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Differential Recovery of Auxotrophs After Penicillin Enrichment in *Escherichia coli*

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Various auxotrophs are recovered from a penicillin enrichment cycle with differing efficiencies. Reconstruction experiments indicate that, under starvation conditions in the presence of penicillin, most auxotrophs undergo some death, whereas prolineless mutants are virtually immune to penicillin-induced killing.

A common method for isolating auxotrophic mutants of *Escherichia coli* involves the use of the antibiotic, penicillin (4, 5, 11). Penicillin acts on growing cells by preventing a transpeptidation reaction involved in the formation of cross linkages in the cell wall (15, 21). Cells growing in the presence of penicillin become osmotically fragile and lyse (12). If the medium lacks an essential nutrient, the cells will not grow and hence will not become osmotically fragile. In a mixed culture of prototrophs and auxotrophs growing in unsupplemented minimal medium with penicillin, the prototrophs will be preferentially lysed, thus increasing the proportion of auxotrophs among the surviving cells.

It is generally assumed that penicillin enrichment is nonspecific, i.e., that the relative frequencies of various types of auxotrophs obtained reflect their frequencies before the addition of penicillin. This is not the case. In a number of separate attempts to isolate spontaneous amino acid auxotrophs, we obtained proline-requiring strains to the exclusion of other classes.

In reconstruction experiments performed under standard penicillin enrichment conditions in hypotonic medium (3), we find that the viable titer of unstarved cells drops several hundred-fold, of proline auxotrophs remains approximately constant, and of several other amino acid auxotrophs drops several-fold. This results in a greater enrichment for prolineless mutants than for the other classes and is sufficient to account for our preferential recovery of proline auxotrophs.

MATERIALS AND METHODS

Strains. Spontaneous auxotrophs were obtained by penicillin enrichment of W3110 (*thy*$^-$): CB0401, proline requiring (*pro*$^-$); CB0402, methionine requiring (*met*$^-$); and CB0403, leucine requiring (*leu*$^-$).

Media. The complete media used were L agar and L broth, supplemented with 10 μg of thymine per ml (13). The minimal medium used was medium E (20), with the exception of one experiment in which medium ML (2) was used. Supplements were used at the following concentrations: thymine, 10 μg/ml; thiamine, 1 μg/ml; L-proline, 30 μg/ml; L-methionine, 10 μg/ml; and L-leucine, 20 μg/ml, (Calbiochem). Difco agar was used in plates at 1.5%. Glucose was added to a final concentration of 0.5%. Hypertonic medium contained, in addition, 20% sucrose.

Enrichment for mutants using penicillin. The general procedure was previously described (3). A fresh solution of potassium penicillin G (Squibb) at a concentration of 2,000 units/ml was used for each cycle.

Reconstruction experiments. Single colony isolates of the auxotrophs were suspended in 5 ml of fully supplemented minimal medium and grown without aeration for 3 hr at 40 C. The cells were then washed and suspended in medium lacking the amino acids at ca. 10^7 cells/ml. Approximately equal numbers of the three auxotrophs were mixed and distributed to three aeration tubes. Proline was added to one tube, leucine to another, and methionine to the third. Thus, in each tube there were two strains which lacked a needed nutrient and one which was fully fed. The unstarved strain differed from tube to tube.

These cultures were aerated at 40 C for 150 or 180 min, at which time penicillin was added. Just before adding penicillin, and at 30- or 45-min intervals afterwards, the cultures were assayed by plating on L agar. After overnight incubation at 40 C, single colonies were suspended in buffer and spotted on an unsupplemented plate and on individually supplemented plates. The spot plates were scored for growth after 24 hr of incubation.

RESULTS

Effects of amino acid starvation. To determine when cell growth ceased, viable titer was followed during a period of starvation. In 2 hr, CB0402(*met*$^-$), and CB0403(*leu*$^-$), underwent 1 to 1½ doublings, and CB0401(*pro*$^-$) underwent

almost three doublings. All three auxotrophs had exhausted their endogenous pools within 2 hr of starvation and remained constant in titer for at least another 90 min (Fig. 1).

Recovery of auxotrophs from penicillin enrichment cycles. A number of penicillin enrichment cycles were performed with various strains of *E. coli* K-12 to obtain spontaneous auxotrophs requiring proline, leucine, methionine, arginine, isoleucine, valine, or histidine. In each cycle, prolineless mutants were obtained exclusively. The frequency of pro^- auxotrophs was ca. 10^{-5}, which is several-fold higher than most estimates of forward mutant frequency (6). These data could be due to the proline loci being highly mutable or to a differential enrichment of various auxotrophs during a penicillin cycle.

Reconstruction experiments in hypotonic medium E. To test the effect of the penicillin enrichment procedure upon auxotroph frequency, reconstruction experiments were performed utilizing CB0401, CB0402, and CB0403. The amino acid required by one of the strains was added to the medium for each reconstruction experiment. When proline was added, the relative frequencies of the two other auxotrophs varied only slightly during 90 min of exposure to penicillin. However, when either methionine or leucine was added, the ratios of the two remaining auxotrophs shifted drastically. The ratio of pro^- to leu^- dropped

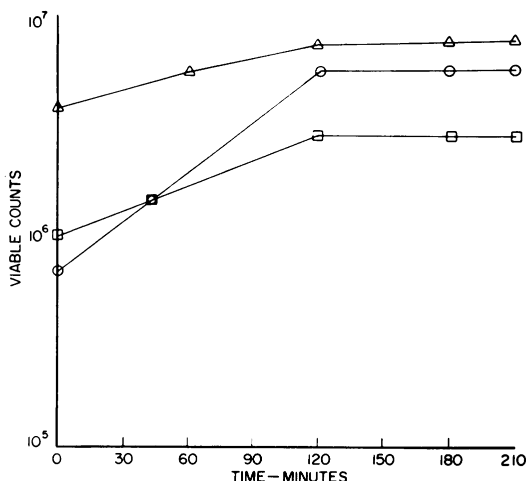

FIG. 1. *Growth of auxotrophs under starvation conditions for required amino acids. Single colony isolates of CB0401 (*pro^-*), CB0402(*met^-*) and CB0403 (*leu^-*) were grown overnight without aeration in L broth at 40 C. Cells were resuspended in supplemented minimal medium and grown for 3 hr with aeration at 40 C. The cultures were washed, suspended in unsupplemented minimal medium, and grown with aeration at 40 C. Symbols:* △, *CB0403(*leu^-*);* ○, *CB0401 (*pro^-*); and* □, *CB0402(*met^-*).*

from 26:21 to 79:1. The ratio of pro^- to met^- dropped from 35:23 to 99:1 (Table 1). This represents a 65-fold decrease in leu^- or met^- relative to pro^- auxotrophs.

The viable titer dropped 4- to 7-fold during the penicillin cycles. This reflects efficient killing (about 100-fold) of the growing strain, and also some killing of the prestarved auxotrophs.

In reconstruction experiments in which there is no growing strain, little or no killing of any of the auxotrophs occurred. This suggests that the auxotroph killing during a penicillin cycle is owing to cross feeding from growing and lysing cells, rather than from the auxotrophic mutation's being leaky.

Reconstruction experiments in hypertonic medium E. When the reconstruction experiments were carried out in hypertonic medium, the relative advantage of proline auxotrophs was lost. In fact, their relative frequencies dropped slightly, i.e., from 30 pro^-:28 leu^- to 46 pro^-:54 leu^-; and from 36 pro^-:20 met^- to 54 pro^-:36 met^- (Table 1). The viable cell titers dropped two- to three-fold during the penicillin cycles. Although the cell death was less in this experiment than in the preceding one, it was not restricted to killing of the supplemented auxotroph. Each of the three strains underwent approximately the same extent of killing when the required amino acid was omitted.

Reconstruction experiments in medium ML. Since it was possible that the differential recovery of proline auxotrophs occurred only in medium E, reconstruction experiments were performed by using medium ML which has a different salt composition (2, 20). As in medium E, proline auxotrophs were recovered preferentially (Table 2). The results with medium ML differ from those with medium E only in that proline auxotrophs had a selective advantage in hypertonic and in hypotonic medium.

DISCUSSION

We found that proline auxotrophs are preferentially recovered over all other auxotrophs which we attempted to isolate (requiring arginine, histidine, isoleucine-valine, leucine, methionine, or threonine) when the penicillin enrichment procedure is used. Reconstruction experiments indicate that this is owing to some killing of other types of auxotrophs during penicillin enrichment. Although many classes of auxotrophs are readily obtained by using penicillin enrichment, the actual efficiency of this method is low for most auxotrophs. Even when the auxotrophs in the culture are fully starved before the addition of penicillin to the medium, a large fraction of most auxotrophs is subsequently lost. In reconstruction

TABLE 1. *Reconstruction experiments in medium E*[a]

Time (min)[b]	Viable cells/ml ($\times 10^6$)	No. of colonies		
		pro[−c]	*met*[−d]	*leu*[−e]
A. Hypotonic medium				
0	30	*56*	12	32
30	6	*18*	40	42
60	5	*0*	30	70
90	4	*0*	30	70
0	30	26	*33*	21
30	10	64	*0*	12
60	9	75	*0*	5
90	8	79	*0*	1
0	30	35	23	*42*
30	10	80	10	*0*
60	8	92	8	*0*
90	5	99	1	*0*
B. Hypertonic medium				
0	9	*34*	28	38
30	4	*8*	32	60
60	4	*4*	28	68
90	3	*0*	36	64
0	8	30	*42*	28
30	7	44	*2*	54
60	5	52	*0*	48
90	4	46	*0*	54
0	9	36	20	*44*
30	5	60	26	*14*
60	4	66	34	*0*
90	3	54	46	*0*

[a] The amino acid required by one strain was added to the medium in each reconstruction experiment. The numbers of colonies recovered from this strain, the "prototroph," are italicized.

[b] Penicillin added at time zero.

[c] CB0401.

[d] CB0402.

[e] CB0403.

experiments carried out in the absence of any growing cells, we find little or no loss in viability in the presence of penicillin. It thus appears that the metabolites which are released from cells growing in the presence of penicillin are scavenged by the starved cells, which then begin growing. The extent of auxotroph lethality would depend on the concentration of metabolites and the efficiency with which the starved cells can accumulate their required metabolites. Since starved proline auxotrophs are practically immune to penicillin killing, the concentration of proline or the ability of these auxotrophs to scavenge must be severely limited.

Penicillin also may interact in some way with proline metabolism such that prolineless mutants are unable to utilize low levels of proline, or proline precursors, rendering these cells unable to grow and, therefore, completely immune to penicillin during an enrichment cycle. Isolated cytoplasmic membrane preparations from *E. coli* catalyze energy-dependent uptake of proline (10). The susceptibility of such transport to inhibition by penicillin should be assayed.

There are three loci in *E. coli* specific for proline biosynthesis (L. J. Charamella and R. Curtiss, Bacteriol. Proc., p. 27, 1966). Mutants defective in genes A or B are blocked before glutamic γ-semi-aldehyde synthesis, and mutants defective in gene C are blocked in the conversion of Δ pyrroline 5-carboxylic acid to proline (18). Since cells which are mutant at all three loci are found after penicillin enrichment (L. J. Charamella and R. Curtiss, Bacteriol. Proc., p. 27, 1966; C. Berg and J. Rossi, *unpublished data*), it is probable that they are equally immune to penicillin killing.

The action of penicillin does not seem to be confined solely to the inhibition of cell wall synthesis. Additional modes of action were suggested. The inhibition of an early step in the proline biosynthetic pathway was shown to be mediated by penicillin, whereas sucrose and sodium chloride prevent this inhibition (1). Plasma membrane disruption (16, 19), the inhibition of amino acid assimilation (7, 8), and protein synthesis

TABLE 2. *Reconstruction experiments in medium ML*[a]

Time (min)[b]	Viable cells/ml ($\times 10^6$)	No. of colonies		
		pro[−c]	*met*[−d]	*leu*[−e]
A. Hypotonic medium				
0	100	6	*79*	16
45	18	27	*0*	23
90	14	80	*0*	20
B. Hypertonic medium				
0	80	31	*34*	35
45	20	91	*0*	9
90	15	97	*0*	3

[a] The amino acid required by one strain was added to the medium in each reconstruction experiment. The numbers of colonies recovered from this strain, the "prototroph," are italicized.

[b] Penicillin added at time zero.

[c] CB0401.

[d] CB0402.

[e] CB0403.

(Curtiss, *personal communication*) are also affected by penicillin. Certain classes of auxotrophs which give concomitant membrane or cell wall alterations cannot be obtained after penicillin enrichment. For example, certain mutants which require tryptophan (*trp*$^-$) result from deletion of part of the tryptophan operon and of an adjacent gene which determines the cell envelope receptor site for bacteriophage T1 (17, 22). These T1-*trp* deletion mutants are not found after penicillin enrichment for *trp*$^-$ (9). Similarly, deletion mutants in the *proB* gene which extend in one direction into a phage receptor site gene are not recovered after penicillin enrichment, whereas deletion mutations in *proB* which extend in the other direction into the *lac* gene are readily obtained (Curtiss, *personal communication*) and furthermore show the same degree of immunity to penicillin killing as probable point mutants (J. Rossi, M.S. Thesis, University of Connecticut, 1971).

Whether an auxotroph is recovered after penicillin enrichment depends not only on which biosynthetic step is affected, but also on whether the mutation is pleiotropic or inactivates adjacent genes. In estimating forward and back mutation rates in histidine genes, Lieb (14) corrected for differences in penicillin killing in the mutants she studied. However, if a significant fraction of histidine auxotrophs had been especially sensitive to penicillin, she would not have found them and, hence, would not have included them in her calculations. Therefore, the calculated rates may be underestimated. Similarly, mutation rates at the tryptophan or proline loci, based upon penicillin enrichment, would be underestimated to the extent that nonenrichable auxotrophs occur. Thus, penicillin enrichment cannot be used in studies designed to quantitate mutation rates.

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