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Cell Density-Dependent Growth of *Myxococcus xanthus* on Casein

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When *Myxococcus xanthus* FB was grown on 0.2% casein it exhibited a phenomenon we call cooperative growth. That is, above 10^4 cells per ml, both strains that were studied exhibited increasing growth rates as a function of increasing cell numbers. Between 10^4 and 10^7 cells per ml, the mean doubling times of strains YS and TNS decreased from 15.2 to 8 h and 26 to 8.5 h, respectively. The extracellular proteinase activity of the two strains was equivalent and directly proportional to cell number. Cooperative growth was correlated with increased concentration of hydrolyzed casein in the medium, suggesting cooperative hydrolysis of casein. At low cell densities neither strain was capable of measurable growth on casein in liquid media, and we have calculated that the average concentration of hydrolyzed casein in the medium was indeed too low to support growth. At low cell densities, growth on hydrolyzed casein (Casitone) was normal and independent of cell concentration. Demonstration of cooperative growth at higher cell densities supports the suggestion that the communal behavior of myxobacteria results in more efficient feeding.

The fruiting myxobacteria are characterized by at least three distinctive properties. (i) The complex life cycle of myxobacteria (19, 22), consisting of intercellular communication, aggregation, fruiting body formation, myxosporulation (or inclusion into macrocysts), and germination, is unique among the procaryotes. (ii) The myxobacteria share with many blue-green algae and certain other bacteria a type of motility described as gliding. The mechanism of gliding motility is completely unknown. (iii) In addition, the myxobacteria have the ability to hydrolyze macromolecules extracellularly and to use the products for growth. Several species of myxobacteria have indeed been found to excrete bacteriolytic and proteolytic enzymes during growth (7, 8, 10, 12, 13, 20, 21). It has been suggested that these three properties are functionally related (5). The relatively slow-gliding motility allows for maximum cell-to-cell interaction and is consistent with the utilization of insoluble macromolecules. The tendency of the myxobacteria to travel as a swarm of densely packed cells may reflect the need to maintain a critical local concentration of extracellular hydrolytic enzymes. If this were so, at least part of the reason for constructing a fruiting body would be to ensure an optimal swarm size on germination. Our results do indeed demonstrate that at high cell densities ($>10^4$ cells/ml) there is cooperative, extracellular hydrolysis of casein resulting in increasing growth rates. At sufficiently low cell densities, the cells in liquid media do not grow on casein at a measurable rate.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. Two phase variants of *M. xanthus* FB were used in these studies: YS (yellow swarmer) and TNS (tan nonswarmer). Using the terminology agreed upon at the first meeting on the Developmental Biology of the Myxobacteria, held at Cold Spring Harbor 16-20 August, 1974, our laboratory strains are designated as follows: FB is MD-1, YS is MD-2, and TNS is MD-4. In this paper the phenotypic designations will be used. Strain YS constructs well-defined fruiting bodies, whereas strain TNS produces only rudimentary aggregates (22). Vegetative cells were maintained by daily transfer into CT medium (4), which contained 2% Casitone (Difco) in 0.01 M potassium phosphate buffer, pH 7.6, plus 8 mM $MgSO_4$. Deionized, distilled water was used for all media. All growth experiments were performed at 32°C with vigorous gyratory shaking. Unless otherwise stated growth media contained 0.2% purified casein, 8 mM $MgSO_4$ and 0.01 M potassium phosphate buffer, pH 7.6. Media were solidified with 1.5% agar (Difco).

Determination of cell number. Total cell number was determined with the use of a Petroff-Hausser counting chamber. Each sample was counted at least three times, yielding a total of more than 300

cells counted per sample. For cell densities below 10^6 per ml, cells were concentrated by being chilled to 0°C, centrifuged at 10,000 × *g* for 20 min, and suspended in an appropriate volume of cold water to yield a final cell concentration of over 10^6 per ml. Viable cell number was determined by plating on CT agar after performing appropriate dilutions in distilled water. Cell density in CT medium was determined turbidimetrically with a Klett-Summerson colorimeter with a no. 54 filter. One Klett unit equals approximately 2.5×10^6 exponentially growing cells per ml. Growth rates in CT medium were determined in the exponential growth phase by periodically diluting the culture with fresh medium so that cell density never exceeded 5% of maximum.

Purification of casein. Ten grams of casein (Difco Laboratories, isoelectric grade) was dissolved in 1 liter of 0.01 M potassium phosphate buffer, pH 7.6, at 100°C. After cooling, the casein was precipitated with 50 ml of cold 100% trichloroacetic acid. The precipitate was washed successively with 5% and 2% trichloroacetic acid, 95% ethanol, ethanol-ether (1:1, vol/vol), and ether. After air drying, the casein was stored at 4°C. Casein-containing media were prepared exactly as previously described with Casitone (4) except that the casein was brought to a boil in the phosphate buffer to aid solution before autoclaving.

Determination of proteinase activity. Proteinase activity was determined by a modification of the casein digestion method (15). A solution containing the enzyme activity plus purified casein (0.2%) in 0.01 M potassium phosphate buffer, pH 7.4, was incubated at 32°C. At timed intervals 3-ml samples were removed and immediately mixed with 2 ml of 10% trichloroacetic acid. After 30 min at 0°C, the mixture was centrifuged at 3,000 × *g* for 10 min. The absorbance of the clear supernatant solution was determined at 280 nm. The readings were corrected for the values of zero time controls.

RESULTS

Colony formation on casein agar. When *M. xanthus* FB was plated on 0.2% casein agar, single cells of substrain YS gave rise to observable colonies in 10 to 12 days, whereas single cells of substrain TNS did not form colonies within 30 days. Both substrains formed colonies on CT agar in the normal time of 5 days. This observation was extended by placing 0.01-ml droplets, containing varying numbers of bacteria, on casein agar and noting the time required for colony formation. This was determined subjectively by noting the time when visible colonies first appeared. Below 10^6 cells per droplet, strain YS grew faster than strain TNS (Fig. 1). In addition to this strain difference, the growth rate of both strains was also dependent upon the initial inoculum size. The higher the initial cell density, the shorter the average doubling time. For example, starting with 1 YS cell per droplet, the average doubling time (calculated on the basis of the time interval between inoculation and visible colony formation) was approximately 12.5 h, whereas starting with 10^4 cells, the average doubling time was 9.3 h. We are assuming that growth was continuous and uniform during this time.

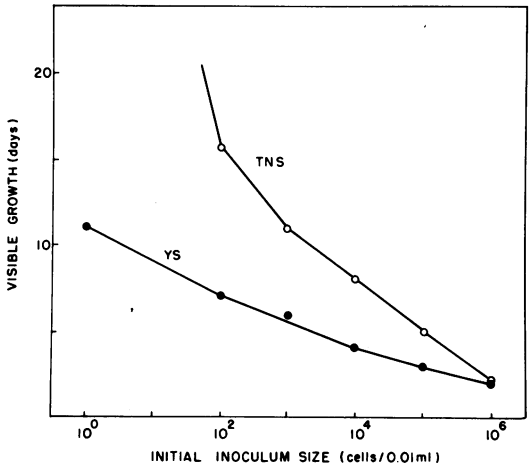

FIG. 1. *Growth of M. xanthus FB, strains YS (●) and TNS (○), on 0.2% casein agar. Cells used for inoculation were grown in 0.2% casein medium, harvested at 10,000 × g for 10 min, and then suspended in cold water. After applying 0.01-ml samples of various dilutions of the cell suspension to the agar, the plates were incubated at 32°C. The diameter of the initial spot was about 1 cm. No visible growth was observed with 1 or 10 cells per sample of strain TNS for up to 30 days.*

These strain and cell density variations in growth rate on casein agar could be due to differences in (i) extracellular proteinase activities, or (ii) utilization of the peptides and amino acids. To study the phenomenon more quantitatively, experiments were performed in liquid media containing purified casein as the sole added nutrient.

Cell density dependent-growth of *M. xanthus* FB in casein medium. Figure 2 summarizes a number of experiments in which the mean doubling time of strain YS in 0.2% casein medium was determined as a function of initial cell density. Above cell densities of 10^4 per ml the growth rate steadily increased, yielding a mean doubling time of about 8 h at 10^8 cells per ml, at which time the culture approached maximum cell density (4.0×10^8 cells per ml).

Strain TNS demonstrated an even stronger dependence of growth rate on cell density (Fig. 3). Between 10^4 and 10^7 cells per ml, the mean doubling time decreased dramatically from 26 to 8.5 h. Below 10^3 cells per ml, no growth of strain TNS or YS was detected in casein medium.

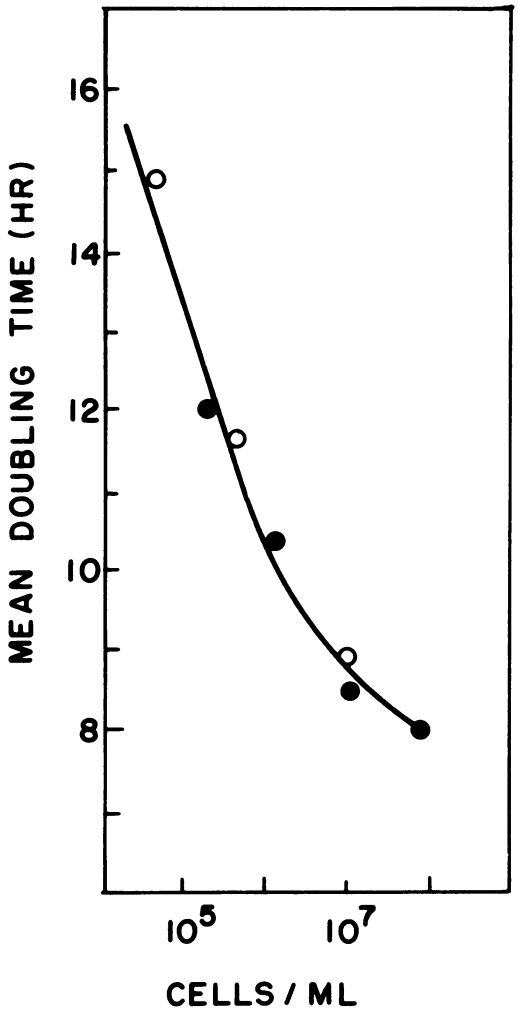

FIG. 2. *Cell density-dependent growth of M. xanthus FB (strain YS) in casein medium. Flasks containing 20 ml of 0.2% casein medium were inoculated with varying quantities of washed cells obtained from an exponentially growing culture (1.0 × 10^8 cells/ml) in 0.2% casein medium. Flasks were incubated at 32°C with vigorous gyratory shaking. At timed intervals, duplicate samples were removed for determination of cell concentration. Average doubling times were determined from viable cell numbers (○) and total cell numbers (●). Between 10^4 and 10^5 cells per ml, total cell numbers were determined on suspensions that had been concentrated 100 times by centrifugation at 10,000 × g for 20 min.*

The data for both strains suggest cooperative interactions for growth on casein medium. When either strain was grown on CT medium (containing enzymatically hydrolyzed casein) the doubling time was independent of cell density over the entire range. In all these experiments cultures were adapted to growth on casein as the sole nutrient by transferring from casein medium to casein medium at least twice, using 1% inocula. Using such adapted cultures, no growth lag was detected at any of the initial cell densities. Attempts to increase the growth rate at low cell densities by supplementation of casein with vitamins and nucleotides were unsuccessful. The remaining experiments were designed to obtain insight into the mechanisms responsible for the cooperative phenomena demonstrated in Fig. 1 and 2.

Extracellular proteinase activity of *M. xanthus* growing on 0.2% casein medium. Myxobacteria growing on macromolecules derive nutrients by the action of secreted extracellu-

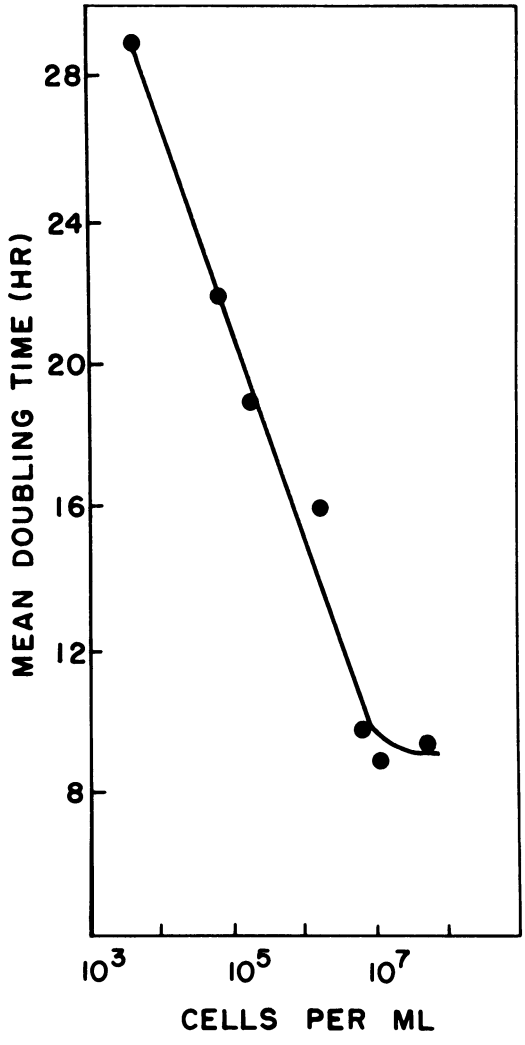

FIG. 3. *Cell density growth of M. xanthus FB (strain TNS) in casein medium. The experiment was performed as described in Fig. 2.*

lar enzymes. Thus, one reasonable explanation for the cooperative growth phenomenon described above is that growth rate is dependent upon the concentration of low-molecular-weight peptides and amino acids in the medium at the bacterial surface. This depends on the balance between the (varying) local rate of production of these nutrients throughout the medium and the rate of diffusion toward or away from the cell surface. The local rate of production is a function of the local concentration of extracellular proteinases. Although the distribution is critical, it is also generally true that the greater the cell density the higher the concentration of proteinases and resulting peptides and amino acids throughout the medium. To test this hypothesis, extracellular proteinase activity was measured at different cell densities (Table 1). The extracellular proteinase activity of both strains is approximately proportional to cell density from 10^5 to 10^8 cells/ml. The assay procedure was not sufficiently sensitive to measure activities of cultures containing less than 10^5 cells per ml. Despite the fact that strain TNS grew more slowly than strain YS on casein at low cell densities, the proteinase activities per cell of the two strains were equivalent. Control experiments with cultures at the highest cell densities of strain YS (3×10^8 cells per ml) and strain TNS (2.35×10^8 cells per ml) indicated that enzyme activity was directly proportional to concentration of the supernatant fluid from undiluted to a 500-fold dilution. Also, activity was constant with time up to 6 h. Where longer incubations were required to obtain measurable hydrolysis rates, data were corrected for decay of enzyme activity (half-life at 32°C under conditions of the assay was about 30 h).

TABLE 1. *Extracellular proteinase activity of M. xanthus FB (strains YS and TNS) growing on 0.2% casein medium*

Strain	Culture density[a] (cells/ml)	Extracellular proteinase[b] (U/ml)	Sp act (U/cell)
YS	3.0×10^8	475	1.6×10^{-6}
	2.5×10^7	42.5	1.7×10^{-6}
	1.5×10^6	2.5[c]	1.7×10^{-6}
	2.2×10^5	0.5[c]	2.3×10^{-6}
TNS	2.35×10^8	510	2.2×10^{-6}
	1.7×10^6	3.7[c]	2.2×10^{-6}

[a] Cell concentration was determined by use of a Petroff-Hausser counting chamber. For cell densities below 10^7/ml, measurements were performed after harvesting 500 ml of culture and suspending the pellet in 5 ml of water.

[b] After chilling to 0°C, cultures were centrifuged at $10,000 \times g$ for 20 min. Supernatant fluids were incubated in the presence of 0.2% casein at 32°C. Three-milliliter samples were removed at various times and precipitated with 2 ml of 10% trichloroacetic acid. After standing in the cold for 30 min, the suspension was centrifuged at $3,000 \times g$ for 10 min. The absorbance of the supernatant was determined at 280 nm. One unit is defined as 0.001 absorbance unit released per 30 min, corresponding to the hydrolysis of approximately 5 μg of casein.

[c] Since these incubations were carried out for more than 8 h, one drop of chloroform was added to prevent contamination, vials were sealed to avoid evaporation, and corrections were made for decay in enzyme activity.

Below 0.5% casein, cell yield was shown to be directly proportional to casein concentration. At 0.2% casein, maximum cell density was 4.0×10^8 cells per ml, or 1 cell per 5×10^{-6} μg of casein. Since the specific activity of the *M. xanthus* proteinase was 2×10^{-6} U/cell (Table 1), or 3.3×10^{-7} μg of casein hydrolyzed/cell per min, it follows that enough casein is hydrolyzed for the production of one cell (5×10^{-6} μg) in only 15 min. Since the measured doubling times of strains YS (Fig. 2) and TNS (Fig. 3) on casein medium varied from 8 to >40 h, the vast majority of peptides and amino acids formed by hydrolysis diffused away from the cell, gradually increasing the concentration of hydrolyzed products in the medium. At high cell densities, growth is thus enhanced by the increasing concentration of hydrolyzed products in the medium.

Determination of hydrolyzed casein in the medium. The prediction that hydrolyzed products should accumulate in the medium during growth on casein was experimentally verified (Fig. 4). Theoretical curves were developed on the basis of a model that took into account the measured rate of production of casein hydrolysate (see Appendix). Consumption of hydrolysate was ignored, and the close agreement between the curves and the data points corroborates the validity of that assumption. The determined values for strain TNS were predictably higher than the values for strain YS since the slower growth rate of strain TNS on casein gave the proteinase a longer time period to act during growth. These data further demonstrated that the slower growth rate of strain TNS was not a result of lower proteinase activity. The difference then seems to be that strain TNS is not able to use low concentrations of nutrients as efficiently as strain YS. This was also shown to be the case with low concentrations of Casitone (Fig. 5).

Cooperative (cell density-dependent) growth began to be significant (10^4 to 10^5 cells per ml) when the average concentration of casein hydrolysate in the medium reached 5 to 20 μg per

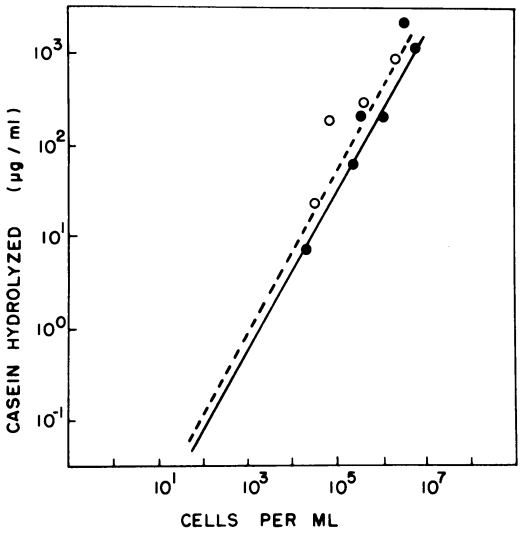

FIG. 4. *Concentration of casein hydrolyzed as a function of cell density. M. xanthus FB, strains YS (●) and TNS (○), were grown in 0.2% casein medium as described in Fig. 2. At various times, 100-ml samples were chilled rapidly and then centrifuged at 10,000 × g for 20 min. The pellets were suspended in cold water for determination of total cell number; 3 volumes of the corresponding supernatant were precipitated with 2 volumes of 10% trichloroacetic acid for determination of acid-soluble amino acids and peptides. The solid line and dashed line are theoretical curves of casein hydrolyzed (c_s) (micrograms per milliliter) by strain YS and TNS, respectively, as a function of cell density calculated from the following equation (Appendix):*

$$c_s = \frac{kqn}{k_c k_d}\left[\frac{1}{(k_c + k_d)}\left(k_d + k_c e^{-(k_c+k_d)t}\right) - e^{-k_c t}\right]$$

where n is the cell number density, q is the rate of enzyme secretion per cell, k is the rate of casein hydrolysis per unit of enzyme, k_c = 0.69/G.T., k_d = 0.69/30 h, and t is time.

ml. From 10^4 to 10^7 cells/ml, the average concentration of hydrolysate increased from 20 to 1,000 μg/ml, and the average doubling time decreased from 15.5 to 8.5 h and 28 to 9 h for strains YS (Fig. 2) and TNS (Fig. 3), respectively.

Below 10^4 cells/ml, the average concentration of hydrolysate was too low to support measurable growth. For example, at 10^3 cells/ml the extracellular proteinase activity was such that it would take weeks to raise the average concentration of hydrolysate to even 1 μg/ml.

Growth of *M. xanthus* FB (strains YS and TNS) on prehydrolyzed casein. The above data demonstrate a strong correlation between growth rate and concentration of hydrolyzed casein. However, it is not possible to conclude from these data that other factors might not also contribute to the cooperative growth phenomenon. Thus, growth rates were determined at low cell densities using prehydrolyzed casein as the nutrient (Table 2). On 0.2% casein hydrolysate both strains grew with doubling times of less than 8 h. Furthermore, growth was independent of cell density.

The specific growth rate as a function of substrate concentration is approximated by the Monod equation (3): $\mu = \mu_{max}\,[S/(K_s + S)]$,

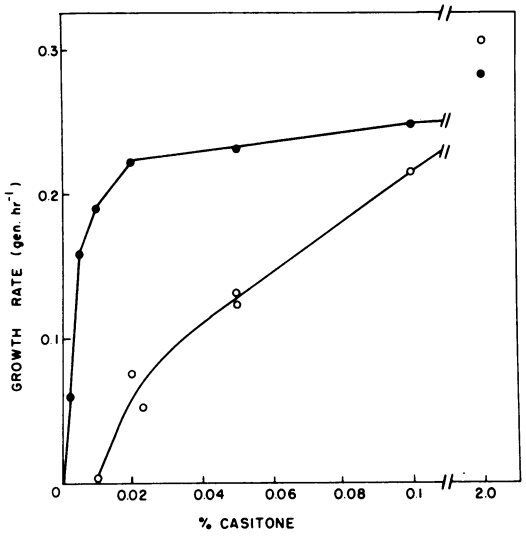

FIG. 5. *Growth rate of M. xanthus FB, strains YS (●), and TNS (○), as a function of Casitone concentration.*

TABLE 2. *Growth of M. xanthus FB (strains YS and TNS) on enzymatically hydrolyzed casein*

Strain	Culture density (cells/ml)	Casein hydrolysate[a] (mg/ml)	Growth rate (gen/h)
YS	2×10^3	2.0	0.137
	5×10^6	2.0	0.135
	2×10^3	1.0	0.098
	2×10^3	0.5	0.062
	2×10^3	0	0
TNS	1×10^3	2.0	0.132

[a] Growth media and culture conditions were as described in Fig. 2 except that various quantities of casein hydrolysate were used in place of casein. The hydrolysate was prepared by harvesting a culture of strain YS growing on 0.2% casein (2×10^8 cells/ml). One volume of the resulting cell-free supernatant was incubated for 16 h at 32°C with 19 volumes of 0.2% casein in 0.01 M potassium phosphate buffer, pH 7.4. A sample of the hydrolysate indicated that over 95% of the casein was soluble in cold 5% trichloroacetic acid. The sterilized hydrolysate was then used for media preparation.

where μ_{max} is the maximum growth rate, S is the substrate concentration (in this case, the concentration of casein hydrolysate) and K_s is a saturation constant. Using the data for strain YS in Table 2, a value for K_s of 1.32 mg/ml and a μ_{max} of 0.227/h are obtained.

DISCUSSION

The microbiological literature contains many examples in which the extent of the lag phase of growth is inversely proportional to the inoculum size. In certain cases such as *Pasteurella tularensis* (11) and several lines of cultured mammalian cells (6), a critical population density was necessary to observe any growth. In several systems that were investigated it was further shown that the lag phase could be shortened and growth initiated by addition of small molecules, such as CO_2 (9), specific amino acids (6), Krebs cycle intermediates (18), and kojic acid (16). More significantly, *Bacillus megaterium* was shown to accumulate an iron-chelating hydroxamic acid during the lag phase of growth (17). This diffusible endogenous siderochrome (1) overcame the inoculum-dependent lag of *B. megaterium* (2) and *Bacillus subtilis* (16). The growth-initiating factor of *P. tularensis* was shown to be a nonhydroxamate Fe [III]-binding compound (11). Another old and well-established observation is the effect of inoculum size on the growth of penicillin-resistant *Bacillus cereus* in media containing penicillin (14). If the inoculum size was too low, insufficient extracellular penicillinase was produced and the growing cells lysed. In these examples the primary need for high cell density is to detoxify or supplement the medium in order to allow growth to proceed. In none of these cases did the growth-initiating substance or high cell density influence the growth rate. Thus, these examples of inoculum-dependent division lag appear to be qualitatively different from the cell density-dependent growth of *M. xanthus* on casein.

Cooperative or synergistic growth is characterized by an increasing growth rate with increasing cell number. At cell densities over 10^4 bacteria per ml, strains YS and TNS both exhibited strong cooperative growth kinetics (Fig. 2 and 3). Cooperative growth rates of *M. xanthus* on casein were correlated with (Fig. 4), and could be accomplished by addition of (Table 2), increased concentrations of hydrolyzed casein in the medium. Although some of the casein hydrolysate produced around an individual cell is utilized by that cell, most of the amino acids and peptides are dispersed in the medium through diffusion away from the cell, slowly increasing the concentration of hydrolyzed products in the medium. However, the extent to which the concentration of hydrolysate is increased around each individual cell depends strongly on the distance between cells; this is a measure of the volume available to each cell in which to disperse the protease and hydrolysate. Whereas the rate of protease production per cell is constant at all cell densities, at cell densities above about 10^5 cells per ml, the protease and the hydrolyzed products are confined to a sufficiently small volume around each cell so that their concentrations begin to increase markedly. This effect becomes more pronounced as cell density increases further and the growth rate continues to increase.

The difference in growth rates between strains TNS and YS on casein was not due to decreased proteinase activity (Table 1 and Fig. 4). At high concentrations of Casitone (Fig. 5) and casein hydrolysate (Table 2) strain TNS grew as rapidly as strain YS. The major difference between the strains appears to be the inability of strain TNS to utilize low concentrations of nutrients (Fig. 5). At 0.01% Casitone, growth of strain TNS was not measurable, whereas strain YS grew at close to its maximum rate. Thus, it is reasonable to suggest that the low (local) concentration of casein hydrolysate produced around individual TNS cells growing on casein was below the threshold needed for growth.

In order for growth to take place with casein as the nutrient, *M. xanthus* must produce extracellular proteinases which hydrolyze the protein to utilizable peptides and amino acids. The proteinase activity of strain YS was approximately proportional to cell density (Table 1), yielding 3.3×10^{-7} μg of casein hydrolyzed/cell per min.

At low cell densities on liquid media containing casein we always observed one to two cell divisions before growth ceased (unpublished observations). The ability of strain YS to form colonies on casein agar from single cells may therefore reflect the formation of a cluster of two to four cells which do not become dispersed and can thus initiate casein hydrolysis, albeit at an extremely low rate. Alternatively, small quantities of various peptides and amino acids present in Difco agar could explain the ability of strain YS to form colonies from single cells on casein agar.

The growth behavior as a function of cell concentration leaves several unresolved questions. These stem from the fact that the variation of doubling time with cell concentration is much weaker than would be predicted on the

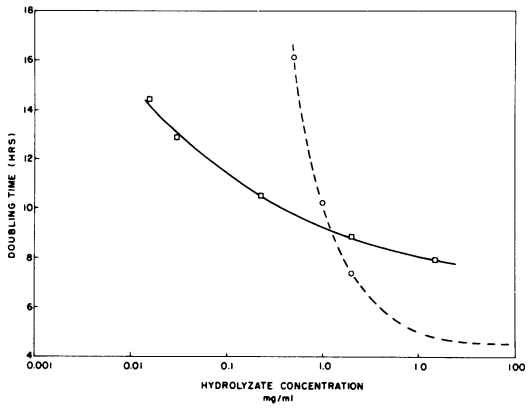

FIG. 6. *Comparison of doubling time as a function of average hydrolysate concentration for strain YS grown on casein hydrolysate* (○) *and grown on casein* (□). *In the casein hydrolysate experiments, concentrations were measured (Table 2); for the casein experiments, concentrations were obtained from Fig. 4 as a function of cell density. The dashed line is a Monod equation plot based on the casein hydrolysate growth data.*

basis of the data on growth in casein hydrolysate (Table 2) and levels of casein hydrolysate found at various cell concentrations (Fig. 4). This is illustrated in Fig. 6 where doubling time is plotted as a function of casein hydrolysate concentration. The hydrolysate concentration is, in turn, a function of the cell concentration. It can be seen that the actual growth rate is much higher than expected at low cell concentrations and slightly lower than expected at high cell concentrations.

In principle, these differences can be explained by the diffusion phenomena occurring in the vicinity of each cell. That is, the concentration of protease should be highest at the cell surface and decrease with distance away from the cell as it establishes a driving force for its diffusion away from the cell. The higher protease activity near the surface would result in more casein being hydrolyzed in the vicinity of the cell than in the medium and thus casein concentration should diminish near the cell surface. Casein hydrolysate production depends on the local concentration of both casein and protease and will exhibit a maximum close to, but not at, the surface. At low-average enzyme levels, this maximum would be very close to the surface and the hydrolysate concentration can be much higher than its average in the medium. At high enzyme concentrations, the maximum would be farther away from the surface and the hydrolysate concentration may, in fact, be lower at the surface than its average value in the medium.

Some preliminary calculations based on these ideas show that the diffusion resistances likely to exist in the liquid medium are not sufficiently high to account for the markedly higher growth rate actually observed at low cell concentrations. We believe, therefore, that it is likely that diffusion resistances in the vicinity of each cell may be higher than those in a normal liquid medium. Such increased resistances could result from a polysaccharide slime, a periplasmic space with significant cell wall resistances, or a clumping phenomenon. We are now initiating experiments to examine this question directly.

Although independent type growth has been studied more extensively in laboratories, it may be that cooperative growth is equally important in certain natural environments. It has been suggested (5) that the complex life cycle of myxobacteria is, at least, partly designed to maintain the potential for high cell density needed for efficient feeding, i.e., a "wolf pack." The experiments presented here have demonstrated that cooperative growth conditions can be established in the laboratory. The underlying mechanisms suggest that cooperative growth kinetics should be demonstrable for any microorganism that must derive its nutrients by the secretion of extracellular enzymes or lytic factors. Whenever the growth substrate is not permeable (which is the most frequent situation in nature, e.g., cellulose, chitin, lignin, most proteins, etc.), then cooperative growth kinetics should prevail. This may partially explain the fact that not only myxobacteria but many soil bacteria exist in nature as cell aggregates.

APPENDIX

The expected relationships among cell number density, average enzyme concentration, and the average casein hydrolysate concentration for an exponentially growing, well-mixed population can be approximated by simplified continuum models. Provided that the doubling time does not change substantially over the period of growth, the number of cells per unit volume, n, is given by:

$$n = n_o e^{k_c t} \quad (1)$$

where n_o is the cell number density at time $t = 0$ and k_c is the cell growth constant (0.69 ÷ doubling time). In the experiments reported here, the change in k_c over a 30-h growth period was relatively small and the maximum error in the value of n predicted from equation 1 using an average value of k_c was always less than 3%.

If the cells secrete enzyme at a rate of q U/(cell) (h) and the enzyme decay constant is k_d (0.69 ÷ half-time), then the differential equation describing the

rate of change of enzyme concentration, c (U/cm^3), is:

$$\frac{dc}{dt} = nq - k_d c \quad (2)$$

Using the expression for n given by equation 1, equation 2 can be solved subject to the initial condition that $c = 0$ at $t = 0$ yielding

$$\frac{c}{n} = \frac{q}{k_d + k_c}[1 - e^{-(k_d + k_c)t}] \quad (3)$$

From equation 3 one can determine the units of enzyme per cell (c/n) at any time, t, after the start of an experiment.

We assume that the rate of production of casein hydrolysate is proportional to the concentration of enzyme and that the amount of hydrolysate consumed by cells is insignificant so that:

$$\frac{dc_s}{dt} = kc \quad (4)$$

where k is the first-order rate constant for hydrolysate production (10 μg/[U][h]) and c_s is the hydrolysate concentration (μg/cm^3). Assuming $c_s = 0$ at $t = 0$, and substituting for c from equation 3, equation 4 can be solved to obtain:

$$\frac{c_s}{n} = \frac{kq}{k_c k_d}\left[\frac{1}{(k_c + k_d)} \cdot (k_d + k_c e^{-(k_c + k_d)t}) - e^{-k_c t}\right] \quad (5)$$

From equation 3 and the data of Table 1, a value for q of $1.77 \pm 0.14 \times 10^{-7}$ U/(cell)(h) is obtained for strain YS. This value shows no trend with increasing culture density, suggesting that the rate of enzyme production is independent of cell doubling time or culture density. From the two data points obtained for strain TNS, a value for q of $1.98 \pm 0.45 \times 10^{-7}$ U/(cell)(h) is obtained.

With q fixed, equation 5 can be used to predict hydrolysate concentration as a function of culture density. In Fig. 4, the solid and dashed lines are predicted values for experimental runs of 30 h.

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