ABSTRACT:

Bacterial isolated from habitats of low nutrient flux generally exhibit low maximum growth rates and an inability to initiate growth in media of high nutrient concentration. We hypothesized that these two properties were interrelated and that both could be accounted for by one physiologic property, viz., that nutrient transport was repressible by moderate, but not oligotrophic levels of substrate. To examine this hypothesis, continuous cultivation in a lactate-limited chemostat was employed to enrich organisms from water of the Great Sippewissett Marsh. The predominant organism in each steady state was organism II, which had been enriched by continuous cultivation at a flux of 1.1 mg lactate-C/ml/hr and a dilution rate (D) of 0.03/hr. Its exponential growth rate was observed to be 0.33/hr over a 40-fold range (1.25-500 mg lactate-C/ml) of initial substrate concentrations. Organism II was enriched at a flux of 4.2 mg lactate-C/ml/hr and D = 0.33/hr, i.e., at an oligotrophic flux, but development a generation time of only 2 hours. Its exponential growth rate was subsequently found to be 0.53/hr. Both isolates grew readily in and on media with low nutrient concentrations, but only organism II was capable of initiating growth on Zobell's medium.
Lactate uptake was determined in 0.1 mM lactate.

The transport capacity to high nutrient concentrations, orthotrophic organisms, the sensitivity of the growth and of transport systems, since selection for a fast-growing organism may be attributable to repressibility of orthotrophs may be attributable to repressibility of high nutrient concentrations generally observed for initial hypotheses that the low growth rate and sensitivity repressively.

These results are consistent with the excess lactate (1.03 and 0.78 nmoles/min/10^6 cells), specificity different for cells grown in limited and not.

In contrast, excess lactate, the velocity was reduced to 0.10. Organism a velocity of 0.61 nmoles/min/10^6 cells for cells grown at lactate-limited cells of organism I accumulated lactate at

This work was supported in part by a grant from the Department of Energy.
Oligotrophic environments are more closely mirrored by this
accumulated nutrient dispersions in time (2,3,6).
Oligotrophic bacteria can be isolated in two ways: 1.)

- By direct plating on dilute media; for growth, cells must
- By chemostat selection at very low nutrient flux; cells must

Other nutrients dispersed in space (1), and 2.) 

This is in fact what was observed.

A selective pressure for altered transport characteristics
were related; selection for fast growth would also serve as
with nutrient concentrations. If these two characteristics
substrate leading to low growth rates and inhibition by
repressed by moderate, but not oligotrophic levels of
for a typical oligotrophic, nutrient transport would be
nutrient transport characteristics. If this were the case,
properties were in fact related physiologically through
purpose of this study was to determine whether these two
Growth in media of high nutrient concentration (3,5). The
exhibit low maximum growth rates and inability to initiate
isolated from habitats of low nutrient flux environments. Bacteria
oceans are typical oligotrophic habitats. Bacteria
carbon flux values not exceeding 0.1 mg/L/day and open
nutrients in these environments (4). Takes with organic
major contributors to the cycling of carbon and other
dilute (from near zero to a fraction of a mg/L/day) and are
From these populations, organisms were chosen for physiological studies on the possible correlation between growth and transport characteristics.
were used in this study. The dilution rates were controlled principally by the difference in working volume between the two units. The C-30 chemostat had a working volume of 6.29 L when agitation was 600 rpm and aeration was 0.6 LPM. The C-32 chemostat had a working volume of 1375 ml when agitation was 400 rpm and aeration was 0.6 LPM. The working volume was maintained at approximately 115 ml/hr in all cases. By changing the concentration of limiting nutrient in the reservoirs of the chemostats, discrete values for nutrient flux were achieved. For example:

\[ \text{Flux} = \frac{\text{Flow Rate} \times \text{Reservoir Nutrient Concentration}}{\text{Working Volume}} \]

The flow rate of medium from the reservoir was approximately 115 ml/hr. The final pH was adjusted to 7.75 just before use by addition of 0.18M NaHCO3. The seawater mixture was diluted with fresh water to 80% its strength and supplemented with Medium-Marine Biological Laboratory artificial sea salts. Medium-Marine Biological Laboratory artificial sea salts was used as a basal medium for enrichments and physiological studies. This consisted of:

- NaCl: 24.72 g
- KC1: 0.67 g
- CaCl2-2H2O: 1.36 g
- MgCl2-6H2O: 4.66 g
- MgSO4-7H2O: 6.29 g
- KH2PO4: 1.36 g
- NaHCO3: 0.18 g/l

The seawater mixture was diluted with fresh water to 80% its strength and supplemented with 12.5 mg or 50 mg of lactate—carbon/liter. Nitrogen was added as NH4Cl and phosphorous was added as KH2PO4 to give a final medium ratio of 10C:4N:1P. This ensured that carbon was the limiting nutrient. Nitrogen was added as NH4Cl and phosphorous was added as KH2PO4 to give a final medium ratio of 10C:4N:1P.
Transport characteristics were determined with:

- Total cell number of cultures growing in 125 ml shake flasks.
- Maximum growth rates were determined by following O.D. and growth curve.
- Substrate concentration at one-half maximum velocity. (Ks)
- Substrate and attempts at measurement of the Ks of selected organisms in batch culture studies. Studies on the transport characteristics of the organisms using radiolabeled substrates in batch culture. Studies included determination of the maximum growth rate (umax) of selected organisms.

Physiological studies—these studies included:

- A wavelength of 600 nm.
- The inoculum was introduced into growth vessels filled to 75% capacity with medium. This was allowed to grow as a batch culture for 36-48 hours before the nutrient pump was activated. The optical density of the culture was 0.06 at a wavelength of 600 nm.
- The inoculum was filtered to remove detritus and larger plankton organisms. The laboratory and filtered through 5 um Millipore membrane.

Sample—water samples for all enrichments were collected at high tide from the mouth of the Great Sippewissett Marsh. The samples were transported back to the laboratory. Transport characteristics studies were done at 25°C.
ucû/uMoi.e)wasai.LuteaWltflIILL01Z.3mMuni.abeL.Lect
Na-lactatein10mMphosphatebufferatpH=7.5. Thisgave
afinalconcentrationof2.5mMoleslactate/1.25mlthat
contained10uCiof14C—lactate, orafinalconcentration
of2.0mMNa-lactate.

Thetransportcharacteristicsofthetwoorganisms
weretestedunderconditionsoflactatelimitation(50
ug/ml) and lactate excess (500ug/ml). Cellsofeach
organismweregrowntolatelogphaseateachcarbon
concentration. Thisensuredthatalltransportsystems
would be expressed and that there would be very little
excess substrate available. The cell suspensions were then
centrifuged, the supernatant was discarded and the pellet
was resuspended in 80% full-strength MBL sea salts
without added nutrients. The final resting cell density of each
culture was recorded.

Uptakeexperiments were done as follows: 50 ul of 2
mM Na-lactate contained 10 UCI of 14C-lactate, or a final concentration
of 2.0 mM Na-lactate. A 100 ul aliquot of cellsuspension
was mixed with 1 ml of cell suspension, allowing a
radioactivity to be determined into 10 ml Aquasol
which contained a lactate concentration of 0.095 mM. A 100 ul aliquot was
withdrawn and placed immediately into 10 ml Aquasol
which contained a lactate concentration of 0.095 mM. A 100 ul aliquot was
removed, and the culture wasthen

The transport characteristiscs of the two organisms
were tested under conditions of lactate immitation (50
ug/ml) and lactate excess (500 ug/ml) Cells of each
organism were grown to late log phase at each carbon
concentration. This ensured that all transport systems
were expressed. The final resting cell density of each
culture was recorded. The final resting cell density of each
organism was determined.
Suspension was tested in the same manner. The samples were repeated for a period of 10 minutes. Each resulting cell
Restric the appearance of protozoa in enrichments carried

the seawater sample was too large (5 µm). This did not

1.) the filter used to remove detritus and protozoa from

Two problems were encountered with the enrichment of

Chemostats.

In the growing culture did not vary during operation of the

that a steady state population had been obtained. The pH

selection of the sample, it was possible to ascertain

and numbers and morphology of colonies on plates after

following optical density (OD), microscopic observations.

were recorded every second volume change. By

(table 2. reviews the populations enriched). Chemostats

were harvested from the seawater samples

rate, four distinct steady state populations of

using the combinations of nutrient flux and dilution

of microorganisms.

growth rate (division rate) selected different populations

experiments (4.2 mL/hr), the four fold difference in

the same nutrient flux was used in two chemostats

Although the division rate of the chemostat, routinely

at very low concentrations and growth at different rates

important in selection ability to assimilate carbon offered

microorganisms are outlined in table 1. Two factors were

the chemostats to enrich for different nutritional types of

methanogenic growth rates and dilution rates used in

the enrichments.
Two isolates were chosen for further study. The prototype to prey upon, and
2. the problem of organism "clumping" in the growth vessel. Thus occurred in the high flux / high dilution rate chemostat enrichment for a fast growing copiotroph. After 36 hours of growth, the key to successfull chemostat experiments is homogeneity in the growth vessel. When clumping occurs, cells in the center of the aggregate are not exposed to the same environment as those at the periphery. Because clumping effects O.D. and plate counts it is impossible to determine if a steady state is achieved. To demonstrate the versatility of the continuous culture approach to enrichment, the slow growing oligotroph B was enriched from the same sample as slow growing oligotroph A. This occurred in the high flux / high dilution rate chemostat enrichment for a fast growing copiotroph which was always present but not favored until the nutritional status of the medium was changed.

Thus the flux (by a factor of 4. a new distinct steady state population was enriched which was always present but
simple by lowering the concentration of all nutrients and enriched from the same sample as slow growing oligotroph A. A new distinct steady state population was enriched which was always present but not favored until the nutritional status of the medium was changed. This occurrence is the problem of organism "clumping" in the growth vessel. Thus occurred in the high flux / high dilution rate chemostat enrichment for a fast growing copiotroph. The key to successful chemostat experiments is homogeneity in the growth vessel. When clumping occurs, cells in the center of the aggregate are not exposed to the same environment as those at the periphery. Because clumping effects O.D. and plate counts it is impossible to determine if a steady state is achieved. To demonstrate the versatility of the continuous culture approach to enrichment, the slow growing oligotroph B was enriched from the same sample as slow growing oligotroph A. This occurred in the high flux / high dilution rate chemostat enrichment for a fast growing copiotroph which was always present but not favored until the nutritional status of the medium was changed.

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Growth of organism II transferred back to oligotrophic conditions. Organism that grew on Zobell's was able to rapidly initiate growth on Zobell's medium. The ability to initiate growth on Zobell's medium, and on media with low nutrient concentrations, only organism II was enriched at a flux of 4.2 ug lactate-C/ml/hr and a dilution rate of 0.33/hr. This organism grew at an oligotrophic flux, but the dilution rate required a growth rate of 0.53/hr. The growth rates of these two organisms were surprisingly close but different. As a test to determine the sensitivity of the organisms to high nutrient concentration, both organisms were plated on Zobell's medium, a rich marine medium, and on media with low nutrient concentrations. Although both organisms grew well on media with low nutrient concentrations, both organisms were plated on Zobell's medium to test the sensitivity of the organisms to high nutrient concentration. The growth rate of organism II was 0.53/hr. The growth rates of these two organisms was determined spectrophotometrically at 600 nm and cell number in a Petroff-Hauser counting chamber. Cell counts give the most reliable estimates of cell density. The growth rate of organism I was 0.33/hr and the growth rates of these two organisms were not significantly different. Lower nutrient levels do not support a significant optical density because the turbidity of the cultures at the lower nutrient concentrations. Exponential growth rates were determined in batch culture over a 40 fold range of substrate concentration (12.5 ug/ml - 500 ug/ml). Growth rates were determined in batch culture over a 40 fold range of substrate concentration (12.5 ug/ml - 500 ug/ml).
Transport characteristics:

Rates of uptake were determined using uniformly labeled 14C-lactate. The transport characteristics of the two organisms were tested under conditions of lactate limitation (50 µg/ml) and lactate excess (500 µg/ml).

The results of the 14C-lactate uptake studies are summarized in Table 4. From the data it is clear that organism A, the fast-growing oligotrophic organism, is not repressed and continues to accumulate lactate at nearly the same rate regardless of lactate concentration. Organism B is repressed in transport by high lactate concentration.

Organisms A and B were grown to late log phase at each carbon concentration. See Materials and Methods. Cells of each organism were grown in Zobell's medium. 

\[
K_s = \frac{s}{(D/y-D)}
\]

During the steady state, the concentration of substrate remaining in the growth vessel is calculated from chemostat experiments by determining the concentration at one-half maximal velocity. This constant, \(K_s\), the substrate concentration at which the rate of translocation equals the rate of translocation at any other concentration, is referred to as the 'transport constant'.

Attempts were made to determine the \(K_s\) for each organism.

\[\text{Dilution Rate} = \frac{D}{y-D}\]

\[s = \text{substrate concentration in the chemostat}
\]

\[D = \text{dilution rate}
\]
3. Preliminary characterization of nutrient transport systems indicates that repressibility of transport may be responsible for the low growth rate and sensitivity to high nutrient concentrations observed in freshly isolated oligotrophic organisms. Selection for fast-growing oligotrophic bacteria selected independently for insensitivity to high nutrient concentrations. Indicating a physiological relationship between these two characteristics.

CONCLUSIONS:

1. Chemostats were very successful for the enrichment of oligotrophic organisms within the range of dilution rates and concentrations of limiting substrate selected.

2. Selection for fast-growing oligotrophic bacteria selected independently for insensitivity to high nutrient concentrations. Indicating a physiological relationship between these two characteristics.

3. Preliminary characterization of nutrient transport systems indicates that repressibility of transport may be responsible for the low growth rate and sensitivity to high nutrient concentrations observed in freshly isolated oligotrophic organisms.

However, due to problems encountered with the lactate assay using lactate dehydrogenase, it was not possible to complete this part of the study.


6. Veldkamp, H., 1976, Continuous culture in microbial physiology and ecology, Meadowfield Press Ltd.


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Flux = (flow rate) (reservoir substrate conc.)

<table>
<thead>
<tr>
<th>Flux Type</th>
<th>Dilution Rate/hr</th>
<th>Dilution Rate/hr</th>
<th>Dilution Rate/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flux = (flow rate) (reservoir substrate conc.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fast Growing Oligotroph A</td>
<td>0.0083/hr</td>
<td>0.0083/hr</td>
<td>0.0083/hr</td>
</tr>
<tr>
<td>Fast Growing Oligotroph B</td>
<td>0.033/hr</td>
<td>0.033/hr</td>
<td>0.033/hr</td>
</tr>
<tr>
<td>Slow Growing Oligotroph</td>
<td>1.00/hr</td>
<td>1.00/hr</td>
<td>1.00/hr</td>
</tr>
</tbody>
</table>

Note: Flux is calculated as the product of flow rate and reservoir substrate concentration. The working volume in the growth vessel is considered in the calculation. The physical type column refers to the different types of growth processes.
Table 2 summarizes steady state chemostat populations.

<table>
<thead>
<tr>
<th></th>
<th>Slow Oligo</th>
<th>Fast Oligo</th>
</tr>
</thead>
<tbody>
<tr>
<td>61%</td>
<td>2.6*10^6</td>
<td>3**</td>
</tr>
<tr>
<td>22%</td>
<td>1.5*10^6</td>
<td>4</td>
</tr>
<tr>
<td>88%</td>
<td>2.5*10^6</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>5</td>
</tr>
</tbody>
</table>
Table 3: Carbon flux and dilution rate are selection parameters.

<table>
<thead>
<tr>
<th>Growth Condition</th>
<th>C Flux (µg/ml/hr)</th>
<th>Dilution Rate (hr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Org. I</td>
<td>4.7</td>
<td>0.33</td>
</tr>
<tr>
<td>Org. II</td>
<td>1.1</td>
<td>0.083</td>
</tr>
<tr>
<td>Org. III</td>
<td>0.8</td>
<td>0.083</td>
</tr>
<tr>
<td>Org. IV</td>
<td>0.5</td>
<td>0.083</td>
</tr>
</tbody>
</table>

Zobell's medium is a rich medium containing approximately 3000 mg carbon per liter. The maximum growth rates of the organisms isolated from the chemostats were determined in batch culture and found not to vary over a 40-fold range of nutrient concentration (1.25 mg/l to 50 mg/l). One organism grew luxuriantly, the other not at all.
Table 4.

Rates of lactate uptake:

<table>
<thead>
<tr>
<th>Conc. lactate velocity</th>
<th>Org. I 50 mg/l</th>
<th>0.613 nmoles/min/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Org. I 500 mg/l</td>
<td>0.10 nmoles/min/10^6 cells</td>
</tr>
<tr>
<td></td>
<td>Org. II 50 mg/l</td>
<td>1.03 nmoles/min/10^6 cells</td>
</tr>
<tr>
<td></td>
<td>Org. II 500 mg/l</td>
<td>0.78 nmoles/min/10^6 cells</td>
</tr>
</tbody>
</table>

Organism I is repressed in uptake by high lactate concentrations. Organism II is virtually unaffected by high nutrient concentrations. Organism II is the fast-growing lactate-utilizing oligotrophic microorganism. Organism I displays the transport characteristics of a "typical" oligotrophic organism.

<table>
<thead>
<tr>
<th>Velocity (nmoles/min/10^6 cells)</th>
<th>Conc. lactate uptake:</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.78</td>
<td>500 mg/l</td>
</tr>
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<td>1.03</td>
<td>50 mg/l</td>
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<tr>
<td>0.10</td>
<td>500 mg/l</td>
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<tr>
<td>0.613</td>
<td>50 mg/l</td>
</tr>
</tbody>
</table>

OrG.