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Growth of an Adherent Mixed Microbial Culture in a Substrate Limited Single State Chemostat

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GROWTH OF AN ADHERENT MIXED MICROBIAL CULTURE IN A SUBSTRATE LIMITED SINGLE STATE CHEMOSTAT

by

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SUMMARY

A steady-state was established between a C. lividum and a Pseudomonas sp at a dilution rate of 0.27 hr^{-1} when the growth limiting substrate was citrate. During both pure and mixed continuous culture studies, the C. lividum adhered to the wall of the chemostat and the Pseudomonas showed no such tendency visually. This system offers a convenient model for studying the importance of bacterial adherence to certain aquatic ecosystems such as river sediments and sewage treatment.

INTRODUCTION

Mixed continuous culture studies have been conducted by numerous investigators (Jannasch, 1967; Tempest, Dicks and Meers, 1967; Mateles and Chian, 1968; Meers and Tempest, 1968; Brunner, Oberzill and Menzel, 1969, Meers, 1971; Veldkamp and Jannasch, 1972) and were reviewed recently (Bungay and Bungay, 1968). Since most environments consist of a variety of microorganisms (Brock, 1966; Jannasch, 1967), research on mixed cultures is pertinent. The importance of continuous culture techniques in studying microbial interactions has been pointed out by Jannasch (1965) "the microbial population in the natural environment must be regarded as an open system." Cell adherence has also been investigated in the chemostat and, in some cases, has been avoided (Jannasch, 1965) and in other situations considered (Dias, Dondero and Finstein, 1968; Topiwala and Hamer, 1971). The attachment of bacterial cells to surfaces or themselves can be applied to an understanding of sewage treatment (Dias, et al, 1968) and bacterial flocculation (Joyce and Dugan, 1970; Pfister, Dugan and Frea, 1971).

The objective of this study was to develop a continuous culture system with two bacterial cultures one of which was adherent to the wall of the chemostat. By developing such a laboratory model, microbial interactions in certain aquatic ecosystems (e.g. river sediments) can be further understood.

METHODS

Microorganisms and Media

The two strains of microorganisms used throughout this study were isolated

from Lake Erie. One organism was identified as Chromobacterium lividum and the other was tentatively classified as a Pseudomonas sp. These were selected for two reasons: both could be grown in the same defined medium and the purple, pigmented C. lividum colonies could be easily differentiated from those of the Pseudomonas sp. The medium for batch and continuous culture (termed citrate medium) contained the following compounds (g/1):$(NH_4)_2HPO_4$, 1.0 g; KH_2PO_4, 1.0 g:$MgSO_4 \cdot 7H_2O$, 0.2g; NaCl, 3.0g; and a specified amount of $Na_3C_6H_5O_7 \cdot 2H_2O$. The pH was adjusted to 6.7 with either 1 N NaOH or 1 N HCl and then autoclaved. Both microorganisms were maintained in static culture, grown in citrate medium (socium citrate concentration was 0.1 g/1) and transferred weekly.

Apparatus

The single-stage continuous culture system (Fig. 1 was similar in design to that described by Jannasch (1967). Media was pumped from the reservoir (5 gallon Nalgene bottle, autoclavable) into the cultivation vessel by a peristalic pump (Buchler). The heater (nichtome wire wrapped six times around pyrex glass tubing and connected to a variable power supply) was maintained at 90 to 95 C and this prevented the motile bacteria in the cultivation vessel from traveling back into the reservoir.

The cultivation vessel (total capacity 1000 ml) was made from pyrex glass and was constructed such that water could continuously circulate through a jacket which surrounded the vessel. The vessel was treated with silicone (Desiocote, Beckman) before each experiment. The fluid level in the culture vessel was kept at a constant level by the continuous input of sterile, humidified air which exited through an overflow tube shaped like a U. When the fluid volume in the cultivation vessel exceeded the desired volume, the liquid would be expelled through the opening in the U tube and exit into the collecting vessel.

Flow rates were measured using two methods. A 10 ml pipette and glass drying tube filled with glass wool (called the air pressure by-pass) were used to measure rates over a short period (1 to 5 minutes). In operating the system, clamp 1 was opened and clamp 2 was closed. Liquid would then collect in the pipette and simultaneously any gases were released. Flow rate was the difference in pipette calibrations divided by the time required to collect the

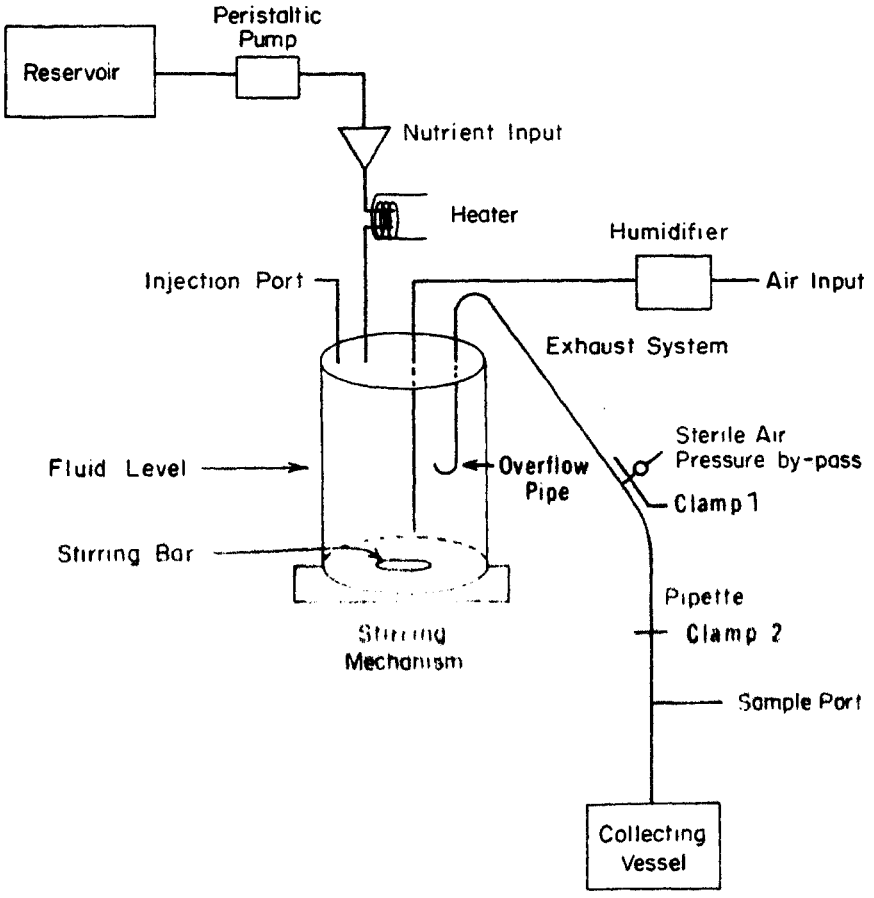

Figure 1

The single stage continuous culture apparatus (chemostat)

measured volume. The flow rate was determined over longer periods (30 to 60 minutes) by attaching a sterile graduated cylinder to the exit line.

Microorganisms were intentionally inoculated into the system through the injection port which was a hypodermic needle (8", 21 guage). Samples for citrate determination and viable bacterial counts were removed at the inoculation port or the same port. The medium was uniformly mixed with a teflon stirring bar rotating at a rate of approximately 300 rpm. Air was pumped (Universal electric air pump) at a rate of 690 ml per minute. The jacketed chemostat was kept at 20± 2 C with a Fermentation Design Refrigeration unit (Fermentation Design, New Brunswick, New Jersey).

Determination of Citrate

A modified method was used to detect citrate at a concentration as low as 10 mg/l (Natelson, Pincus and Logovoy, 1948). Standards were prepared using different concentrations of sodium citrate. Three ml of sample were treated with 0.1 ml of 18 N H_2SO_4 and 0.3 ml of 1 N KBr and incubated at room temperature for 10 minutes. One-half of 5% (w/v) $KMnO_4$ was added and this was incubated for 10 minutes at room temperature. This solution was decolorized with the dropwise addition of 6% (v/v) H_2O_2 (both the 6% H_2O_2 and tubes containing solution were kept in an ice bath). After decolorization 5 ml of petroleum ether was added and mixed for 1 minute on a Vortex. Four ml of the upper layer containing the petroleum ether were added to 4.5 ml of 4% (w/v) thiourea solution (pH 9.2), mixed 1 minute on a Vortex, and the optical density was determined on the aqueous layer at 445 nm.

Determination of Viable Bacteria

Samples which were removed from the chemostat were immediately diluted in phosphate buffered saline, termed PBS (pH 7.3, 0.85% NaCl buffered with 0.067 M K_2HPO_4 and KH_2PO_4); and 0.1 ml volumes were spread over the surface of starch agar plates (Difco). Each dilution was plated in triplicate. After 36 hours of incubation at 25 C, colonies were counted on a Quebec colony counter. Total cell number was determined using a Petroff Hauser counting chamber.

Differentiation of Bacterial Colonies

Incubation of starch agar plates at 25 C provided a convenient method for distinguishing between the Pseudomonas sp and C. lividum. Purple pigmented C. lividum colonies were readily distinguished from cream colored Pseudomonas sp colonies within 48 hours to 72 hours. Also, only areas of starch hydrolysis were seen around the Pseudomonas sp colonies. By adding several drops of 0.02% tincture of iodine to starch agar plates, non-hydrolyzed areas stained purple and hydrolyzed areas were clear.

Differentiation of Bacteria Microscopically

The indirect fluorescent antibody technique (Coons and Kaplan, 1951) was used for staining cells. Since both microorganisms were Gram negative rods, it was impossible to distinguish between them using phase contrast optics. Samples removed from the chemostat were placed on microscope slides (etched with 1 mm^2 area), air dried, and fixed in acetone for 10 minutes. The slides were placed in a moisture chamber and several drops of gamma gloublin (specific for the Pseudomonas sp) were added to the slides. After 10 minutes incubation at room temperature, the slides were washed three times in PBS and returned to the moisture chamber. Several drops of goat anti-rabbit fluorescein isothiocynate (Microbiological Associates, Bethesda, Maryland) were added to the slides, incubated 10 minutes at room temperature, and again washed three times in PBS. Glycerol was placed on the slide and the 1 mm^2 area was examined with Carl Zeiss phase contrast fluorescent microscope at a magnification of 1000 X. The field was examined with both phase contrast and fluorescent optics. The number of bacteria were counted, using both optical systems, at 20 different locations within the 1 mm^2 area and the number averaged. Cells observed with phase contrast optics represented both Pseudomonas sp and C. lividum. When the field was examined with fluorescent optics, only the Pseudomonas sp cells fluoresced. The exciter filter was a #1 and the barrier filter unit was #50. Photographs were taken on Tri-X film (Kodak-400 ASA) with exposures up to 1 minute.

Determination of Maximum Specific Growth Rates (μ_{max})

Batch culture studies were performed in the chemostat vessel. The growth conditions were as follows: Citrate medium (5.0 g/l sodium citrate), the pH

was 6.7 and the temperature was 20 C. The maximum specific growth rate was determined using the following formula:

$$\mu_{max} = \frac{0.67}{t_d}$$

The t_d is the doubling time which was calculated from the growth curve during logrithmic growth. The t_d for the Pseudomonas sp was 2.2 hr and the μ_{max} was 0.30 hr^{-1} and the t_c for the C. lividum was 2.3 hr and the μ_{max} was 0.29 hr^{-1}.

RESULTS

Before conducting continuous culture studies, a batch culture experiment was performed in which the two microorganisms were grown separately and then in mixed culture (Table I). The viable cell number of the C. lividum remained nearly unchanged (except at 48 hours) under both sets of conditions while the Pseudomonas sp was inhibited in the mixed culture after 48 hours.

The first continuous culture experiment (Table 2) was conducted at an initial concentration of 0.05 g/l sodium citrate (initial substrate concentration will be termed S_r). The viable cell count for the C. lividum was recorded only at several retention times (retention time will be termed R and is the time to change completely the chemostat volume e.g. R10 would indicate that the volume has been changed 10 times) due to the visible at R10. The population varied from 1.5 to 16 x 10^5 bacteria/ml and this variation was attributed to the wall growth. The population for the Pseudomonas sp was examined more frequently and ranged in value from 2.1 to 7.9 x 10^6 bacteria/ml. During this period the Pseudomonas sp cells showed no tendency towards adherence to the chemostat wall or clumping.

The subsequent conditions culture experiment (Table 3) was also performed at an S_r value of 0.05 g/l sodium citrate. The Pseudomonas sp was first grown in the chemostat and at R28 (assuming that the Pseudomonas sp achieved a steady-state), the C. lividum (grown in batch culture) was injected into the chemostat. At R34 colonies of C. lividum were not present on the following dilutions: 10^{-4}, 10^{-5}, and 10^{-6}. However, by R36 several purple colonies were observed on the 10^{-4} plate and the number of C. lividum continued to increase until a cell volume of 7 x 10^5 bacteria/ml occurred at R48. During this experiment the microbial population was monitored on starch agar plates and microscopically

Table 1

time (Hrs)	1	24	48	72
Microorganism and culture conditions				
Pseudomonas sp grown separately	0.17	1.50	22.5	135
C. lividium grown separately	0.15	0.17	23.4	20.0
Pseudomonas sp and *C. lividium* in mixed culture	0.17	0.98	13.1	13.0
	0.16	0.16	3.0	17.0

Batch cultures of the *Pseudomonas sp*, *C. lividum* and an equal mixture of the two microorganisms were grown in citrate medium containing 0.1 g/l sodium citrate at 20 C. The counts are the viable bacteria / ml x 10^6.

Table 2

R	0	5	7	10	15	17	22
Pseudomonas sp pH 6.7 mo/ml x 10^6	0.3	6.9	4.9	4.2	2.1	4.3	7.9[b]
C. lividium pH 6.7 mo/ml x 10^6	0.1	-	-	-[a]	0.15	-	1.7[b]

a bacterial growth on the chemostat wall was apparent
b samples used for electron microscopy
c noted much clumping
- samples were not removed

Continuous cultivation of the Pseudomonas sp and of C. lividum in separate chemostats with an initial sodium citrate concentration of 0.05 g/l (this will be referred to as Sr). The other parameters which remained constant were: T=20 C, pH=6.7, and dilution rate referred to as D=0.20 hr^{-1}. The counts for the microorganisms represent the viable bacteria per ml x 10^6. The retention time will be referred to as R.

Table 3

R	0	15	20	24	26	28	29	34	36	38	40	42	48
Pseudomonas sp plate counts mo/ml x 10^6	0.3	12	8.7	8.7	8.1	9.9	13	9.0	6.9	6.4	7.5	8.1	6.6
C. lividium plate counts mo/ml x 10^6							1.0	-	.03	0.1[a]	0.2	0.3	0.7
Ratio: *Pseudomonas* sp to *C. lividium*							13	-	230	64	37	27	9.4
Pseudomonas sp fluorescent counts per microscopic field	25	25	24	21	34	31	31	28	24	28	28	20	20
C. lividium fluorescent counts per microscopic field							0.7	0	0	0	0	0	0.6
Ratio: *Pseudomonas* sp to *C. lividium*							43	-	-	-	-	-	42

a bacterial growth on the chemostat wall was apparent
- samples were not removed

Continuous cultivation of the *Pseudomonas sp* in citrate medium followed by an inoculation of *C. lividum* from batch culture. During this experiment the indirect fluorescent antibody technique was employed. (Sr=0.05 g/liter; T=20 C; pH=6.7; D=0.27 hr^{-1})

with the aid of the indirect fluorescent antibody technique. As shown in the table, wall growth was observed at R38.

The S_r value was reduced from 0.05 g/l to 0.01 g/l sodium citrate and mixed culture studies were again performed (Tables 4 and 5). For this experiment two chemostats were operated simultaneously. The indirect fluorescent antibody technique was not used during these experiments since the population density was reduced to such an extent that, microscopically, it was too difficult to obtain accurate cell counts. A steady-state population of the Pseudomonas sp was first established, then an inoculum of C. lividum (grown simultaneously in the other chemostat) was added at R38 such that the ratio of Pseudomonas sp to C. lividum was 1500. The ratio increased at R43 and then began to approach 1 (R60). This ratio remained rather constant after R60 indicating that the two microorganisms had achieved a simultaneous steady-state. As indicated, wall growth was clearly visible at R52 and persisted throughout the remainder of the experiment.

In the next table (5) C. lividum was initially established in the chemostat and the Pseudomonas sp (grown simultaneously in the other chemostat) was added at R 38. Following the addition of the Pseudomonas sp the number of Pseudomonas sp began to decrease while the number of C. lividum remained unchanged. But, as also observed in the previous experiment (Table 4), a mixed culture was again established; however, this time the C. lividum outnumbered the Pseudomonas sp by approximately 20 to 1.

DISCUSSION

As shown the C. lividum adhered to the submerged portion of the wall of the chemostat (which was treated with silicone) whereas the Pseudomonas sp showed no such tendency visibly. Attachment of the C. lividum cells to the chemostat wall provided a unique 'niche' for this microorganism. Thus the two microorganisms occupied different locations and this eliminated bacterial competition except in the liquid portion where both microorganisms would compete for substrate. The results obtained by Dias et al (1968) also stress the importance of bacterial adherence to continuous culture. When Sphaerotilus natans was grown in mixed conditions, the S. natans continued to adhere to the continuous culture device even at high dilution rates. The paper by

Table 4

R	0	7	11	12	17	19	24	37	38	43	45	48
Pseudomonas sp mo/ml x 10^5	2.0	1.4	13.2	14	50	5.5	14.2	50[b]	15	50	5.5	10.8
C. lividium mo/ml x 10^5									.01	.002	.07	.35
Ratio: Pseudomonas sp to C. lividium									1500	25,000	78	31

R	51	52	55	60	64	68	73	77	79	83	87
Pseudomonas sp mo/ml x 10^5	24.8	13.1	27	10	3.5	13	3.5	7.4	7.2	8.5	13
C. lividium mo/ml x 10^5	0.70	.60[a]	1.8	3.5	3.5	5.0	2.5	5.0	2.5	5.0	6.0
Ratio: Pseudomonas sp to C. lividium	35	22	15	3	1	2.6	1.4	1.4	2.9	1.7	2.2

a bacterial growth on the chemostat wall was apparent
b sample used to inocculate continuous culture of C. lividum (see Table 5)

Continuous cultivation of the Pseudomonas sp in citrate medium followed by the inoculation of C. lividum. The C. lividum was removed from continuous culture at R 37 (see Table 5)
(Sr-0.01 g/liter; T=20 C; pH=6.7; D=-.27 hr^{-1})

Table 5

R	0	7	11	12	17	19	24	37	38	43	45	48
C. lividium mo/ml x 10^5	0.7	.15	3.4	8.5	19.8[a]	7.5	5.3	5.2[b]	4.9	11	2.5	2.5
Pseudomonas sp mo/ml x 10^5									0.5	.08	.03	.03
Ratio: C. lividium to Pseudomonas sp									10	137	83	83

R	51	52	55	60	64	68	73	77	79	83	87
C. lividium mo/ml x 10^5	11	3.5	4.5	1.7	4.4	8.5	1.5	4.1	2.5	2.8	6.2
Pseudomonas sp mo/ml x 10^5	.08	.05	.09	.2	.11	.2	.11	.13	.13	.14	.24
Ratio: C. lividium to Pseudomonas sp	137	70	50	34	49	42	13	31	19	20	26

a bacterial growth on the chemostat wall was apparent
b sample used to inoculate continuous culture of Pseudomonas sp (see Table 4)

Continuous cultivation of C. lividum in citrate medium followed by the inoculation of Pseudomonas cells. The Pseudomonas sp inoculum was taken from the continuous culture at R37 (see Table 4) (Sr-0.01 g/liter; T=20 C; pH=6.7; D=0.27 hr^{-1})

Brunner et al (1969) examined the growth of two non-adherent bacteria in continuous culture and demonstrated that mixed cultures could mutually persist over a wide range of dilution rates. This coexistence was attributed to variability of generation times in the microbial population.

Based on the finding that the open ocean has about 10 mg/l of dissolved organics (Veldkamp and Jannasch, 1972), the growth of microorganisms at very low concentrations of substrate, which represented nutrient conditions in this paper, provided an environment comparable to those expected in many aquatic ecosystems. Recently bacterial adherence was studied in the chemostat (Topiwala and Hamer, 1971); and, when the initial limiting substrate (S_r) was low, then K (a constant value determined by the total mass of adhering cells) was higher. Thus the adherent microorganism (C. lividum) could persist in continuous culture beyond the calculated maximum specific growth rate (μ_{max}).

The achievement of a steady-state for two microorganisms competing for a single substrate was, at first, difficult to rationalize when taking into consideration the theoretical model of competition for a single growth-limiting substrate (Veldkamp and Jannasch, 1972). In this study the coesixtence of both microorganisms in a steady-state was accomplished since both microorganisms exhibited similar maximum growth rates and, moreover, one microorganism adhered to the wall of the chemostat. These findings could be further applied to river sediments, sewage treatment and other aquatic systems where adherence could be an important factor.

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