Chemical Studies on Some Bacteria which Utilize Gaseous Unsaturated Hydrocarbons

By J. A. M. DE BONT,^{1†} S. B. PRIMROSE,^{1*} M. D. COLLINS²
AND DOROTHY JONES²

¹Department of Biological Sciences, University of Warwick, Coventry CV4 7AL

²Department of Microbiology, University of Leicester, Leicester LE1 7RH

(Received 3 September 1979)

Lipid and DNA studies on eight Gram-positive bacteria capable of utilizing gaseous unsaturated hydrocarbons as sole carbon sources indicated that the one strain which utilized acetylene as sole carbon source was a member of the genus *Rhodococcus*. The remaining seven strains which utilized ethylene and/or propylene as sole carbon sources all appeared to be members of the genus *Mycobacterium*.

INTRODUCTION

The ability of micro-organisms to oxidize gaseous unsaturated hydrocarbons has been detected in few bacteria to date. Although the Gram-negative methanotrophs can co-oxidize ethylene (de Bont & Mulder, 1974) the only organisms isolated so far which can utilize it for growth are Gram-positive rods which resemble mycobacteria (de Bont, 1976; S. B. Primrose, unpublished observations). A bacterium which grew on acetylene as sole carbon source was identified as a mycobacterium by Birch-Hirschfeld (1932). Recently, we have isolated additional ethylene-utilizing bacteria as well as a bacterium which can use propylene, but not ethylene, and another which can use acetylene, but not ethylene, as sole carbon sources. With the exception of the acetylene-utilizer, all the bacteria isolated by us appeared to be morphologically similar. In an attempt to characterize the acetylene-utilizer, and to investigate the apparent taxonomic homogeneity of the other isolates, we have employed DNA-DNA hybridization and lipid analyses, techniques previously shown to be of value in the classification of similar bacteria (Minnikin & Goodfellow, 1976; Bradley & Mordarski, 1976).

METHODS

Bacteria. Details of the bacteria used in this study are given in Table 1. The isolation of E20 and T1 has been described previously (de Bont, 1976). The other strains were obtained by the same methods except for A1 and Py1 where ethylene was replaced by acetylene and propylene, respectively.

Escherichia coli K12, *Nocardia brasiliensis* ATCC 19296 and *Rhodococcus rhodochrous* (R. E. Gordon, No. 21) were from the culture collections of either the University of Warwick or the University of Leicester.

Cultivation of bacteria. Bacteria were cultivated on a glucose/salts medium of the following composition (per litre distilled water): 2 g glucose, 2 g NH₄Cl, 0.2 g (NH₄)₂SO₄, 0.85 g KH₂PO₄, 1.55 g K₂HPO₄, 0.25 g MgSO₄·7H₂O, 0.5 g yeast extract, 0.02 g CaCl₂·2H₂O, 6.6 mg FeCl₃, 15 mg Na₂EDTA, 0.2 g NaCl, 0.16 mg ZnSO₄·7H₂O, 0.2 mg Na₂MoO₄, 0.25 g H₃BO₃, 0.2 mg MnSO₄·4H₂O, 0.02 mg CuSO₄·5H₂O and 1 µg CoCl₂·6H₂O. A solid medium was prepared by the addition of Oxoid Ionagar (1.5%, w/v).

Preparation of DNA. High molecular weight DNA, suitable for use in the model E analytical ultra-

† Permanent address: Department of Microbiology, Agricultural University, Wageningen, The Netherlands.

centrifuge, was prepared by the following method. Bacteria were grown to the late-exponential phase in 500 ml glucose/salts medium, harvested by centrifugation and resuspended in 2 ml 10 mM-Tris pH 8.0 containing 1 mM-EDTA. Crystalline lysozyme (Sigma; 10 mg) was added and the suspension was incubated at 37 °C. After 1 h, 0.1 ml RNAase (Sigma; 1 mg ml⁻¹, boiled for 10 min) and 2 ml Sarkosyl [Sigma; 5 mg ml⁻¹ in 0.1 × SSC buffer containing 10 mM-EDTA (SSC buffer pH 7.0 is 0.15 M-NaCl/0.015 M-trisodium citrate)] were added and incubation was continued till lysis occurred. Solid CsCl and distilled water were added to the lysate to make 20 ml having a density of 1.71 g l⁻¹. The mixture was centrifuged at 45000 rev. min⁻¹ for 40 h in an angle head rotor. The centrifuge tubes were punctured and the viscous DNA band was collected and dialysed extensively against 0.1 × SSC.

DNA for use in hybridization studies was prepared as follows. Bacteria were grown to the late-exponential phase in 500 ml glucose/salts medium, harvested by centrifugation, resuspended in 10 ml 0.25 M-sodium phosphate buffer pH 6.8 containing 8 M-urea and 0.8 % (w/v) sodium dodecyl sulphate, and passed through a French pressure cell at 6 kPa. The DNA was then purified from the cell lysate by hydroxylapatite chromatography as described by Britten *et al.* (1970) and dialysed against 0.1 × SSC. The DNA was concentrated by precipitation with cold ethanol and redissolved in the minimal volume of 0.01 M-sodium phosphate buffer pH 6.8.

Analysis of base compositions. The mol % G+C was determined from the buoyant density in CsCl (Mandel *et al.*, 1968) and the ultraviolet absorbance-temperature profile (Mandel & Marmur, 1968). *Escherichia coli* K12 DNA was used as a standard.

Preparation of radioactive DNA for homology studies. Purified DNA was labelled *in vitro* with high concentration, carrier-free ¹²⁵I (The Radiochemical Centre, Amersham) by the method of Commerford (1971). After iodination, the DNA was purified by extraction once with an equal volume of phenol saturated with distilled water and precipitated from the aqueous phase with 2 vol. cold ethanol in the presence of 0.2 M-NaCl. The precipitated DNA was dissolved in a small volume of 0.1 × SSC and dialysed overnight at 4 °C against 0.01 M-sodium phosphate buffer pH 6.8.

The labelled DNA was sonicated in an ice bath at a frequency of 24 kHz (six periods of 15 s with 30 s intervals).

Hybridization. Labelled DNA (1 µl) was mixed with 0.072 A₂₆₀ units of unlabelled DNA in a 0.75 ml plastic micro-centrifuge tube. The volume was adjusted to 0.1 ml with distilled water and the tube was sealed and placed in a boiling water bath for 3 min. The tube was cooled and 10 µl 4.8 M-sodium phosphate buffer pH 6.8 was added to the contents which were incubated for 16 h at 70 °C (*C₀t* = 140). The hybridization mixture was cooled on ice and diluted with 0.3 ml distilled water and 1.6 ml 0.14 M-sodium phosphate buffer pH 6.8 containing 0.2 % (w/v) sodium dodecyl sulphate.

Hydroxylapatite (DNA grade, Bio-Rad Laboratories; 0.5 g) was suspended in 5 ml 0.14 M-sodium phosphate buffer pH 6.8 containing 0.2 % sodium dodecyl sulphate and packed under pressure in a jacketed 1 cm diam. chromatography column. The hybridization mixture was layered carefully on to the hydroxylapatite, heated to 70 °C and forced through under pressure. The eluate was collected in a scintillation vial. The column was washed with 2 ml 0.14 M-sodium phosphate buffer pH 6.8 and the eluate was collected in the same vial. The column was then washed with a further 4 ml of similar buffer and the eluate was collected in a second vial. The double-stranded DNA retained by the column was then eluted with two successive washes of 4 ml 0.3 M-sodium phosphate buffer pH 6.8 which were collected in separate vials. All samples were counted in an LKB 1280 Ultragamma counter.

The percentage hybridization was calculated from the ratio of counts in the last two vials to the total number of counts eluted.

Whole-organism methanolysis and thin-layer chromatography. Dried bacteria (50 mg) were degraded using the acid methanolysis procedure described by Minnikin *et al.* (1975). Hexane extracts of the methanolysates were examined by one-dimensional thin-layer chromatography on Merck Kiesel gel 60 F₂₅₄ aluminium-backed sheets (10 × 10 cm, 0.2 mm thick) using petroleum ether (b.p. 60 to 80 °C)/diethyl ether (85:15, v/v) as developing solvent. The lipids were revealed by spraying the chromatograms with 10 % (w/v) molybdo-phosphoric acid in ethanol followed by heating to 125 °C for 15 min.

Extraction, purification and analysis of isoprenoid quinones. Dried organisms (50 mg) were examined using the procedure described by Collins *et al.* (1977). Purified isoprenoid quinones were further examined by reverse phase thin-layer chromatography (t.l.c.) using Whatman KC₁₈F reverse phase thin-layer plates and a polar developing mixture of acetone/water (97:3, v/v) (Collins *et al.*, 1979). Structures were confirmed by mass spectrometry, the spectra being recorded on an AEI MS9 instrument using a direct insertion probe, an ionizing voltage of 70 eV and a temperature range of 200 to 230 °C.

Fig. 1. Thin-layer chromatography of whole-organism methanolysates of the test strains: FAME, fatty acid methyl esters; MAME, mycolic acid methyl esters.

RESULTS

Cultural and staining properties. All the ethylene-utilizing strains and the propylene-utilizer, strain Py1, were Gram-positive, acid-fast rods which did not exhibit branching. The acetylene-utilizer, strain A1, was quite different from the other strains in having Gram-positive, non acid-fast, branching filaments. When plated on solid media none of the strains showed aerial mycelium. Particularly noteworthy is the fact that six of the eight strains grew poorly or failed to grow in the presence of natural or tungsten light (Table 1).

Whole-organism methanolysis. The patterns obtained by chromatography of methanolysates of the alkene- and alkyne-utilizers are shown in Fig. 1. Strain A1, the only acetylene-utilizer, produced a single spot with an R_F value similar to mycolic acid methyl esters obtained from an authentic strain of *R. rhodochrous*. The methanolysates of the other strains produced multi-spot patterns in accordance with previous studies on mycobacteria (Minnikin *et al.*, 1975; Minnikin & Goodfellow, 1976). Four different patterns of mycolic methyl esters could be discerned: the EU2 pattern which was also produced by EU3 and E20, the EU1 pattern which was also produced by Py1, the T1 pattern and the E44 pattern.

Analysis of isoprenoid quinones. Components that co-chromatographed with vitamin K were the only isoprenoid quinones detected in the extracts of the strains examined. As expected from published data (Azerad & Cyrot-Pelletier, 1973), the mass spectra of the menaquinone samples showed intense peaks at m/e 187 and 225 derived from the naphthoquinone nucleus as well as peaks corresponding to molecular ions (M^+). The

Table 1. *Properties of test strains*

Strain	Origin	Gases utilized as sole carbon source for growth			Pigmentation		G + C (mol %)	Acid-fastness		Major menaquinone isoprenologue†
		Acetylene	Ethylene	Propylene	Light absent	Light present		After 2 d	After 12 d	
EU1	English soil	—	+	—	White	No growth	67	+	±	MK-9(H ₂)‡
EU2	English soil	—	+	—	Yellow	Orange*	68	+	+	MK-9(H ₂)‡
EU3	English soil	—	+	—	Yellow	Orange*	67	+	+	MK-9(H ₂)‡
E20	Dutch soil	—	+	+	Yellow	Orange*	69.5	+	+	MK-9(H ₂)‡
E44	Dutch ditch water	—	+	—	White	No growth	69	+	+	MK-9(H ₂)‡
T1	Turkish soil	—	+	NT	Creamy	Yellow	68	+	+	MK-9(H ₂)‡
Py1	Dutch soil	—	—	+	Yellow	Orange*	68.5	±	—	MK-9(H ₂)‡
A1	Dutch soil	+	—	—	Creamy-beige	Creamy-beige	67	—	—	MK-8(H ₂)§

NT, Not tested.

* Extremely poor growth.

† MK-9(H₂), *m/e* 786; MK-8, *m/e* 716; MK-8(H₂), *m/e* 718; MK-7(H₂), *m/e* 650; MK-6(H₂), *m/e* 582.‡ Strains EU1, EU2, EU3, E44, E20, T1 and Py1 contained minor amounts of MK-9 and MK-8(H₂).§ Strain A1 contained minor amounts of MK-8, MK-7(H₂) and MK-6(H₂).

Table 2. DNA homology of alkene- and alkyne-utilizing bacteria

Source of unlabelled DNA	Percentage hybridization with labelled DNA from:				
	E20	EU2	T1	E44	Py1
EU1	—	22	16	35	26
EU2	90	100	17	13	32
EU3	30	29	18	25	23
E44	—	25	18	100	26
E20	100	85	29	21	21
T1	—	21	100	15	26
Py1	—	23	26	24	100
A1	—	12	10	15	14
<i>R. rhodochrous</i> No. 21	—	10	7	13	13
<i>E. coli</i>	—	1	11	8	3

—, Not determined.

results of the mass spectral analyses of the menaquinones (Table 1) were confirmed by reverse phase t.l.c.

DNA studies. The values of the mol % G+C as calculated from the buoyant density of the DNA in CsCl are shown in Table 1. The degree of DNA-DNA hybridization between DNA from different strains is shown in Table 2. The average value for the extent of the hybridization in the homologous reactions was 70% and the values for these homologous reactions have been normalized to 100%. With the exception of DNA from EU2 and E20 there was no significant DNA homology between the different isolates nor between any of them and *R. rhodochrous* (R. E. Gordon, No. 21) or *E. coli* K12.

DISCUSSION

The recovery of dihydrogenated menaquinones with eight isoprene units together with the presence of mycolic acid methyl esters of a similar R_F value to those from an authentic rhodococcus suggests that strain A1 is a member of the genus *Rhodococcus* (Minnikin & Goodfellow, 1976; Collins *et al.*, 1977). The only previous report known to us of an acetylene-utilizing bacterium is that of Birch-Hirschfeld (1932). Although this author considered her organism to be a mycobacterium, from the description given, it is likely that it was a member of the genus *Rhodococcus* as described by Goodfellow & Alderson (1977).

The multi-spot patterns of the mycolic acid methyl esters and the presence of MK-9(H₂) in the other seven isolates examined suggests that they are all members of the genus *Mycobacterium* (Minnikin *et al.*, 1975; Minnikin & Goodfellow, 1976; Collins *et al.*, 1977) and the mol % G+C of these strains is in accord with this conclusion.

The identical mycolic acid patterns of strains EU2 and E20 is in keeping with the degree of DNA homology demonstrated between these two strains. The absence of DNA homology between the other strains, together with the variation in the mycolic acid patterns, indicate that the ethylene- and propylene-utilizing bacteria studied by us represent probably six different species in the genus *Mycobacterium*. Further work is required to establish whether they are members of currently described species or whether they represent new taxa. Although DNA reassociation studies yield extremely valuable information on the taxonomic relationships between strains, they can only be used sensibly when the probable taxonomic groupings have been established by other methods.

In an attempt to characterize the isolates further, their photochromogenicity was investigated. To our surprise, six of the eight strains were photophobic. There was no correlation between this photophobic effect and the mycolic acid pattern indicating that

this phenomenon was not peculiar to any one group of our bacteria. To our knowledge, this is the first report of this effect amongst mycobacteria and merits further investigation. Regardless of its cause this photophobic phenomenon could explain the difficulties we have encountered in isolating ethylene-oxidizing bacteria.

One of us (J.A.M.B.) wishes to thank V. Waddell and C. S. Dow for patient instruction in numerous techniques. M.D.C. and D.J. thank the Medical Research Council for support (grant G977/613/S).

REFERENCES

- AZERAD, R. & CYROT-PELLETIER, M. O. (1973). Structure and configuration of the polyprenoid side chain of dihydromenaquinones from myco- and corynebacteria. *Biochimie* **55**, 591–603.
- BIRCH-HIRSCHFELD, L. (1932). Die Umsetzung von Acetylen durch *Mycobacterium lacticola*. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene (Abteilung II)* **86**, 113–128.
- BONT, J. A. M. DE (1976). Oxidation of ethylene by soil bacteria. *Antonie van Leeuwenhoek* **42**, 59–71.
- BONT, J. A. M. DE & MULDER, E. G. (1974). Nitrogen fixation and co-oxidation of ethylene by a methane-utilizing bacterium. *Journal of General Microbiology* **83**, 113–121.
- BRADLEY, S. G. & MORDARSKI, M. (1976). Association of polydeoxyribonucleotides of deoxyribonucleic acids from nocardioform bacteria. In *The Biology of the Nocardiae*, pp. 310–336. Edited by M. Goodfellow, G. H. Brownell & J. A. Serrano. London: Academic Press.
- BRITTEN, R. J., PAVICH, R. & SMITH, J. (1970). A new method for DNA purification. *Carnegie Institute of Washington Year Book* **68**, 400–402.
- COLLINS, M. D., PIROUZ, T., GOODFELLOW, M. & MINNIKIN, D. E. (1977). Distribution of menaquinones in actinomycetes and corynebacteria. *Journal of General Microbiology* **100**, 221–230.
- COLLINS, M. D., GOODFELLOW, M. & MINNIKIN, D. E. (1979). Isoprenoid quinones in the classification of coryneform and related bacteria. *Journal of General Microbiology* **110**, 127–136.
- COMMERFORD, S. L. (1971). Iodination of nucleic acids *in vitro*. *Biochemistry* **10**, 1993–1998.
- GOODFELLOW, M. & ALDERSON, G. (1977). The actinomycete genus *Rhodococcus*: a home for the 'rhodochrous' complex. *Journal of General Microbiology* **100**, 99–122.
- MANDEL, M. & MARMUR, J. (1968). Use of ultraviolet absorbance–temperature profile for determining the guanine plus cytosine content of DNA. *Methods in Enzymology* **12B**, 195–206.
- MANDEL, M., SCHILDKRAUT, C. L. & MARMUR, J. (1968). Use of CsCl density gradient analysis for determining the guanine plus cytosine content of DNA. *Methods in Enzymology* **12B**, 184–195.
- MINNIKIN, D. E. & GOODFELLOW, M. (1976). Lipid composition in the classification and identification of nocardiae and related taxa. In *The Biology of the Nocardiae*, pp. 160–219. Edited by M. Goodfellow, G. H. Brownell & J. A. Serrano. London: Academic Press.
- MINNIKIN, D. E., ALSHAMAONY, L. & GOODFELLOW, M. (1975). Differentiation of *Mycobacterium*, *Nocardia* and related taxa by thin-layer chromatographic analysis of whole-organism methanolysates. *Journal of General Microbiology* **88**, 200–204.