Biochemical Patterns of Some Heterotrophic Marine Bacteria Grown in Defined Media

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SUMMARY

Three heterotrophic marine bacteria, isolated from the Tyrrhenian sea on the basis of their relative growth requirements for acetate and ammonium, were studied mainly in regard to their biochemical and nutritional characters. Data about the amino acid pools, protein amino acids, $\text{CO}_2:\text{O}_2$ ratios, enzyme activities and sensitivities to metal ions and antibiotics, are reported. The utility of an index obtained from the study of the oxygen uptake with various organic-C concentrations and the use of defined media for a more satisfactory discrimination of the various strains, is shown.

INTRODUCTION

In the marine ecosystem, bacteria play a very important role as consumers and producers of dissolved and particulate organic matter (Wood, 1965). The importance of marine bacteria was pointed out many years ago by ZoBell (1946) and more recently by Wood (1963, 1965) and Kriss (1963); but only in the last few years have marine microbiologists become interested in the more specific problems about the physiology of these micro-organisms. Recently, a considerable amount of work has been done by workers from various countries, because of the interest that marine microbiology has in many different fields (Scholes & Shewan, 1964). In general, the most recent work of physiological interest deals with the activity of enzyme systems in certain species isolated from the sea (MacLeod & Hori, 1960; Burton & Morita, 1963; Holmes & Halvorson, 1965; Shieh, 1965; Campbell, Hellebust & Watson, 1966; Gundersen, 1966), with their nutritional requirements (MacLeod, Onofrey & Norris, 1954; Tyler, Bielling & Pratt, 1960; Brown, 1960) and with their particular environments (Brown, 1964; MacLeod, 1965). This type of research assumes obviously the identification of isolated strains and in this field, too, significant results have been obtained (Shewan, Hobbs & Hodgkiss, 1960; Shewan, 1963; Hendrie, Hodgkiss & Shewan, 1964; Colwell, Citarella & Ryman, 1965; Pfister & Burkholder, 1965) without, however, reaching a definition of the problem (Wood, 1967). The present work reports the first results from a systematic study of heterotrophic bacteria of the Mediterranean basin with special reference to the Tyrrhenian sea. The main purpose has been the study of some biochemical characters of the micro-organisms, not only in the method of isolation (which has been achieved on the basis of relative specificity towards defined substrates such as acetate-C and ammonium-N) but also as useful criteria for their identification.

METHODS

Sampling. The sampling of the sea water was done along the Tyrrhenian coast, 20 miles out from Torvaianica (Rome), where the depth was 150 m.

The samples were taken by using ZoBell type bottles at the surface (0 m.) and at 25 and 100 m. depths; in the latter case stainless-steel bottles with internal glass container were used. The samples were kept between 10° and 15° and used in the laboratory within about 3 hr.

Isolation of bacteria. To 500 ml. of the sea water sample were added 5 ml. of aged natural sea water (ANSW) containing: 10 mg.-atom organic-C as $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$; 5 mg.-atom $\text{NH}_4\text{-N}$ as $(\text{NH}_4)_2\text{SO}_4$ and 10 $\mu\text{g.-atom PO}_4\text{-P}$ as K_2HPO_4 . The sample was kept at 18° in a 1 l. flask and after 72 hr a suitable dilution from it was seeded on 1.5% agar medium containing the same substrates as above. After a few days of incubation the colonies grown were examined and the strains isolated were maintained at 18° by weekly transfer on the same medium. Three of the strains isolated are reported on here; they were labelled as follows: TS/0-3, TS/25-1, TS/100-1 (TS = Tyrrhenian sea; 0, 25 and 100 = depth in metres; 3 and 1 = sample number). In the present paper the strains will be indicated by these initials, as our purpose was the study of peculiar physiological characters rather than a taxonomic classification of the species isolated. However, considering the morphological characters reported below and according to the scheme for the identification of Gram-negative rods proposed by Hendrie, Hodgkiss & Shewan (1964), TS/100-1 can be included in *Pseudomonas* group II, and TS/0-3 and TS/25-1 in *Pseudomonas* group IV.

Studies on nutritional requirements. The culture media were prepared in ANSW by adding the appropriate substrates and sterilizing at 115° for 15–20 min. These solutions were adjusted to pH 7.5 (glass electrode). After sterilization the pH value rose to pH 7.8–8.1. Heat-labile substrates were sterilized by filtration through Millipore filters (0.45 μ pore size).

The composition of various defined media used will be reported in the Results section, where the concentration of substrates will be also expressed in mg.-atom or in $\mu\text{g.-atom}$ of C, N, P per litre according to results of oceanographic analysis (Riley & Skirrow, 1965).

Media were inoculated by adding 0.1 ml. of cultures grown for 72 hr in acetate + ammonium + phosphate medium. These inoculated cultures were incubated at 18° for 80–90 hr, without agitation, and the degree of growth was measured by extinction at 500 m μ in cuvettes of 2 cm. light-path in a PMQ II Zeiss spectrophotometer.

Morphological studies. The three micro-organisms were incubated at 18° for 60 hr with aeration in ANSW containing acetate-C, $\text{NH}_4\text{-N}$ and $\text{PO}_4\text{-P}$. The examination of colonies was made after incubation for 72 hr on the acetate + ammonium + phosphate agar already described.

The observation of bacteria by an Orthomat Leitz phase-contrast microscope was preceded by treatment with a drop of 40% formaldehyde to immobilize motile micro-organisms.

The electron-microscope observations were done with a Siemens Elmiskop 1A using a drop of bacterial suspension placed on a microscope grid with Formvar membrane with drop of 1.5% phosphotungstic acid (pH 7.0) added.

Qualitative amino acids chromatography. Eight-litre batches of bacterial cultures

incubated in acetate + ammonium + phosphate ANSW medium at 18° for 72 hr with aeration were used. The bacteria were centrifuged at low temperatures, washed three times with ANSW and disintegrated for 10 min. in distilled water at 0° by ultrasonic treatment in a MSE 60 W. disintegrator. The suspension was then heated at 100° for 10 min., cooled and a drop of 6 N-HCl added. After centrifugation the clear supernatant fluid (A = amino acid pool) was used for ion-exchange resin purification, while the precipitated material, washed three times with distilled water, was hydrolysed with 6 N-HCl at 110° for 24 hr in a sealed tube. This coloured solution, dried at reduced pressure, suspended in distilled water and filtered through Whatman no. 42 paper, was subsequently purified (B = protein amino acids).

Five ml. each of A and B solutions were placed, separately, on the tops of the two chromatographic columns (1 cm. diameter) containing Amberlite CG-120 200–400 mesh in the H⁺ form (40 cm.); 100 ml. of freshly distilled water were passed through each column to remove impurities and then 150 ml. of 2 N-NH₄OH were used to elute the amino acids. The eluates, dried at reduced pressure, dissolved in 0.5 ml. distilled water and filtered through a micro-sintered-glass filter, were used for paper chromatography: 0.1 ml. was applied to Whatman no. 1 paper and the chromatograms developed according to the method of Mizzel & Simpson (1961).

Manometric measurements. In all experiments the micro-organisms were grown at 18°, with aeration, in 2 l. flasks containing: 1000 ml. ANSW, 20 mg.-atom total organic-C (acetate + pyruvate + maltose), 10 mg.-atom NH₄-N and 20 µg.-atom PO₄-P. After 50 hr the micro-organisms were centrifuged down, washed twice and suspended in 50 ml. of sterilized ANSW + PO₄-P and kept for 12 hr at 18° with aeration to deplete the endogenous respiration.

Before use the washed organisms were resuspended in fresh ANSW + PO₄-P so that, when diluted 1/10, the $E_{500\text{ m}\mu}^{1\text{ cm.}}$ value ranged between 0.4 and 0.5.

The oxygen uptake and CO₂ evolution were measured in a Warburg apparatus at 18° ± 0.1, over the entire experimental period, following the principle of Dickens & Simer (see Umbreit, Burris & Stauffer, 1957), but using a different technique. The pH value in the reaction vessels was about pH 7.8, at which value practically all the CO₂ was in the bicarbonate form (Riley & Skirrow, 1965). The amount of CO₂ normally present in sea water was determined in separate vessels and then subtracted from the other experimental results.

Crude-extract preparations. Aerated cultures grown at 18° for 50 hr in 2 l. ANSW containing acetate + ammonium + phosphate, as described, were centrifuged and the deposit washed twice with ANSW. The pellets suspended in 7 ml. ANSW + 3 ml. 0.2 M-tris buffer (pH 7.4) were treated in the MSE disintegrator for 5 min. at 0°. The debris was centrifuged down at 30,000 g for 7 min. and the opalescent supernatant fluid used as crude extract for enzyme assays.

Enzyme assays. The oxidation and reduction of nicotinamide adenine nucleotides with different substrates was done at room temperature by measuring the change in $E_{340\text{ m}\mu}^{1\text{ cm.}}$ in spectrophotometer cuvettes (Hilger-Gilford equipment). In a similar way fumarase activity was measured at 300 mµ by using 10–20 µmole fumarate and 1 ml. crude extract in 0.05 M-tris buffer (pH 7.4).

Inhibition by heavy metals and antibiotics. The media and inocula were prepared as described for nutritional requirements. The experiments with antibiotics were done in test-tubes containing 4 ml. 0.1 % nutrient broth (Difco) in ANSW sterilized at

115° for 20 min. Penicillin, tetracycline and streptomycin were added in a volume of 1 ml. of acidified medium at pH 4 to obtain a final pH of 7.2: 0.1 ml. inocula from serial dilutions of a culture aerated for 36 hr at 18° with $E_{500\text{ m}\mu}^{1.0\text{m}} = 0.1$ were obtained by dilution with the same medium.

Substrates and reagents. The amino acids and carbohydrates were purchased from British Drug Houses Ltd. or Mann Res. Lab. Inc., ketoacids and nucleotides were from Sigma or Boehringer, while all the other reagents were from Merck. The sea water for the media was taken in the sampling area and aged in the dark for about 30 days before being used.

RESULTS

The three strains isolated showed these common characters: straight or curved rods (Pl. 1, fig. 4-6), motile with a polar flagellum (Pl. 2, fig. 7-9), Gram-negative.

Colonial appearances (Pl. 1, fig. 1-3) on acetate+ammonium+phosphate agar were as follows.

Organism TS/0-3: colonies round with an entire margin (Pl. 1, fig. 1) of yellow colour due to a not diffusible pigment, transparent to transmitted light and opaque to incident light, with a delicate circular structure.

Organism TS/25-1: colonies round with an entire margin, colourless, transparent to transmitted light and opaque to incident light (Pl. 1, fig. 2).

Organism TS/100-1: colonies round with an entire margin, and a dark central core with internal scattered black dots (Pl. 1, fig. 3); on acetate+ammonium+phosphate slope a diffusible dark-brown pigment produced.

Nutritional requirements

Carbon sources. The results obtained with ammonium as nitrogen source and with different carbon substrates are given in Table 1, in which the data are expressed in arbitrary units of amount of growth related to the extinction. The results show good growth of all three isolates with the following organic-C compounds: starch, glycogen, trehalose, maltose, acetate, pyruvate, oxalacetate. None of the three micro-organisms utilized malic acid and α -ketoglutaric acid. Organisms TS/0-3 and TS/25-1 did not grow in the presence of the other intermediates of the Krebs cycle, whereas strain TS/100-1 grew in the presence of citrate, succinate or fumarate. TS/0-3 metabolized maltose but not glucose, TS/25-1 gave growth with all carbohydrates tested, and TS/100-1 utilized agar as a carbon source. The negative response to pentoses was typical of all strains, although TS/25-1 sometimes grew slightly.

Nitrogen sources. The data about the organic and inorganic nitrogen utilization in the presence or in absence of a carbon source are shown in Tables 2 and 3. The results show that in the presence of acetate-C the three organisms grew with glycine, alanine, valine, leucine, isoleucine and norleucine (leucines), proline, aspartic acid, glutamic acid, arginine or asparagine, but not with norvaline, glutamine or α -amino butyric acid.

With inorganic-N, organism TS/0-3 did not utilize $\text{NO}_2\text{-N}$ and $\text{NO}_3\text{-N}$ in the presence of acetate-C but, in common with the other strains, it multiplied in ANSW with gelatine added and liquified gelatine in nutrient broth. Less indicative were data obtained in absence of organic-C by reason of the poor growth reached with single amino acids as source of C and N.

General metabolic characteristics

Amino acids chromatography. A reference chromatogram and two typical chromatographic patterns of strain TS/25-1 are shown on Pl. 4, fig. 10-12.

By substituting acetate-C with glucose-C in the culture media, identical results were obtained with the same strain.

Table 1. *Growth of three heterotrophic marine bacteria on different carbon sources*

The organisms were grown in test-tubes at 18° in 10 ml. of ANSW containing: 10 mg.-atom/l. of organic-C; 5 mg.-atom/l. of $\text{NH}_4\text{-N}$ and 20 $\mu\text{g.}$ atom/l. of $\text{PO}_4\text{-P}$. The results are means of duplicate readings. Initial pH = 8.0; the final pH values are in parentheses.

Carbon sources	Organism		
	TS/0-3	TS/25-1	TS/100-1
Starch	2 (7.23)	4 (6.50)	4 (6.30)
Inulin	0	2 (7.28)	0
Glycogen	2 (7.31)	2 (7.27)	3 (6.50)
Agar	0	0	3 (5.80)
Sucrose	0	4 (6.0)	3 (6.60)
D(+)Cellobiose	0	3 (6.54)	3 (6.80)
D Trehalose	3 (7.22)	3 (6.60)	3 (6.80)
Lactose	0	3 (7.27)	2 (7.31)
Maltose	2 (7.28)	4 (7.00)	3 (6.57)
D(+)Glucose	0	4 (6.21)	3 (6.94)
D(+)Mannose	1 (7.27)	1 (7.40)	0
D(+)Galactose	0	3 (6.24)	4 (5.60)
D(-)Fructose	0	4 (6.25)	0
D(-)Mannitol	0	2 (6.21)	3 (6.74)
D(+)Xylose	0	1 (7.00)	0
D(-)Ribose	0	1 (7.20)	0
L(+)Arabinose	0	1 (7.20)	0
Acetate	2	3	2
Pyruvate	1	4	4
Citrate	0	0	4
α -Ketoglutarate	0	0	0
Succinate	0	0	4
Fumarate	0	0	2
DL-Malate	0	0	0
Oxalacetate	2	2	3

Key to the numbers, $E_{600}^{2.0\text{ cm.}}$. 0-0.03 = 0: 0.03-0.1 = 1: 0.1-0.2 = 2: 0.2-0.3 = 3: > 0.3 = 4.

The amino acid composition of all three bacteria are given in Table 4. It will be noted that aspartic acid and glutamic acid were present in large amounts when acetate-C and $\text{NH}_4\text{-N}$ were the only nutrients present in the medium. Strain TS/0-3 differed from the others in that histidine and arginine were absent; TS/25-1 had the largest number of amino acids in the pool.

Biochemical activity as measured in the Warburg apparatus

When nitrogen sources were absent, the consumption rate of oxygen by the starved micro-organisms was substantially different for acetate-C, pyruvate-C and maltose-C (Fig. 1, 2).

As described in Methods, when the micro-organisms were starved for 12 hr at 18°

Table 2. *Growth of three heterotrophic marine bacteria on different nitrogen sources in presence of acetate-C*

10 ml. of ANSW containing: 10 mg.-atom/l. of acetate-C; 5 mg.-atom/l. of $\text{NH}_4\text{-N}$ or $\text{NO}_3\text{-N}$ or $\text{NO}_2\text{-N}$ or protein-N and 20 $\mu\text{g.-atom/l. of PO}_4\text{-P}$.

Nitrogen and carbon sources	Organism		
	TS/0-3	TS/25-1	TS/100-1
Acetate +			
Glycine	2	3	4
Alanine	1	2	3
Valine	1	1	1
Norvaline	0	0	0
Leucine	2	2	2
Isoleucine	2	2	3
Norleucine	0	2	1
Serine	0	2	3
Threonine	0	2	4
Phenylalanine	2	4	3
Tryptophan	2	2	0
Cysteine	0	0	2
Methionine	0	1	3
Proline	2	2	3
Aspartic acid	2	4	3
Glutamic acid	3	4	4
Histidine	0	2	3
Arginine	2	3	2
Glutamine	0	0	0
Asparagine	2	3	4
α -Aminobutyric	0	0	0
NH_4^+	2	4	2
NO_2^-	0	2	1
NO_3^-	0	3	2
Gelatine	4	4	4
Gelatine-liquef. + nutrient broth	+ (90 h)	+ (48 h)	+ (48 h)

Key and other conditions: see Table 1. DL amino acids have been used.

Table 3. *Growth of three heterotrophic marine bacteria on amino acids as nitrogen and carbon sources*

10 ml. of ANSW containing: 5 mg.-atom/l. of $\text{NH}_4\text{-N}$ and 20 $\mu\text{g.-atom/l. PO}_4\text{-P}$

Nitrogen and carbon sources	Organism		
	TS/0-3	TS/25-1	TS/100-1
Alanine	0	1	2
Valine	0	1	0
Isoleucine	0	1	0
Serine	0	1	0
Threonine	0	1	0
Phenylalanine	0	2	0
Proline	2	0	2
Aspartic acid	0	0	2
Glutamic acid	2	0	1
Arginine	2	0	0
Asparagine	0	1	0

Key and other conditions: see Table 1. The other amino acids gave a negative response.

Fig. 1. Influence of various organic-C concentrations on the oxygen uptake of three heterotrophic marine bacteria. Each Warburg flask (19 ml.) contained: in the bottom compartment, 2 ml. of bacterial suspension of calculated extinction from 4.0 to 5.0, and 0.25 ml. of organic-C in ANSW; in the centre well, 200 μ l. of 20 % KOH in distilled water. The final volume was made up to 3 ml. with ANSW. Endogenous oxygen has been subtracted. ●—●, Acetate-C; ○—○, pyruvate-C; ●—●, maltose-C.

Table 4. *Chromatographic amino acids composition of three heterotrophic marine bacteria*

First dimension: n-butanol+acetic acid+water, 25+6+25. Second dimension: methylethylketone+n-butanol+water, 2+2+1 by vol.

	Amino acid pools			Protein amino acids		
	TS/0-3	TS/25-1	TS/100-1	TS/0-3	TS/25-1	TS/100-1
Glycine	+	—	—	+	+	++
Alanine	+	++	++	++	+	++
Valine	—	+	—	+	+	+
Leucines*	+	++	+	++	++	++
Serine	—	+	—	+	+	++
Threonine	+	—	—	+	+	++
Phenylalanine	—	+	—	+	+	+
Methionine	+	++	+	+	++	++
Proline	+	+	+	+	+	+
Aspartic acid	+	++	+++	+++	+++	+++
Glutamic acid	+++	+++	+++	+++	+++	+++
Histidine	—	+	—	—	+	+
Lysine	—	++	+	+	++	++
Arginine	—	—	++	—	++	++
Tyrosine	—	+	—	+	+	+
Number of unknowns	0	1	0	3	0	0

Key:—, absence; +, present in small amounts; ++, moderate amounts; +++, large amounts.

* Leucines=leucine, isoleucine, norleucine. These amino acids do not appear well separated, sharing the same tone of blue colour in presence of cyclohexylamine according to Mizzel & Simpson (1961). Therefore it was not possible to profit by the differences in the colours as for the other amino acids (e.g. glutamic acid, aspartic acid, glycine, arginine, and so on).

with aeration, the endogenous oxygen uptake was very low, showing that the bacteria had exhausted their reserves; when an organic-C source was added, oxygen uptake increased rapidly. Acetate-C and pyruvate-C always caused more oxygen to be taken up than maltose (Fig. 1), but for organism TS/100-1 this happened only at high carbon-source concentration. Other experiments showed that when $\text{NH}_4\text{-N}$ and $\text{NO}_3\text{-N}$ were added to the Warburg vessels the oxygen uptake was the same, while with $\text{NH}_2\text{-N}$ (as glycine) more oxygen was taken up.

Fig. 2. Oxygen consumption rates, at fixed organic-C concentration, of three heterotrophic marine bacteria. Each Warburg flask (19 ml.) contained: in the bottom compartment, 2 ml. of bacterial suspension ($E_{800\text{nm}}^{1\text{cm}} = 3.5$) and $25 \mu\text{g.}$ -atom of organic-C in 0.25 ml. of ANSW; in the centre well, 200 $\mu\text{l.}$ of 20 % KOH in distilled water. The final volume was made up to 3 ml. with ANSW. Endogenous oxygen has been subtracted. ●—●, Acetate-C; ○—○, pyruvate-C; ●—●, maltose-C; ---, endogenous oxygen.

Fig. 3. Enzyme activity on NADH_2 , NADH_2 + oxalacetate and NADH_2 + pyruvate, by cell-free extracts of three heterotrophic marine bacteria. Each spectrophotometer cuvette contained: 1 ml. of crude extract; $1.5\text{--}3 \mu\text{mole}$ of NADH_2 ; $10 \mu\text{mole}$ of substrate and 0.05 M tris buffer at pH 7.4 in a total volume of 3.5 ml. with distilled water. ○—○, NADH_2 ; ●—●, NADH_2 + pyruvate, ●—● NADH_2 + oxalacetate.

Table 5. Ratio $\text{CO}_2:\text{O}_2$ and carbon sources for three heterotrophic marine bacteria

For CO_2 evolution each Warburg flask (7.5 ml.) contained: in the bottom compartment, 50 $\mu\text{l.}$ of KOH collected from the centre well of the flasks used for O_2 uptake, and 0.45 ml. of distilled water. In the side arm, 0.5 ml. of 3 N-HCl. After equilibrium, the HCl was transferred to the bottom compartment and CO_2 evolved after 20 min. was recorded. For O_2 uptake, see Fig. 2.

Carbon sources	TS/0-3		TS/25-1		TS/100-1	
	S.v.*	M.v.†	S.v.	M.v.	S.v.	M.v.
Acetate	1.88 2.42 1.46	1.92 ± 0.27	1.08 1.06 1.27	1.13 ± 0.07	0.78 0.90 1.08	0.92 ± 0.09
Pyruvate	2.14 2.35 1.96		1.48 1.64 1.91		1.51 1.31 1.27	
Maltose	0.78 0.90 1.08		1.25 1.24 1.40	1.30 ± 0.16	0.71 0.67 0.59	0.65 ± 0.01

* Single value. † Mean value $\pm$ standard error.

Fig. 4. Enzyme activity on NAD, NADP, NADH₂, NADP + malate and NADP + citrate, by cell-free extract of strain TS/25-1. Experimental conditions as in Fig. 3.

Table 6. *Enzyme activities in sonicated cell-free extracts of three heterotrophic marine bacteria*

Each cuvette contained in 3.5 ml.: 1 ml. of crude extract, 10 μ mole of substrate, 1.5–3 μ mole of nucleotide in 0.05 M-tris buffer at pH 7.4.

Coenzymes and substrates	Micro-organisms		
	TS/0-3	TS/25-1	TS/100-1
NADH ₂	+++	+++	+++
NADPH ₂	+++	+++	+
NAD	++	++	++
NADP	—	+	+
NADH ₂ + pyruvate	+++	+++	+++
NADH ₂ + oxalacetate	+++	+++	+++
NAD + malate	++	+++	—
NAD + α -ketoglutarate	—	—	—
NADP + citrate	—	+++	—
NADP + malate	—	++	—
NADP + α -ketoglutarate	—	—	—
Fumarate	—	+	—

Key: + + +, high activity; + +, moderate activity; +, low activity; —, absence of activity.

The values for CO₂:O₂ ratios of the three organic-C sources (Table 5) were obtained from the experiments illustrated in Fig. 2. These results show that the ratios were higher for pyruvate-C and lower for maltose-C for organisms TS/0-3 and TS/100-1, but, for TS/25-1 this latter value was intermediate between acetate-C and pyruvate-C.

Enzyme activities in crude extract experiments. In Fig. 3 the rates of change in extinction (ΔE) at 340 m μ obtained with cell-free extracts of the three bacteria are shown. The results show that when oxalacetate or pyruvate were added to sonicated soluble fractions, the rate of NADH₂ oxidation was greater than in the controls. Data about the NAD and NADP reduction and NADPH₂ oxidation by cell-free extracts of organism TS/25-1 are given in Fig. 4, which shows the enzyme activity

towards citrate or malate. The results of all experiments on enzyme activity in crude extracts are summarized in Table 6.

Effect of some inhibitors. From the data presented in Table 7 it can be seen that organism TS/0-3 was the most sensitive to inhibition by heavy metal ions; in general, Cu^{2+} and Zn^{2+} were the most inhibitory ions. None of the metals tested stimulated the growth of the three organisms under the experimental conditions adopted. As shown in Fig. 5, the three organisms were in general fairly resistant to antibiotics under the described conditions. Organisms TS/0-3 and TS/25-1 were sensitive to penicillin when the inoculum was small; with larger inocula all the organisms showed a clear resistance; especially TS/25-1 and TS/100-1 towards penicillin, and TS/0-3 towards tetracycline.

Fig. 5. Minimum inhibitory concentration (M.I.C.) of penicillin, tetracycline and streptomycin, on three heterotrophic marine bacteria, at various inoculum densities. Experimental conditions, see methods. The values represent the mean of duplicate readings. ○—○, penicillin; ●—●, tetracycline; *—*, streptomycin.

Table 7. *Effect of heavy metal ions on growth of three heterotrophic marine bacteria*

Metal ions	$\mu\text{g.-atom/l.}$	Micro-organisms		
		TS/0-3	TS/25-1	TS/100-1
		2	3	2
Cu^{2+}	0.1	2	3	2
Cu^{2+}	1.0	1	3	1
Cu^{2+}	10	0	2	0
Fe^{3+}	0.1	2	3	2
Fe^{3+}	1.0	2	3	2
Fe^{3+}	10	2	3	2
Mn^{2+}	0.1	1	3	2
Mn^{2+}	1.0	2	3	2
Mn^{2+}	10	1	3	2
Zn^{2+}	0.1	1	3	2
Zn^{2+}	1.0	1	3	2
Zn^{2+}	10	0	2	1
MoO_4^{3-}	0.1	1	3	2
MoO_4^{2-}	1.0	1	3	1
MoO_4^{2-}	10	1	3	2
Co^{2+}	0.1	1	3	2
Co^{2+}	1.0	1	3	2
Co^{2+}	10	1	3	1

Key and other conditions: see Table 1.

DISCUSSION

The conditions used in the isolations of the bacteria were based on the principle of selection under minimal nutrient conditions (acetate + ammonium + phosphate) at the temperature of the sea water in the sampling area (15 to 18°). In this way all the strains isolated shared the relative specificity of a nutritional character. Although the specificity towards a substrate might depend upon the physiological conditions of the micro-organism at time of sampling, and the use of such a principle for strain isolation might be questionable (Hungate, 1962; Hayaishi, 1955), the bacteria isolated in this way have kept, during the time of the research (16 months), the property of multiplying on acetate-C and ammonium-N. This property has proved to be stable. Future work will test whether this principle might be of use in grouping different strains of marine bacteria according to their relative specificity towards defined substrates.

Morphological characters. The photographs given were chosen as the most typical; but very often, especially for organisms TS/0-3 and TS/100-1, very different shapes were observed. In fact, curved forms were frequently observed and this could have been considered as characteristic of the genus *Vibrio*. However, in accord with the Hendrie scheme, based on behaviour of micro-organisms towards glucose-containing medium and the pteridine O/129, this possibility was excluded and the organisms recognized as belonging to the genus *Pseudomonas*. However, further morphological observations, made on different strains successively isolated, showed that the vibrio shape was attributable to a stage in division in which the bacteria showed an inflexion before dividing in two. These data are in agreement with the criticism made by Rhodes (1965) and by Wood (1967) on the morphological characters of bacteria used in taxonomic studies. One character, on the contrary, which has shown a noticeable constancy, at least in the experimental conditions used, is the shape and pigmentation of the colonies on acetate + ammonium agar: organism TS/0-3 yellow; TS/25-1 colourless; TS/100-1 black-brown, although the appearance of colourless variants was occasionally noted.

Nutritional characters. Results obtained from study of nutritional requirements were distinctive under the experimental conditions used. Organism TS/100-1 possessed characters quite different from the other two organisms in that it used agar, citrate, succinate, fumarate and, with the exception of lactose, produced acid from the carbohydrates. Organism TS/0-3 had the singular character of using maltose but not glucose and it did not use $\text{NO}_3\text{-N}$ and $\text{NO}_2\text{-N}$. An interesting property common to the three organisms was the non-utilization of malate, α -ketoglutarate or glutamine. Apart from the consideration of substrate permeability, the non-utilization of glutamine could be due to the lack of glutaminase, which hydrolyses glutamine to glutamic acid and ammonia (Cohen & Sallach, 1961; Roberts, 1960).

Hence clear differences in cultural behaviour exist among the three microscopically similar organisms.

General biochemical characters. This little-known aspect of marine bacteria was studied to compile biochemical properties of eventual general interest and to try to relate some positive or negative characters common to the three organisms with their relative specificity to acetate and ammonium. Obviously the data given here are not sufficient to clarify the metabolic activities of the organisms for the various substrates, but their utility in bringing out the differences is evident. Chromatography of pools

of amino acids has been used sometimes for taxonomic studies (Holden, 1962). Taking into account the experimental conditions used for growth and extraction, some significant differences were found in the amino acid pools of the three strains (Table 4). These differences cannot be easily interpreted biochemically, but should be useful, when a sufficient variety of marine bacteria have been examined, to judge whether the composition of amino acid pools is helpful in their classification.

The Warburg experiments clearly indicate differences among the three strains in the rates of oxygen uptake from various carbon sources, particularly with acetate. Starved cells were used because endogenous respiration was then very low; with not-starved cells, endogenous respiration was so high that the differences in the oxygen uptake with different substrates were less marked.

Assuming that the micro-organisms did not multiply in the Warburg flasks, Fig. 1 may be used to calculate a substrate affinity constant resembling Michaelis-Menten's kinetic constant for the enzymes. Half-maximum reaction rates correspond, for the three strains with acetate-C, to values (in molar units) of: TS/0-3, $K_{\text{acet.}} = 1.6 \times 10^{-3}$; TS/25-1, $K_{\text{acet.}} = 5.6 \times 10^{-3}$; TS/100-1, $K_{\text{acet.}} = 2.7 \times 10^{-3}$. The reproducibility of these values was indicated by other experiments made with similar strictly controlled experimental conditions: for pyruvate (25 $\mu\text{g.}$ /Warburg vessel) the oxygen uptakes, over 60 min., were respectively: 58-66-55 $\mu\text{l.}$ for TS/0-3; 116-134-123 $\mu\text{l.}$ for TS/25-1; 165-198-170 $\mu\text{l.}$ for TS/100-1, where the values are the means of duplicate tests in three independent experiments.

In spite of the approximate nature of these values it appears that differences in the substrate affinity are sufficient to differentiate the three strains despite their specificity for the same substrate. The ratios $\text{CO}_2:\text{O}_2$ obtained with the same substrates are also significantly different for the three micro-organisms, which presumably reflects differences in the metabolism of these substrates under starved conditions. Values below 1 indicate a prevalence of oxidative processes and agree very well with the pH values for various strains shown in Table 1. The $\text{CO}_2:\text{O}_2$ ratio is thus important in marine bacterial respiration, but it is not yet clear whether such values will be useful in taxonomic studies as proposed by Katznelson & Robinson (1956) for halophilic strains.

The redox potential of these systems did not correlate with $\text{CO}_2:\text{O}_2$ ratios. With TS/100-1 strain, for example, the initial value of E_h was +330 mV. at pH 8.0 (slightly different from the value of +322 mV. at 20° and at pH 8.15 for the sea water in the sampling area at 0 m. Of course, the E_h of this bacterial system changed during the experiments and after 24 hr it was +160 mV. with little differences for acetate, pyruvate and maltose.

Experiments on cell-free extracts have been recently employed by some authors (De Vries & Stouthamer, 1967; Joyner & Baldwin, 1966) to get indications on the taxonomy and ecology of many non-marine micro-organisms, but the significance of these researches depends strictly on the use of well-defined experimental conditions. The synthesis of many enzymes in bacteria represent an adaptation phenomenon (Daron, 1967) and the nutritional conditions influence the enzyme synthesis of the tricarboxylic acid cycle (Hanson & Cox, 1967). Despite the limited purification of the extracts and the possible differences in the optimal conditions required for the similar enzyme activities in various strains, the experiments reported here with cell-free extracts have shown their utility by demonstrating the presence of some enzyme activities which were not recognizable with Warburg experiments. For example, in

preliminary experiments, suspensions of sonicated marine bacteria did not take up any oxygen with pyruvate, citrate or succinate, whereas by direct spectrophotometry these enzyme activities were indicated. Presumably some enzyme systems of the terminal electron transport mechanisms were inactivated by ultrasonics or removed by centrifugation.

A particular substrate and pyridine nucleotides evoked enzyme activities related to Krebs cycle in the TS/25-1 extract. The apparent absence of comparable activities in extracts of TS/0-3 and TS/100-1 was probably because the majority of these enzymes do not link to NAD or NADP and require an artificial acceptor for the measurement of activity. As already emphasized by Ochoa & Stern (1952) and Barret & Kallio (1953), such experiments as these do not prove that the Krebs cycle is really the route used *in vivo* for terminal carbohydrate metabolism. The results of nutritional and cell-free extract experiments indicate that the non-utilization of citrate, succinate, fumarate and malate by TS/25-1 as sole carbon source in the presence of ammonium nitrogen could be attributable to inducible permeation systems (Kogut & Podoski, 1953; Barret & Kallio, 1953).

Growth inhibitors. Antibiotic sensitivity has been recently adopted by Pfister & Burkholder (1965) as a discriminating character among marine strains and Shewan (1963) used pteridine o/129 as a differential test of Gram-negative asporogenous marine rods.

The experiments with metal ions reported in Table 7 show a significant difference between strain TS/25-1, which is practically insensitive, and strain TS/0-3, which is the most sensitive. The experiments with antibiotics were carried out in a complex medium, instead of a defined one as generally used with non-marine strains. Only penicillin showed a clear difference in sensitivity between TS/25-1 and the other two bacteria; this strain was, however, sensitive when the inoculum was low.

All three strains showed apparent resistance to tetracycline and streptomycin, but the ease with which these drugs, especially tetracycline, form complexes with Ca^{2+} and Mg^{2+} that lower antibacterial activity (Laskin, 1967), suggests that these two antibiotics are of little use in sea-water media which contain Mg^{2+} and Ca^{2+} ions.

Finally, a common characteristic of the three strains was their incapability of multiplying with malic acid or α -ketoglutaric acid. This property was not shown by some terrestrial strains of pseudomonas, classified, according Hendrie *et al.* (1964), into groups I (three strains), II (two strains) and IV (one strain), all of which grew very well after 24 hr at 37° with ammonium nitrogen and malic acid or α -ketoglutaric acid. This interesting nutritional characteristic of these marine bacteria has been confirmed by the isolation of eight other strains which behave similarly. Hence the use of particular defined media for the isolation of marine bacteria sharing characteristic biochemical properties is important and may in future assist the identification of newly isolated strains.

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EXPLANATION OF PLATES

PLATE I

- Fig. 1. Micrograph of colonies of strain TS/0-3.
Fig. 2. Micrograph of colonies of strain TS/25-1.
Fig. 3. Micrograph of one colony of strain TS/100-1.
Fig. 4. Phase-contrast micrograph of strain TS/0-3. $\times 1500$.
Fig. 5. Phase-contrast micrograph of strain TS/25-1. $\times 1500$.
Fig. 6. Phase-contrast micrograph of strain TS/100-1. $\times 1500$.

PLATE 2

- Fig. 7. Electron micrograph of strain TS/0-3.
Fig. 8. Electron micrograph of strain TS/25-1.
Fig. 9. Electron micrograph of strain TS/100-1.

PLATE 3

- Fig. 10. Amino acids standard chromatogram.
Fig. 11. Amino acids pool chromatogram of strain TS/25-1.
Fig. 12. Protein amino acids chromatogram of strain TS/25-1.