The Isolation and Characterization of Agarolytic Bacteria from a Lowland River

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Several strains of agar-degrading bacteria were isolated from the River Wey, Guildford, during the summer of 1976. All were Gram-negative rods and could be divided into two groups. Those which only softened the agar belonged to the genus *Cytophaga* and those which caused extensive liquefaction of the agar have been referred to the genus *Alteromonas*. Attempts to isolate, purify and characterize the enzymes showed some differences between the two taxa. The strains of *Cytophaga* produced at least two enzyme complexes, one cell-free and the other cell-bound, and hydrolysed agar with the formation of oligo-saccharides. The strains of *Alteromonas*, on the other hand, readily released 'agarase' into the medium yielding monosaccharides as major end-products. The agar-degrading enzymes of both groups were inducible, not only by agar, but also by other galactans and polysaccharides associated with plants. The enzyme preparations also hydrolysed a wide range of plant-derived polysaccharides, including some associated with terrestrial and freshwater plants rather than with marine algae. These results suggest that there is no special ecological reason for the presence of agarolytic bacteria in fresh water but that their activity reflects the wide substrate spectrum of the polysaccharides of such organisms. The agar-softening strains are considered to belong to a new species for which the name *Cytophaga saccharophila* is proposed.

INTRODUCTION

Agar is a complex polysaccharide produced by marine red algae from genera such as *Gelidium*, *Gracilaria* and *Ceramium*. It contains a neutral agarose fraction and an ionic agaropectin fraction (Araki, 1937) but both fractions are thought to consist of alternating units of (1→3)-β-D-galactopyranoside and (1→4)-3,6-anhydro-α-L-galactoside. In the ionic agaropectin fraction the galactoside units may be methylated or contain pyruvic or sulphuric acid residues (Araki, 1956; Duckworth & Yaphe, 1971*a, b*; Izumi, 1972).

The firm gel formed when a 1.5 to 2.0% (w/v) solution of agar in boiling water is allowed to cool is believed to be a matrix of double helical polymers held together by hydrogen bonds (Ng Ying Kin & Yaphe, 1972). The gel may be degraded by micro-organisms in three ways. (1) Disruption of the double helical structure without breakdown of the polymer. This does not generally produce any visible changes in the gel and such limited activity can only be detected by the failure to form the brown colour that the normal gel forms with iodine (Gran, 1902). This colour is thought to depend on the integrity of the double helical structure (Ng Ying Kin & Yaphe, 1972). (2) Cleavage of the α-linkages of the agar molecule, giving rise to oligosaccharides of the agarobiose series with 3,6-anhydro-L-galactose at the reducing end. (3) Cleavage of the β-linkages, giving rise to the neo-

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agarobiose series of oligosaccharides with D-galactose at the reducing end. The enzymes involved in (2) and (3) are called α - and β -agarases, respectively; most agarolytic bacteria so far described are considered to produce only β -agarase (Yaphe, 1966; Turvey & Christison, 1967; Vattuone *et al.*, 1975; van der Meulen & Harder, 1975). Visible changes caused by cleavage of the agar molecule range from softening of the surface of the gel to the formation of craters and eventual complete liquefaction.

Although many isolates associated with agarolytic activity have been named in the literature, the 8th edition of *Bergey's Manual* (Buchanan & Gibbons, 1974) retains very few fully described species. The Gram-negative species occur in the genera *Cytophaga* and *Flavobacterium* while many isolates previously placed in the genera *Vibrio* and *Pseudomonas* are no longer accepted as belonging to those genera. Most agarolytic bacteria have been isolated from marine habitats but recent isolations from other sources have been reported by v. Hofsten & Malmqvist (1975), van der Meulen *et al.* (1974) and Hunger & Claus (1978).

During a study of the bacteria which contribute to the degradation of the macromolecular organic materials of plant origin in the River Wey, a number of agar-degrading organisms were isolated. The present study was undertaken in an attempt to characterize these agarolytic isolates and to understand their role in a freshwater habitat.

METHODS

Sampling. Water samples were collected from the River Wey at Guildford over a 9 month period. During any one session, seven samples were collected simultaneously in sterile 500 ml bottles from a range of depths in the river using the device illustrated in Fig. 1. Viable bacteria were estimated by spreading 0.1 ml of dilutions of each sample on plates of quarter-strength nutrient broth solidified with 1.5% (w/v) Davis agar (0.25NA) and counting colonies after 7 d incubation at 25 °C.

Enrichment and isolation of agarolytic bacteria. Erlenmeyer flasks (250 ml) containing sterile river water (100 ml), to which 0.1% (w/v) Oxoid no. 3 agar had been added, were inoculated with river water samples (20 ml) and incubated at 25 °C on a rotary shaker for 4 d. Samples (0.1 ml) of the cultures were then plated on a mineral medium containing (g l⁻¹ in deionized water): KH₂PO₄ (1.0); NH₄Cl (0.5); MgSO₄·7H₂O (0.5); CaCl₂·2H₂O (0.1); Oxoid no. 3 agar (15); adjusted to pH 7.2 before autoclaving at 121 °C for 15 min. Plates were incubated at 25 °C and examined daily for agarolytic activity, assessed by liquefaction or shallow depressions appearing around the colonies. After 7 d, plates were flooded with Lugol's iodine and the appearance of pale-yellow zones around colonies against a reddish-violet background was considered indicative of some agar-degrading activity in the absence of the visible signs already referred to. All colonies showing liquefaction or depressions in the agar were picked off and purified by streaking out on mineral or nutrient agar.

Representative agar-degrading strains were maintained in Dubos' solution containing (g l⁻¹): NaNO₃ (0.5); K₂HPO₄ (0.1); MgSO₄·7H₂O (0.5); FeSO₄·7H₂O (0.01); adjusted to pH 7.2, dispensed into Bijou bottles and sterilized at 121 °C for 15 min.

Strains used for detailed examination. Eleven strains representing the non-pigmented, motile, agar-liquefying isolates and six representing the pigmented, agar-softening isolates were examined along with agarolytic strains NCIB 9491 (Mitchell *et al.*, 1969) and NCMB 1914 (v. Hofsten & Malmqvist, 1975), obtained from the National Collections of Industrial and Marine Bacteria, Torrey Research Station, Aberdeen. *Pseudomonas putrefaciens* (NCIB 10472) and *P. rubescens* (NCIB 8768), obtained from the culture collection of the Department of Microbiology, University of Surrey, were included as non-agarolytic reference strains. *Pseudomonas putrefaciens* and *P. rubescens* are considered to be synonyms of *Alteromonas putrefaciens* (Lee *et al.*, 1977).

All tests were carried out at 25 °C unless stated otherwise.

Colonial and cell morphology. Colonial characteristics and pigmentation were studied on plates of nutrient agar, with and without 30% (v/v) skim milk (Shewan *et al.*, 1960), on river water agar, mineral medium agar and the media of King *et al.* (1954).

Motility (hanging drop), Gram-staining (modification of Preston & Morrel) and examination for microcyst formation in yellow-pigmented isolates (Stanier, 1942) were done on bacteria grown in peptone (1.5%, w/v) water for 48 h. Cell morphology and flagellation were studied using a Jeol transmission electron microscope (JEM 100B). Bacteria grown on nutrient agar plates for 24 to 96 h were harvested

Fig. 1. River sampling apparatus: A, vacuum reservoir; B, rack of 500 ml sampling bottles; C, hollow rigid plastic column to support sampling tubes; D, sterilizable plastic tubing (the lengths were adjusted to provide an equivalent sampling rate from each depth; the dead space of the tubing was considered to be negligible compared with the sample size); E, metal base plate.

in sterile distilled water, centrifuged at 40 *g* for 30 min, washed once with 10% (v/v) aqueous dimethyl sulphoxide to remove extracellular polysaccharide and twice in distilled water. The washed bacteria were suspended in 1 vol. 0.025% (w/v) bovine serum albumin and mixed with 2 vol. 2% (w/v) aqueous phosphotungstic acid previously adjusted to pH 7.2 with 1 M-KOH. After standing for 30 to 60 min, drops of the stained suspension were pipetted on to Formvar-coated copper grids, dried and examined in the electron microscope at an accelerating voltage of 80 kV.

Physiological tests. Growth at temperatures between 4 and 50 °C was tested on slopes of 0.25NA, or in tubes of 0.25NB, incubated in an aluminium temperature gradient block. Growth on slopes was assessed visually and that in broth spectrophotometrically at 546 nm (Unicam SP600).

Growth at pH values between 4 and 9 was tested in mineral medium containing 0.2% (w/v) agar, adjusted with either 0.2 M- K_2HPO_4 or 0.2 M- KH_2PO_4 .

Sodium chloride tolerance was tested on plates of a medium containing (g l⁻¹): peptone (Oxoid), 1.25; yeast extract (Oxoid), 0.5; agar (Oxoid no. 3), 15; and NaCl up to 10% (w/v). Growth was assessed after 5 d.

The ability to grow anaerobically was tested by incubating plates of nutrient agar in anaerobic jars with an atmosphere of hydrogen for 5 d.

Biochemical tests. Strains were tested for catalase, indole (Kovacs' method), H_2S (method 3) and phosphatase (method 1) production, methyl red and Voges-Proskauer (Barritt's method) reactions, nitrate reduction (method 1) and gelatin hydrolysis (method 1 at 20 °C for 7 d) (all according to Cowan & Steel, 1974), oxidase activity (Kovacs, 1956), DNAase activity (Jeffries *et al.*, 1957) using Bacto DNAase test agar (Difco), arginine dihydrolase production (Thornley, 1960) and decarboxylation of arginine, ornithine and lysine (Møller, 1955). Tests were examined after 5 and 10 d except where otherwise indicated.

The O/F test (Cowan & Steel, 1974) was done using the basal medium of Hugh & Leifson (1953) containing 1% (w/v) glucose. The agar-liquefying strains were further examined on plates of the same medium, incubating one set aerobically and the other set in an anaerobic jar with hydrogen. Both tubes and plates were examined after 7 and 14 d.

Starch hydrolysis was examined by incubating plates of yeast extract agar (Difco) containing 0.2% (w/v) soluble starch (Fisons) for 4 d and then flooding with Lugol's iodine. Alternatively, where liquefaction of the agar was a nuisance, tubes of peptone water containing 0.1% (w/v) K_2HPO_4 and 1.0% (w/v) soluble starch were used. After 14 d, residual starch was tested for by adding a drop of Lugol's iodine. Cellulose

Table 1. *Characters used for numerical taxonomy and other compounds tested as sole carbon source*

<i>Numerical taxonomy</i>		
Non-diffusible pigment		Utilization of:
Diffusible pigment		Glycine
Rapid motility		Isoleucine
Gliding motility		Threonine
Polar flagella		Aspartate
Hugh & Leifson:		Arginine
O (acid)		Proline
O (alkaline)		Malate
F		Glycerol
No growth		Glucose
Growth at:		Ribose
37 °C		Xylose
41 °C		Arabinose
Growth with:		Galactose
0% (w/v) NaCl		<i>N</i> -Acetylglucosamine
2% (w/v) NaCl		Glucosamine
3% (w/v) NaCl		Trehalose
7% (w/v) NaCl		Raffinose
10% (w/v) NaCl		Benzoate
Indole production		<i>p</i> -Hydroxybenzoate
Voges-Proskauer		<i>m</i> -Hydroxybenzoate
Methyl red		Sensitivity to:
DNAase		Tetracycline (10 µg)
Phosphatase		Erythromycin (15 µg)
Amylase		Chloramphenicol (10 µg)
Liquefaction of gelatin		Polymyxin (300 units)
Liquefaction of agar		Streptomycin (10 µg)
Softening of agar		Fucidin (5 µg)
Catalase		Novobiocin (5 µg)
Oxidase		Penicillin G (2 units)
Arginine dihydrolase		Penicillin G (10 units)
Arginine decarboxylase		Methicillin (10 µg)
Ornithine decarboxylase		Neomycin (10 µg)
Lysine decarboxylase		Trimethoprim (1.25 units)
Trimethylammonium oxide reduction		Sulphafurazole (100 µg)
Nitrate to nitrite		0/129 (10 µg)
Nitrate to nitrogen		
Acid from inositol		
Gas from glucose		
<i>Further compounds tested as sole carbon source for isolates 049/1 and 024</i>		
Fructose	L-Rhamnose	Mannose
Sucrose	Maltose	Cellobiose
Lactose	Melibiose	Stachyose (Sigma)
Agar (Oxoid)	Agarose (Sigma)	Starch (Fisons)
Pectin (BDH)	Carrageenan (Sigma)	Arabinogalactan (Sigma)
Gum arabic (Sigma)	Gum xanthan (Sigma)	Galactomannan
Succinate		

decomposition was tested on plates of nutrient agar overlaid with a thin layer of cellulose powder suspended in nutrient agar. Plates were incubated for 14 d and examined for clearing of the cellulose layer around developing colonies. Trimethylamine oxide reduction was tested by the method of Laycock & Regier (1971) except that 20% (w/v) KOH was used in place of 20% (w/v) K₂CO₃.

Carbon compounds utilized for growth. Strains were tested for their ability to utilize a range of organic compounds as sole carbon and energy source. The compounds chosen (see Table 1) included many of those used by Lee *et al.* (1977), Lewin (1969), Stanier (1942) and van der Meulen *et al.* (1974) for the differentiation of aerobic Gram-negative rods. They were added to the basal mineral medium to give a final concentration of 0.2% (w/v) and autoclaved at 121 °C for 15 min, with the exception of pentose sugars which were sterilized by filtration and added aseptically to autoclaved basal medium. Those organisms unable to grow on or hydrolyse gelatin were also tested on plates of the carbon source media solidified with 15% (w/v) gelatin. Tubes were incubated at 25 °C and plates at 20 °C and examined daily for growth.

Antibiotic sensitivity. Antibiotic Multodisks (Oxoid) and discs containing the vibriostat 0/129 (10 µg) were applied to surface-inoculated dried plates of CM3 medium (Oxoid). Details of the antibiotics used are given in Table 1.

DNA analysis. DNA was extracted and purified by the method of Mandel *et al.* (1971); the T_m was determined using the method of Marmur & Doty (1962) and the mol % GC was calculated using the equation of De Ley (1970).

Numerical taxonomy. The results of the tests and observations listed in Table 1 were coded as binary data and the similarity matrix of similarities, calculated using the simple matching coefficient (Sneath, 1957), was sorted using McQuitty's techniques (McQuitty, 1967). Cluster analysis was carried out using the Clustan 1A package of Wishart (1969) on the University of Surrey ICL 1905F computer.

Assay of agarase activity. Selected isolates were cultured in 250 ml Erlenmeyer flasks containing 150 ml basal mineral medium supplemented with 0.2% (w/v) agar (Oxoid no. 3) and incubated at 25 °C for 5 to 10 d on a rotary shaker (150 rev. min⁻¹). Cultures were then centrifuged to remove bacteria, the supernatant was treated with 0.2 vol. 2.5% (w/v) Cetrimide and the resulting precipitate was removed by centrifuging. The supernatant was treated with 2 vol. acetone at 0 °C and allowed to stand at 4 °C for 30 min. The precipitate was collected by centrifuging and redissolved in 5 mM-KH₂PO₄/Na₂HPO₄ buffer (pH 6.4).

The activity of this crude extracellular agarase was assayed with a solution of agarose (Sigma; 1.25 mg ml⁻¹) in 5 mM-KH₂PO₄/Na₂HPO₄ buffer (pH 6.4). The agarase preparation (0.5 ml) was mixed with 0.5 ml agarose solution and 2 ml 10 mM-KH₂PO₄/Na₂HPO₄ buffer (pH 6.4) and incubated at 28 °C for 1 h. The reaction was stopped by adding 1 ml 2.5% (w/v) Cetrimide and the reducing sugars were measured by the method of Dygert *et al.* (1965) except that any precipitate of undegraded agarose was removed by centrifuging (2600 g) for 15 min. Blanks of substrate with no enzyme and enzyme with no substrate were treated in the same way. One unit of agarase activity was defined as the release of reducing groups equivalent to 1 µmol D-galactose in 1 h at 28 °C, pH 6.4.

The products of the action of the crude agarase were examined qualitatively using agarose (Sigma) as a substrate. Agarose (2.5 mg ml⁻¹) was dissolved in boiling 10 mM-KH₂PO₄/Na₂HPO₄ buffer (pH 6.4), cooled to 28 °C, added to an equal volume of agarase preparation and incubated at the same temperature for 5 h. The reaction mixture was then added to 3 vol. ethanol at 0 °C, any precipitate was removed by centrifuging, and the mixture was concentrated under reduced pressure. The products were analysed on unactivated plates of cellulose MN300 (Sigma), prepared by spreading a thin layer of an aqueous suspension of cellulose (15%, w/v) on grease-free glass plates (20 × 20 cm) and drying at room temperature for 3 d. D-Galactose was used as a marker. Plates were developed with 1-butanol/ethanol/water (3:2:2, by vol.), dried and sprayed with the modified naphthoresorcinol reagent of Duckworth & Yaphe (1970).

Release of reducing compounds from polysaccharides other than agar. Solutions of polysaccharides (2.5 mg ml⁻¹) were prepared in 10 mM-KH₂PO₄/Na₂HPO₄ buffer (pH 6.4) (see Table 7). The substrate (0.5 ml) was added to either a preparation of crude agarase (0.5 ml) or buffer (0.5 ml) and incubated at 28 °C for 1 h. The reaction was stopped by adding 2.5% (w/v) Cetrimide (1 ml), and the mixture was clarified by centrifuging and assayed for reducing compounds by the method of Dygert *et al.* (1965).

Assay of α- and β-galactosidase activities. The enzyme preparation (0.5 ml) was mixed with 0.5 ml of either 15 mM-*o*-nitrophenyl-α-D-galactopyranoside or 20 mM-*o*-nitrophenyl-β-D-galactopyranoside in 5 mM-KH₂PO₄/Na₂HPO₄ buffer (pH 7.0) and incubated at 28 °C. The reaction was stopped after 1 h, or in some cases after 24 h, by adding 5 drops of 1.0 M-Na₂CO₃, and the yellow colour of the *o*-nitrophenol released was measured at 420 nm. Blanks containing substrate but no enzyme were treated in the same manner.

Repression and induction of agarase. The production of extracellular agarase by the isolates 049/1 and 024 was examined using a number of different carbohydrates and carbohydrate derivatives as carbon sources (see Table 6). Media were prepared by adding 10 ml of an aqueous solution or homogenized suspension of the carbon source (2%, w/v, except gum xanthan which was 1%, w/v) to 100 ml of a basal medium (in 250 ml Erlenmeyer flasks) containing (% w/v): KNO₃ (0.02); MnSO₄·4H₂O (0.02); MgCl₂ (0.02); FeSO₄·7H₂O (0.001); adjusted to pH 7.2 with 0.2 M-K₂HPO₄ and autoclaved at 121 °C for 15 min (except arabinogalactan which was sterilized separately by filtration before adding to autoclaved basal medium). Flasks were inoculated with 2 ml of 48 h culture in peptone water (0.05%, w/v) and incubated on a rotary shaker (150 rev. min⁻¹) at 25 °C.

Samples (5 ml) were removed after 1, 2, 5 and 7 d, centrifuged to remove bacteria, the supernatant was treated with 1 ml 2.5% (w/v) Cetrimide and any precipitate was removed by centrifuging. The supernatant was then treated with 2 vol. cold (4 °C) acetone and the resulting precipitate was recovered by centrifuging and redissolved in 2.5 ml 5 mM-KH₂PO₄/Na₂HPO₄ buffer (pH 6.4). This preparation was assayed for agarase activity as previously described.

RESULTS

The total viable count varied from 10^4 to 10^5 colony-forming units during the sampling period; the maximum counts were recorded from samples taken near the silt/water interface (Table 2). Agar-degrading bacteria were detected in relatively few enriched samples, mostly in those taken near the silt/water interface during summer (Table 3). No strains able to liquefy or soften agar were isolated from the surface waters. Isolates changing the structure of agar so that it no longer turned a deep reddish violet with Lugol's iodine, but not softening or liquefying it, were found at all depths. Because this character was found to be unstable, such isolates were not studied in more detail.

When the mutual similarity indices of Sneath (1957), based on 70 morphological and biochemical observations, were plotted as a dendrogram (Fig. 2), the organisms divided into three groups at the 80% similarity level. Group A included all the non-pigmented, agar-liquefying isolates with NCMB 1914 and NCIB 9491, group B contained only the yellow-pigmented, agar-softening isolates and group C contained NCIB 10472 and NCIB 8768.

Colourless, agar-liquefying strains (group A)

Apart from minor differences, all 11 non-pigmented, agar-liquefying isolates were very similar and almost certainly belong to the same species. Colonies were white and rapidly produced a crater of digested agar on nutrient agar plates, in the manner illustrated by v. Hofsten & Malmqvist (1975). Moist nutrient agar plates were completely liquefied in about 10 d at 25 °C. Colonies produced no diffusible pigment and growth occurred over a wide temperature range (4 to 40 °C) with the optimum at 25 °C.

All strains were aerobic, oxidase, DNAase and phosphatase positive, methyl red, Voges-Proskauer and arginine dihydrolase negative, resistant to the vibriostat 0/129 and 2 units of benzylpenicillin, sensitive to 10 units of benzylpenicillin, polymyxin (300 units), novobiocin (5 µg) and chloramphenicol (10 µg), utilized D-xylose, L-arabinose, L-rhamnose, D-glucose, D-galactose, D-mannose, D-fructose, sucrose, trehalose, maltose, cellobiose, lactose, melibiose, raffinose, glucosamine, N-acetylglucosamine, agar, agarose, carrageenan, arabinogalactan, galactomannan, gum arabic, gum xanthan and cellulose as sole carbon source, and reduced nitrate to nitrite. No strain hydrolysed gelatin or starch, produced indole or H₂S or reduced trimethylamine oxide.

Some strains (3/11) were weakly catalase positive, the rest being negative, and their reaction in the traditional O/F test was ambiguous, difficult to assess and was usually scored as weakly oxidative or non-reactive. Distinct acid production was observed on plates of Hugh & Leifson medium incubated aerobically, but not on plates incubated anaerobically on which no growth occurred.

Strains were vigorously motile and had a single polar flagellum. They showed no special requirement for Na⁺ and were inhibited in the presence of more than 2% NaCl. Considered as pseudomonads, these isolates most closely resemble phenon E of Lee *et al.* (1977) except that they were unable to reduce trimethylamine oxide or produce H₂S. The mol % GC of three representative strains was 50.8, 50.3 and 50.8. It is suggested that the strains of group A belong to the genus *Alteromonas* as redefined by Lee *et al.* (1977). Despite some significant differences, our isolates are very closely related to, and probably belong to the same species as, NCMB 1914.

Vibrio agarliquefaciens (Berget *et al.*, 1934), first described as *Microspira agarliquefaciens* by Gray & Chalmers (1924), is of uncertain affinity and might also be con-specific with the isolates described in this paper. Thus we decline from proposing a new name for them because the existing epithet '*agarliquefaciens*' may be applicable. We have not examined *V. agarliquefaciens* and further work is required to establish whether it belongs to *Alteromonas*. A representative of our isolates (049/1) has been deposited with the National

Table 2. Counts of colony-forming units (c.f.u.) on 0.25NA at different depths of some of the deeper, and possibly stratified, stretches of the River Weir during the period March to November 1976

Depth (cm)	10 ⁻⁴ × Mean* no. of c.f.u. ml ⁻¹ during each month									
	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	
30	20.3	16.3	9.3	7.5	4.5	4.9	4.0	2.5	4.8	
60	15.8	13.0	8.3	5.0	4.1	2.9	ND	1.9	ND	
90	7.8	11.5	6.9	2.5	2.9	2.2	2.6	1.8	2.3	
120	7.3	4.3	5.5	2.1	2.1	ND	1.8	1.8	2.5	
150	20.0	1.5	9.0	2.8	2.8	2.9	3.2	ND	2.9	
180	23.0	21.1	12.3	8.2	5.3	ND	ND	3.0	ND	
Bottom	45.0	40.5	37.3	15.1	10.7	11.7	11.6	25.0	11.8	

ND, Not determined.

* Each value is based on at least 10, and usually 15, samples from each depth.

Table 3. Number of samples (taken from the River Weir) from which agar-degrading bacteria were detected after enrichment

Depth (cm)	Reaction†	No. of positive samples* during each month									
		Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Total
30	G	0	4	1	0	0	2	2	0	0	9
60	S	0	0	0	2	0	0	1	0	1	4
	G	2	0	2	3	2	1	3	0	3	16
120	S	0	0	1	1	0	0	0	1	0	3
	G	0	2	1	4	1	0	2	2	0	12
180	L	0	0	0	2	1	0	0	0	0	3
	S	0	1	0	3	2	3	2	2	1	14
	G	3	4	3	8	5	2	2	2	2	31
Bottom	L	0	0	2	4	0	0	ND	0	0	6
	S	0	0	2	6	1	1	ND	0	0	10
	G	6	4	8	7	5	4	ND	3	2	39

ND, Not determined.

* Number of samples from each depth was 10 in March and April and 15 at all other times.

† L, Liquefaction; S, softening; G, 'gelase' reaction with iodine. No samples from < 180 cm depth showed liquefaction and no samples from < 60 cm showed softening.

Fig. 2. Dendrogram based on mutual similarity indices (*S*) computed from 70 observations on 17 isolates and 4 culture collection strains.

Table 4. *Comparison of the agar-liquefying isolates NCMB 2062 (this paper), NCMB 1914 (v. Hofsten & Malmqvist, 1975) and Microspira agarliquefaciens (Gray & Chalmers, 1924)*

Character	NCMB 2062 (049/1)	NCMB 1914	<i>Microspira</i> <i>agarliquefaciens</i> *
Shape	Straight to curved rods	Slightly curved rods	Short curved rods
Size (μm)	0.5–0.7 $\times$ 2.0–3.0	0.5 $\times$ 1.7	0.6–1.2 $\times$ 1.4–3.0
Flagella	Single polar	Single polar	Single polar
O ₂ requirement	Highly aerobic	Highly aerobic	Highly aerobic
NO ₃ [−] $\rightarrow$ NO ₂ [−]	+	+	+
Growth at:			
16 °C	+	?	+
25 °C	++	++	++
37 °C	+	+	−
Cellulolysis	+	−	+
Gelatin liquefaction	−	−	−
Amylase	−	−	+
Growth on:			
Glucose	+	+	+
Sucrose	+	?	+
Lactose	+	+	+
Maltose	+	+	+
Glycerol	−	−	−

* Data from Gray & Chalmers (1924).

Collection of Marine Bacteria, Torry Research Station, Aberdeen, as NCMB 2062. Some properties of this isolate, those of NCMB 1914, and those described by Gray & Chalmers (1924) for *Microspira agarliquefaciens* are compared in Table 4.

Pigmented, agar-softening isolates (group B)

Two of the six original isolates in this group (025 and 026) lost their agarolytic activity after several subcultures and the following description is based on the remaining four (023, 024, 028 and 030). All strains produced discrete yellow colonies surrounded by a shallow depression on nutrient agar. After 5 to 10 d the agar gel was obviously softened but did not liquefy. Colonies were positive in the iodine test, a character which was reproducible after many subcultures (Fig. 3). Colonies remained circular and discrete on nutrient agar but limited spreading was observed on river water agar and basal mineral medium agar if the agar surface was sufficiently moist. Strains grew between 4 and 30 °C with the optimum at 25 °C.

The bacteria were Gram-negative, non-motile and showed considerable pleomorphism with coccoid, long slender, and constricted forms occurring in all strains (Fig. 4). The constricted forms were reminiscent of those illustrated by Mitchell *et al.* (1969) and considered by them to be characteristic of species of *Cytophaga*. Pleomorphism was more common in cultures from solid media and long slender forms predominated in cultures in liquid media. Bacteria taken from 24 h-old colonies grown on solid media frequently showed periplasmic strands similar to structures observed on cell surfaces of *Cytophaga johnsonae* by Follett & Webley (1965). Strains did not show flexing movement in hanging drop preparations but very slow gliding motility was observed at areas of contact between the meniscus of the drop and the supporting cover slip. These bacteria did not stain well and microcysts were never observed in stained preparations.

All isolates grew well in media free of NaCl, poorly with 2% NaCl, and not at all at higher concentrations. The four isolates were sufficiently similar to be considered members of the same species and, despite the difficulty in observing gliding motility coupled with the production of compact, discrete colonies rather than thin spreading colonies, they fit well into the genus *Cytophaga*. With the exception of the freshwater isolate *Cytophaga*

Fig. 3. Nutrient agar plate inoculated with *Cytophaga saccharophila* before (a) and after (b) flooding with Lugol's iodine. Depressions in the agar can just be seen before treatment with iodine which exposes the extent of the 'gelase' reaction.

Fig. 4. Electron micrographs of *Cytophaga saccharophila* negatively stained with 2% (w/v) phosphotungstic acid. Bar markers represent 1 µm.

flevensis (van der Meulen *et al.*, 1974), all adequately described agarolytic species of *Cytophaga* have been isolated from marine habitats. Our isolates differ from *C. flevensis* in their ability to hydrolyse starch, gelatin and carrageenan and to utilize cellobiose and trehalose as sources of carbon and energy. *Flavobacterium pectinovorum* (Dorey, 1959), considered by Mitchell *et al.* (1969) to be a species of *Cytophaga* and by Christensen (1977a) to be synonymous with *C. johnsonae*, hydrolyses both starch and gelatin but not agar.

Flavobacterium meningosepticum, which may also be a species of *Cytophaga* (Mitchell *et al.*, 1969), and *Flavobacterium* group IIb (Owen & Lapage, 1974) have a mol % GC of 36 to 38, which is close to that of the isolates described in this paper, but they differ from our isolates in a number of other respects (Table 5). *Flavobacterium* sp. NCIB 9491, considered by Mitchell *et al.* (1969) to be of uncertain taxonomic affinity, has a mol % GC of 51.2 and, in our experience, is actively motile and liquefies agar. It seems to be more closely related to *Alteromonas* than to *Cytophaga* (Fig. 2) and produces rather dull, greyish-yellow colonies.

In view of these differences from adequately described species, and the wide range of carbohydrates which our isolates are able to utilize, we suggest that they belong to a distinct species for which we give the following name and description.

Cytophaga saccharophila sp. nov.

sac.cha.ro.phi'la Gr.n. *sacchar* sugar; Gr.adj. *philus* loving; M.L.fem.adj. *saccharophila* sugar loving.

Gram-negative rods, 2.5 to 6.0 × 0.5 to 0.7 µm occurring singly or in small clumps. In older cultures, especially on solid media, coccoid and constricted forms occur. In liquid culture, slender rods and filamentous forms predominate. Microcyst formation has not

Table 5. *Comparison of Cytophaga saccharophila with Flavobacterium meningosepticum and Flavobacterium group IIb*

Character	<i>Flavobacterium</i> group IIb NCTC 10797*	<i>Flavobacterium</i> <i>meningosepticum</i> NCTC 10588*	<i>Cytophaga</i> <i>saccharophila</i>
Base composition (mol % GC)	36.1	36.1	36.1–36.3
Anaerobic growth	+	+	—
Aerobic growth	+	+	+
Pigment	Orange–yellow	Yellow	Yellow
Growth at 22 °C	+	+	+
Growth at 37 °C	+	+	—
Indole	+	+	—
Phosphatase	+	+	+
H ₂ S production	+	+	+
Sensitivity to:			
Penicillin (2 units)	—	—	—
Polymyxin (300 units)	—	—	—
Nitrate reduction	—	+	+
Starch hydrolysis	—	+	+
Gelatin hydrolysis	—	—	+
Agarolytic activity	—	—	+

* Data from Owen & Lapage (1974).

been observed. Flagella are absent but gliding motility may be observed when cells are in contact with surfaces.

Colonies on nutrient agar are 1 to 2 mm in diameter, convex, glistening, slimy, yellow and opaque becoming translucent with age, especially at the periphery. On moist media spreading may be observed but normally colonies are sunken in shallow hollows as a result of their agarolytic activity.

Obligately aerobic, oxidase positive, strongly catalase positive, metabolizing glucose oxidatively in the Hugh & Leifson O/F test. Acetylmethylcarbinol and indole are not produced and the methyl red test is negative. Starch and gelatin are both hydrolysed and all strains examined are phosphatase positive and DNAase negative. Hydrogen sulphide is produced from triple sugar iron agar.

The following carbohydrates may be used as sole carbon and energy source: D-glucose, D-galactose, D-fructose, D-mannose, D-xylose, L-arabinose, L-rhamnose, lactose, sucrose, maltose, cellobiose, melibiose, trehalose, raffinose, stachyose. A number of polysaccharides may also form the sole carbon and energy source: agar, agarose, starch, pectin, carrageenan, arabinogalactan, gum arabic, gum xanthan and galactomannan.

Nitrate is reduced to nitrite by 3 out of 4 strains. All isolates so far studied are sensitive to tetracycline (10 µg), streptomycin (10 µg), fucidin (5 µg), novobiocin (5 µg), O/129 (10 µg) and benzylpenicillin (10 units) but are resistant to polymyxin B (300 units), chloramphenicol (10 µg) and neomycin (10 µg). Concentrations of antibiotic are given as quantities per disc using a Multodisk assay.

Agarolytic activity

The agarolytic activity of both types of organism was repressed in the presence of glucose or galactose. Agarase appeared to be inducible, not only by agar, but by a range of polysaccharides (Table 6).

The crude extracellular agarase preparations from the *Alteromonas* and *Cytophaga* species differed in the nature of the most readily demonstrable end products. The *Alteromonas* species gave a product with the same chromatographic properties as galactose whereas the *Cytophaga* species gave a complex mixture of breakdown products corresponding to oligosaccharides in their chromatographic behaviour. Both preparations could release reducing compounds from a number of polysaccharides other than agar (Table 7). Studies

Table 6. Induction of 'agarase' activity in *Alteromonas* sp. (049/1) and *Cytophaga saccharophila* (024) by a number of carbohydrate derivatives when present as the sole carbon source

Carbon source	Agarolytic activity relative to that produced when agar was the carbon source	
	<i>Alteromonas</i> sp. (049/1)	<i>Cytophaga</i> <i>saccharophila</i> (024)
Agar	1.000	1.000
Glucose	0.000	0.000
Starch	0.045	2.577
Galactomannan	0.824	1.080
Pectin	0.094	2.577
Cellulose	0.211	0.998
Stachyose	0.059	0.748
Raffinose	0.056	1.000
Melibiose	0.069	0.582
Arabinogalactan	0.978	2.995
Gum arabic	0.640	1.000
Gum xanthan	0.577	1.496
Oakwood leachate	0.282	3.159

Table 7. Production of reducing compounds, as determined by the neocuproine method, by the 'agarases' of *Alteromonas* sp. (049/1) and *Cytophaga saccharophila* (024)

Substrate	Reducing material released from 1.25 mg substrate (as $\mu\text{mol galactose h}^{-1}$) by the partially purified agarase of:	
	<i>Alteromonas</i> sp. (049/1)	<i>Cytophaga</i> <i>saccharophila</i> (024)
Agar	33.33	8.57
Agarose	34.29	5.71
Galactomannan	0.48	8.57
Starch	0.09	5.24
Pectin	0.00	6.67
Carboxymethylcellulose	17.14	3.81
Gum arabic	22.86	3.81
Gum xanthan	24.76	4.29
Stachyose	1.43	3.33
Arabinogalactan	23.80	3.00
Melibiose	0.00	1.90
Raffinose	0.95	5.24
<i>o</i> -Nitrophenyl- α -D-galactoside	0.00	8.57
<i>o</i> -Nitrophenyl- β -D-galactoside	0.00	1.90
<i>o</i> -Nitrophenyl- β -D-glucoside	0.00	1.90

using preparations of 'agarases' from these freshwater isolates, which have been fractionated by a variety of methods, have confirmed that they retain their ability to hydrolyse a range of polysaccharides other than agar although it is still possible that such preparations contain a mixture of polysaccharidases rather than a single enzyme with a wide substrate specificity.

The two *o*-nitrophenylgalactosides were very poor substrates although both α - and β -galactosidase activities could be detected in preparations from both organisms. The results of studies on the purification and properties of the two types of 'agarase' activity will be reported in a further communication.

DISCUSSION

Although most agarolytic bacteria described have been isolated from marine sources, there are reports that they also occur in soil and freshwater, e.g. *Cytophaga flevensis* from water and *Bacillus agar-exedens* from soil. Indeed, Nicol (1931), having noticed the presence of agar-softening, yellow-pigmented bacteria from a swampy soil in Palmer's Green, London, observed that it seemed likely that the discovery of such bacteria would be more widespread if they were looked for.

There can be little doubt that agar-degrading bacteria play an important role in the recycling of organic material at the edges of the seas and oceans where agar forms a significant constituent of the polysaccharides of the red algae. When such organisms have been isolated from non-marine habitats, attempts have been made to rationalize their presence by looking for agar in the environment or by relating the environment to a recent marine origin. Thus, v. Hofsten & Malmqvist (1975) isolated their strongly agarolytic organism from a sewage treatment plant which they suggest receives quantities of agar laboratory wastes. Although agar as an environmental pollutant could have some selective effect it is probable that agarolytic organisms were present, and had a role to play, in the habitat before the appearance of the pollutant. The isolation of *Cytophaga flevensis* from the IJsselmeer is discussed by van der Meulen (1975) in the context of the recent marine origin of the IJsselmeer and the presence of *Bangia fuscopurpurea*, a red alga adapted to fresh water. Like the organisms described in this paper, the isolates from the Swedish sewage effluent and from the IJsselmeer cannot grow in the presence of more than 2% NaCl and show no particular salt requirement. As Christensen (1977*b*) pointed out, 'This low salt tolerance would argue against it (*C. flevensis*) being a relict from the marine period and indicates instead that it is more likely to be the first non-marine agar-decomposing *Cytophaga*'.

In lowland rivers, such as the River Wey, a large part of the organic material will be polymers of plant origin, both allochthonous material from terrestrial plants including trees, and autochthonous material from aquatic plants including algae. Although in several respects the structure of agar is unique, it is one of many plant polysaccharides containing D-galactose. Such compounds occur in the hemicellulose fraction of plant cell walls, the pectin fraction of the middle lamella and the gums of plant exudates. None of these contains the derivatives of L-galactose present in the polysaccharides of agar but it is conceivable that some of the micro-organisms able to degrade these polymers could also degrade agar.

Our studies have shown that the 'agarases' from two taxonomically distinct agar-degrading isolates from freshwater can also degrade other polysaccharides and are inducible by the presence of polysaccharides other than agar.

Van der Meulen & Harder (1975, 1976*a, b*) found that the agarase of *Cytophaga flevensis* was very specific, hydrolysing agar, agarose and agaropectin but not porphyran, carrageenans, pectin, polygalactoaraban, inulin or amylose. The formation of agarase by this organism is, however, induced by melibiose as well as by agar and the oligosaccharides associated with it (van der Meulen & Harder, 1975). The 'agarase' of *Cytophaga saccharophila* would seem to differ from that of *C. flevensis*, both in its range of substrates and in being inducible by a number of polysaccharides and oligosaccharides. Further study is needed to discover whether the polysaccharidase activity of *C. saccharophila* is associated with the same enzyme complex or is a reflection of the presence of a mixture of distinct enzymes.

It is unlikely that *C. saccharophila* and the agarolytic *Alteromonas* sp. had been in contact with agar before their isolation in the laboratory. Their low salt tolerance rules out the possibility that they are marine organisms recently adapted to a freshwater

environment. We therefore propose the alternative hypothesis that the agarolytic activity of these isolates is a reflection of their ability to break down a wide range of polysaccharides including many heteropolysaccharides containing D-galactose.

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