

Acetylene degradation by new isolates of aerobic bacteria and comparison of acetylene hydratase enzymes

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Abstract

Aerobic acetylene-degrading bacteria were isolated from soil samples. Two isolates were assigned to the species *Rhodococcus opacus*, two others to *Rhodococcus ruber* and *Gordona* sp. They were compared with known strains of aerobic acetylene-, cyanide-, or nitrile-utilizing bacteria. The acetylene hydratases of *R. opacus* could be measured in cell-free extracts only in the presence of a strong reductant like titanium(III) citrate. Expression of these enzymes was molybdenum-dependent. Acetylene hydratases in cell-free extracts of *R. ruber* and *Gordona* spp. did not require addition of reductants. No cross-reactivity could be found between cell-free extracts of any of these aerobic isolates and antibodies raised against the acetylene hydratase of the strictly anaerobic fermenting bacterium *Pelobacter acetylenicus*. These results show that acetylene hydratases are a biochemically heterogeneous group of enzymes.

Keywords: *Rhodococcus*; *Gordona*; *Pelobacter acetylenicus*; Acetylene hydratase; Acetylene degradation

1. Introduction

The unsaturated hydrocarbon acetylene (ethyne) is used as carbon and energy source for growth by several aerobic bacteria, e.g. *Mycobacterium lacticola* [1], *Nocardia rhodochrous* [2], *Rhodococcus* A1 [3], *Bacillus* sp. [4], and *Rhodococcus rhodochrous* [5]. *Pelobacter acetylenicus* was isolated as the first strictly anaerobic, fermenting bacterium degrading acetylene [6]. In all these bacteria, acetylene is attacked by a hydration reaction forming acetaldehyde

as the first detectable intermediate [1,3,6,8]; they are attacked in a similar fashion as nitriles and cyanides, therefore ([7], and references therein). Other possible reaction types, such as epoxidation or hydroxylation by oxygenase reactions, or reduction to the respective alkane [9], do not apply to acetylene.

Acetylene hydratase activity has been demonstrated in cell-free extracts of *Rhodococcus* A1 [3] and *P. acetylenicus* [10]. In the latter case, the enzyme could be detected only under strict exclusion of oxygen, in the presence of a strong reducing agent such as titanium citrate. Unequivocal proof of acetylene hydratase could not always be provided [8]. The aim of the present study was to compare aerobic

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and anaerobic acetylene-degrading bacteria and their acetylene-hydrating enzymes, including described bacterial strains and new isolates.

2. Materials and methods

2.1. Organisms and growth conditions

Strains MoAcy1, MoAcy2, TueAcy1 and TueAcy3 were enriched in liquid mineral medium with acetylene as sole carbon and energy source from various dry soil samples taken near Tübingen, Germany. Strains were isolated by subsequent streaking on agar (1.5% v/v) plates with mineral medium incubated under air with 10% (v/v) acetylene at 30°C. All strains were deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig (DSMZ), see Table 1. *Gordona rubropertincta* (DSM 43197), *G. terrae* (DSM 43249) *R. rhodochrous* (DSM 43241) and *R. fascians* (DSM 20131) were provided by DSMZ, Braunschweig, Germany, *R. rhodochrous* NCIMB 11216, *Nocardia rhodochrous* LL100-21, and *Brevibacterium* R312 by Dr. C.J. Knowles, University of Kent, Canterbury, UK, strain KS-7D by Dr. H.-J. Knackmuss, University of Stuttgart, Germany.

Strains MoAcy1, MoAcy2, TueAcy1, and TueAcy3 were cultivated in mineral medium that contained (in g/l): NaCl 1.0; MgCl₂·6H₂O 0.4; KH₂PO₄ 0.2; NH₄Cl 0.25; KCl 0.5; CaCl₂·2H₂O 0.15; (NH₄)₂SO₄ 0.66; MOPS 5.23, pH 7.2. After autoclaving and cooling, 1 ml trace element solution SL 10 [11], 1 ml selenite-tungstate solution [11], and 1 ml 7-vitamins solution [12] were added per liter. Mineral medium for cultivation of *R. rhodochrous* NCIMB 11216, *N. rhodochrous* LL100-21, *Brevibacterium* R312, *G. rubropertincta*, *G. terrae*, *R. rhodochrous*, *Brevibacterium* R312, *R. fascians* and strain KS-7D was prepared after [13], with 1 ml trace element solution SL 10 and 1 ml 7-vitamins solution. All strains were cultivated in Erlenmeyer flasks under air with 5% (v/v; equivalent to 2 mM in liquid medium) acetylene, which were closed with butyl rubber stoppers, on a rotary shaker at 200 rpm in the dark. Acetylene was added to the cultures with sterile syringes. Cell material for fatty and mycolic acid and 16S rDNA sequence analyses was harvested from cultures

grown on TSB agar (3% (w/v) Trypticase soy broth (BBL), 1.5% (w/v) Bacto-Agar (Difco)) for 4 days at 28°C. For chemical examination, all strains were cultivated on GYM agar (0.4% (w/v) D-glucose, 0.4% (w/v) yeast extract, 1% (w/v) malt extract, 1.2% (w/v) Agar No. 1 (Oxoid) for 3 days at 28°C. Cell material for cell wall analysis was obtained from growth in Trypticase soy broth (BBL) for 4 days at 28°C on a rotary shaker (90 rpm), harvested by centrifugation, and washed twice with distilled water.

2.2. Strain characterization

Carbon source utilization and quantitative enzyme tests were performed in standard microtiter plates (F-forms, Greiner, Germany) according to [14]. The amino acid and sugar analysis of whole cell hydrolysates followed described procedures [15]. Isoprenoid quinones were extracted and purified using a small-scale integrated procedure [16] and analyzed as published [17,18]. Polar lipids were extracted, separated by two-dimensional thin-layer chromatography and identified [16]. Fatty acid methyl esters were prepared from 40–80 mg wet cells according to [19] with minor modifications.

Genomic DNA was extracted and the 16S rRNA gene amplified by PCR as described [20] and sequenced (*Taq* DyeDeoxy Terminator Cycle Sequencing Kit, Applied Biosystems, Inc., Foster City, CA). Sequences were manually aligned with representatives from mycolic acid containing taxa using the 'ae2 editor' [21] and the full 16S rDNA sequences of all *Rhodococcus* and *Gordona* species [22].

2.3. Characterization of acetylene hydratases

Cells were harvested by centrifugation at 13 000 × g at 4°C, washed in 50 mM potassium phosphate buffer, pH 7.0, and disrupted by 4 passages through a French pressure cell (Aminco, Silver Springs, MD, USA) at 136 MPa. Cell debris was removed by centrifugation at 30 000 × g. Acetylene hydratase activity was determined in a coupled photometric assay with yeast alcohol dehydrogenase [10], in the presence or absence of 2 mM titanium(III) citrate [23] or other reducing agents. The reaction was started by addition of gaseous acetylene to a final concentration of about 20 mM.

Cross-reactivity of cell-free extracts of the aerobic strains with antibodies against purified acetylene hydratase from *P. acetylenicus* [10] was tested in Western blots.

All chemicals were of reagent grade quality and obtained from Merck (Darmstadt), Fluka (Neu-Ulm), and Sigma (Munich), yeast alcohol dehydrogenase from Boehringer (Mannheim), and gases from Sauerstoffwerke (Friedrichshafen).

3. Results

3.1. Isolation and identification of isolated strains

With acetylene as sole carbon and energy source, six aerobic bacterial strains were isolated from soil. Four strains (MoAcy1, MoAcy2, TueAcy1, TueAcy3) were characterized further. The cell walls of these strains contained arabinose and galactose as major cell wall sugars. Meso-diaminopimelic acid was the only diamino acid found in all four strains. The polar lipids were composed of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides and phosphatidylethanolamine. The fatty acid pattern of all strains contained straight chain saturated and unsaturated fatty acids with significant amounts of tuberculostearic acid (10-methyl octadecanoic acid). The combination of these chemical markers is diagnostic for members of the CMN group (*Corynebacterium*, *Mycobacterium*, *Nocardia*) including *Dietzia*, *Rhodococcus* and *Tsukamurella*. Table 1 lists the predominant menaquinones and mycolic acid chain lengths which identify strains MoAcy1, TueAcy1 and Tue-

Acy3 as members of the genus *Rhodococcus* (MK-8(H₂) and C₃₀-C₅₄), and strain MoAcy2 as a *Gordona* sp. (MK-9(H₂) and C₄₈-C₆₆). Strains MoAcy1 and TueAcy1 could be further identified to the species level as belonging to *R. opacus*, comparing the qualitative and quantitative results of the fatty acid and mycolic acid analyses with our data bases and by the physiological reaction profile. These data are in good accordance with the results of the 16S rDNA analyses. Strain TueAcy1 was identified by 16S rDNA analyses as *Rhodococcus zopfii* [24]. This strain could not be identified by chemotaxonomy and physiology to species level because no data were available for this species. The classification of strain MoAcy2 to one of the known *Gordona* species by chemical markers is difficult. Based on the fatty acid results, strain MoAcy2 could be identified as *G. rubropertincta* with only low similarity. It differs in mycolic acid chain length from *G. rubropertincta* (C₅₄-C₆₀) in 2 carbon atoms and could therefore not be identified by the mycolic acid pattern. Although the relationship to *G. rubropertincta* is quite low by chemical markers, 16S rDNA sequence analyses showed clearly (Table 1) that *G. rubropertincta* is the nearest relative to strain MoAcy2.

3.2. Growth with acetylene and acetylene hydratase activities

Strains MoAcy1 and TueAcy1 grew with 2 mM acetylene as sole carbon and energy source in liquid mineral medium with doubling times of 3.6 h. Strains MoAcy2 and TueAcy3 could grow in liquid mineral medium with acetylene only in the presence of a small amount of yeast extract (0.1% w/v). Acetylene

Table 1
Chemotaxonomic, physiological and phylogenetic identification of acetylene-utilizing strains isolated from soil

Strain	DSM number	meso-DAP arabinose+ galactose	Mycolic acid chain length	Mena-quinone	Identity based on			
					Fatty acid analysis	16S rDNA sequence similarity	Physiological tests	Mycolic acid chain length
MoAcy1	44186	+	C ₄₈ -C ₅₄	MK-8 (H ₂)	<i>Rh.o.</i>	99.7% to <i>Rh.o.</i>	<i>Rh.o.</i>	<i>Rh.o.</i>
TueAcy1	44188	+	C ₄₈ -C ₅₄	MK-8 (H ₂)	<i>Rh.o.</i>	99.2% to <i>Rh.o.</i>	<i>Rh.o.</i>	<i>Rh.o.</i>
TueAcy3	44189	+	C ₄₂ -C ₄₈	MK-8 (H ₂)	<i>Rh.sp.</i> ^a	99.7% to <i>Rh.z.</i>	no match ^b	<i>Rh.sp.</i>
MoAcy2	44187	+	C ₅₂ -C ₅₈	MK-9 (H ₂)	<i>G.r.</i>	99.2% to <i>G.r.</i>	no match	<i>G.sp.</i>

^a*Rhodococcus zopfii* not included in current fatty acid database.

^b*Rhodococcus zopfii* not included in current physiological database.

Rh.o., *Rhodococcus opacus*; *Rh.z.*, *Rhodococcus zopfii*; *Rh.sp.*, *Rhodococcus* sp.; *G.r.*, *Gordona rubropertincta*; *G.sp.*, *Gordona* sp.

Table 2

Specific acetylene hydratase activity of aerobic acetylene-utilizing strains isolated from soil, and of *Gordona rubropertincta* DSM 43197

Strain	Growth conditions (mineral medium with the following)	Assay conditions (phosphate buffer with the following)	Specific enzyme activity ($\mu\text{mol min}^{-1} \text{mg protein}^{-1}$)
MoAcy1	acetylene	titanium(III) citrate (2 mM)	1.1
		dithionite (2 mM)	0.2
		dithiothreitol (10 mM)	0
		cysteine (10 mM)	0
		no reducing agent	0
		oxic	0
TueAcy1	acetylene	titanium(III) citrate	1.30
		no reducing agent	0.01
		oxic	0
MoAcy2	acetylene+yeast extract	titanium(III) citrate	0.30
		oxic	0.30
TueAcy3	acetylene+yeast extract	titanium(III) citrate	0.28
		oxic	0.28
<i>G. rubropertincta</i>	acetylene+yeast extract	titanium(III) citrate	0.50
		oxic	0.50
	yeast extract	titanium(III) citrate	0.20
		oxic	0.20

hydratase activity was demonstrated in cell-free extracts of all strains (Table 2), and was higher after growth with acetylene plus yeast extract than with yeast extract only.

G. rubropertincta (DSM 43197), *G. terrae* (DSM 43249), *R. rhodochrous* (DSM 43241), and *R. fascians* (DSM 20131) as well as the nitrile or cyanide degrading strains *R. rhodochrous* NCIMB 11216, *N. rhodochrous* LL100-21, *Brevibacterium* R312, and KS-7D, were tested for their ability to grow with acetylene and for acetylene hydratase activity. Only for *G. rubropertincta* could growth on mineral agar with 10% (v/v) acetylene in the gas phase be proven, and a specific acetylene hydratase activity of $0.5 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$ was measured in cell-free extracts after growth in liquid mineral medium with acetylene and 0.1% (w/v) yeast extract ($0.2 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$ after growth with 0.1% yeast extract alone).

Acetylene hydratase activity in strains MoAcy1 and TueAcy1 was highest if the enzyme assay was performed in the presence of a strong reducing agent such as 2 mM titanium(III) citrate. In the presence of 2 mM dithionite, only 20% of this activity was found. With 10 mM dithiothreitol or 10 mM cysteine, in the absence of a reducing agent, or under air, no acetylene hydratase activity could be demonstrated. For the other strains that showed acetylene

hydratase activity, i.e. MoAcy2, TueAcy3, and *G. rubropertincta*, acetylene hydratase activity was the same in measurements either in the presence of titanium(III) citrate or under air.

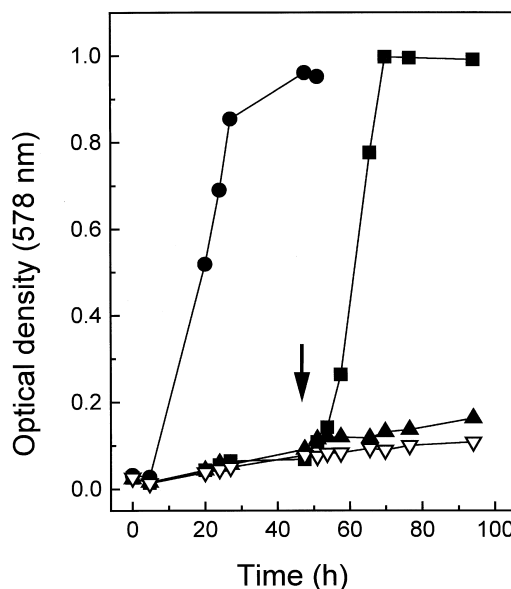


Fig. 1. Growth of *Rhodococcus opacus* strain TueAcy1 in mineral medium with 150 nM molybdate and 12 nM tungstate (●) and in medium without molybdate and tungstate. After 48 h (arrow), 100 nM molybdate (■) or 100 nM tungstate (▲) was added. No molybdate or tungstate was added to the control (▽).

With strains MoAcy1 and TueAcy1, growth in defined liquid mineral medium depended on the presence of molybdate (Fig. 1). In the absence of molybdate and tungstate, growth was barely detectable. After addition of 100 nM molybdate to the medium, growth started immediately. Addition of 100 nM tungstate had no effect, and only little acetylene hydratase activity (less than 10% of molybdate-grown cells) was detected after growth with tungstate only.

Cell-free extracts of strains MoAcy1, TueAcy1, MoAcy2, TueAcy3, and *G. rubropertincta* were tested for cross-reactivity with antibodies raised against purified acetylene hydratase from the anaerobic bacterium *P. acetylenicus*. No cross-reactivity could be demonstrated with extracts of any of the aerobic strains.

4. Discussion

Acetylene hydratase activity was demonstrated recently for the strictly anaerobic fermenting bacterium *P. acetylenicus*. The enzyme was purified and characterized. It was shown to be a tungsten iron-sulfur protein [10], and enzyme expression depended on the presence of tungstate in the growth medium. Also the acetylene hydratases of the newly isolated strains MoAcy1 and TueAcy1, which were classified as *R. opacus*, could be demonstrated only in the presence of a strong reducing agent, similar to the acetylene hydratase activity of *P. acetylenicus* [10]. Growth of these strains with acetylene depended on the presence of molybdate. Thus, acetylene hydratases of strains TueAcy1 and MoAcy1 are very likely to be molybdenum-containing enzymes, different from acetylene hydratase of *P. acetylenicus*. Tungstate was not necessary for growth of these strains.

With the other strains that showed acetylene hydratase activity, i.e. MoAcy2, TueAcy3, and *G. rubropertincta*, the enzyme reaction could be seen in the presence of reducing agents or under air at identical levels, indicating that these enzymes are basically different from those of *P. acetylenicus* and *R. opacus*. A possible dependence of these strains on molybdate or tungstate was not tested since growth was possible only in the presence of yeast extract which would provide sufficient amounts of trace metals.

Although acetylene hydratase activity of strains MoAcy1 and TueAcy1 was similar to that of *P. acetylenicus* with respect to requirement of a reducing agent in the assay, cell-free extracts of these or of the other aerobic strains did not cross-react with antibodies against purified acetylene hydratase of *P. acetylenicus*, indicating again that these acetylene-converting enzymes are structurally different proteins.

The natural function of acetylene hydratase (beyond acetylene utilization) remains unknown. It has been speculated that the enzyme might be involved in detoxification of cyanide or nitriles [6]. Cyanide hydratases and cyanidases as well as nitrile hydratases and nitrilases have been described [7,13,25–31]. However, bacterial strains that possess cyanidase or nitrile hydratase activity, such as strain KS-7D, *R. rhodochrous* NCIMB 11216, *N. rhodochrous* LL100-21, and *Brevibacterium* R312, did not grow with acetylene, and cell-free extracts of those strains after growth with cyanide or propionitrile did not show acetylene hydratase activity. This indicates that acetylene hydratase and cyanide or nitrile hydratases again are different types of enzymes. Nonetheless, it is striking that strains described to convert acetylene mostly belong to the mycolic acid-containing Actinomycetales [1–3,5] (this study). Especially bacteria of the genus *Rhodococcus* are known to degrade short-chain hydrocarbons [32], and the same group of Gram-positive bacteria is prominent as well in cyanide and nitrile degradation.

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