The Influence of Extracellular Products on the Behaviour of Mixed Microbial Populations in Magnesium-limited Chemostat Cultures

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SUMMARY

Magnesium-limited mixed cultures, each containing two microbial species, were produced by exchange of small numbers of organisms between magnesium-limited chemostat cultures of *Aerobacter aerogenes*, *Pseudomonas fluorescens*, *Bacillus subtilis*, *B. megaterium*, *Staphylococcus epidermis* and *Torula utilis*. The fate of each species in these mixed cultures was determined following cross-inoculation. Gram-negative organisms invariably outgrew the Gram-positive ones, but the ability of *B. subtilis* and *B. megaterium* to outgrow each other, or to outgrow the yeast, depended on the inoculum size. This dependence resulted from the presence of specific extracellular products in the *Bacillus* cultures which stimulated their growth and uptake of magnesium. The concentration of these extracellular growth-promoting substance(s) in magnesium-limited cultures of *B. subtilis* varied with population density. Thus, when the magnesium concentration in the medium was decreased from 0.9 to 0.15 $\mu\text{g./ml.}$ (thereby causing the steady-state population density to be decreased to one-eightieth the initial value), *B. subtilis* could no longer maintain itself against proportionately low concentrations of *T. utilis*. But addition of extracellular fluids from dense magnesium-limited *B. subtilis* cultures to magnesium-limited *T. utilis* cultures enabled small numbers of the *Bacillus* now to outgrow the yeast. From these two observations it is concluded that magnesium assimilation by *B. subtilis* is more dependent on extracellular substance(s) than is magnesium assimilation by either *T. utilis* or the Gram-negative organisms. Modification of the basic theory of microbial growth in a chemostat culture, to take account of product-stimulated substrate-assimilation, is suggested.

INTRODUCTION

It is generally assumed (Monod, 1950; Herbert, Elsworth & Telling, 1956; Powell, 1965) that the relationship between the growth rate of organisms and the concentration of growth-limiting nutrient, in a chemostat culture, can be represented adequately by a Michaelis–Menten type of equation:

$$[D =] \mu = \mu_{\max} \left(\frac{s}{K_s + s} \right), \quad (1)$$

where μ is the specific growth rate, $\mu_{\max}$ the maximum rate of growth of the organisms in the medium, s the extracellular concentration of growth-limiting substrate in the culture (Mg^{2+} in most of the experiments to be described here) and K_s a saturation constant (numerically equal to the extracellular concentration of growth-limiting

nutrient at $0.5 \mu_{\max}$). Under steady-state conditions in a chemostat, μ is equal to the dilution rate (D).

In the above equation there are two variables (μ and s) and when these are plotted against one another for a pure culture of an organism (A), a curve is obtained as shown in Fig. 1. With cultures of a different organism (B) a plot of similar shape would be obtained, but it would not be identical to the curve for organism A unless both of the constants ($\mu_{\max}$ and K_s) were also identical. Now, assuming the saturation curves for two organisms to be exactly as shown in Fig. 1, and that these were grown in separate chemostats under identical (magnesium-limited) conditions at a dilution rate of $D_1 \text{ hr}^{-1}$, then the extracellular concentration of Mg^{2+} in the culture of A -type organisms would be s_A , whereas that in the culture of B -type organisms would be s_B . If a small number of A -type organisms were now transferred to the chemostat containing a steady-state population of B -type organisms, then (assuming no lag period) A -type organisms would begin growing at a rate μ_A (since the growth-limiting substrate concentration would be s_B ; Fig. 1). Because μ_A is greater than D_1 , the concentration of A -type organisms in the culture would increase. Their growth would cause the growth-limiting substrate concentration to decrease towards the value s_A ; at this latter concentration B -type organisms could grow only at a rate μ_B which, being less than D_1 , would result in them being washed completely from the culture. Thus, organism A would totally displace organism B from the chemostat culture and this should occur irrespective of the initial concentration of each organism in the mixed culture and, in this case, the dilution rate.

We described previously (Tempest, Dicks & Meers, 1967) how *Aerobacter aerogenes* outgrew several Gram-positive organisms in Mg^{2+} -limited mixed culture experiments in a manner predicted by the above theory. But mixed cultures of *Torula utilis* and *Bacillus subtilis* behaved paradoxically: neither organism outgrew the other species unless the inoculum population exceeded a certain minimum concentration. The present paper reports the results of experiments designed to explain the unexpected behaviour of these mixed cultures. A preliminary report has been published (Meers & Tempest, 1968).

METHODS

Organisms. *Aerobacter aerogenes* (NCTC 418); *Pseudomonas fluorescens* (strain KB 1, from the University of Sheffield); *Bacillus subtilis* var. *niger* (ATCC 9372); *B. megaterium* (strain KM, from the University of Edinburgh); *Staphylococcus epidermidis* (isolated by Mr F. A. Dark, Porton). Each organism was maintained on tryptic meat digest agar slopes. *Torula utilis* (NCYC 321) was maintained on yeast extract peptone glucose agar slopes.

Growth conditions. Continuous cultures of organisms were maintained in 0.25 l. chemostats (designed by Dr D. Herbert) without pH control. The magnesium-limited medium was basically that described previously (Tempest *et al.*, 1967), but the magnesium content was varied as indicated in the Results section. The potassium-limited medium was that described by Tempest, Dicks & Hunter (1966), but with the potassium concentration decreased to 0.15 mM; the carbon-limited medium was that of Tempest, Hunter & Sykes (1965) but with glucose (1 g./l.) replacing the glycerol. The same magnesium-limited medium, with the magnesium concentration adjusted to 0.02 mM, was used for batch culture experiments.

In some experiments the medium was supplemented with supernatant fluids from previous chemostat cultures. These fluids were obtained by rapid filtration of the culture through a sintered glass filter (no. E5), to eliminate most of the organisms, and then through a membrane filter (Oxoid; standard grade) to effect their complete removal. Where larger volumes of culture were to be treated, the bulk of organisms were removed by centrifugation (5000g for 5 min.), before sterilization by filtration as above.

The technique for growing mixed cultures of organisms in the chemostat was that described by Tempest *et al.* (1967). For batch culture experiments, 20 ml. volumes of medium (contained in 350 ml. conical flasks) were inoculated with organisms from magnesium-limited chemostat cultures and aerated by shaking, on a reciprocating shaker, at 37°. At appropriate intervals of time, 3 ml. samples were withdrawn from each culture and measurements made of the extinction at 540 m μ (Bausch and Lomb 'Spectronic 20' spectrophotometer with tube of 0.5 in. (1.2 cm.) internal diameter) and culture population density (by counting directly the number of organisms in a known volume of culture, by using a Thoma Haemocytometer of 0.02 mm. depth). The results obtained by both methods correlated closely.

Magnesium determinations were made using an EEL (model 140) atomic absorption spectrophotometer on samples prepared as described previously (Tempest *et al.* 1967).

RESULTS

The magnesium-limited mixed culture experiments reported previously (Tempest *et al.* 1967) were extended to include most the possible combinations of two species from the following group of organisms: *Aerobacter aerogenes*, *Pseudomonas fluorescens*, *Bacillus subtilis*, *B. megaterium*, *Staphylococcus epidermidis* and *Torula utilis*. It was found that the Gram-negative bacteria invariably outgrew the Gram-positive organisms rapidly, irrespective of the initial population densities of the two competing species in the mixed cultures. Thus it can be concluded that both *A. aerogenes* and *P. fluorescens* have more efficient mechanisms for the uptake of magnesium from their environment than the Gram-positive organisms listed above. It is important to point out that *S. epidermidis* would grow only in simple salts media that had been supplemented with Casamino acids (1 %, w/v) and yeast extract (0.001 %, w/v), each of which contain substances that stimulated growth and the uptake of magnesium by *B. subtilis* (Tempest *et al.* 1967) and presumably also by the *Staphylococcus* organisms. Nevertheless, *S. epidermidis* cultures still were rapidly outgrown by either *A. aerogenes* or *P. fluorescens* organisms.

The ability of *Bacillus megaterium* and *B. subtilis* to outgrow each other or to outgrow *Torula utilis* in magnesium-limited mixed cultures depended on the relative amounts of each species in the culture immediately after mixing the two populations. Thus, when magnesium-limited mixed cultures of either *B. megaterium* and *T. utilis* or *B. megaterium* and *B. subtilis* were prepared, the ultimate fate of each species in each mixed culture depended on their concentrations immediately after cross-inoculation (Fig. 2). These results, which were identical to that with *B. subtilis* and *T. utilis* (Tempest *et al.* 1967), cannot be predicted from the theory outlined in the Introduction, since equation (1) contains no factor for population density.

The most obvious explanation for these paradoxical take-over patterns is that the

Gram-positive organisms produced substances that accumulated in the culture extracellular fluids and inhibited the growth of the competing species. To investigate this possibility, batch cultures of *Bacillus subtilis*, *B. megaterium* and *Torula utilis* were grown in low-magnesium simple salts media which had been supplemented with equal volumes of the supernatant fluids from magnesium-limited chemostat cultures of the three species. The resulting growth curves (Fig. 3) showed no evidence of growth inhibition. On the contrary, the growth curves indicated clearly the presence

Fig. 1

Fig. 2

Fig. 1. Hypothetical saturation curves for two microbial species (*A* and *B*) growing in magnesium-limited chemostat cultures. At a fixed dilution rate (D_1) the growth-limiting substrate concentration in a culture of *A* organisms would be s_A and of *B* organisms s_B . It is assumed that the maximum growth rate ($\mu_{\max}$) for *A* organisms is greater than for *B* organisms and that the saturation constants (K_s values) bear a similar relationship.

Fig. 2. Growth of *Bacillus megaterium* and *Torula utilis* in magnesium-limited simple salts medium, in a chemostat. In the experiment represented by the solid lines the initial concentration of *B. megaterium* (●) was 9×10^7 orgs/ml. and *T. utilis* (○) 3×10^7 orgs/ml., i.e. 75% and 25% of the initial mixed population, respectively. Also recorded (broken lines) are the results of a similar experiment in which the initial concentration of *B. megaterium* (▲) was increased to 1.8×10^8 orgs/ml. and that of *T. utilis* (△) lowered to 1×10^7 orgs/ml., i.e. 95% and 5% of the total population, respectively. In both experiments the dilution rate was 0.3 hr^{-1} and the temperature 33° . The pH value was not controlled but did not vary beyond the range 6.1 to 6.5 in each experiment.

of growth-promoting substances in the culture extracellular fluids. Some specificities were apparent since the growth of each of the *Bacillus* species was promoted to the greatest extent by its own extracellular fluid (Fig. 3). It seemed possible, therefore, that the effect of population density on the growth patterns of Gram-positive organisms in magnesium-limited mixed cultures resulted from the presence of specific substances, secreted into the culture fluids, which facilitated magnesium assimilation.

If the above hypothesis is correct, then addition of extracellular fluid from a magnesium-limited *Bacillus* culture to the medium supplying a mixed culture of that *Bacillus* species and, for example, yeast organisms, possibly could enable small numbers of the bacilli now to outgrow the yeast. When sterile supernatant fluid from a magnesium-limited *Bacillus subtilis* culture which had contained 5×10^8 bacilli/ml.

was mixed (50 %, v/v) with a magnesium-limited simple salts medium ($0.9 \mu\text{g. Mg}^{2+}/\text{ml.}$, final concentration) and used as the medium supplying a magnesium-limited chemostat culture containing *Torula utilis* (4.5×10^7 organisms/ml.) and *B. subtilis* (2×10^8 bacilli/ml., initial concentration), the bacilli now outgrew the yeast organisms (Table 1).

Fig. 3

Fig. 4

Fig. 3. Growth of (A) *Bacillus subtilis*, (B) *Bacillus megaterium* and (C) *Torula utilis* in simple salts media that were supplemented (50 %, v/v) with cell-free fluid from magnesium-limited cultures of *B. subtilis* (●), *B. megaterium* (▲) and *T. utilis* (△); growth of organisms in an unsupplemented medium is shown by the open circles. The cultures were grown in 350 ml. conical flasks, incubated at 37° on a reciprocating shaker. All the media contained $0.5 \mu\text{g. Mg}^{2+}/\text{ml.}$ and in each case the inoculum was from a magnesium-limited chemostat culture of the appropriate organism.

Fig. 4. Growth of *Bacillus subtilis* in a simple salts medium containing $0.5 \mu\text{g. Mg}^{2+}/\text{ml.}$ that had been (△) supplemented (50 %, v/v) with cell-free fluid from a magnesium-limited culture of *B. subtilis* which had contained about 5×10^8 orgs/ml., (▲) supplemented (5 %, v/v) with the same cell-free fluid, and (●) supplemented (50 %, v/v) with cell-free fluid from a magnesium-limited *B. subtilis* culture that had contained about 1×10^8 orgs/ml. The open circles refer to growth of *B. subtilis* in an unsupplemented medium. The cultures were grown in 350 ml. conical flasks, incubated at 37° on a reciprocating shaker. The inoculum was from a magnesium-limited chemostat culture ($D = 0.3 \text{ hr}^{-1}$, 33° , pH 6.5).

Table 1. Growth of *Bacillus subtilis* and *Torula utilis* in a magnesium-limited chemostat culture supplemented with magnesium-limited *B. subtilis* extracellular culture fluid

The culture temperature was controlled at 33° ; the pH value was not controlled but varied only from 6.1 to 6.4 during the experiment. The magnesium-limited simple salts medium was mixed (50 %, v/v) with cell-free fluid from a magnesium-limited *B. subtilis* culture which had contained about 5×10^8 bacilli/ml.; the total magnesium concentration was adjusted to $0.9 \mu\text{g.}/\text{ml.}$ The dilution rate was fixed at 0.3 hr^{-1} .

Time after mixing (hr)	No. of orgs/ml. ($\times 10^{-7}$)		Proportion of total no. of orgs (%)	
	<i>B. subtilis</i>	<i>T. utilis</i>	<i>B. subtilis</i>	<i>T. utilis</i>
0	0.2	4.5	5	95
3	0.3	5.6	5	95
8	0.7	5.0	12	88
13	4.6	5.0	48	52
21	13.2	2.2	86	14
26	20.0	1.8	92	8
29	39	1.6	96	4

The nature of these growth-promoting substances has not been established, but their concentration in a magnesium-limited culture of *Bacillus subtilis* varied with population density. Thus, when the concentration of magnesium in a culture of *B. subtilis* was decreased to a value ($0.3 \mu\text{g. Mg}^{2+}/\text{ml.}$) such that the steady-state culture population density was about 1×10^8 bacilli/ml., the growth-promoting capacity of the resulting extracellular fluid was decreased correspondingly (Fig. 4). At this lower bacterial concentration small numbers of yeast organisms still could not outgrow the bacilli, but when the culture bacterial population density was decreased further, to 6×10^6 bacilli/ml. (culture magnesium concentration, $0.15 \mu\text{g.}/\text{ml.}$), proportionately small numbers of the yeast organisms (about 1×10^7 orgs/ml., initially) now outgrew the bacilli (Table 2) but only slowly. Presumably the very small population of *B. subtilis* organisms could not produce a sufficient concentration of extracellular growth promoting substance(s) to facilitate magnesium uptake by the bacilli to an extent that would allow them to compete effectively with the yeast organisms for the available magnesium.

Table 2. *Growth of Bacillus subtilis and Torula utilis at low population densities in a magnesium-limited culture*

The magnesium-limited simple salts medium contained $0.15 \mu\text{g. Mg}^{2+}/\text{ml.}$; 0.1 ml. of a *T. utilis* chemostat culture, growing in this low-magnesium medium, was transferred to a *B. subtilis* culture also growing in this medium. The dilution rate was maintained throughout at 0.3 hr^{-1} and the temperature controlled at 33° . The pH value (uncontrolled) was 6.4 and did not vary.

Time after mixing (hr)	No. orgs/ml. ($\times 10^{-6}$)		Proportion of total no. orgs (%)	
	<i>B. subtilis</i>	<i>T. utilis</i>	<i>B. subtilis</i>	<i>T. utilis</i>
0	6	< 0.1	99	1
3	6	< 0.1	99	1
6	6	< 0.1	99	1
23	6	0.3	95	5
30	2.2	3.6	38	62
49	< 0.1	9.4	1	99

The inability of small numbers of *Bacillus subtilis* or *B. megaterium* organisms to outgrow *Torula utilis* in a simple salts medium did not obtain when magnesium was present in excess of requirement and growth was limited either by the availability of potassium or glucose. In both these cases the bacilli rapidly outgrew the yeast, irrespective of the initial population densities. This finding suggests that the growth-promoting substances were associated specifically with magnesium assimilation and not with cation uptake in general.

DISCUSSION

The above results provide an explanation for the unpredictable behaviour of some magnesium-limited mixed cultures described here and previously (Tempest *et al.* 1967); clearly magnesium uptake by both *Bacillus subtilis* and *B. megaterium* was facilitated by substances that were secreted into the extracellular fluids when the bacilli were growing under conditions where availability of magnesium limited growth. Since the concentration of these growth-promoting substances varied with population density, so did the ability of the bacilli to compete with other organisms

in magnesium-limited chemostat mixed cultures. As yet we have no precise knowledge of the nature of these growth-promoting substances but the active material present in a magnesium-limited culture of *B. subtilis* was found to survive heating at 100° for 30 min., diffuse through a dialysis sac and separate on a Sephadex column (G-10) with material of molecular weight greater than 700. Since both Casamino acids and yeast extract promoted growth of *B. subtilis* and *B. megaterium* in low-magnesium environments (but had no specificity), the growth-promoting substances possibly may be polypeptides or polynucleotides; they do not appear to be teichoic acids. It may be significant that products of RNA degradation are usually, if not invariably, found in extracellular fluids from non-growing suspensions of bacteria (Strange, Dark & Ness, 1961; Postgate & Hunter, 1962; Strange, Wade & Ness, 1963; Dawes & Ribbons, 1964), particularly when the extracellular magnesium concentration is low (Strange & Hunter, 1967), and nucleotides have been found to be secreted by cultures of *B. subtilis* growing in defined media (Demain, Burg & Hendlin, 1965). Also of interest in this connexion is the observation of Byers, Powell & Lankford (1967) that *B. megaterium* could secrete a substance (Schizokinen) that actively initiated cell division by chelating iron and facilitating its transport into the cell.

Several workers have observed an effect of population density on the growth of bacteria in both 'batch' and 'continuous' cultures. Bail (1929) proposed the concept of a bacterial space requirement to account for inhibition of growth at high population densities, and Contois (1959) provided evidence that assimilation of NH_4^+ , by *Aerobacter aerogenes* growing in an NH_4^+ -limited chemostat culture, was less efficient at high population densities than at low ones. On the other hand, Jannasch (1962, 1963) showed with carbon-limited chemostat cultures of *Spirillum serpens* that assimilation of growth-limiting substrate (lactate) was inefficient at low population densities; this he ascribed in part to lack of reducing power in cultures of low population density. Our results suggest, like Jannasch's, that assimilation of growth-limiting substrate (magnesium) was less efficient in the cultures of low population density. Thus, although the method used for determining extracellular magnesium was insufficiently sensitive to detect small changes ($< 0.05 \mu\text{g. Mg}^{2+}/\text{ml.}$) in the steady-state growth-limiting substrate concentration, the mixed culture experiments indicated clearly that for *Bacillus subtilis* and *B. megaterium*, at least, the relationship between growth-limiting substrate concentration (s) and growth rate (μ) varied with population density when the availability of magnesium limited growth.

Although the Michaelis-Menten type of equation (1) may describe accurately the relationship between μ and s in many real chemostat cultures, clearly it is inadequate for cultures in which growth is affected by bacterial products which accumulate in the environment. In such cases a 'population factor' (or, more accurately, a 'population-product factor') must be incorporated in the growth equation. But how equation (1) should be modified to accommodate such a factor is a matter for conjecture, since it is not certain whether its effect is primarily on the saturation constant (K_s) or on the maximum growth rate value (μ_{max}) or on both. Recent work by Dr E. O. Powell (personal communication), using a turbidostat to determine values of μ_{max} , showed that magnesium-limited *Bacillus subtilis* organisms secreted a substance (or substances) which increased the maximum growth-rate of *B. subtilis* growing in a low magnesium medium; whether the saturation constant (K_s) was also affected could not be determined. If the growth-promoting substance affected μ_{max} only,

then the simplest relationship that can be derived to accommodate its effect on growth is as follows:

$$\mu = \mu_{\max} \left(\frac{1 + \lambda p}{1 + p} \right) \left(\frac{s}{K_s + s} \right), \quad (2)$$

where p represents the concentration of extracellular growth-promoting metabolite and λ is a constant; $\mu_{\max}$ is defined as the maximum growth rate when $p = 0$. Thus, plotting the relationship between s and μ for magnesium-limited chemostat cultures of *B. subtilis* that differed only in the concentration of magnesium in the input medium (and hence in culture population density and concentration of extracellular growth-promoting substance) would produce a family of curves, similar to those shown in Fig. 1, each describing the relationship between s and μ at a particular culture population density. At low population densities the concentration of growth-promoting substance would be negligible, and then the relationship between growth rate and growth-limiting substrate concentration given by equation (2) would reduce to that given by equation (1). At high population densities, on the other hand, the concentration of growth-promoting substance would reach a 'saturation' value at which it would exert its maximum effect; thus $(1 + \lambda p)/(1 + p)$ would approximate to λ and the maximum growth rate approach a value equal to $\lambda \mu_{\max}$.

Powell (1958) and Jannasch (1967) concluded that the fate of a contaminant organism in a chemostat culture depended on many factors including the nature of the growth-limiting component of the medium, the dilution rate, temperature and culture pH value. Our results show that under some conditions at least, the culture population density is a further factor.

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