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NOTES

Impact of Growth in Benzoate and *m*-Toluate Liquid Media on Culturability of *Pseudomonas putida* on Benzoate and *m*-Toluate Plates

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Pseudomonas putida **grown in continuous culture on benzoate or *m*-toluate lost the ability to grow on benzoate or *m*-toluate plates. A similar effect was not seen with a glucose continuous culture. Cells carrying and expressing a TOL plasmid rapidly lost their ability to grow on benzoate solid medium.**

Direct plating of culture samples onto selective media is routinely used to quantify cell concentrations. The usefulness of the technique depends on the supposition that all viable cells that are capable of growing on the substrate(s) present in the plating media actually form colonies that are visible to the naked eye. In fact, plating onto selective media has severe limitations, especially for cultures isolated from carbon-limited environments. For example, it has been shown that plate counts often underestimate the number of viable cells in cultures (12, 16) and that selective plate counts do not necessarily represent the genetic potential of microbial cultures (14). Various reasons have been suggested for the limited culturability of otherwise viable cells. Of specific interest in this note is the culturability of microorganisms grown in continuous culture under carbon-limited conditions. A case is presented which demonstrates the generation of a viable nonculturable state of *Pseudomonas putida* during continuous culture on benzoate (BA) or *m*-toluate (TA). Recognition of such a state is important to the proper interpretation of experiments investigating instability of the TOL plasmid.

Media. A mineral salts medium (pH 6.8) was used for selective plates, as described elsewhere (2) except that ammonium was provided as NH_4Cl (1.0 g/liter) and phosphate was provided as a buffer by using equimolar amounts of K_2HPO_4 (4.35 g/liter) and KH_2PO_4 (3.4 g/liter). BA, TA, and glucose (GL) plates were made by adding filter-sterilized sodium BA (1.25 mM), *m*-toluic acid (1.73 mM) or GL (3.91 mM), and disodium EDTA (0.5 mM), respectively, to heat-sterilized mineral salts (final concentrations). Rich broth (RB) medium contained, per liter, 10 g of tryptone, 5 g of NaCl, and 1 g of yeast extract. The mineral salts medium used for continuous cultures contained the same minerals as that used for the plates except in different proportions: phosphate was added for metabolic purposes only (rather than as a buffer) as KH_2PO_4 at a concentration of 136 mg/liter, and all other minerals were diluted to 20% of the concentration used in the plates. BA, TA, or GL alone or in combination at a final concentration of 100 mg/liter as chemical oxygen demand (COD) was added as a limiting substrate for continuous culture experiments. COD is a measure of available electrons in an organic compound and is expressed in terms of the quantity of oxygen required to accept the electrons during complete oxidation of the compound to carbon dioxide and water. The quantities of substrate providing 100 mg of COD per liter are 0.347 mmol of TA, 0.418 mmol of BA, and 0.521 mmol of GL.

Bacterial strains and culture conditions. *P. putida* PaW164 was the parent species for these experiments and is designated X^+ herein. It contains the TOL plasmid pWW0-164 and is capable of metabolizing BA by either the chromosomally encoded *ortho* or the plasmid-encoded *meta* pathway and TA by the *meta* pathway. An ampicillin resistance gene is contained on the plasmid outside the catabolic gene region and assists in distinguishing between cell types. Thus, PaW164 is Ben^+ Tol^+ Amp^r. Derivatives of PaW164 can form because of plasmid instability mechanisms (1) and include PaW174 pWW0-174 (Ben^+ Tol^- Amp^r) (17) and plasmid-free PaW85 (Ben^+ Tol^- Amp^s), which are designated X^- and X^0, respectively. All continuous culture experiments were begun with X^+ cells, were maintained at 30°C, and were conducted at a dilution rate (D = flow rate ÷ reactor volume) of either 0.10 or 0.17 h^{-1}.

Culture enumeration methods. All enumeration plates were incubated at 30°C. Total cell counts were obtained from RB or BA solid medium spread plates. During plasmid instability experiments, X^- cells were generated from X^+ cells (very few X^0 cells were formed). The fraction of X^+ cells was estimated by applying the catechol drop method (17) to BA or RB plates. Plate recovery is defined as the concentration of cells determined by selective plates (BA, GL, or TA) normalized to the concentration as determined by RB plates.

Miniature slide cultures were prepared in a manner similar to that described by Postgate (11), using sterile serological glass slides containing 13-mm (inside diameter) ceramic rings into which solid medium was placed; details of slide culture preparation are given elsewhere (7). Sample aliquots (30 μl) from continuous cultures were immediately added to prepared slide plates and were topped with a sterile glass coverslip (15-mm diameter). Slide cultures were placed into a petri dish with a wet sponge for moisture, which allowed the medium to last up to 24 h without drying out. The growth of individual cells on the slide cultures over time was monitored with a Wild phase-contrast microscope (magnification, ×500) during incubation in a 30°C constant temperature room. Viable cells were

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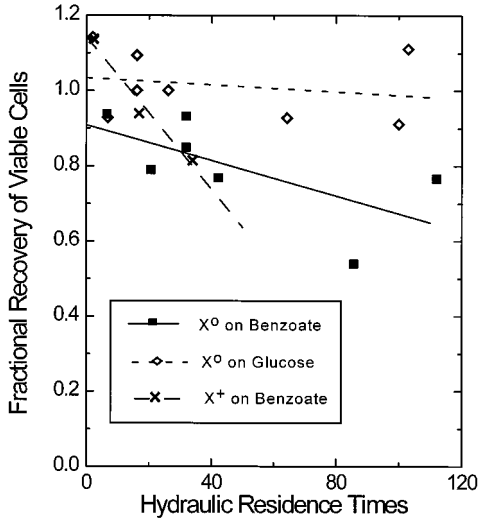

FIG. 1. Change in fractional recovery of viable cells on BA medium over time as observed with slide plates. Data are presented for BA- and GL-grown continuous cultures at a D of 0.1 h^{-1}. Lines represent linear regressions of data. One hydraulic residence time is 10 h.

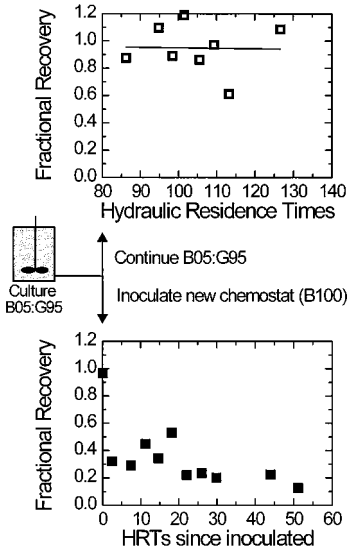

FIG. 2. Impact of BA liquid medium on the culturability of bacteria on BA solid medium. The effluent from a continuous culture fed 5% BA:95% GL (B05:G95) at 80 hydraulic retention times was used to inoculate a new continuous culture being fed 100% BA (B100). Changes in the fractional recovery of viable cells on BA medium are given (D = 0.17 h^{-1}). One hydraulic residence time (HRT) is 6 h.

considered to be those that divided at least once on RB or GL medium. Estimates of total viable cell counts on these two media were statistically equal. Recovery for slide plates is defined as the fraction of cells that divide on selective medium (BA or TA) normalized to the fraction of viable cells on RB or GL medium.

Culturability experiments. Results from three slide culture experiments are presented in Fig. 1. X^0 and X^+ cells were grown in continuous culture on BA, and X^0 cells were grown on glucose. As shown, the fraction of cells that divided at least once on BA slide plates decreased relative to the fraction dividing on GL or RB slide plates for both of the BA-grown cultures whereas the fraction remained relatively constant for the GL-grown culture. Therefore, with time, an increasing fraction of viable cells in BA continuous cultures became nonculturable on BA medium. Glucose continuous cultures, on the other hand, did not experience a similar decline in culturability on BA medium. Similar results were obtained from spread plates (data not shown). Since both X^0 and X^+ cultures grown on BA demonstrated a shift towards nonculturability, the phenomenon did not depend on the presence of the plasmid, although the rate of decline in culturability was more rapid for the plasmid-carrying X^+ cells.

Cells from a continuous culture fed 5% BA and 95% GL (as COD, experiment B05:G95) were used to inoculate a new continuous culture receiving 100% BA (experiment B100). Experiment B05:G95 had been operated for 116 generations (80 hydraulic residence times) at the point of inoculation, and the culture composition was relatively stable at approximately 75% X^+ cells and 25% X^- cells. Prior to inoculation of the new culture, BA plate recoveries of cells grown in the B05:G95 continuous culture hovered around 1.0, indicating no adverse impact of the medium on the culturability of viable cells onto BA plates. As illustrated in Fig. 2, the culturability on BA plates of the cells from the continuous culture receiving 5% BA and 95% GL continued unchanged, whereas the culturability on BA plates of cells from the new culture receiving only BA decreased from almost 100% to around 30% within four generations of growth. The composition of the culture changed from approximately 75% X^+ to almost 90% X^- in the same time frame.

The inability of cells from BA continuous cultures to grow well on solid BA medium appeared to be slightly greater for X^+ cells than for other cell types. During an experiment on plasmid instability, X^+ cells were used to inoculate a continuous culture receiving a feed containing BA and GL at a COD ratio of 62%:38%. The loss of plasmid function (resulting in the generation of X^- or X^0 cells) was measured by estimating the fraction of the total culture that was composed of X^+ cells (designated p^+) by the catechol drop technique on either BA or RB plates. As seen in Fig. 3, p^+ as estimated by using BA plates dropped faster than p^+ as estimated on RB plates, thereby suggesting that X^+ cells were impacted more strongly than X^- or X^0 cells by the BA medium.

TA-grown continuous cultures also demonstrated a loss of culturability on BA and TA plates on the basis of the recov-

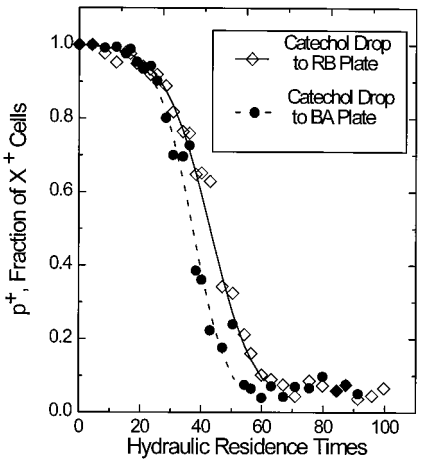

FIG. 3. Estimates for p^+, the fraction of X^+ cells in a culture, over time as a function of the solid medium (RB or BA) used for total plate counts. Data are from a continuous culture fed 62% BA:38% GL at a D of 0.17 h^{-1}. One hydraulic residence time is 6 h.

eries measured during an experiment in which cells were fed 100% TA. A TA-fed continuous culture can only support growth of X^+ cells, so the total cell concentration based on TA plates should have been comparable to concentration estimates obtained from RB plates. However, during the experiment, the TA plate recovery measured at the beginning of the experiment was 96%, whereas the recovery after 100 generations was 73%; the BA plate recovery for the same sample was 75%. It appears, therefore, that TA liquid medium also impacted continuous culture-grown *P. putida*'s ability to grow on solid TA and BA media.

The impact of growth in BA liquid medium on the culturability of *P. putida* on BA plates appeared to be reversible. A BA-grown X^0 culture was plated on both BA and RB plates, with a resulting recovery of less than 10% on BA plates. Thirty colonies from an RB plate were transferred onto a BA plate by making a 1- to 2-cm streak with a sterile metal probe. If the loss of culturability had been due to an irreversible mutation, then only 10%, or approximately three, of the colonies should have been able to grow on the BA plate. Instead, every colony that was streaked grew well on the BA plate. This suggests that the loss of culturability on BA was not due to an irreversible mutation and was more likely to have been due to a reversible physiological response.

A hypothesis is offered to explain the loss of culturability experienced. In general, bacteria have been shown to contain dual affinity enzyme systems that enhance the rate of substrate uptake under carbon-limited conditions (5, 10). Additionally, it has been demonstrated that the transient response of bacteria to carbon-sufficient conditions following carbon-limited conditions is an immediate increase in specific substrate uptake rate (2, 15). This response might be detrimental in some situations because upon being shifted to carbon-sufficient conditions, intracellular concentrations of substrate and/or intermediates may increase to inhibitory levels before an organism has had enough time to adjust its macromolecular composition sufficiently to handle the increased flux of those materials. Several examples of such a response have been reviewed by Tempest and Neijssel (15).

During these experiments, *P. putida* cells were taken from continuous cultures fed BA alone or in combination with GL or TA. Under these conditions, the BA catabolic enzymes were well induced despite the fact that the residual BA concentration surrounding the cells was quite low. When sufficient BA was suddenly made available, as on the solid medium plates, it is possible that intracellular concentrations of BA and/or intermediates reached fairly high levels. It has been demonstrated that catechol, an intermediate of BA degradation via both the plasmid-encoded *meta* and chromosomally encoded *ortho* pathways, is quite inhibitory to cells growing on aromatic compounds via the *meta* pathway (9). Furthermore, since catechol is more inhibitory in the presence of an active *meta* pathway than in an *ortho* pathway (8), X^+ cells would be more impacted and respond with lower recoveries than X^- and X^0 cells, as observed in our experiments. TA-grown cells experienced some loss of culturability, but to a lesser extent than BA-grown cells (data not shown); however, the reason for this is not clear. Perhaps 3-methyl catechol, the equivalent *meta* pathway intermediate for TA, is less toxic to the *meta* pathway than catechol.

Three pieces of evidence support the hypothesis that the effect was due to a temporary imbalance in the metabolic machinery of affected cultures. First, GL-grown cells did not experience a loss of culturability on BA solid medium. When GL-grown cultures were transferred to BA plates, the BA degradative pathways had to be induced, thereby preventing rapid scavenging of BA and high intracellular concentrations of intermediates such as catechol. Second, the loss of culturability was not permanent. Third, the loss of culturability was seen in every BA continuous culture and the degree of the effect diminished as the BA fraction of feed COD decreased.

A consequence of using BA and TA plates to quantify the relative X^+, X^-, and X^0 compositions in *P. putida* cultures grown in BA- or TA-limited environments is that inaccurate estimates of p^+ may be generated. Several researchers have used plating to RB (3, 13) or BA (4) solid medium followed with catechol dropping or selective plating to TA medium (6) to enumerate X^+ *P. putida* strains from low-growth-rate environments. On the basis of the results presented here, it is clear that the carbon source used in the solid media, as well as the growth history of the culture to be enumerated, is an important factor to consider when selecting a plating technique. Consequently, it is suggested that researchers working with BA- and TA-degrading bacterial cultures perform control experiments to confirm the fraction of viable cells that actually grow on BA and TA plates if such plates are to be used to characterize the composition of cultures.

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