Journal of General Microbiology (1981), **123**, 75–80. *Printed in Great Britain*

A Rapid Procedure for the Detection of Archaebacterial Lipids in Halophilic Bacteria

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(*Received 19 September 1980*)

Whole-organism acid methanolysates of strains of halophilic bacteria were examined for glycerol diether moieties and other long-chain constituents by thin-layer chromatography. Glycerol diether moieties were detected only in acid methanolysates of archaebacterial halophiles. Eubacterial halophiles contained only fatty acid methyl esters. Spots on thin-layer plates attributed to diether moieties were further identified by infrared spectroscopy. Thin-layer chromatographic analysis of whole-organism methanolysates provides a simple and rapid method of distinguishing archaebacterial halophiles from eubacterial halophiles. The majority of archaebacterial halophiles produced only one chromatographically distinct glycerol diether moiety, but certain strains, including the recently described alkalophilic halophiles, produced two distinct moieties.

INTRODUCTION

Molecular genealogical analysis based upon transfer and ribosomal RNA sequence homologies has revealed that the bacteria do not constitute a phylogenetically monolithic grouping (see Woese & Fox, 1977). The kingdom *Procaryotae* actually comprises two phylogenetically distinct groups – the 'archaebacteria', and the 'eubacteria' including the cyanobacteria. The first organisms recognized to be archaebacteria were the methanogens (Woese & Fox, 1977) and subsequent studies indicated that certain thermoacidophiles (*Caldariella*, *Sulfolobus* and *Thermoplasma*) and extreme halophiles (*Halobacterium* and *Halococcus*) should also be included in this group (Woese *et al.*, 1978). Amongst other distinctive features of the archaebacteria are the absence of cell wall peptidoglycan (Brown & Cho, 1970; Kandler & Hippe, 1977) and the presence of ether-linked lipids (deRosa *et al.*, 1974, 1980; Kates, 1978; Tornabene & Langworthy, 1978; Balch *et al.*, 1979).

At present, archaebacteria have only been isolated from a few restricted environments and there is considerable interest in establishing whether they occur elsewhere. The assignment of a particular isolate to the archaebacteria is currently a laborious procedure. During recent investigations into the taxonomic status of alkalophilic halophiles from Kenyan soda lakes (Tindall *et al.*, 1980; W. D. Grant, unpublished results) we have developed a rapid screening procedure for detecting archaebacteria, based on the presence of the ether-linked lipids. The ether lipids of halophilic bacteria have been the subject of extensive investigation by Kates and associates (Sehgal *et al.*, 1962; Kates *et al.*, 1966; Joo *et al.*, 1968; Kates, 1978), but no simple procedure existed for the detection of these lipids. This paper demonstrates that the pattern of spots corresponding to glycerol diether moieties obtained by thin-layer chromatographic (t.l.c.) analysis of whole-organism methanolysates (Minnikin *et al.*, 1975) provides a means of distinguishing archaebacterial halophilic taxa from eubacterial halophilic

0022-1287/81/0000-9545 $02.00

taxa. The method described is rapid, simple, requires no expensive equipment and should be applicable in routine laboratories.

METHODS

Cultures and cultivation. Details of the test strains and their sources are shown in Table 1. *Actinopolyspora halophila* and members of the genera *Halobacterium* and *Halococcus* were grown at 37 °C in liquid shake culture on the medium of Payne *et al.* (1960) with the exception of *Halobacterium volcanii* which was grown in the

Table 1. *Grouping of test strains based on chromatographic analysis of whole-organism methanolysates*

(*a*) *Halophilic bacteria lacking fatty acid methyl esters but containing glycerol diether moieties*

Strain	Source*
Halobacterium cutirubrum	NCMB 763
Halobacterium halobium	NCMB 736
Halobacterium halobium	CCM 2090
Halobacterium halobium	NCMB 777
Halobacterium halobium	NCMB 2080
Halobacterium saccharovorum	NCMB 2081
Halobacterium salinarium	NCMB 764
Halobacterium salinarium subsp. *proteolyticum*	NCMB 786
Halobacterium salinarium	CCM 2148
Halobacterium simoncinii subsp. *neapolitanum*	NCMB 773
Halobacterium sp. (Cagliari)	NCMB 768
Halobacterium trapanicum	NCMB 784
Halobacterium volcanii	NCMB 2012
Halococcus morrhuae	NCMB 787
Halococcus morrhuae	NCMB 749
Halococcus morrhuae	CCM 2526
Sarcina morrhuae†	NCMB 761
Sarcina litoralis†	NCMB 757
Sarcina sreenivasani†	NCMB 776
Alkalophilic halophiles: SP1	W. D. Grant
SP2	W. D. Grant
MS3	W. D. Grant

(*b*) *Halophilic bacteria containing fatty acid methyl esters but lacking glycerol diether moieties*

Strain	Source*
Actinopolyspora halophila	ATCC 27976
Brevibacterium halotolerans	ATCC 25096
Ectothiorhodospira halophila	DSM 244
Ectothiorhodospira sp. (S1)	W. D. Grant
Flavobacterium halmephilum	NCMB 1971
Micrococcus halobius	CCM 2526
Micromonospora halophytica subsp. *halophytica*	NRRL 2998
Micromonospora halophytica subsp. *nigra*	NRRL 3097
Paracoccus halodenitrificans	NCMB 700
Planococcus halophilus	ATCC 27964
Pseudomonas beijerinckii	NCIB 9041
Vibrio costicola	NCMB 701

* ATCC, American Type Culture Collection, Rockville, U.S.A.; CCM, Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia; DSM, Deutsche Sammlung von Mikroorganismen, Munich, W. Germany; NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland; NCMB, National Collection of Marine Bacteria, Aberdeen, Scotland; NRRL, Northern Utilization Research and Development Division, U.S. Department of Agriculture, Illinois, U.S.A.; W. D. Grant, Department of Microbiology, University of Leicester, Leicester.

† Now classified as *Halococcus morrhuae* by Kocur & Hodgkiss (1973).

medium of Mullakhanbhai & Larsen (1975). *Flavobacterium halmephilum*, *Micrococcus halobius*, *Micromonospora halophytica*, *Paracoccus halodenitrificans*, *Pseudomonas beijerinckii* and *Vibrio costicola* were grown in liquid shake culture in nutrient broth no. 2 (Oxoid) supplemented with 10% (w/v) NaCl at 30 °C. *Brevibacterium halotolerans* was grown in nutrient broth no. 2 (Oxoid) supplemented with 5% (w/v) NaCl at 30 °C. The alkalophilic halophiles (SP1, SP2, MS3) and *Ectothiorhodospira* strains (*E. halophila*, S1) were grown in the media of Tindall *et al.* (1980) and Imhoff & Trüper (1977), respectively, at 37 °C. *Planococcus halophilus* was grown at 30 °C in liquid medium containing tryptone (5 g l^{-1}), proteose peptone (5 g l^{-1}) and NaCl (58·4 g l^{-1}). Cultures were checked for purity at maximum growth, harvested by centrifugation (10000 ***g***) and freeze-dried.

Whole-organism acid methanolysis. Dry cells (100 mg) were mixed with methanol (3 ml), toluene (3 ml) and conc. H_2SO_4 (0·1 ml) and heated at 50 °C for 15 to 18 h as described by Minnikin *et al.* (1975). The long-chain components were extracted from this mixture by adding 1·5 ml hexane (Minnikin *et al.*, 1975). Hexane extracts were chromatographed on Merck silica gel $60F_{254}$ aluminium-backed thin-layer plates (10 × 10 cm) using petroleum ether (b.p. 60 to 80 °C)/diethyl ether (85 : 15, v/v) as developing solvent (Minnikin *et al.*, 1975). Further resolution of glycerol diether moieties was achieved by developing the t.l.c. plate in one dimension using petroleum ether (b.p. 60 to 80 °C)/acetone (95 : 5, v/v) followed by redevelopment in the same direction using toluene/acetone (97 : 3, v/v). Confirmation of the results obtained from the whole-organism methanolysis method of Minnikin *et al.* (1975) was achieved by using the anhydrous methanolic HCl procedure of Kates *et al.* (1966) on the total lipid fraction of the test strains. Lipids were revealed by spraying the t.l.c. plates with 10% dodecamolybdophosphoric acid in absolute ethanol and heating for 15 min at 150 °C. All lipids appeared as dark blue spots on a yellow background.

RESULTS AND DISCUSSION

The patterns obtained by chromatographic analysis [using petroleum ether (b.p. 60 to 80 °C)/diethyl ether; 85 : 15, v/v] of methanolysates of halophilic bacteria are shown in Fig. 1.

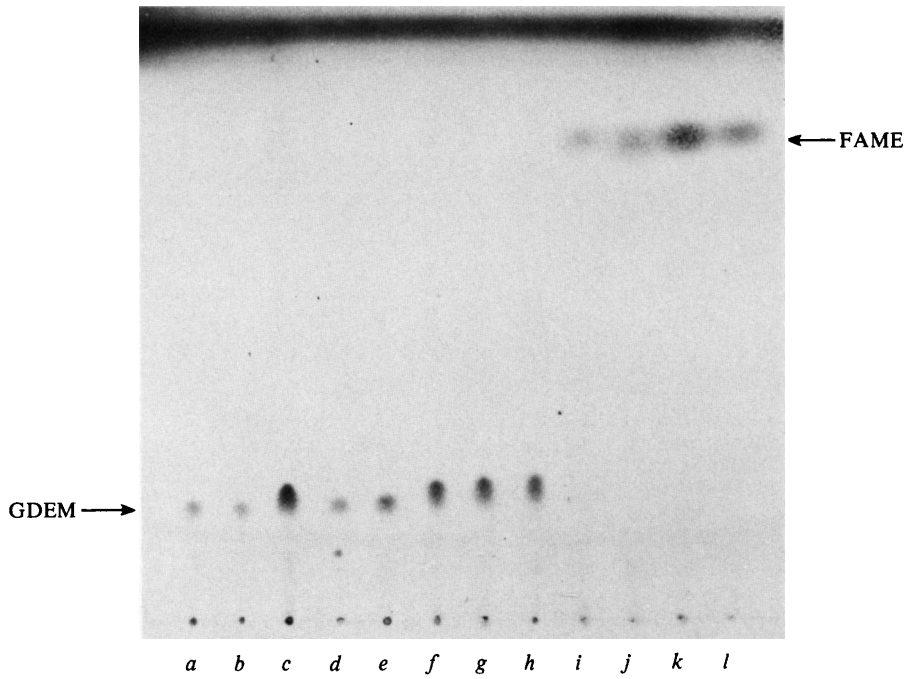

Fig. 1. Thin-layer chromatographic analysis of whole-organism methanolysates of (*a*) *Halococcus morrhuae* NCMB 787, (*b*) *Halococcus morrhuae* NCMB 749, (*c*) *Halobacterium halobium* NCMB 777, (*d*) *Halobacterium salinarium* NCMB 764, (*e*) *Halobacterium volcanii* NCMB 2012, (*f*) SP1, (*g*) SP2, (*h*) MS3, (*i*) *Planococcus halophilus* ATCC 27964, (*j*) *Actinopolyspora halophila* ATCC 27976, (*k*) *Vibrio costicola* NCMB 701 and (*l*) *Pseudomonas beijerinckii* NCIB 9041. Plates were developed in petroleum ether (b.p. 60 to 80 °C)/diethyl ether (85 : 15, v/v). FAME, fatty acid methyl esters; GDEM, glycerol diether moieties.

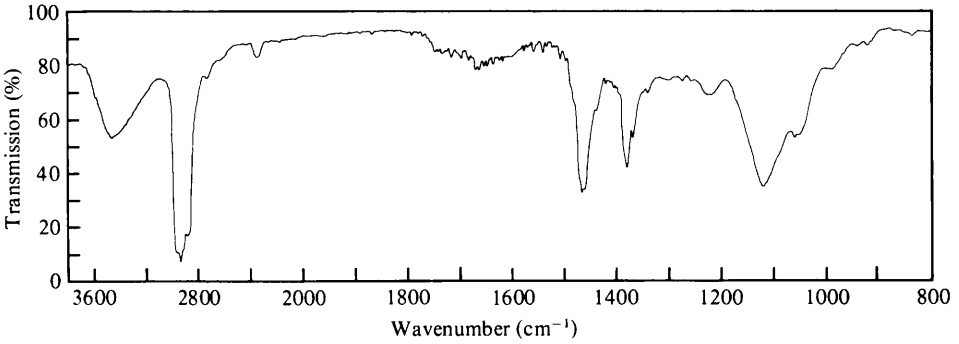

Fig. 2. Infrared spectrum of the purified glycerol diether moiety from *Halobacterium cutirubrum* NCMB 763.

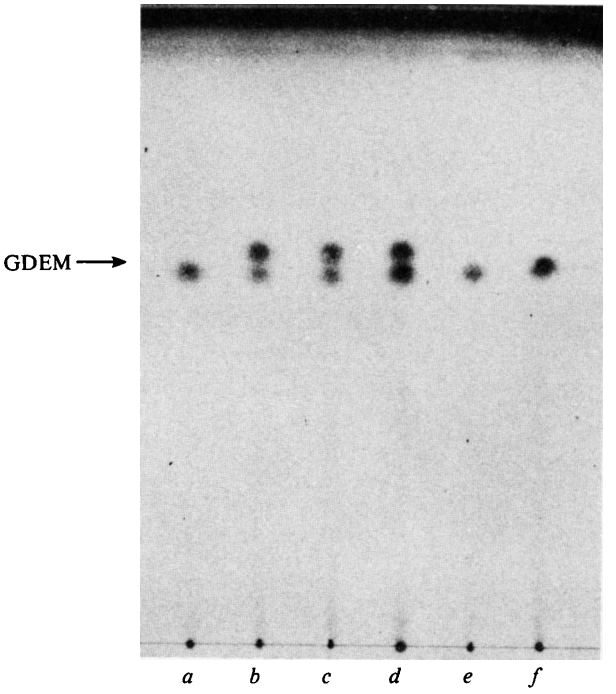

Fig. 3. Thin-layer chromatographic analysis of whole-organism methanolysates of (*a*) *Halobacterium cutirubrum* NCMB 763, (*b*) SP1, (*c*) SP2, (*d*) MS3, (*e*) *Halobacterium salinarium* NCMB 764 and (*f*) *Halococcus morrhuae* NCMB 749. Plates were first developed in petroleum ether (b.p. 60 to 80 °C)/acetone (95 : 5, v/v), then dried and redeveloped in the same direction in toluene/acetone (97 : 3, v/v). GDEM, glycerol diether moieties.

Spots with R_F values of about 0·2 were attributable to glycerol diether moeities (GDEM) whereas components having R_F values greater than 0·6 corresponded to methyl esters of non-hydroxylated fatty acids (FAME) (Minnikin *et al.*, 1975, 1978). On the basis of their chromatographic patterns, the test strains were divided into two broad groups: taxa containing GDEM and lacking FAME (Table 1 *a*), and taxa containing FAME only (Table 1 *b*). The absence of ester-containing lipids in the archaebacterial halophiles (Table 1 *a*) was demonstrated by a negative reaction with the hydroxamate/$FeCl_3$ ester spray reagent (Skidmore & Entenman, 1962; Kates, 1975). This reagent unequivocally distinguishes

glycerol diether moieties from ester-containing components such as hydroxylated fatty acid methyl esters, which have similar R_F values (about 0·2). The presumptive diether moieties, purified by preparative t.l.c., were further confirmed by infrared spectroscopy. Notable was the complete absence of ester absorption in the range 1730 to 1750 cm^{-1} (Fig. 2). However, the spectra did show strong absorption bands indicative of long-chain groups (2960, 2940, 2880, and 1460 cm^{-1}), OH groups (3400 cm^{-1}, broad) and ether C–O–C groups (1120 cm^{-1}) (Fig. 2). Doublets at 1365 to 1380 cm^{-1} were indicative of isopropyl units.

It was noted that the glycerol diether spots of certain archaebacterial strains (spots *c*, *f*, *g* and *h*, Fig. 1) were elongated. An attempt to resolve these components further was made by developing the t.l.c. plate in one dimension using petroleum ether (b.p. 60 to 80 °C)/acetone (95 : 5, v/v) followed by redevelopment in the same direction in toluene/acetone (97 : 3, v/v). Using this procedure, two separate spots were observed, suggesting two distinct glycerol diether moieties (Fig. 3). Further confirmation that these strains contain two chromatographically distinct glycerol diether moieties was obtained by applying the alternative acid methanolysis procedure of Kates *et al.* (1966). Chromatographic analysis of these extracts yielded identical results. This is the first report of two distinct glycerol diether moieties within the extreme halophiles. The extensive investigations of Kates and associates were confined to strains (notably *Halobacterium cutirubrum*) which possess only one major glycerol diether moiety (Fig. 3). Work is in progress to elucidate the structural relationship between these two chromatographically distinct diether moieties.

We believe that thin-layer chromatographic analysis of whole-organism methanolysates provides a simple and effective way of distinguishing archaebacterial halophiles from eubacterial halophiles. In addition, the methanolysis procedure of Minnikin *et al.* (1975) can be scaled down to about 15 to 20 bacterial colonies and thus provides a useful screening procedure for archaebacterial halophiles.

H. N. M. Ross and B. J. Tindall are grateful to the Science Research Council for studentship grants. W. D. Grant is grateful to the Royal Society for support.

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