ISOLATION OF SPONTANEOUS MUTANT STRAINS OF PSEUDOMONAS PUTIDA

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SUMMARY

A simple but powerful technique for the enrichment of spontaneous mutant strains of Pseudomonas putida is described. Repeated counter-selection of wild type cells in the presence of both D-cycloserine and penicillin increases the relative frequency of mutant organisms to over 1% of the population. The method has been used to isolate strains with either catabolic or biosynthetic dysfunctions.

Investigation of the bacterium Pseudomonas putida has revealed diverse metabolic pathways and novel regulatory mechanisms (1-4). Hence, a wealth of biochemical information has been opened to genetic analysis by the recent development of a system for transduction within this species (5). Extensive genetic studies will require the isolation of stable mutant strains that are blocked in each step of complex biochemical sequences. Furthermore, intensive genetic investigation of specific loci may be facilitated greatly by the isolation of strains that have undergone chromosomal deletion. Since spontaneous mutations usually are stable and often occur by deletion, we have developed a technique for the isolation of mutant cells without the use of artifical mutagenesis.

METHODS

Bacterial strains. Two strains of Pseudomonas putida were employed in this study: PpG2 (ATCC 17452, strain 76 in the Berkeley collection

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(1)) and PRS2000, a mutant strain of PRS1 (ATCC 12633, strain 90 in the Berkeley collection) that is freely permeable to cis,cis-muconate. Both PpG2 and PRS1 have been classified as biotype A of P. putida (1).

Growth conditions. Liquid cultures were grown with continuous shaking at 30° in 15 ml screw cap culture tubes containing 5 ml of medium. The minimal growth medium contained 50 mM Na_2HPO_4, 100 mM KH_2PO_4, 2 gm/liter $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 100 μM $CaCl_2$ and 10 μM $FeSO_4$. The pH of this solution was 6.2. Carbon sources were prepared as 1 M solutions, sterilized and aseptically added to sterile minimal medium. Solid medium used in petri dishes contained 1% (w/v) ionagar (Oxoid).

In all cases lytic medium contained D-cycloserine (Sigma) (0.1 mg/ml) and penicillin G (Squibb) (1×10^4 units/ml). The antibiotics were dissolved in minimal medium as 10 times concentrated solutions and sterilized by filtration immediately prior to use.

Selection of mutant strains - General procedure. Repeated transfers of cells between growth medium (which permitted growth of both mutant and wild type cells) and lytic medium (which favored lysis of wild type cells) led to the progressive enrichment of mutant organisms. To assure the selection of independent mutant strains, several cultures derived from isolated clones were treated in parallel.

RESULTS

Isolation of strains blocked in cis,cis-muconate catabolism. Cultures of PRS2000 utilize cis,cis-muconate as a growth substrate via the steps depicted in Fig. 1. Two reactions,catalyzed by muconate lactonizing enzyme and muconolactone isomerase, convert muconate to β-ketoadipate enol-lactone, which is also an intermediate in the dissimilation of p-hydroxybenzoate. Strains blocked in the synthesis of these two enzymes were selected by alternating cycles of non-selective growth with p-hydroxybenzoate and of

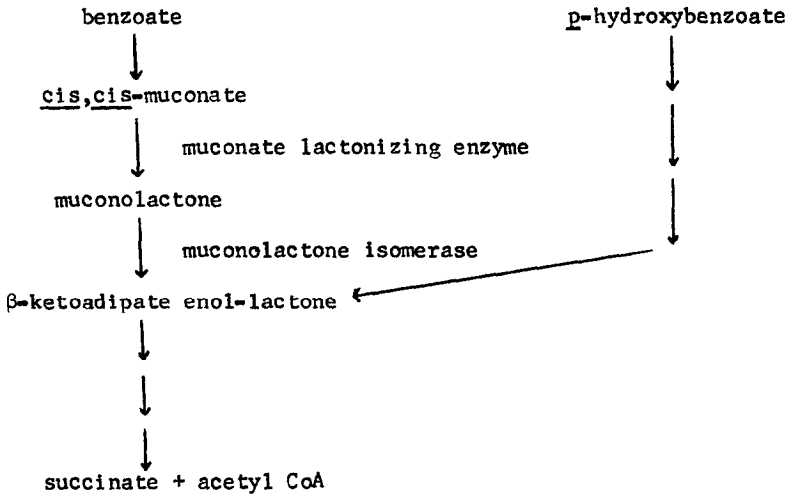

Fig. 1. The metabolism of cis,cis-muconate by P. putida.

lysis in the presence of muconate.

Ten independent cultures of strain PRS2000 were grown into stationary phase overnight with 0.2 mM p-hydroxybenzoate. In the morning p-hydroxybenzoate was added to a final concentration of 5 mM and the cultures were shaken at 30° for thirty minutes. At that time benzoate, a precursor of muconate, was added to a final concentration of 5 mM in order to induce enzymes of the muconate pathway. After an additional hour of shaking, the cells were harvested, washed and resuspended in minimal medium which contained both benzoate and muconate at a final concentration of 5 mM. The cell mass of strain PRS2000 doubles about every 50 minutes in the presence of these growth substrates. The cultures were incubated for thirty minutes, D-cycloserine and penicillin G were added and the cells were shaken for an additional four to six hours. At this time substantial lysis was observed. The cells were harvested, washed, resuspended in distilled water and shaken for twenty minutes to effect further lysis. The overall lytic treatment led to a 10^2 to 10^3 fold reduction in viability. A 0.1 ml portion of the culture was transferred to minimal medium containing 0.5 mM p-hydroxybenzoate, allowed to grow overnight, and subjected to a new cycle of lysis the next day.

After treatment with the antibiotics, washed cells were diluted 10^{-1}, 10^{-2} and 10^{-3} from the distilled water suspension into minimal medium; aliquots of 0.1 ml of the diluted cells were spread on agar plates containing 10 mM succinate. The plates were incubated at 30° for 36 hours; plates containing from 30 to 300 colonies were replicated onto agar plates supplemented with 10 mM benzoate or 10 mM succinate. No mutant organisms were detected after the first round of lysis, but after three lytic cycles, mutant strains that could not utilize benzoate were found with a frequency of greater than 1% in eight of the ten cultures. Subsequent analysis revealed that all of the mutant organisms tested were blocked in the synthesis of either muconate lactonizing enzyme or muconolactone isomerase; some strains did not form either enzyme. Since the third cycle of lysis was as effective as the first, it is unlikely that antibiotic resistant strains were substantially enriched by the cycloserine-penicillin procedure.

Isolation of strains blocked in arginine biosynthesis. A strictly analagous procedure led to the isolation from strain PpG2 of organisms that had lost the ability to form arginine. Cells were grown at the expense of succinate; this carbon source permits a doubling of cell mass every 45 minutes. Growth medium was supplemented with arginine (15μg/ml); lytic medium contained succinate alone. After growth overnight with 0.2 mM succinate supplemented with 15 μg/ml arginine, succinate was added to a final concentration of 5 mM and cells were shaken for thirty minutes. They were then harvested, washed and resuspended in minimal medium containing 5 mM succinate alone. After an additional thirty minutes of shaking, D-cycloserine and penicillin G were added and the shaking was continued for one to two hours. As before, the overall lytic treatment led to a 10^2 to 10^3 fold decrease in the viable count. A 0.1 ml portion of the partially lysed culture was transferred to a fresh tube containing 0.5 mM succinate and 15 μg/ml arginine and grown out overnight for another cycle of lysis.

After three treatments with the antibiotics, eight of the ten tubes

that were examined contained arginine auxotrophs at a frequency higher than 1%. In some tubes, the mutant cells represented a majority of the population. Most of the arginine auxotrophs were blocked at steps prior to the formation of citrulline. In a subsequent experiment strains unable to convert citrulline to arginine were readily isolated by including citrulline (20 μg/ml) in the lytic medium; this modification caused lysis of those cells that could convert citrulline to arginine.

DISCUSSION

Repeated selection with D-cycloserine and penicillin G is an effective method for isolation of spontaneous mutant strains of P. putida. If spontaneous mutant cells are present at a frequency of 10^{-8}, then they must be enriched by a factor of 10^6 in order to be detected readily. This goal appears to be achieved by three sequential enrichments, each of which probably increases the mutant frequency by 10^2 to 10^3. If necessary, the lytic treatments could be repeated further in order to select infrequently occurring mutant strains. The probability of selecting undesired antibiotic resistant organisms is decreased substantially by including two inhibitors of cell wall synthesis in the lytic medium: strains resistant to one antibiotic lyse during growth in the presence of the other.

The mutant strains derived thus far have been extremely stable and hence will be useful subjects for transductional analyses which, in turn, should reveal those strains that have undergone chromosomal deletions.

Lessie (personal communication) has shown that growth in the presence of D-cycloserine alone causes lysis of cultures of Pseudomonas multivorans, Pseudomonas acidovorans and Pseudomonas aeruginosa; he has used single treatments with cycloserine to select isoleucine auxotrophs of P. multivorans (6). Accordingly, we believe that the D-cycloserine-penicillin procedure will be applicable to many Pseudomonas species, thus facilitating the genetic investigation of the members of this versatile genus. In addition, the technique may

permit the ready isolation of spontaneous mutant organisms from other bacterial genera.

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