Growth of Mixed Cultures of Bacteria on Methanol

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The bacterium Pseudomonas C was grown in a chemostat on methanol as sole source of carbon and energy. At a dilution rate of 0.1 h⁻¹, other methanol-utilizing bacteria (Pseudomonas 1 and Pseudomonas 135), when added separately at a steady state, became dominant in the fermenter and Pseudomonas C was excluded. At a dilution rate of 0.3 h⁻¹, however, Pseudomonas C dominated and the other bacteria were excluded. When various bacteria unable to utilize methanol were added to the chemostat during a steady state growth of Pseudomonas C, they remained in the fermenter independent of the dilution rate, but as a very low percentage of the total population (about 1%). When pathogenic bacteria (Staphylococcus aureus and Salmonella typhimurium), which are unable to utilize methanol as a sole carbon source, were added separately to a pure culture of Pseudomonas C in a chemostat, they too remained in the fermenter independent of the dilution rate. However, they constituted less than 1% of the population in the culture broth but a high percentage of the population on the fermenter wall. When added to a mixture of Pseudomonas C and bacteria unable to utilize methanol, the pathogenic bacteria could not be found in the fermenter after a few medium changes.

The results suggest that operation of a continuous culture of Pseudomonas C at high dilution rates serves to prevent contamination with other methylotrophs that may have lower yields. A mixture of Pseudomonas C and heterotrophs from soil is relatively resistant to invasion by pathogens.

INTRODUCTION

Although the production of single-cell protein (SCP) by continuous culture using methanol as a substrate is usually based on pure cultures of micro-organisms (Terui et al., 1973; MacLennan et al., 1974; Mateles et al., 1976; Häggström, 1977; Chen et al., 1977), a number of reports have suggested the desirability of working with mixed cultures (Snedecor & Cooney, 1974; Häggström, 1969; Harrison et al., 1976; Cremieux et al., 1977; Ballerini et al., 1977), and a review of mixed cultures has appeared recently (Harrison, 1978). The mixed culture may offer several possible advantages compared with a pure culture, e.g. (1) the mixed culture may grow at a faster rate or give a higher yield than the pure culture, or (2) the mixed culture may be able to maintain itself in the face of accidental contamination more easily than the pure culture, thus permitting substantial savings in capital and operating costs associated with sterilization.

The mixed culture may consist of organisms competing for growth on methanol itself or on C₁ compounds derived from the oxidation of methanol, or may involve a single methanol-utilizer and one or more other organisms growing on compounds (other than C₁) excreted by the methanol-utilizer or arising from its partial lysis.

The purpose of this work was to investigate the relationship of Pseudomonas C grown in continuous culture to various types of competing organisms, both methanol-utilizers and
bacteria unable to utilize methanol, and to establish the extent to which \textit{Pseudomonas C} was able to remain dominant in mixed culture. \textit{Pseudomonas C} represents the group of methanol-utilizers which assimilate methanol in high yield (Goldberg et al., 1976) via the ribulose monophosphate pathway (Stieglitz \\& Mateles, 1973) without the need for other species to be present, and it is this group of organisms which is of immediate interest to SCP production (MacLennan et al., 1974; Terui et al., 1973; Mateles et al., 1976).

\section*{Methods}

\textit{Bacteria.} Three of the bacteria used in this study were able to utilize methanol as a sole carbon source: \textit{Pseudomonas C} utilized methanol via the ribulose monophosphate pathway of formaldehyde fixation (Chalfan \\& Mateles, 1972; Stieglitz \\& Mateles, 1973); \textit{Pseudomonas 1} and \textit{Pseudomonas 135} used the serine pathway for methanol assimilation (Goldberg \\& Mateles, 1975; Rock et al., 1976). \textit{Escherichia coli} was obtained from the Fermentation Unit, Hebrew University, Jerusalem, Israel and \textit{Staphylococcus aureus} (coagulate positive) and \textit{Salmonella typhimurium} 4734–11804 were obtained from the Israel Ministry of Health. Bacteria characterized as a \textit{Moraxella} sp. and a \textit{Bacillus} sp. had been isolated as adventitious contaminants from earlier continuous cultures of \textit{Pseudomonas C} on methanol. They were characterized using the methods of Cowan \\& Steel (1965) and were incapable of growth on methanol. Loamy garden soil was used as an inoculum in certain experiments to provide a wide variety of bacteria to compete in the continuous cultures.

\textit{Medium and growth conditions.} Bacteria were grown in continuous culture on M-3 medium (Mateles \\& Battat, 1974) containing \(\text{NaH}_2\text{PO}_4\), \(\text{H}_2\text{O} (0.45 \text{g l}^{-1})\) as the sole phosphate source. Operation of the continuous culture has been described previously (Goldberg et al., 1976).

\textit{Viable counts.} Samples of broth were taken at different times, diluted in 0-9 \%(w/v) \text{NaCl} and spread on nutrient agar (Difco) and methanol (0-2 \%, w/v) agar plates. Plates were incubated at 35 °C for 48 h before counting. Methanol-utilizing bacteria were counted on methanol agar plates. (\textit{Pseudomonas 1} and \textit{Pseudomonas 135} had a distinct pink colour on these plates as compared to \textit{Pseudomonas C} which was off-white.) Methanol non-utilizers were counted on nutrient agar plates. \textit{Moraxella} sp., \textit{Bacillus} sp., \textit{E. coli}, \textit{Staph. aureus} and \textit{S. typhimurium} were first plated as pure cultures and the appearance of colonies on these plates served as a control when counting samples from mixtures. A few days after adding soil to the fermenter, different colonies appeared on the nutrient agar plates; these were differentiated on the basis of colour and morphology. The plates prepared from a continuous culture were incubated for 48 h and then stored at 4 °C for counting after the run was terminated so that colonies from samples taken at different times could be compared.

\textit{Wall growth.} Samples for estimation of wall growth were taken after fermentations were terminated. The cell suspension in the fermenter was removed and fresh sterile medium was added to the growth vessel. The fermenter was opened and a sterile brush was used to disperse the organisms present on the wall of the growth vessel into the medium. The cell suspension was homogenized and samples were plated as described above.

\textit{Analyses.} For the estimation of dry weight, samples of cell suspension were centrifuged (10000 g, 4 °C, 10 min), and the pellets obtained were washed twice with distilled water, dried at 65 °C for 20 h and weighed. Methanol was determined using gas-liquid chromatography as described by Chalfan \\& Mateles (1972). Total carbon content was measured in a total organic carbon analyser (Oceanography International Corp., College Station, Texas, U.S.A.) according to Wölfel \\& Sontheimer (1974). Analyses were performed on the whole cell suspension and on the culture supernatant obtained after centrifugation (17000 g, 4 °C, 15 min). The carbon content of the bacteria was calculated from the difference in these measurements.

\textit{Growth yield.} The growth yield was calculated as described by Goldberg et al. (1976).

\section*{Results}

\textit{Mixtures of Pseudomonas C and other methanol-utilizing bacteria}

\textit{Pseudomonas C} was grown in a chemostat at \(D = 0.1 \text{ h}^{-1}\) until a steady state was established, and then a small inoculum of \textit{Pseudomonas 1} was added to the growth vessel (Fig. 1). \textit{Pseudomonas 1} increased in concentration and reached a plateau whereas \textit{Pseudomonas C} decreased. When \textit{Pseudomonas 1} dominated in the fermenter the yield value \(Y\) of the mixed culture was 0-35 g bacterial dry wt (g methanol utilized)\(^{-1}\) (Table 1), similar to or slightly less than the yield for a pure culture of \textit{Pseudomonas 1} \(Y = 0.38; \) Goldberg et al., 1976). When the dilution rate was changed to 0.3 h\(^{-1}\), \textit{Pseudomonas C} increased in concen-
Mixed cultures on methanol

Table 1. Yield for different mixed cultures

<table>
<thead>
<tr>
<th>Bacteria in the culture</th>
<th>Dilution rate (h⁻¹)</th>
<th>Yield [g bacterial dry wt (g methanol)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas C</td>
<td>0.3</td>
<td>0.54*</td>
</tr>
<tr>
<td>Pseudomonas C + Pseudomonas 1</td>
<td>0.1</td>
<td>0.35</td>
</tr>
<tr>
<td>Pseudomonas 1</td>
<td>0.14</td>
<td>0.38*</td>
</tr>
<tr>
<td>Pseudomonas C + Pseudomonas 135</td>
<td>0.1</td>
<td>0.35</td>
</tr>
<tr>
<td>Pseudomonas 135</td>
<td>0.1</td>
<td>0.38*</td>
</tr>
<tr>
<td>Pseudomonas C</td>
<td>0.1</td>
<td>0.43</td>
</tr>
<tr>
<td>Pseudomonas C + Moraxella sp.</td>
<td>0.1</td>
<td>0.43</td>
</tr>
<tr>
<td>Pseudomonas C + Bacillus sp.</td>
<td>0.1</td>
<td>0.35</td>
</tr>
<tr>
<td>Pseudomonas C + Bacillus sp.</td>
<td>0.3</td>
<td>0.40</td>
</tr>
<tr>
<td>Pseudomonas C + Bacillus sp.</td>
<td>0.4</td>
<td>0.48</td>
</tr>
<tr>
<td>Pseudomonas C + E. coli</td>
<td>0.1</td>
<td>0.35</td>
</tr>
<tr>
<td>Pseudomonas C</td>
<td>0.3</td>
<td>0.50†</td>
</tr>
<tr>
<td>Pseudomonas C + Soil bacteria</td>
<td>0.3</td>
<td>0.50†</td>
</tr>
</tbody>
</table>

* Results from Goldberg et al. (1976).
† Yield determined from the total organic carbon assay, assuming the cell composition of *Pseudomonas* C to be C<sub>3.6</sub>H<sub>7.0</sub>N<sub>2.5</sub>O<sub>2.2</sub> (calculated from results of Goldberg et al., 1976).

tation and nearly regained its initial value, while *Pseudomonas* 1 decreased. Similar results were obtained when *Pseudomonas* 135 was inoculated into a *Pseudomonas* C culture grown at *D* = 0.3 h⁻¹ in a chemostat (results not shown). A mixed culture composed of *Pseudomonas* 135 and *Pseudomonas* C at *D* = 0.1 h⁻¹ gave *Y* = 0.35 (Table 1), similar to or slightly less than the yield for a pure culture of *Pseudomonas* 135 (*Y* = 0.38; Goldberg et al., 1976).

Mixtures of a methanol-utilizing bacterium with bacteria unable to utilize methanol

A number of continuous culture experiments were carried out in which *Pseudomonas* C was grown at a steady state in the chemostat, and a methanol non-utilizing bacterium, which had contaminated the fermenter in an earlier run, was added. In one case the contaminating bacterium was a *Moraxella* sp. and it was inoculated at a high concentration (5.5 x 10⁸ bacteria ml⁻¹) into a culture of *Pseudomonas* C (2.5 x 10⁹ bacteria ml⁻¹) grown at *D* = 0.1 h⁻¹. The concentration of the *Moraxella* sp. steadily decreased throughout the fermentation, until it comprised less than 1% of the total population, while *Pseudomonas* C remained constant. The yield was determined before and after the addition of the *Moraxella* sp. and showed no change (Table 1). In another experiment a *Bacillus* sp., unable to grow on methanol as a sole carbon source, was inoculated at a relatively low concentration (2 x 10⁶ bacteria ml⁻¹) into a culture of *Pseudomonas* C (2.5 x 10⁹ bacteria ml⁻¹) grown at *D* = 0.3 h⁻¹. The dilution rate was changed to 0.1 h⁻¹ before the *Bacillus* sp. was washed out of the growth vessel, at which point its concentration increased. Measurements of yield values at different dilution rates and at a steady state are given in Table 1 which shows that the yield fell somewhat upon the addition of the *Bacillus* sp.

*Pseudomonas* C was grown at *D* = 0.3 h⁻¹ to a steady state and then soil was added to the fermenter (Fig. 2) to provide a high number of different bacterial strains. In addition to *Pseudomonas* C, four strains of bacteria, distinguishable by colonial morphology, established themselves in the fermenter. Their total number, however, never increased beyond 1% of the total population. None of these bacteria could utilize methanol as a sole carbon source and, although there were changes in the proportions of these bacteria, their total number remained fairly constant throughout the fermentation (Fig. 2). The yield was determined several times during the fermentation (Table 1) but no difference was found between growth of *Pseudomonas* C in pure culture and growth in mixed culture. At the end of the fermentation period the bacterial growth on the walls of the fermenter was examined; contaminating bacteria comprised 75% of the population on the wall (Table 2), but only about 2% of the
bacteria in suspension. Total organic carbon was determined for the whole broth and the supernatant liquid when *Pseudomonas* C had been grown as a pure culture, as well as when the system had reached an approximate steady state after the addition of soil. Total soluble organic carbon in the supernatant decreased from 28 to 16 mg l\(^{-1}\) after addition of the soil, indicating that organic material produced by *Pseudomonas* C had been utilized by the added bacteria. The amount of additional growth of various organisms supported by the utilization of 12 mg organic carbon l\(^{-1}\) was not sufficient to cause a noticeable change in biomass (Table 1).

**Mixture of Pseudomonas C and an indicator of pathogenic bacteria**

When *Escherichia coli* was inoculated into a culture of *Pseudomonas* C in steady state at \(D = 0.1\) h\(^{-1}\), it reached a concentration of 5% of the population but had no apparent effect on the numbers of *Pseudomonas* C (Fig. 3), although the yield decreased slightly after the addition (Table 1). The concentration of *E. coli* decreased to 2% when the dilution rate was changed from 0.1 to 0.3 h\(^{-1}\) (Fig. 3). Before the addition of *E. coli* the amount of total organic carbon was 49 mg l\(^{-1}\); after the addition this decreased to 27 mg l\(^{-1}\), equivalent to 70 mg methanol l\(^{-1}\).

In another experiment *Staphylococcus aureus* was inoculated into a fermenter in which *Pseudomonas* C was growing at a steady state at \(D = 0.1\) h\(^{-1}\). *Staphylococcus aureus* decreased to less than 1% of the total population, and its concentration remained constant when the dilution rate was increased to 0.3 h\(^{-1}\). When the fermentation was terminated, a representative sample from the wall of the fermenter was taken. The concentration of *Staph. aureus* on the wall was eight times higher than the concentration of *Pseudomonas* C (Table 2) whereas in the broth *Staph. aureus* constituted only 0.1% of the total population.

The same experiment was performed with *Salmonella typhimurium* inoculated when *Pseudomonas* C grew at a steady state at \(D = 0.3\) h\(^{-1}\) (Fig. 4). *Salmonella typhimurium* constituted 7% of the total population and its concentration did not change when the
Table 2. Concentration of bacteria in the broth and on the fermenter wall at the end of continuous fermentation

The contaminating bacteria were added to steady state cultures of Pseudomonas C and continuous culture was maintained for a further 100 to 200 h.

<table>
<thead>
<tr>
<th>Bacteria in the culture</th>
<th>Broth (ml⁻¹)</th>
<th>Wall (cm⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas C⁺</td>
<td>9 × 10⁸</td>
<td>0.5 × 10⁷</td>
</tr>
<tr>
<td>Soil bacteria</td>
<td>2 × 10⁷</td>
<td>1.6 × 10⁵</td>
</tr>
<tr>
<td>Pseudomonas C⁺</td>
<td>1 × 10⁹</td>
<td>0.2 × 10⁷</td>
</tr>
<tr>
<td>Staph. aureus</td>
<td>1 × 10⁶</td>
<td>1.6 × 10⁵</td>
</tr>
<tr>
<td>Pseudomonas C⁺</td>
<td>2.5 × 10⁹</td>
<td>0.3 × 10⁷</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>2.7 × 10⁷</td>
<td>1.6 × 10⁵</td>
</tr>
<tr>
<td>Pseudomonas C⁺</td>
<td>1 × 10⁹</td>
<td>0.3 × 10⁷</td>
</tr>
<tr>
<td>Soil bacteria*⁺</td>
<td>1 × 10⁹</td>
<td>3.7 × 10⁷</td>
</tr>
<tr>
<td>Staph. aureus</td>
<td>&lt; 1 × 10⁶</td>
<td>&lt; 1 × 10⁴</td>
</tr>
<tr>
<td>Pseudomonas C†⁺</td>
<td>2.5 × 10⁹</td>
<td>0.4 × 10⁷</td>
</tr>
<tr>
<td>Soil bacteria†⁺</td>
<td>5 × 10⁷</td>
<td>4.2 × 10⁷</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>&lt; 1 × 10³</td>
<td>&lt; 1 × 10⁴</td>
</tr>
</tbody>
</table>

* These bacteria were grown together for 1150 h at a dilution rate of 0.1 h⁻¹, and then 1.5 × 10⁷ Staph. aureus ml⁻¹ were added and the dilution rate was raised to 0.3 h⁻¹. Bacteria were counted after an additional 144 h.

† These bacteria were grown together for 800 h at a dilution rate of 0.1 h⁻¹, and then 3.5 × 10⁷ S. typhimurium ml⁻¹ were added and the dilution rate was raised to 0.3 h⁻¹. Bacteria were counted after an additional 168 h.

Fig. 3. Growth of Pseudomonas C (○) and Escherichia coli (●) in a mixed culture in a chemostat at two different dilution rates.

dilution rate was increased to 0.4 h⁻¹. A sample taken from the wall of the growth vessel showed that there were five times more S. typhimurium than Pseudomonas C on the wall, while in the broth 93% of the total population was Pseudomonas C (Table 2).

To examine whether the relatively 'sticky' soil bacteria might be able to reduce the tendency of the pathogens to 'stick' to the wall, experiments were carried out in which Pseudomonas C, together with the mixture of soil bacteria, was cultivated in a chemostat for some hundreds of hours. Then, when it might be assumed that a steady state had been reached and the walls were saturated with whatever organisms would grow on them, an inoculum of pathogen was added and its concentration in the broth was measured after several days. Results of two experiments of this type with Staph. aureus and S. typhimurium showed that the concentration of pathogen in the broth declined to several orders of magnitude lower than the level obtained when the pathogen alone competed with Pseudomonas C (Table 2). Further-
more, the concentration of the soil bacteria on the wall and in the broth was more or less the same as was obtained in the absence of the pathogens, but the concentration of pathogens on the wall was at least five orders of magnitude lower than when the soil bacteria were not established in the system.

DISCUSSION

In this study, methanol was the single carbon substrate for the growth of *Pseudomonas* C and other bacteria. Two classes of mixed cultures were examined: (1) cultures in which the component micro-organisms competed for the primary substrate, and (2) cultures in which one component grew on the primary substrate and produced organic compounds which served as substrates for other components of the system.

A third type, where complex interactions of component micro-organisms occur owing to supplementation of nutritional deficiencies or mutual antagonism, was not found. Such a type of mixed culture has been reported for growth on methane (Wilkinson et al., 1974; Linton & Buckee, 1977).

In the first class, involving competition for methanol between *Pseudomonas* C and *Pseudomonas* 1 or *Pseudomonas* 135, either organism could dominate the culture depending on the dilution rate. At \( D = 0.1 \) h\(^{-1}\) *Pseudomonas* 1 or *Pseudomonas* 135 dominated, whereas at \( D = 0.3 \) h\(^{-1}\) *Pseudomonas* C prevailed. These results are not surprising as the \( K_s \) for methanol is lower for *Pseudomonas* 1 and *Pseudomonas* 135 than for *Pseudomonas* C (Goldberg et al., 1976), thus providing the first two organisms with an advantage at low dilution rates, but as their maximal growth rate was lower than the higher dilution rate used they washed out at \( D = 0.3 \) h\(^{-1}\).

Our results differ from those of Cremieux et al. (1977) in that we did not obtain steady states with both methylotrophs present in high concentrations. Cremieux et al. (1977) reported growth in a chemostat of four methylotrophs on methanol as the sole carbon and energy source. The three methylotrophs in the minority comprised a constant 20% of the total population over 2 months. We observed no such steady state mixed population.

When contaminants of pure cultures of *Pseudomonas* C were isolated, purified, and added back both in low and high concentrations to a pure culture of *Pseudomonas* C growing at a steady state, they always remained, but only at about 1% of the total population. When unsterilized soil was added to *Pseudomonas* C growing at \( D = 0.3 \) h\(^{-1}\), four new colony types emerged which grew on the organic compounds produced by *Pseudomonas* C without influencing the growth of *Pseudomonas* C.

Although we found that adding other bacteria to *Pseudomonas* C did not increase the
yield, it should be noted that the pure culture had a comparatively high yield 0.54 g bacterial dry wt (g methanol) \(^{-1}\). Harrison et al. (1976) reported a yield of 0.30 for the pure methanol-utilizer ‘EN’ improving to 0.54 on addition of four strains unable to utilize methanol. Recently, Wren (1978) reported that optimizing the medium and growth conditions increased the yield of the pure culture to 0.46. Ballerini et al. (1977) reported a growth yield of 0.44 for a mixture of four methanol-utilizing bacteria, but reported no data for the yields of the individual components in pure culture.

The difference in yield of Pseudomonas C of 0.54 at \(D = 0.3 \text{ h}^{-1}\) and 0.43 at \(D = 0.1 \text{ h}^{-1}\) can be accounted for by maintenance requirements. For Pseudomonas C growing on methanol, a true molar yield of 19.5 g bacterial dry wt (mol methanol)\(^{-1}\) and a maintenance coefficient of 2.5 mmol (g bacterial dry wt)\(^{-1}\) h\(^{-1}\) was found (Rokem et al., 1978). Using these values at dilution rates of 0.3 and 0.1 h\(^{-1}\) gives yields of 0.52 and 0.41, respectively, very close to the values measured in this investigation. The relatively high yield at \(D = 0.3 \text{ h}^{-1}\), together with the beneficial effects on productivity, is a strong motive for operating commercial SCP processes at high dilution rates rather than lower ones. This is in addition to considerations of resistance to contaminants.

A possibility which might arise during SCP production is contamination with pathogenic micro-organisms. When Escherichia coli, an indicator of faecal contamination, was added to a culture of Pseudomonas C growing at \(D = 0.1 \text{ h}^{-1}\), it remained at 5% of the total population. At \(D = 0.3 \text{ h}^{-1}\) E. coli decreased to about 2% of the total population. The pathogens, Staphylococcus aureus and Salmonella typhimurium, however, remained in the growth vessel independent of the dilution rate. Although the influence of wall growth on the populations of bacteria cannot be measured directly, the generation time of Staph. aureus on the wall is probably several hours, and if the newly formed daughter cells were detached from the wall when complete, the concentration of bacteria on the wall might be enough to account for most of the 0.1% of Staph. aureus found in the culture. Even in a large fermenter (100 m\(^3\)) where the wall area/volume ratio is much smaller, thousands of bacteria ml\(^{-1}\) would still be present in the broth.

When substantial inocula of these pathogens were added to continuous cultures of Pseudomonas C which had been growing with soil bacteria, the pathogens failed to establish themselves either in the broth or on the walls. This suggests that the niche of the pathogen had been filled by other heterotrophic bacteria, making the mixed culture less susceptible to invasion by pathogens.

The propensity of a particular micro-organism to stick to a surface varies with the nature of the surface (Munson & Bridges, 1964; Topiwala & Hamer, 1971). Our experiments were carried out in glass fermenters, and it would be necessary to perform tests in steel fermenters to reach conclusions on the relevance of these results to industrial processes.

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REFERENCES


