Nitrogen Enrichments for Certain Moderately Halophilic Bacteria Indigenous in a Saline Lagoon

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In continuing studies of the lagoonal system of the Central East Coast of Florida (NEVIN, et.al. 1973), it has been a usual observation that combined nitrogen could not be detected in the waters by the methods employed. The noted deficiency may be explained plausibly by any of a number of arguments. Few of these, however, admit of ready experimental evaluation.

An approach to the problem described herein employes the indigenous heterotrophic, moderately halophilic bacteria. These creatures are expected to have relatively simple but somewhat exacting requirements for nitrogenous nutrilities (WAKSMAN, et.al., 1933; ZOBELL, 1946; BRISOU and VARGUES, 1963; WOOD, 1965; BURKHOLDER and BORNSIDE, 1957, STEVENSEN, 1966; DUNDAS and HALVORSEN, 1966; NORBERT and HOFSTEN, 1969). They should, therefore, assimilate rapidly any soluble nitrogenous compounds made available.

METHODS AND MATERIALS

Samples of lagoonal waters, 6 to 10 liters each, were collected by immersing clean, sterile half-liter bottles 3 to 6 inches below the surface, removing the cap and allowing the bottle to fill. While still below the surface the cap was replaced, and the collected samples were stored in the cold. They were ordinarily used within 24 hours. The temperature, pH and salinity of the water were determined at the sample site in order to ensure that no extreme conditions were encountered.

Several 100 ml aliquots of the sample were dispersed into sterile amber glass bottles, and each was enriched with 10 mg of a single amino acid (NB Co., Cleveland, Ohio). In all, 20 amino acids were so used. The enrichment cultures were then incubated at room temperature (25 degrees C) in the dark for 72 hours. Infrequently, the incubation was continued for an additional 24 hours in the hope that a

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greater cell crop would be obtained. The appearance of visible turbidity indicated a successful enrichment, and relative amounts of growth were estimated by percent transmittance at 600mu in a Bausch & Lomb Spectronic 20 colorimeter. Gram stained preparations of each enrichment culture were examined microscopically. Those amino acids which proved to be growth supporting enrichments were then studied in all combinations to determine any growth enhancing effect of multiple additions.

Four control cultures were carried in parallel. These were 100 ml amounts of lagoonal water enriched with: 5 ml of sterile reconstituted nutrient broth (Difco) which was replaced by a peptone solution in later experiments; or 0.05 mgs. of yeast extract (NB Co.); or 7.6 mgs. of NH_4Cl; or unenriched as a control on endogenously supported growth.

The most probable number of organisms which would grow upon enrichment with amino acids was estimated using raw lagoonal water serially diluted from 10^{-2} through 10^{-6} with sterilized (121 C, 15 lbs pressure) lagoonal water. Aliquots of each dilution were then inoculated into each of 5 tubes containing a sterile lagoonal water solution of the best growth producing combination of amino acids. Eight isolates were obtained in pure culture during these experiments and have been tentatively identified at the genus level.

A mixture of vitamins including thiamine, pyridoxine, p-aminobenzoic acid, pantothenic acid, niacinamide, biotin and folic acid were used as enrichments of the lagoonal water and were also added to single amino acid enrichments to establish their effect if any. So too were an ethanol-ether-chloroform soluble fraction of yeast extract, the insoluble residue, and an acid hydrolysate of the residue. The Biuret and Molisch test (CLARK, 1964) were used to determine residual peptides and carbohydrates respectively.

RESULTS

Visible evidence of the bacterial growth (cloudiness) occurred when any one of the amino acids: tyrosine, histidine, proline, serine, cystine, methionine or glycine was used as an enrichment in lagoonal water. Several varieties of gram negative rod shaped organisms, both motile and non-motile, as well as cocci and spirilla were always found. The nutrient broth enriched and yeast extract enriched controls, although yielding denser growth did not appear to support a broader variety of organisms.

Of the possible combination of the 7 amino acids, the following groups produced 5% or greater reduction in light transmission at 600 mu: serine, methionine, proline; cystine, proline, histidine, glycine; serine, histidine, cystine, glycine, methionine. Glycine seemed to inhibit growth when either, or both, cystine and tyrosine were present, but the inhibition was not apparent when serine and histidine were also present. The addition of either cystine or methionine to any other non-inhibiting combination of amino acids always seemed to enhance growth, suggesting a need for supplemental organic sulfur.

The most probable number of organisms which could be grown by amino acid enrichments of water samples taken from 6 different sample sites are presented in Table I.

Table I

MPN[1] of organism in 100 ml of river enriched with 6[2] amino acids

Sample #	MPN x 10^3
S - 25	2.8
S - 26	9.2
S - 27	0.8
S - 28	22.0
S - 29	35.0
S - 30	35.0

[1] Previous data also indicate 10^3 to 10^4 organisms per 100 ml when MPN's were determined in Thioglycollate medium prepared with distilled water, but these were gram positive spore formers (BEAZLEY, et. al., 1974)

[2] Glycine, methionine, proline, histidine, cystine, serine.

That they are present in appreciable numbers is evident; however, only 4 genera were tentatively distinguished: Vibrio, Benecka, Agarbacterium, and Pseudomonas according to criteria in Bergeys' Manual of Determinative Bacteriology (7th Ed.).

The cell crop was improved by substituting more complex materials for the amino acids. The results of these experiments are presented in Table II. At this point, a

when the acid soluble fraction was used. These data are presented in Table III.

Table III

Influence of Yeast Extract derived enrichments on the growth of lagoonal halophils

Enrichment	Probable Major Component			Growth
	Peptide (Biuret)	CH_2O (Molisch)	Lipid (Sudan IV)	% Trans. (600 Mu)
EEC[1] sol.	tr. (3)	tr.	str.	93
EEC insol.	str. (3)	str.	tr.	61
Acid hydrolyzed[2] EEC insol.				
Acid sol.	neg. (3)	neg.	n.d. (3)	100
Acid sol. + glucose	neg.	tr.	n.d.	95
Acid insol.	tr.	neg.	n.d.	86.5
Acid insol. + glucose	tr.	tr.	n.d.	39

1) Ethanol-Diethyl Ether-Chloroform (3,1,1)
2) 12 N. HCl, 100 C, 4 Hours
3) tr. = trace; str. = strong; neg. = negative; n.d. = not done.

DISCUSSION

The waters of the East Coast lagoonal system are notably nitrate and ammonia deficient (NEVIN, et. al., 1973). It was anticipated therefore, that any source of nitrogen in available form would serve as an enrichment for the cultivation of indigenous moderately haloduric microbes. This did in fact happen but the response was disappointingly small when amino acids were added singly or in several combinations. The addition of a mixture of known vitamins to the amino acid medium did little to increase the cell yield. This too was disappointing since green plants (Manatee grass, algae, etc.) abound in the area waters and would be expected to provide natural enrichments thereof. Glucose, however, did improve cell crop when 10 mg/100 ml was added.

Yeast extract (BBL) and a commercial peptone preparation (BBL) proved to be relatively good enrichments. Since yeast extract is a recognized source of many nutrilites, the effects of several identifiable components were studied. Upon extraction with ethanol-chloroform-ether, a lipid fraction was obtained which enhanced growth slightly. The non-lipid fraction yielded excellent growth and was strongly Biuret and Molisch positive. Following acid hydrolysis of the non-lipid fraction, however, response to both tests was markedly reduced, indicating sharp decreases in both peptides and carbohydrates. A filtrate of the hydrolysate yielded no growth, even with added glucose. The insoluble residue, however, which was weakly Biuret positive and Molisch negative, did support some growth, and when supplemented with glucose, supported excellent growth.

It is known that many organisms which must be supplied with amino acids frequently grow much more prolifically if intact peptide bonds are also supplied (GUIRARD and SNELL 1962). The greatly improved bacterial growth when either peptones or yeast extract was furnished can therefore be related to the availability of peptide bonds, since growth was generally poor when a Biuret negative acid hydrolysate of yeast extract was used as an enrichment.

The ability of these organisms to utilize glucose is apparent when amino acids are available but greatly increased cell crops seem dependent upon the availability of peptide bonds since no measureable growth ensued when the acid soluble fraction of the yeast extract hydrolysate was supplemented with the sugar. Whereas excellent growth resulted when the acid insoluble (presumably incompletely hydrolyzed) fraction was supplemented.

The roles of peptide metabolism and glucolysis have not been established during this study. Their probable importance, however, is almost self-evident. Firstly, the highest Most Probable Numbers of bacteria in the water were obtained at sample sites in the lagoon which are constantly enriched by run-off from the adjacent land masses. Secondly, soluble plant and animal materials dispersed in the water as a result of predatory actions are probably rapidly utilized by these bacteria, and are thereby removed from the water and conserved for the food chain. Thirdly, it seems most probable that these organisms, in common with most bacteria, live in intimate contact with particulates or often the outer tissue layers of indigenous plants and animals (PSHENIN, L., 1959, LISTON, J., 1960). The required nutrilites can then be assimilated about as fast as they become available from the host, before they can reach the open water. Thus no measureable amounts are found free in the water.

commercial peptone was substituted for more complex nutrient broth hitherto used as a control. Yeast extract also proved an excellent growth enhancing substance. Further, the indigenous organisms do not use NH_4+ even when supplied with glucose as a source of organic carbon although glucose, when added to an amino acid mixture did enhance growth. It is, in fact, evident that NH_4+ is distinctly inhibitory to these organisms since their growth is greatly diminished when the ion is added to an otherwise adequate enrichment.

Table II

Substitutions for or additions to amino acids as lagoonal water enrichments

Substituent or Additive	Growth as % transmission at 600mu
Peptone	86
Peptone + NH_4+	93
Glucose	98
Glucose + NH_4+	98
6 Amino acids	95
6 Amino acids + glucose	86
6 Amino acids + yeast extract	70
Yeast extract	60
Yeast extract + NH_4+	80
Unenriched control	100

In order to gain some insight into the growth enhancing effect of yeast extract, a mixture of known water soluble vitamins was added to the amino acid enrichment, but this had no demonstrable effect. An ethanol-ether-chloroform extraction of the yeast extract was also carried out. The "lipid" fraction, so obtained, was then added to several combinations of enrichments of sterile lagoonal water. The resulting media were inoculated with pure cultures of 6 of the 8 isolates with only slight enhancement of growth.

The extracted residue however, yielded excellent growth both of the pure cultures, and upon enrichment of freshly collected lagoonal water. This effect was obviated when the residue was hydrolyzed with 12 Normal Hydrochloric acid at 100 for 4 hours. The Biuret (for peptides) and Molisch tests (for carbohydrates) which were rather strong before hydrolysis, were greatly diminished in intensity afterwards. Upon the addition of either of the two products of the acid hydrolysis, exceptionally good growth was obtained with the acid insoluble (presumably unhydrolized) residue, but no significant effect was noted

Obviously glucolysis, which may also imply broader saccharolytic activities, would have been more notable by its failure to appear. The fact that it is associated with peptide metabolism, however, supports the concept of intimate associations of the bacteria with indigenous plants and animals.

The inhibitory effect of NH_4+ was at first surprising in view of the characteristic absence of measureable amounts of this ion in the water. However, the paucity of NH_4+ in the area waters may have been a selective factor for more exacting organisms which assimilate amino acids and peptides.

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