A SIMPLE SCHEME FOR THE IDENTIFICATION OF MARINE HETEROTROPHIC BACTERIA

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ABSTRACT
A scheme has been proposed to identify the marine heterotrophic bacterial genera based on the minimum biochemical tests. Fifteen gram negative and six gram positive marine bacterial genera could be identified with this proposed scheme which involves staining, morphology, motility and some major biochemical tests

INTRODUCTION
A number of reports are available on key characters for the identification of heterotrophic bacteria (Simidu and Aiso, 1962; Baumann et al., 1972; Buchanan and Gibbons, 1974; Starr et al., 1981; Sneath, 1986), and in particular for the marine heterotrophic bacteria (Baumann et al., 1972; Oliver, 1982). But, most of the schemes are based on several time consuming biochemical tests. In spite of performing all the laborious biochemical tests, sometimes, the isolates are misidentified. Oliver (1982) proposed a key for the identification of gram-negative bacteria and Jensen and Fenical (1995) for gram-positive bacteria and these are widely followed. More bacterial genera have been reported from the marine environment, and these are not included in those schemes. Hence, in the present study an attempt has been made to simplify and modify these schemes to include the recently reported marine heterotrophic bacteria and to propose a collated scheme for the identification of marine bacterial genera (both gram negative and gram positive) based on minimum biochemical tests. The scheme relies on the information given by Shewan (1963), Okami and Okazaki (1972), Buchanan and Gibbons (1974), Starr et al. (1981), Oliver (1982), Jensen and Fenical (1995) and Cappuccino and Sherman (2001).
MATERIALS AND METHODS/ RESULTS AND DISCUSSION

The scheme starts with the gram staining, developed by Hans Christian Gram to separate the bacteria into two groups: gram +ve and gram –ve; based on the cell-wall structure. But commercially available kit for the gram staining requires time and involves morphological changes of bacteria during heat fixation. The non-staining KOH method originally proposed by Fluharty and Packard (1967) and later modified by Buck (1982), is a simple and rapid method to determine gram reaction where small colonies could also be identified (Jensen and Fenical, 1995). The advantage with respect to time, ease and cost of this technique is obvious. To perform this test, a drop (10µl) of 3% aqueous KOH (Potassium hydroxide) is to be placed on a slide. As many as 8 to 10 tests per slide can be done conveniently. Using a sterile loop, visible amount of bacterial growth from an agar culture is to be transferred to the drop of KOH. The cells and KOH are to be mixed thoroughly on the slide, constantly stirred over an area about 1.5 cm in diameter. If the bacterium-KOH suspension becomes markedly viscid or gels within 50-60 seconds, the isolate is gram negative. If no gelling is observed, the isolate is gram positive. The best way to determine viscosity is to raise the loop about 1 cm from the slide. If an obvious stringiness is present, then the culture is gram negative.

The scheme for gram-negative isolates is presented in Figure 1. The gram-negative rods which are without spores (rods only have spores) are to be tested for motility in the semi-solid nutrient agar medium (1.5% agar) by stabbing the bacterial culture. Gram negative, heterotrophic bacteria, which are motile by means of flagella, are readily isolated from the ocean and appear to comprise a major component of the bacterial flora of the sea (Baumann et al., 1972). The non-motile isolates
are then to be examined for pigment production in Seawater Yeast Extract Peptone agar media (Peptone-5g; Yeast extract- 3g; Aged seawater- 750ml; Distilled water- 250 ml; pH- 8; Agar- 15g) (Schneider and Rheinheimer, 1988). The pigment producing bacteria show yellow or orange pigmentation and these pigmented bacteria are subjected to Catalase test further with 3% H2O2. The catalase negative isolates are tentatively identified as Flexibacter and the catalase positive isolates are tested for polymyxin B sensitivity and the sensitive isolates belong to Flavobacterium and non-sensitive isolates belong to Cytophaga genus.

The non-pigmented isolates are to be tested for glucose dissimilation in Hugh-Leifson’s basal medium (Hugh and Leifson, 1953) with glucose as substrate. The basal medium is prepared with the composition of Peptone-2g; Sodium chloride- 5g; Dipotassium orthophosphate- 0.3g; Bromothymol blue (1% aqueous solution)- 3 ml; Distilled water- 1000ml; pH- 7.1 and Agar- 3g with filter sterilized 100ml glucose (10% aqueous solution). The required amount of medium is to be disposed in to the test tubes into which Durham’s tubes are placed to trap the gas bubbles. The result may be read as: Yellow (aerobic and anaerobic) - Fermentative; Yellow (aerobic) - Oxidative; Green/ No change- No reaction/Alkaline.

The fermentative strains are then subjected to Kovac’s oxidase test. A filter paper is to be soaked in 1% aqueous tetramethyl-p-phenylene diamine dihydrochloride solution and dried. Young cultures (less than 18 hrs) of the isolates are to be streaked on this filter paper and the results are read as follows: Pink or purple- Positive; No colour- Negative. The oxidase negative luminescent strains are Photobacter and non-luminescent strains are Enterobacteriaceae (Klebsiella). The oxidase positive strains are then subjected to pteridine test with 0/129 and the sensitive strains are Photobacterium and the resistant strains are Aeromonas.

The oxidative/alkaline/no reaction strains are also to be tested for oxidase production. The negative oxidase isolates belong to Acinetobacter and the positive oxidase isolates are checked for penicillin sensitivity (1.5 IU). The sensitive strains are Moraxella and the resistant strains are Pseudomonas.

The other parts of motility test, i.e. motile isolates are subjected to Kovac’s Oxidase test and the isolates showing negative results are Enterobacteriaceae. The positive oxidase isolates are then examined for glucose dissimilation as described earlier. The results may be recorded as follows: Fermentative (F), Oxidative (OX), oxidative/Negative (OX/N) and No growth/
Negative (NG/N). The isolates showing the last fraction of the result are considered as Alcaligenes (Kakimoto et al., 1974). The isolates showing OX and OX/N belong to Pseudomonas and Alteromonas respectively. But to have confirmed identification of these two group’s, isolates are to be examined for penicillin sensitivity and the sensitive strains are Pseudomonas and the resistant strains are Alteromonas (Lee et al., 1977). The fermentative (F) strains are then treated in Kovac’s Oxidase test and the negative strains are Enterobacteriaceae. If gas is evolved from the positive isolates the isolates are Photobacterium. The negative gas producing strains are then treated with vibriostatic pteridine. 0/129 (2, 4- diamino-6, 7 diisopropyl pteridine) can be used as saturated solution on filter paper disks to test for vibriostatic action according to the method of Collier et al. (1950). The sensitive strains are Vibrio and the resistant strains are Aeromonas.

The scheme for gram-positive isolates is presented in Figure 2. The gram-positive strains are looked under light microscope (400x) for the morphology. The gram-positive bacilli (rod shaped) spores are to be stained then. After fixing the smear in a glass slide, malachite green may be added and steamed. After draining, safranin should be added as counterstain and the results are: spores- green colour and without spores- red colour. The endospore forming isolates are Bacillus and without spores are Corynebacterium.

The gram-positive cocci (round shaped) are tested for catalase production and the negative catalase isolates are Streptococcus. The catalase positive spherical isolates are Arthrobacter and the clumped isolates are tested for glucose dissimilation. The oxidative strains are Micrococcus and the fermentative strains are Staphylococcus.

In any ecological study on bacteria, identification of the isolates gives an exact picture about the community structure, thereby, enabling to understand the role of those genera in the biogeochemical process in the marine environment. Although, almost all contemporary marine microbiologists have been flipped over to molecular approaches for the identification, the present key would be useful for tentative identification of bulk samples where the molecular approaches would not be cost effective. With this proposed key, the generic identification of heterotrophic bacteria would be rather easier and faster and would be a boon for the marine microbial ecologists.

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