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Isolation and characterization of alkene-utilizing *Xanthobacter* spp.

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Abstract. Yellow-pigmented bacteria showing typical characteristics of *Xanthobacter* spp. were isolated from enrichments with propene and 1-butene, using classical techniques. The generation time for growth on propene and 1-butene of these bacteria ranged from 5 to 7 h. A NADH-dependent mono-oxygenase was identified in cell-free extract of *Xanthobacter* Py2. This mono-oxygenase was not influenced by potential inhibitors tested indicating that propene mono-oxygenase is different from other hydrocarbon mono-oxygenases described until now. Nitrogenase activity could be measured using the acetylene reduction assay with propene as energy source, because acetylene did not inhibit the mono-oxygenase activity.

Key words: Propene – 1-Butene – *Xanthobacter* – Mono-oxygenase – Nitrogen fixation

The production of 1,2-epoxyalkanes from alkenes in biotechnological processes has been studied with several micro-organisms including methane-utilizing bacteria (Higgins et al. 1979, 1980; Hou et al. 1979), propane- and butane-utilizing micro-organisms (Hou et al. 1983; Patel et al. 1983), heptane-utilizing *Pseudomonas* (van der Linden 1963) and the octane-utilizing *Pseudomonas oleovorans* (de Smet et al. 1981, 1983). The formation of 1,2-epoxyalkanes by these alkane-grown bacteria is due to the non-specific oxidation of alkenes by the alkane-hydroxylase. The formation of 1,2-epoxyalkanes has also been studied with alkene-utilizing bacteria like *Nocardia corallina* (Furahashi et al. 1981) and ethene-utilizing *Mycobacterium* strains (Habets-Crützen et al. 1984). Growth of micro-organisms on propene was first reported by Cerniglia et al. (1976) and later by de Bont et al. (1980). *Mycobacterium* PY1 isolated by de Bont oxidized propene via a mono-oxygenase to 1,2-epoxypropane and this specific alkene-monooxygenase was also involved in the formation of 1,2-epoxyethane (de Bont et al. 1983). The alkene-utilizing *Mycobacterium* strains organisms isolated until now are relatively slow growing. While trying to isolate faster growing micro-organisms on propene and 1-butene we repeatedly isolated *Xanthobacter* spp.

In this report we describe the isolation and characterization of these alkene-utilizing *Xanthobacter* spp.

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Materials and methods

Chemicals. Gaseous alkenes, ethyne and 1,2-epoxyethane were obtained from Hoek Loos, Amsterdam, The Netherlands. NADH and NADPH were purchased from Boehringer, Mannheim, FRG. All other chemicals were obtained from Janssen Chimica, Beerse, Belgium.

Micro-organisms. The *Xanthobacter* strains were isolated by methods described previously (Wiegant and de Bont 1980) except that ethene was replaced by higher concentrations (5%) of propene or 1-butene. *Xanthobacter autotrophicus* strain 7C and strain JW 33 were obtained from Deutsche Sammlung von Mikroorganismen (DSM), Göttingen, FRG.

Maintenance and cultivation of the micro-organisms. The bacteria were maintained on yeast/glucose slopes. The micro-organisms were cultivated in mineral medium supplemented with the appropriate gaseous alkenes as described by Wiegant and de Bont (1980). The gas phase contained only 10% (v/v) oxygen when the organisms were grown under nitrogen-fixing conditions in which case $(NH_4)_2SO_4$ and NH_4Cl in the medium were replaced by $MgSO_4$ and NaCl, respectively. Growth on other carbon and energy sources and the determination of the doubling time has been described by Habets-Crützen et al. (1984).

Analyses. Gaseous alkenes were determined as described by de Bont et al. (1979). Ethene concentrations were measured during the acetylene reduction test in the same way as otherwise except that the oven temperature was 60° C instead of 180° C. Protein concentrations were determined as described by Habets-Crützen et al. (1984). The detection of mycolic acids was determined by thin layer chromatographic analysis of whole organisms methanolysates as described by Minnikin et al. (1975). GC content was determined as described by Mandel and Marmur (1968) and poly-β-hydroxybutyric acid was determined by the method described by Jüttner et al. (1975).

Preparation of washed cell suspensions and cell-free extracts. The preparation of washed cell suspensions and cell-free extracts has been described previously (de Bont and Harder 1978; de Bont et al. 1979). Cell-free extract was dialysed by eluating the extract over a G-25 Sephadex column.

Determination of kinetic constants. A washed cell suspension (0.5 mg protein) of propene-grown cells was incubated in screw-cap bottles in a shaking bath at 30° C. To measure

Table 1. Kinetic constants for propene of *Mycobacterium* Py1 and two *Xanthobacter* strains

Bacteria	V_{max} nmol/min × mg protein	K_m [a] vpm
Mycobacterium Py1	15	100
Xanthobacter Py2	70	280
Xanthobacter Py10	65	230

[a] K_m has been related to the concentration in the gas phase

Michaelis-Menten constants, the reaction was started by injecting 500 pm propene in the gas phase. Samples from the gas phase were withdrawn at regular intervals for gaschromatographic analysis. Michaelis-Menten constants were calculated by fitting the integrated Michaelis-Menten equation to the measured concentration-time data by means of a computer program.

Acetylene reduction test. Cells of a culture grown in a nitrogen-free medium under reduced oxygen tension were injected directly in Hungate tubes containing the appropriate gaseous mixtures. The tubes were incubated in a shaking bath at 30°C. The gas phase was analysed for ethene at regular intervals.

Propene mono-oxygenase. Oxidation of propene by cell-free extracts was carried out as described by de Bont and Harder (1978). Inhibitors were injected just before starting the reaction with propene.

Results

Isolation

Bacteria able to grow on either propene or 1-butene were enriched by incubating various soil and water samples in an atmosphere of 5% alkene in air. Organisms were isolated from such enrichments in the presence of either propene or 1-butene by subculturing in a liquid mineral medium and by streaking cells to purity on agar plates of mineral medium. In this way, six strains were isolated from enrichments in the presence of propene (Py2, Py3, Py7, Py10, Py11, Py17) and one strain was isolated in the presence of 1-butene (By2). The specific growth rate of these strains on propene and 1-butene ranged from 0.14 to 0.10 h^{-1} which is about 5 times faster than the specific growth rate of *Mycobacterium* Py1 on 2% propene in air.

Mycobacterium Py1 was originally isolated using low concentrations of propene, while the newly isolated strains were obtained using high concentrations of alkene. The isolation of the new strains might therefore be a consequence of different affinities towards alkenes. Therefore, the K_m and V_m for propene of washed cell suspensions of strain Py2 and *Mycobacterium* Py1 were measured (Table 1).

Characterization

The seven strains were all immotile, irregularly-shaped rods which divided by snapping. The organisms were pleomorph as illustrated by Fig. 1 showing strain Py2 grown on propene (A), succinate (B) and 1-propanol (C), respectively. They formed round, slimy, yellow colonies when plated on yeast/glucose medium. Slime-free mutants could be isolated easily from a carbon limited chemostat culture. The strains were all able to use other substrates for growth as for instance H_2/CO_2, methanol, ethanol, 1-propanol, propanal, acetone, 2-propanol, 1,2-propanediol, propionate, 1,2-epoxypropane, pyruvate, 1,2-epoxybutane, 1-butanol, 1,2-butanediol, glucose, fructose and glutamate. These strains were also able to grow on ethene, albeit with doubling times of 20 h or more. No growth was observed on ethane, propane, butane or galactose. The strains isolated could utilize NH_4^+ or NO_3^- as nitrogen source and fixed atmospheric nitrogen at reduced oxygen levels. No mycolic acid could be detected in either strain Py2 and strain Py10. It was shown that poly-β-hydroxybutyric acid and catalase were present in strain Py2 and strain Py10. The GC content of strain Py2 was 70.0%. The physiological and morphological data of strain Py2 and *Xanthobacter autotrophicus* are summarized in Table 2. On basis of these properties the bacteria were assigned to the genus *Xanthobacter*. For comparison *Xanthobacter autotrophicus* strains 7C and JW33 were also tested for growth on alkenes but these bacteria were not able to grow on either ethene, propene or 1-butene.

Acetylene reduction with propene

The reduction of acetylene to ethene is a measure for the rate of nitrogen fixation by bacteria. Acetylene reduction by *Xanthobacter* Py2 was only possible at reduced oxygen levels. Figure 2 shows that 20% of oxygen in the gas phase totally inhibited acetylene reduction. Acetylene reduction occurred with propene as energy source and the rate of acetylene reduction was not influenced by the propene concentration (Fig. 2).

Propene mono-oxygenase

The disappearance of propene was measured in vitro to establish the nature of the enzyme involved in the conversion of propene. The oxidation of propene by a cell-free extract was supported by NADH in the presence of oxygen indicating that the enzyme involved is a mono-oxygenase (Fig. 3). NADH could be replaced by NADPH but not by other electron donors like $FADH_2$ or ascorbic acid. Some potential inhibitors of hydrocarbon mono-oxygenases were tested but only in few cases an effect on propene oxidation was recorded (Table 3). CN^- at higher concentrations, and allylthiourea inhibited the propene mono-oxygenase for only 30% and also ethyne inhibited the mono-oxygenase only slightly.

Discussion

Bacteria growing on either ethene, propene or 1-butene have been described by several authors (de Bont 1976; de Bont et al. 1980; Cerniglia et al. 1976; Heyer 1976) but so far only strains belonging to the genus *Mycobacterium* and an unidentified strain were isolated on these alkenes. The isolation of these *Mycobacteria* was generally achieved by methods involving low concentrations of alkene in the gas phase. We

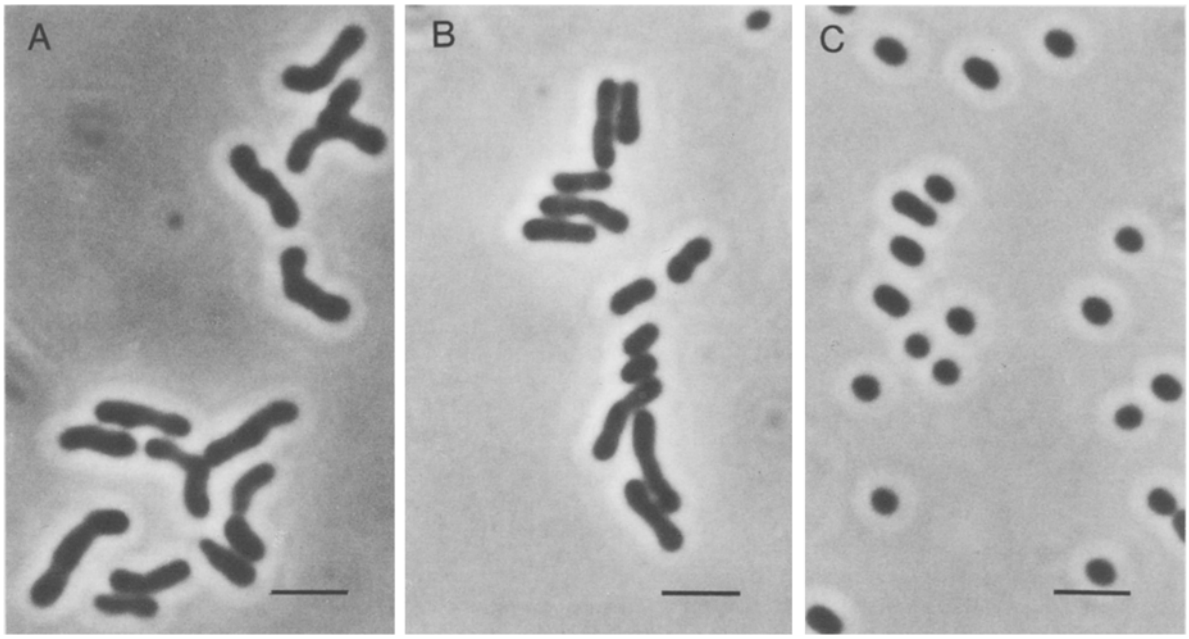

Fig. 1. Phase-contrast light micrograph of *Xanthobacter* Py2 grown on succinate **A**, propene **B** and 1-propanol **C**. The bar in the figures represents 1 μm

Table 2. Characteristics of *Xanthobacter* Py2 and *Xanthobacter autotrophicus* JW 33

Characters	*Xanthobacter autotrophicus* JW33	*Xanthobacter* Py2
G + C content	68.9	70
Cyst formation	−	−
Storage material	PHB	PHB
Refractile bodies	+	+
Slime production	+	+
Colony color	yellow	yellow
Cell size	0.4−1.0	0.4−1.0
Pleomorphism	+	+
Motility	−	−
Catalase production	+	+
Gram reaction	variable	variable

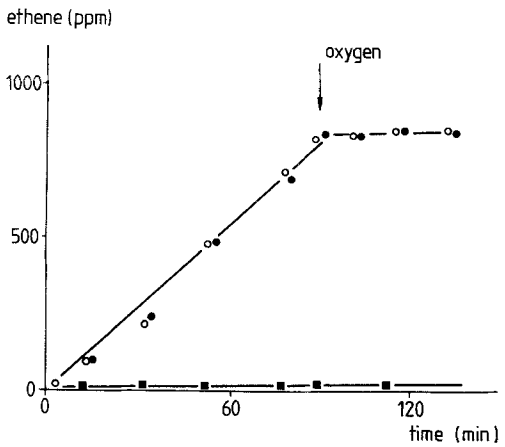

Fig. 2. Acetylene reduction by propene-grown *Xanthobacter* Py2 as influenced by propene and oxygen. The oxygen concentration in the gas-phase (5%) was increased to 20% after 90 min of incubation. The propene concentration in the gas phase was 0% (■), 5% (○) and 19% (●)

have now used higher concentrations of propene and 1-butene in the gas phase (5%) while enriching for alkene-utilizers resulting in the isolation of faster growing *Xanthobacter* spp. It seems that so far only the slower growing *Mycobacterium* were isolated on propene because these organisms have a higher affinity towards the alkene than the *Xanthobacter* strains. An indication that *Mycobacteria* have a higher affinity towards propene than the newly isolated *Xanthobacter* is the difference in the K_m for propene of *Xanthobacter* Py2 and *Mycobacterium* Py1.

The carbohydrate utilization, colony and cell morphology of the newly isolated strains are in agreement with previous published data for *Xanthobacter* spp. The pleomorphism of the strains, along with the copious slime production, are also similar to other *Xanthobacter* spp. Also, the isolation of slime-free mutants of *Xanthobacter* spp. has been described by several authors. *Xanthobacter* Py2 grown on succinate is showing a typical branched cell formation and cells grown on 1-propanol show coccoid cell formation as described by Wiegel et al. (1978). Wiegel et al. (1978) also described that their *Xanthobacter* did not possess mycolic acids. Mycolic acids could not be detected in our strains, while a multi spot pattern was shown on the thin layer chromatography plates of the propene-utilizing *Mycobacterium* Py1 (de Bont et al. 1980). The GC content of *Xanthobacter* Py2 is in agreement with data published by Wiegel et al. (1978). Growth of *Xanthobacter* on alkenes is not a general property of these bacteria since the type strains JW 33 and 7C did not utilize these compounds.

The alkene-utilizing *Xanthobacter* are all able to fix atmospheric nitrogen at reduced oxygen levels. Acetylene

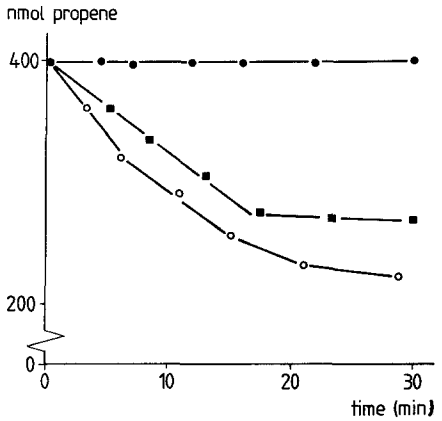

Fig. 3. Oxidation of propene by dialysed cell-free extract (13 mg protein) of *Xanthobacter* Py2 grown on propene without cofactor (●) and in the presence of NADH (○) or NADPH (■)

Table 3. Effect of potential inhibitors of propene mono-oxygenase activity on propene oxidation by cell-free extract of *Xanthobacter* Py2

Inhibitor	Concentration	Relative activity (%)
None	–	100
Imidazole	0.1 mM	97
2-Mercaptoethanol	0.1 mM	118
CN^-	1 mM	67
CN^-	0.1 mM	81
CN^-	0.01 mM	101
8-Hydroxyquinoline	1 mM	99
8-Hydroxyquinoline	0.1 mM	126
Thiourea	0.1 mM	126
Allylthiourea	0.1 mM	77
CO	10%	105
Ethyne	4%	72

was also reduced to ethene with propene as energy source which is surprising because other hydrocarbon-utilizing bacteria as for instance methane-oxidizing bacteria cannot reduce acetylene. The inability to detect nitrogen fixation in methane-oxidizing bacteria with the acetylene reduction test was caused by the inhibition of methane mono-oxygenase by acetylene (de Bont and Mulder 1976).

The propene mono-oxygenase of *Xanthobacter* Py2 resembles the alkene mono-oxygenase of *Mycobacterium* Py1 and *Mycobacterium* E20 in that it only oxidizes unsaturated hydrocarbon bonds to 1,2-epoxyalkanes and does not hydroxylate alkanes, and in that only NADH and NADPH acted as electron donors. To preliminary characterize the enzyme, some potential inhibitors of hydrocarbon mono-oxygenases were tested. Acetylene, an inhibitor of other hydrocarbon mono-oxygenases did not inhibit the propene mono-oxygenase, and furthermore other potential inhibitors did not act on the mono-oxygenase with an exception for CN^- at higher concentrations. This suggests that the propene mono-oxygenase is different from other hydrocarbon mono-oxygenases described to date (Cardini and Jurtshuk 1970; Colby and Dalton 1976; McKenna and Coon 1970; Tonge et al. 1977). Further research with the alkene-utilizing strains will concentrate on epoxide formation by these organisms.

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References

Bont JAM de (1976) Oxidation of ethylene by soil bacteria. Antonie van Leeuwenhoek J Microbiol Serol 42:59–71

Bont JAM de, Harder W (1978) Metabolism of ethylene by *Mycobacterium* E20. FEMS Microbiol Lett 3:89–93

Bont JAM de, Mulder EG (1976) Invalidity of the acetylene reduction assay in alkane-utilizing nitrogen fixing bacteria. Appl Environ Microbiol 31:640–674

Bont JAM de, Attwood MM, Primrose SB, Harder W (1979) Epoxidation of short-chain alkenes in *Mycobacterium* E20; the involvement of a specific mono-oxygenase. FEMS Microbiol Lett 3:89–93

Bont JAM de, Primrose SB, Collins MD, Jones D (1980) Chemical studies on some bacteria which utilize gaseous unsaturated hydrocarbons. J Gen Microbiol 117:97–102

Bont JAM de, Ginkel CG van, Tramper J, Luyben KChAM (1983) Ethylene oxide production by immobilized *Mycobacterium* Py1 in a gas/solid bioreactor. Enzyme Microbiol Technol 5:55–60

Cardini G, Jurtshuk P (1970) The enzymatic hydroxylation of n-octane by a *Corynebacterium* sp strain 7 ELC. J Biol Chem 245:2789–2796

Cerniglia CE, Blevins WT, Perry JJ (1976) Microbial oxidation and assimilation of propylene. Appl Environ Microbiol 6:764–768

Colby J, Dalton H (1976) Some properties of a soluble methane mono-oxygenase from *Methylococcus capsulatus* strain Bath. Biochem J 157:495–497

Furahashi K, Taoka A, Karube I, Suzuki S (1981) Production of 1,2-epoxyalkanes from 1-alkenes by *Nocardia corallina* B276. Eur J Appl Microbiol Biotechnol 20:39–45

Habets-Crützen AQH, Brink LES, van Ginkel CG, de Bont JAM, Tramper J (1984) Production of epoxides from gaseous alkenes by resting-cell suspensions and immobilized cells of alkene-utilizing bacteria. J Appl Microbiol Biotechnol 20:245–251

Heyer J (1976) Mikrobielle Verwertung von Äthylen. Z Allg Mikrobiol 16:633–637

Higgins IJ, Hammons RC, Saraislani FS, Best DJ, Davis MM, Tryhorn SE, Taylor F (1979) Biotransformations of hydrocarbons and related compounds by whole organisms of *Methylosinus trichosporium* OB3b. Biochem Biophys Res Commun 89:671–677

Higgins IJ, Best DJ, Hammons RC (198) New findings in methane-utilizing bacteria highlight their importance in the biosphere and their commercial potential. Nature 286:561–564

Hou CT, Patel RN, Laskin AI (1979) Microbial oxidation of gaseous hydrocarbons by methylotrophic bacteria. Appl Environ Microbiol 38:127–134

Hou CT, Patel P, Laskin AI, Barnabe N, Barist I (1983) Epoxidation of short-chain alkenes by resting-cell suspensions of propane-grown bacteria. Appl Environ Microbiol 46:171–177

Jüttner RR, Lafferty RM, Knackmuss HJ (1975) A simple method for the determination of poly-β-hydroxybutyric acid in microbial biomass. European J Appl Microbiol 1:233–237

Linden AC van der (1963) Epoxidation of olefins by heptane-grown *Pseudomonas* cells. Biochim Biophys Acta 77:157–159

Mandel M, Marmur J (1968) Use of ultraviolet absorbance-temperature profile for determining the guanine plus cytosine content of DNA. In: Grossmann L, Moldave K (eds) Methods in enzymology, vol XII, part B. Academic Press, New York London, pp 195–206

McKenna EJ, Coon MJ (1970) Enzymatic ω-oxidation. IV. Purification and properties of the ω-hydroxylase of *Pseudomonas oleovorans*. J Biol Chem 245:3882–3889

Minnikin DE, Alshamaohy L, Goodfellow M (1975) Differentiation of *Mycobacterium, Nocardia* and related taxa by thin layer chromatographic analysis of whole organism methanolysates. J Gen Microbiol 88:200–204

Patel RN, Hou CT, Laskin AI, Felix A, Derelanko P (1983) Epoxidation of n-alkanes by organisms grown on gaseous alkanes. J Appl Biochem 5:121–131

Smet MJ de, Wijnberg H, Witholt B (1981) Synthesis of 1,2-epoxyoctane by *Pseudomonas oleovorans* during growth in a two-phase system containing high concentrations of 1-octene. Appl Environ Microbiol 42:811–816

Smet MJ de, Kingma J, Witholt B (1983) *Pseudomonas oleovorans* as a tool in bioconversions of hydrocarbons; growth morphology and conversion characteristics in different two-phase systems. Enzyme Microbiol Technol 5:352–360

Tonge GM, Harrison DEF, Higgins IJ (1977) Purification and properties of methane mono-oxygenase enzyme system from *Methylosinus trichosporium* OB3b. Biochem J 161:333–344

Wiegant WM, Bont JAM de (1980) A new route for ethylene glycol metabolism in *Mycobacterium* E44. J Gen Microbiol 120:325–331

Wiegel J, Wilke D, Baumgarten J, Opitz R, Schlegel HG (1978) Transfer of the nitrogen fixing hydrogen bacterium *Corynebacterium autotrophicum* Baumgarten et al. to *Xanthobacter* gen. nov. Int J Syst Bacteriol 28:573–581

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