ABSTRACT:

Bacteria 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 hypothhesized 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 isolated, and two isolates were studied in detail. Organism I had been enriched by cultivation at a flux of 1.1 ug lactate-C/ml/hr and a dilution rate (D) of 0.083/hr. Its exponential growth rate was observed to be 0.33/hr over a 40-fold range (12.5-500 ug lactate-C/ml) of initial substrate concentration. Organism II was enriched at a flux of 4.2 ug lactate-C/ml/hr and D=0.33/hr, i.e., at an oligotrophic flux, but demanding a gneration time of only 2 hrs. 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. In

of lactate uptake was determined in 0.1 mM lactate. Lactate-limited cells of organism I accumulated lactate at a velocity of 0.61nmoles/min./$10^6$cells; for cells grown in excess lactate, the velocity was reduced to 0.10. Organism II, in contrast, exhibited velocities that were not significantly different for cells grown in limited and excess lactate (1.03 and 0.78 nmoles/min./$10^6$cells, respectively). These results are consistent with the initial hypothesis that the low growth rate and sensitivity to high nutrient concentrations generally observed for oligotrophs may be attributable to repressibility of transport systems, since selection for a fast-growing oligotroph eliminated the sensitivity of its growth and of its transport capacity to high nutrient concentrations.

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dilute (from near zero to a fraction of a mg/L/day) and are major contributors to the cycling of carbon and other nutrients in these environments (4). Lakes with organic carbon flux values not exceeding 0.1 mg/L/day and open oceans are typical oligotrophic habitats. Bacteria isolated from habitats of low nutrient flux generally exhibit low maximum growth rates and inability to initiate growth in media of high nutrient concentration (3,5). The purpose of this study was to determine whether these two properties were in fact related physiologically through nutrient transport characteristics. If this were the case, for a typical oligotroph, nutrient transport would be repressed by moderate, but not oligotrophic levels of substrate leading to low growth rates and inhibition by high nutrient concentrations. If these two characteristics were related, selection for fast growth would also serve as a selective pressure for altered transport characteristics. This is in fact what was observed.

Oligotrophic bacteria can be isolated in two ways: 1.) by direct plating on dilute media; for growth, cells must gather nutrients dispersed in space (1), and 2.) by chemostat selection at very low nutrient flux; cells must accumulate nutrients dispersed in time (2,3,6). Oligotrophic environments are more closely mirrored by this

affinities. From these populations, organisms were chosen for physiologic 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 330 ml when agitation was 400 rpm and aeration was 0.6 LPM and the C-32 chemostat had a working volume of 1375 ml when agitation was 600 rpm and aeration was 1.0 LPM. The flow rate of medium from the reservior 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:

Flux= (flow rate)(reservoir nut. conc.) / working volume

F= (115 ml/hr)( 50 ug/ml) / 1375 ml = 4.18 ug/ml/hr

Medium- Marine Biological Laboratory artificial sea salts was used as a basal medium for enrichments and physiologic studies. This consisted of: NaCl 24.72g, KCl 0.67g, $CaCl_2$-$2H_2O$ 1.36g, $MgCl_2$-$6H_2O$ 4.66g, $MgSO_4$-$7H_2O$ 6.29g, in 1 liter of water. The final pH was adjusted to 7.75 just before use by addition of 0.18g/l $NaHCO_3$. The seawater mixture was diluted with fresh water to 80% its full strength and supplemented with 12.5 mg or 50 mg of lactate-carbon/liter. Nitrogen was added as $NH_4Cl$ and phosphorous was added as $KH_2PO_4$ to give a final medium ratio of 10C:4N:1P. This ensured that carbon was the

autoclaving. The agar medium with the same nutrient composition was used for subsequent population counts and isolation of organisms. All enrichments were done at 25° C.

Sample- Water samples for all enrichments were collected at high tide from the mouth of the Great Sippewisset Marsh. The samples were transported back to the laboratory and filtered through 5 um millipore membrane filters to remove detritus and larger plankton organisms. 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.

Physiological studies- These studies included determinaton of the maximum growth rate ($u_{max}$) of selected organisms in batch culture, studies on the transport characteristics of the organisms using radiolabelled substrate, and attempts at measurement of the $K_s$ (the substrate concentration at one-half maximum velocity). Maximum growth rates were determined by following O.D., and total cell number of cultures growing in 125 ml sidearm flasks.

Transport characteristics were determined with

uCi/uMole) was diluted with 1 ml of 2.25 mM unlabelled Na-lactate in 10 mM phosphate buffer at pH=7.5. This gave a final concentration of 2.5 uMoles lactate/1.25 ml that contained 10 uCi of 14C-lactate, or a final concentration of 2.0 mM Na-lactate.

The transport characteristics of the two organisms were tested under conditions of lactate limitation (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 would be expressed and that there would be very little excess substrate available. The cell suspensions were then centrifuged, the supernatent 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.

Uptake experiments were done as follows: 50 ul of 2 mM $^{14}$C-lactate was mixed with 1 ml of cell suspension, giving a lactate concentration of 0.095 mM. A 100 ul aliquot was withdrawn and placed immediately into 10 ml Aquasol scintillation cocktail. This determined exactly how much radioactivity was in the resting cell suspension. To determine substrate taken up by the cells with time, 100 ul samples of the cell/lactate suspension were filtered at one minute intervals through a 0.45 uM millipore filter and

repeated for a period of 10 minutes. Each resting cell suspension was tested in the same manner. The samples were then counted in a scintillation counter and corrected for background. Uptake of the substrate was linear over the time period of the experiment. The rate of uptake in nanomoles of lactate/ min/ $10^6$ cells was calculated from the slope of the uptake lines for each population of cells. See results section.

the chemostats to enrich for different nutritional types of microorganisms are outlined in table 1. Two factors were important in selection ability to assimilate carbon offered at very low concentrations and growth at different rates governed by the dilution rate of the chemostat. Although the same nutrient flux was used in two chemostat experiments (4.2 ug/ml/hr), the four fold difference in growth rate (dilution rate) selected different populations of microorganisms.

Using the combinations of nutrient flux and dilution rate, four distinct steady state populations of microorganisms were enriched from the seawater samples (table 2. reviews the populations enriched). Chemostats were aseptically sampled every second volume change. By following optical density (O.D.), microscopic observations, and numbers and morphhology of colonies on plates after serial dilution of the sample, it was possible to ascertain that a steady state population had been obtained. The pH in the growing culture did not vary during operation of the chemostat.

Two problems were encountered with the enrichment: 1.) the filter used to remove detritus and protozoa from the sea water sample was too large (5 um). This did not restrict the appearance of protozoa in enrichments carried

the protozoa to prey upon, and 2.) the problem of organism "clumping" in the growth vessel. This occured in the high flux / high dilution rate chemostat enrichment for a fast growing copiotroph after 36 hours of growth. 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.

Among the advantages of the chemostat is sub-selection of populations. In our studies, all but one chemostat experiment was started with a fresh inoculum. To demonstrate the versatilty of the continuous culture approach to enrichment, the slow growing oligotroph B was enriched from the same sample as slow growing oligotroph A simply by lowering the concentration of all nutrients (and thus the flux) by a factor of 4. A new distinct steady state population was enriched which was always present but not favored until the nutritional status of the medium was changed.

Physiological Studies:

Two isolates were chosen for further study. Their

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 oligotroic flux, but the dilution rate required a generation time of only 2 hrs. Both of these organisms were dominant in their respective populations. Exponential growth rates were determined in batch culture over a 40 fold range of substrate concentration (12.5 ug/ml - 500 ug lactate-C/ml) by monitoring O.D. spectrophotometrically at 600 nm and cell number in a Petroff-Hauser counting chamber. Cell counts give the most reliable estimates of growth rates because the turbidity of the cultures at the lower nutrient levels do not support a significant optical density. The growth rate of organism I was 0.33/hr and the growth rate of organism II was 0.53/hr. The growth rates of these two organisms was surprisingly close but significantly different. As a test to determine sensitivity of the organisms to high nutrient concentrations, 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, only organism I had the ability to initiate growth on Zobell's medium. The organism that grew on Zobell's was able to rapidly initiate growth if transfered back to oligotrohic conditions.

Zobell's medium.

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 ug/ml) and lactate excess (500 ug/ml). Cells of each organism were grown to late log phase at each carbon concentration. See Materials and Methods.

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

Attempts were made to determine the $K_s$, the substrate concentration at one-half maximal velocity. This constant is calculated from chemostat experiments by determining the concentration of substrate remaining in the growth vessel during the steady state:

$$s = K_s \, (D/u_m - D)$$

s= substrate concentration in the chemostat

D= dilution rate

The next step in our study would have involved a competition experiment between our isolates. The chemostats were run and all samples were collected. However, due to problems encountered with the lactate assay using lactate dehydrogenase, it was not possible to complete this part of the study.

CONCLUSIONS:

1. Chemostats were very successful for the enrichment of oligotrohic 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.

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TABLE 1.

| Flux | Dilution Rate | Physiological Type |
|---|---|---|
| 16.8 ug/ml/hr | 0.33 /hr | Fast Growing Copiotroph |
| 4.2 " | 0.083 /hr | Slow Growing Oligotroph A |
| 4.2 " | 0.33 /hr | Fast Growing Oligotroph |
| 1.04 " | 0.083 /hr | Slow Growing Oligotroph B |

$$\text{Flux} = \frac{\text{(flow rate) (reservoir substrate conc.)}}{\text{(working volume in growth vessel)}}$$

| | | | | |
|---|---|---|---|---|
| Fast Copio | 5 | --- | 0.04 | --- |
| Slow Oligo A | 5 | $2.5*10^6$ | 0.04 | 88% |
| Fast Oligo* | 4 | $1.5*10^6$ | 0.01 | 72% |
| Slow Oligo B** | 3 | $2.6*10^6$ | 0.01 | 61% |

Table 2 summarizes steady state chemostat populations. Colony morpholoy indicates the number of different colony types in the populations. Cell number was determined by serial dilution and plating. The percent dominance indicates what percentage of the colonies on the plate were of the dominant colony morphology. The flux and growth rate requirements had an obvious influence on size and number of the cells in the culture. Oligotrohic cells were highest in number and very small. The surface area to volume ratio increased the absortive capacity of the cells. Populations not stressed in this fashion were much larger but an order of magnitude lower in number. Measurments of optical density are included but are not significant at these low readings.

* Fast-growing oligotroph = organism II

** Slow-growing oligotroph = organism I

TABLE 3.

| | Enrich. Condition C flux | D | $u_{max}$ | Growth on Zobells |
|---|---|---|---|---|
| Org. I | 1.1 ug/ml/hr | 0.083 | 0.33 | no growth |
| Org. II | 4.2 ug/ml/hr | 0.33 | 0.53 | growth |

Carbon flux and dilution rate are selection parameters predetermined for the chemostat experiment. 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 (12.5 mg/l-500 mg/l). Zobell's medium is a rich medium containing approximately 3000 mg carbon/ liter. One organism grew luxuriantly, the other not at all.

TABLE 4.

Rates of lactate uptake:

| | Conc. lactate | velocity |
|---|---|---|
| Org. I | 50 mg/l | 0.613 nmoles/min/$10^6$ cells |
| Org. I | 500 mg/l | 0.10 nmoles/min/$10^6$ cells |
| Org. II | 50 mg/l | 1.03 nmoles/min/$10^6$ cells |
| Org. II | 500 mg/l | 0.78 nmoles/min/$10^6$ cells |

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-utilizng oligotrophic microorganism. Organism I displays the transport characteristics of a "typical" oligotrophic organism.