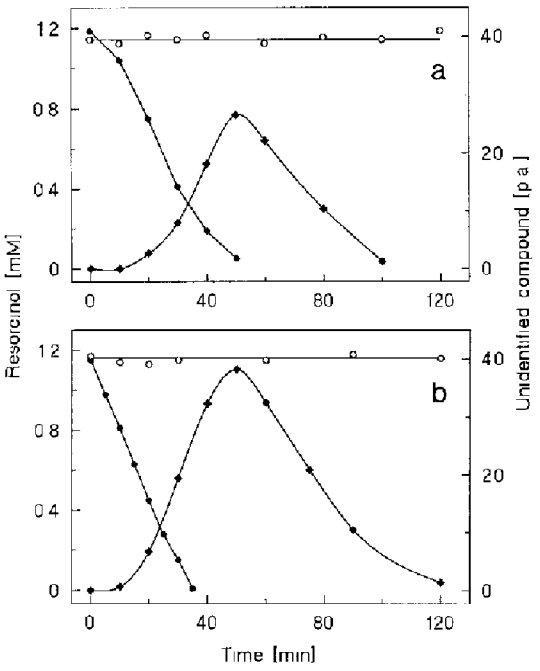

Fig. 3a, b. Degradation of resorcinol by dense cell suspensions with 8 mM nitrate. **a** Strain LuBRes1; **b** strain LuFRes1. [p.a.] peak area at 270 nm. *Symbols.* ● resorcinol; ◆ unknown compound; ○ resorcinol without nitrate or with CTAB

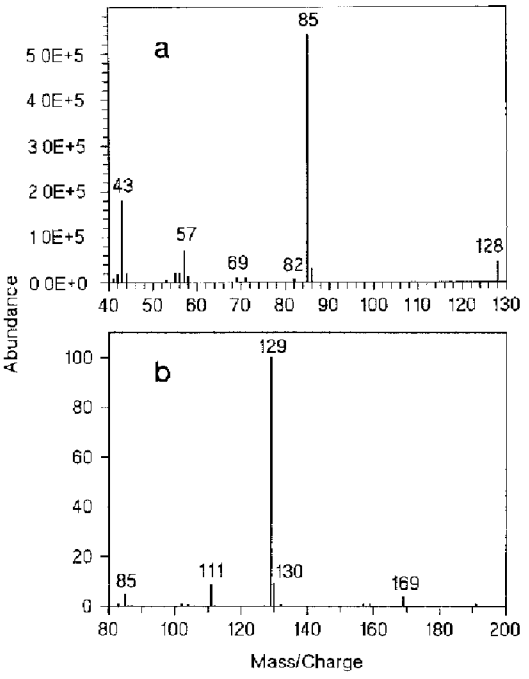

Fig. 5a, b. Mass spectra of lyophilized supernatant extract from cell suspensions. **a** Electron impact ionisation spectrum; **b** chemical ionisation spectrum

followed under various conditions. The results are shown in Fig. 3. Strain LuBRes1 degraded resorcinol at an activity of 0.09 µmol per min and mg protein and strain LuFRes1 at 0.36 µmol per min and mg protein. Both strains accumulated an unidentified compound to various amounts (see below). This compound was completely degraded during the further course of the experiment. Resorcinol was not degraded in the absence of an electron acceptor or if cells were permeabilized by addition of CTAB. Fluoroacetate (1 mM) inhibited degradation of both compounds completely.

Identification of a degradation intermediate

During cell suspension experiments, an unidentified compound with a characteristic absorption maximum at 270 nm wavelength accumulated in the culture liquid

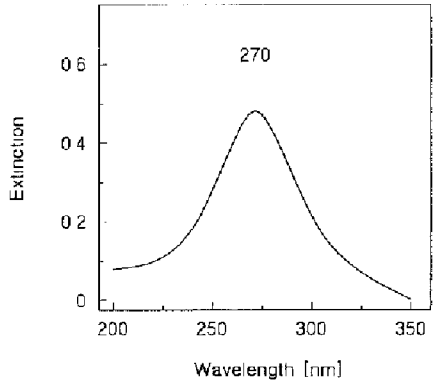

Fig. 4. UV-absorption spectrum of the unidentified compound in 100 mM ammonium phosphate buffer, pH 2.6

(Fig. 4). No further absorption maxima at lower wavelengths were detected. A molecular mass of 128 was determined for this compound by electron impact ionisation analysis (Fig. 5a) and was confirmed by a corresponding M + 1 base peak at a m/z value of 129 in the chemical ionisation spectrum (Fig. 5b). A fragment with 43 m/z (85 m/z resp.), indicating α-cleavage of a methylketone (CH_3CO), accounts for 5-oxo-hexenoic acid. The location of the double bond should be between C2 and C3 for the following reasons: α-cleavage of the methylketone, now in allylic position, is easy to conceive, and would also account for a stronger signal at 85 m/z instead of 43 m/z. 5-Oxo-2-hexenoic acid is the only compound for which all these mass spectrometric data fit.

Enzyme activities

In extracts of strain LuBRes1 and strain LuFRes1 cells grown with resorcinol, no resorcinol reductase or 1,3-cyclohexanedione hydrolase activity could be detected. No acyl CoA synthetase activity with benzoate, 2-hydroxybenzoate, 4-hydroxybenzoate, 2,4-dihydroxybenzoate, or 2,6-dihydroxybenzoate as substrate was found either. No coenzyme A-dependent or hydrolytic cleavage of resorcinol could be detected in cell extracts.

3-Hydroxyacyl CoA dehydratase (E.C. 4.2.1.55.) activity in resorcinol-grown cells of strain LuFRes1 was detected at 5.2 U per mg protein. 3-hydroxybutyryl CoA dehydrogenase (E.C. 1.1.1.35) activity at 0.26 U per mg protein and β-ketothiolase (E.C. 2.3.1.16) activity at 6.2 U per mg protein. In resorcinol-grown cells of strain LuBRes1, the following enzyme activities were detected: 3-hydroxyacyl CoA dehydratase at 2.5 U per mg protein, 3-hydroxybutyryl CoA dehydrogenase at 0.03 U per mg protein and β-ketothiolase at 1.6 U per mg protein.

Discussion

An obligately nitrate-reducing bacterium

In the present paper, we report on enrichment and isolation of denitrifying bacteria able to grow anaerobically with resorcinol and nitrate. Two strains were obtained, one from activated sludge, and the other one from anoxic sewage sludge. Both reduced nitrate to nitrite and lateron to molecular nitrogen but, in contrast to strain LuBRes1, strain LuFRes1 was unable to carry out any kind of energy metabolism in the absence of oxidized nitrogen compounds. Since no other electron acceptor was used and fermentative substrate degradation did not occur either strain LuFRes1 has to be considered as an obligately nitrate-reducing bacterium. All other nitrate-reducing bacteria described so far are either facultatively aerobic, or are fermenting bacteria. The latter reduce nitrate either to nitrite or to ammonia such as *Clostridium* strains or some sulfate-reducing bacteria (Krieg and Holt 1984). Even *Thiomicrospira denitrificans* is able to carry out an oxidative metabolism with oxygen as terminal electron acceptor if the pO_2 is lower than 0.5% (Staley et al. 1989).

Strain LuFRes1 is the first bacterium that depends obligately on oxidized nitrogen compounds for growth, and converts them finally to dinitrogen as reduced product. Isolation of this bacterium was possible only because we used strictly anoxic cultivation techniques throughout; in other labs, enrichment for nitrate reducers are usually performed in the presence of considerable amounts of leftover oxygen in the media. There may be many more obligate nitrate reducers out in nature that have been overlooked so far because they can be isolated only under strictly anoxic conditions.

Resorcinol degradation through hydrolysis

Experiments with dense cell suspensions showed rapid degradation of resorcinol (Fig. 3). Simultaneously, an unknown compound accumulated which reached a maximum concentration when resorcinol was almost completely degraded. No resorcinol degradation was found in the absence of an electron acceptor, or if cells were permeabilized by CTAB. Obviously, resorcinol degradation and production of the unknown compound were possible only with intact cells able to carry out energy metabolism. The unknown compound exhibited a single absorption maximum at 270 nm in the UV-spectrum (range from 200 to 350 nm) and could be distinguished well from resorcinol or any other aromatic compound. Results of mass spectrometric analysis supported the assumption that a non-aromatic compound is produced by these cell suspensions. The M + 1-ion (129 m/z) obtained from chemical ionisation (Fig. 5b) indicates a molecular weight of 128. Direct hydrolytic cleavage of resorcinol would gain a compound with this molecular weight. Theoretically, two different compounds are conceivable as cleavage products: 5-oxo-2-hexenoic acid and 5-oxo-3-hexenoic acid (Fig. 6). 5-Oxo-3-hexenoic acid is an α,β-unsaturated ketone which would correspond to

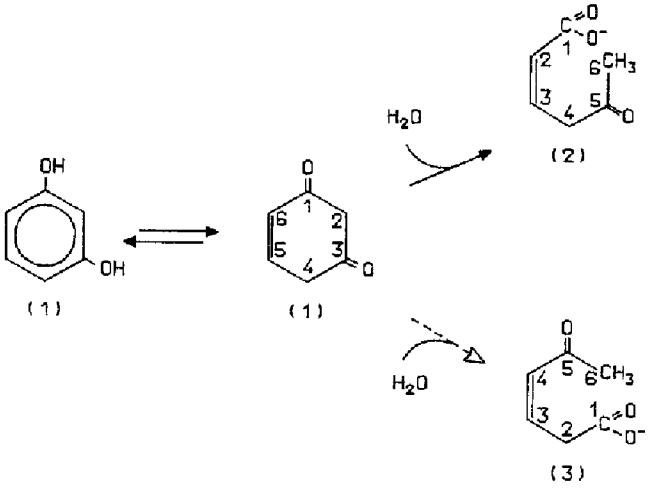

Fig. 6. Initial reaction of resorcinol degradation by denitrifying bacteria as indicated by the present study: *(1)* resorcinol, *(2)* 5-oxo-2-hexenoic acid, *(3)* 5-oxo-3-hexenoic acid

a strong absorption maximum at about 215 nm (π–π^*-transition) and a weak one around 320 nm (n π^* transition). On the other hand, a molecule with an isolated oxo-group absorbs neither between 200 and 230 nm, nor between 300 and 340 nm. Since the UV-spectra showed only a single absorption maximum at 270 nm within the range of 200 to 350 nm, the unknown compound should be the 5-oxo-2-hexenoic acid.

The mass spectrum fragmentation pattern obtained by electron impact ionisation (Fig. 5a) confirms the photo-spectrometrical data. The prominent ions had 43 and 85 m/z. An α-cleavage (removing a non-binding electron from a heteroatom and cleaving in α-position to this heteroatom) between C-atom 4 and C-atom 5 would lead to these fragments. In case of 5-oxo-2-hexenoic acid, ions with 43 and 85 m/z would be the predominant ones; in contrast, 5-oxo-3-hexenoic acid would create only small amounts of the latter, if at all. Therefore all results obtained from spectrophotometrical and mass spectrometrical analysis point to 5–oxo-2-hexenoic acid as the unknown compound produced by cell suspensions. Unfortunately, there is no standard reference compound available to further confirm this statement.

Resorcinol reductase and 1,3-cyclohexanedione hydrolase could not be detected in extracts of the denitrifying strain LG 245 (Kluge et al. 1990). The same is true for our strains LuBRes1 and LuFRes1. Since 1,3-cyclohexanedione and 5-oxo-caproate were not used either as growth substrates we assume that denitrifying bacteria use a pathway for anaerobic resorcinol degradation different from the reductive pathway of fermenting bacteria.

There was no indication either of resorcinol degradation via benzoyl-CoA: degradation did not depend on CO_2, and extracts of both strains activated neither benzoate nor 2-hydroxybenzoate, 4-hydroxybenzoate, 2,4-dihydroxybenzoate or 2,6-dihydroxybenzoate in the presence of CoASH, ATP and MG^{2+}.

Results from cell suspension experiments indicate that the new pathway suggested here starts with direct hy-

drolytic cleavage of resorcinol to form 5-oxo-2-hexenoic acid as first product. Unfortunately, this activity could not be demonstrated in cell-free extracts. Perhaps the aromatic ring is cleaved after intermediate reduction and subsequent reoxidation, through an enzyme system that is destroyed during cell disintegration. After activation by enoyl CoA synthetase, further degradation of 5-oxo-hexenoic acid would be possible by enzymes of the well-known pathway of fatty acid β-oxidation. High activities of 3-hydroxyacyl CoA dehydratase, 3-hydroxyacyl CoA:NAD oxidoreductase and β-ketothiolase were detected indicating that this pathway is used by our isolates.

Taxonomy

Because of its strictly oxidative metabolism, gram-negative cell wall architecture, peritrichous flagellation, positive catalase reaction, and a guanine-plus-cytosine content of the DNA of 69.4 ± 0.4%, strain LuBRes1 can be grouped with the genus *Alcaligenes*. Since adipate was used as growth substrate and nitrite as terminal electron acceptor, affiliation to the species *Alcaligenes denitrificans* appears justified (Krieg and Holt 1984). Strain LuFRes1 cannot be attributed to any known species because it reduces nitrate to dinitrogen, and is not able to grow aerobically. Experiments in test tubes with agar medium indicated that even with low oxygen concentrations no growth occurred, possibly due to the observed lack of catalase activity. The phenomenon of an obligately nitrate reducing metabolism requires further study in the future.

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