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A SELECTIVE AND DIAGNOSTIC MEDIUM FOR AMMONIA OXIDISING BACTERIA

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1. Introduction

Pure culture isolations of ammonia oxidisers are made by variations of the terminal dilution [1] or colony picking [2] methods. The former method is tedious and requires a high enrichment of ammonia oxidisers for reliable results; though the colony-picking method is more reliable, it requires visual identification of colonies. Because of variation in size and morphology, identification of colonies is uncertain and may be biased towards those which are readily recognisable.

The medium described in this paper is selective for ammonia oxidisers because of the exclusion of organic nutrients and the addition of antibiotics. The dye neutral red performs the dual role of pH indicator and vital stain, colouring acidogenic colonies deep red and heterotrophic colonies pale orange.

2. Materials and Methods

2.1. Composition of media

Basal medium: (litre^{-1}) $(NH_4)_2SO_4$ 0.5 g, KH_2PO_4 0.2 g, $CaCl_2$ 0.02 g, $MgSO_4 \cdot 7H_2O$ 0.04 g, FeNaEDTA 0.1 ml of 3.8% solution, phenol red 2 ml of 0.05% solution. pH was adjusted to 8.0 after autoclaving by dropwise addition of 6% Na_2CO_3 solution to give a pink phenol red end point. The medium was solidified with Difco Noble agar 15 g and supplemented with $CaCO_3$ 25 g, cycloheximide 0.05 g or chloramphenicol 0.05 g when required. When neutral red was added, phenol red was omitted and the medium was buffered by chalk. 8 ml of 1% aqueous neutral red was added to media for *Nitrosomonas* and *Nitrosolobus* and 3 ml of the same solution for *Nitrosospira.* All chemicals were obtained from BDH Ltd; neutral red was stain grade, other compounds were analytical reagent grade. All cultures were incubated at 25°C.

2.2. Selectivity of inhibitors

Liquid basal medium without chalk or neutral red was amended with dyes and antibiotics (Table 1). These media received identical liquid inocula of *Nitrosomonas europaea* ATCC 163 277. Nitrite concentration was measured colorimetrically after 6 days by coupling *N*-(1-naphthyl)ethylenediamine dihydrochloride and diazotised sulphanilamide (3).

2.3. Electron microscopy

Colonies were removed from solid media in hand-pulled micro-pipettes under a dissection microscope (32× magnification) after at least 5 weeks incubation (25°C). They were suspended in a drop of water and placed on a copper grid coated with collodion. After excess liquid was removed, the cells were negatively stained with 2% aqueous potassium phosphotungstate (Fig. 1).

3. Results

3.1. Medium constituents

Of the inhibitors tested as potential selective medium amendments (Table 1), only the anti-

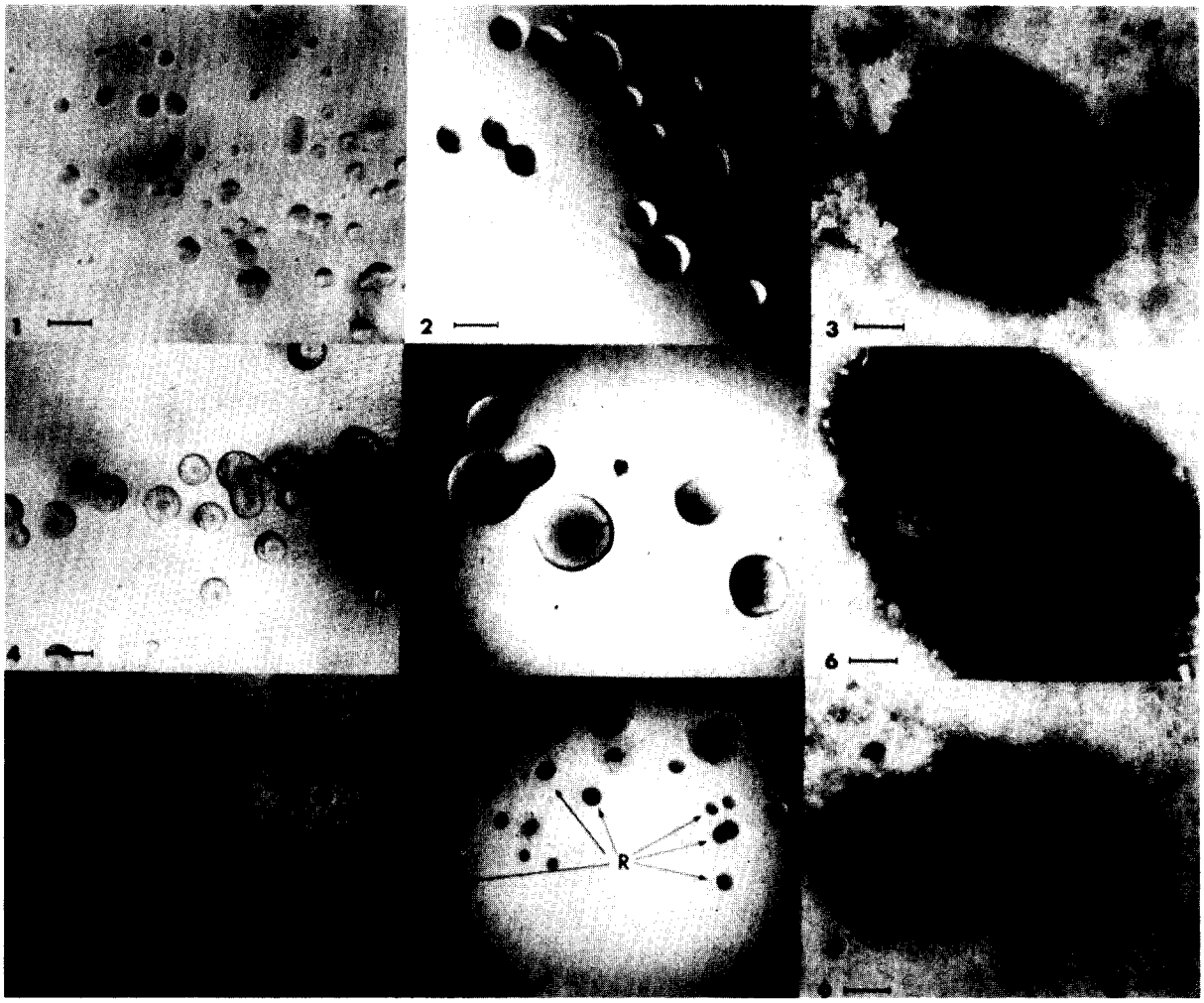

Fig. 1. Ammonia oxidisers growing on basal and neutral red media. 1 and 2. *N. europaea* ATCC 163,277 colonies after 5 wk incubation on basal and neutral red medium respectively. 3. Appearance of the bacterium from the colonies in 2. 4 and 5. *Nitrosolobus* enrichment on basal and neutral red media. 5 weeks incubation. 6. The bacterium from the colonies shown in 4. 7 and 8. *Nitrosospira* enrichment after 5 weeks incubation on basal and neutral red media. 9. The bacterium from the red colonies in 8 (R). Bars in 1, 2, 4, 5, 7 and 8 = 50 μm, in 3, 6 and 9 = 0.1 μm. In all cases contrast between colonies and agar is greatest on neutral red media; in 8, dark *Nitrosospira* colonies are clearly distinguishable from non-pigmented heterotroph colonies.

biotics novobiocin (30 ppm), chloramphenicol (50 ppm), cycloheximide (50 ppm) and benzyl penicillin (1 ppm) were useful. Chloramphenicol and cycloheximide were chosen for routine use because of their resistance to autoclaving.

Addition of neutral red to chalk-buffered solid media caused acidogenic colonies to become deep red (Fig. 1). Plates with inocula of *N. europaea* ATCC 163 277 mixed with *Bacillus megatherium, B. licheniformis, Streptomyces* sp., *Arthrobacter* sp., *Pseudomonas* sp. or *Klebsiella pneumoniae* clearly differentiated the brightly coloured ammonia oxidiser colonies from the pale orange heterotroph colonies. 0.008% neutral red was optimal for colour development in *Nitrosomonas* and *Nitrosolobus* colonies; higher concentrations were inhibitory. *Nitrosospira* was inhibited by >0.003% neutral red. Optimal colour development occurred only in the

TABLE 1
Sensitivity of *Nitrosomonas europaea* ATCC 163,277 to inhibitors

Inhibitor	Concentration (ppm)	Concentration of NO_2^- (ppm)
Benzyl penicillin	1	87
Cholramphenicol	50	89
Cycloheximide	50	95
Neomycin	20	48
Novobiocin	30	88
Rose bengal	30	50
Brilliant green	13	50
Crystal violet	1.6	43
Basic fuchsin	20	53
$KTeO_3$	35	36
Tween 80	1000	8
Pyronin	5	54
None (control)		84

largest colonies and was most obvious after 5 or more weeks incubation. Intensity of colouration varied between isolates of different genera; *Nitrosospira* was commonly the most intensely coloured.

3.2. Use of the medium

An ammonia oxidising enrichment was prepared in liquid basal medium from a grassy guano deposit collected from the Ivrgsuartât Islands (Greenland). When the culture ceased to produce acid, it was plated on basal medium with chalk and 0.003% neutral red. After 5 weeks, red colonies were conspicuous (Fig. 1); electron microscopy revealed that they were a *Nitrospira* sp. Fig. 1 also shows the colonial appearance of *N. europaea* ATCC 163 277 and a *Nitrosolubus* enrichment (from Park Grass, Rothamsted) on neutral red-containing and normal agar media.

Use of chloramphenicol and cycloheximide enabled larger inocula to be plated from enrichments than would otherwise have been possible. This meant that ammonia oxidisers could be identified in enrichment cultures more quickly than normal. The potential generic diversity of the crude cultures was thus maintained by reducing the degree of enrichment necessary for subculturing or identification.

4. Discussion

Pure cultures of ammonia oxidisers may be readily obtained from natural environments on the medium described. The advantages of this medium over those currently in use are:

(1) Colonies of ammonia oxidisers can be differentiated from those of heterotrophs and can be removed for subculture or identification by electron microscopy.

(2) The time taken to isolate pure cultures from soil is reduced to a period of weeks rather than months.

(3) Because the degree of enrichment necessary for generic identification is reduced, isolates may be selected more according to their concentration in their environment rather than their growth rate in the enrichment.

The growth of pure cultures of different genera of ammonia oxidisers on solid media has been studied. Media and isolation procedures, which may allow direct isolation of specific genera of ammonia oxidisers without enrichment will be reported.

References

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