PRODUCTION OF A MUTANT-SPECIFIC BACTERICIDE BY THE ACTION OF PENICILLIN ON *ESCHERICHIA COLI*[1]

THOMAS J. BIRD AND JOSEPH S. GOTS

Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia 4, Pennsylvania

Received for publication October 24, 1955

When *Escherichia coli* strain B is plated with an excess of T_1 coliphage, two main types of T_1 resistant mutants can be isolated (Demerec and Fano, 1945). One type, designated *B/1,5*, is also resistant to T_5 coliphage and, like the phage-sensitive wild type, it shows no specific requirement for growth. The other type, *B/1,trp* is sensitive to T_5 and is nutritionally different from the wild type in that tryptophan is required for growth (Anderson, 1946).

Thus, a tryptophan-requiring mutant of *E. coli* strain B may be isolated by selection with T_1 coliphage. Another method of obtaining tryptophan-requiring mutants of this strain is by selection for auxotrophy with penicillin (Davis, 1949). Tryptophanless mutants so obtained have been shown to be different from the *B/1,trp* type, not only in their sensitivity to T_1 phage but in nutritional response patterns and types of intermediates accumulated (Gots *et al.*, 1954). Isolation of *B/1,trp* by selection for its tryptophan requirement could not be accomplished even from populations in which they were known to be heavily concentrated as determined by fluctuation analysis and selection for phage resistance.

The inability to isolate these mutants by the penicillin method of auxotrophic selection presents a paradox. An investigation into the nature of this paradox has revealed that the *B/1,trp* mutants cannot be isolated by the penicillin method because the action of the penicillin on the parent wild type population creates an environment which is deadly for the *B/1,trp* mutants. A description of this unique phenomenon is the subject of this paper.

MATERIALS AND METHODS

Medium. The synthetic medium used consisted of the following: Na_2HPO_4, 6 g; KH_2PO_4, 2 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; NaCl, 1 g; NH_4Cl, 1 g; glucose (autoclaved separately), 2 g; H_2O, 1000 ml; pH 7.2.

Organisms. The *B/1,trp* mutants were obtained from the survivors of approximately 2×10^8 *E. coli* strain B cells plated with sufficient T_1 coliphage to yield confluent lysis. Other tryptophan auxotrophs, e. g., strain *B-82*, were obtained by the penicillin method (Davis, 1949). Washed bacterial suspensions served as the source of inoculum for all experiments. These were prepared from 24 hr cultures in nutrient broth which were washed by centrifuging and suspending in sterile saline. *B/1,trp* suspensions were prepared from a previous suspension in the synthetic medium which had been aerated for 3 hr to exhaust any stored-up nutrients.

Experimental methods will be described for each experiment.

RESULTS

Table 1 depicts the results of a reconstruction experiment with varying population mixtures of wild type "*B*" bacteria and *B/1,trp* mutants. Synthetic media containing penicillin (1,000 units per ml) were inoculated with washed suspensions of the bacteria to give the series of final cell concentrations shown in the table. The *B:B/1,trp* ratio varied from 0.001 to 1,000. After 24 hr of incubation no visible growth was evident. *B/1,trp* survivors were determined by plating on nutrient agar containing sufficient T_1 coliphage to give confluent lysis of the parent wild type. Wild type survivors were determined by plating on unsupplemented synthetic agar medium. The survivor frequency of the wild type after penicillin action varied from 10^{-5} to 10^{-6}. Phage resistant mutants arising spontaneously from the wild type population were not evident at this low survival rate.

Though the penicillin technique is based on the ability of non-proliferating bacteria to survive the sterilizing action of penicillin, the recovery of

[1] This work has been aided by a grant from the United States Atomic Energy Commission AEC Contract AT(30-1)-1342.

TABLE 1

Reconstruction experiment for the recovery of B/1,trp by the penicillin technique

Initial Concentration		B / B/1,trp Ratio	Recovery after Penicillin Action	
B	B/1,trp		B/1,trp	B
(*bacteria/ml*)	(*bacteria/ml*)		(*bacteria/ml*)	(*bacteria/ml*)
0	10^7	$-\infty$	2.3×10^6	—
10^4	10^7	10^{-3}	1.1×10^6	0
10^5	10^7	10^{-2}	7.1×10^5	0
10^6	10^7	10^{-1}	3.3×10^5	<10
10^7	10^7	10^0	1.5×10^5	<100
10^7	10^6	10^1	5.0×10^4	<100
10^7	10^5	10^2	1×10^3	<100
10^7	10^4	10^3	0	<100
10^7	0	∞	—	60

B/1,trp from media lacking tryptophan was never 100 per cent. In the absence of added wild type bacteria the recovery of *B/1,trp* varied from 7 to 40 per cent. When the wild type organisms were present, the recovery of *B/1,trp* progressively decreased with increased concentration of wild type cells until a *B:B/1,trp* ratio of 1,000 was reached, whereupon no *B/1,trp* could be recovered. The killing effect of penicillin on the parent B type was not affected by the presence of *B/1,trp*. This experiment explains why the penicillin method has failed in allowing the isolation of this particular type of tryptophan-requiring mutant. Similar reconstruction experiments with other tryptophan auxotrophs which were originally isolated by the penicillin method did not show an enhancement of the killing action of penicillin by the addition of wild type organisms. This serves to rule out the possibility that tryptophan is liberated during autolytic death of the wild type, thus allowing the growth which is necessary for the bactericidal action of penicillin. Other experiments to be described also make this an untenable explanation.

The following experiment will show that the action of penicillin on the wild type creates an environment which is more lethal for *B/1,trp* than is penicillin alone. Synthetic medium containing 500 units of penicillin per ml was inoculated with wild type B organisms to a final concentration of 1×10^7 per ml. After 24 hr of incubation the survivors (approximately 100 per ml) were removed from half the culture by filtration through a bacterial filter and from the other half by boiling for 10 min. The sterilized media were then inoculated with a suspension of *B/1,trp* organisms to give an initial concentration of 10^5 per ml. Synthetic media with and without penicillin were similarly inoculated. Plating for survivors of the added *B/1,trp* organisms was done on nutrient agar at intervals of ½ hr.

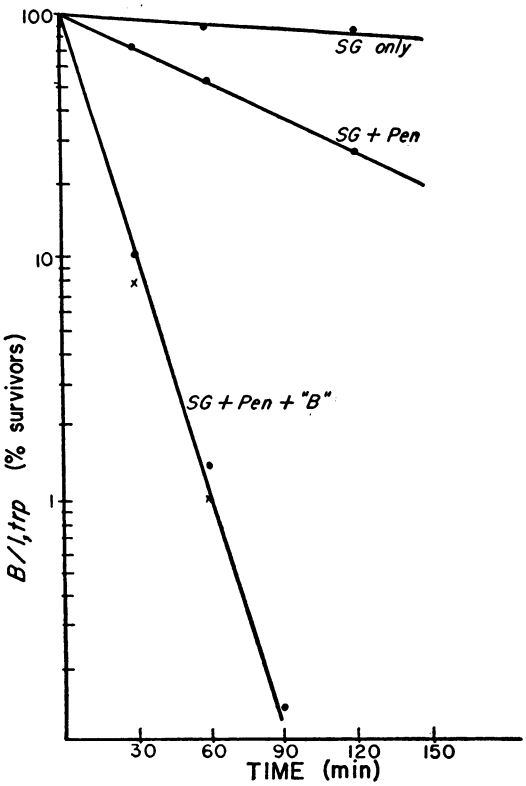

Figure 1. Killing effect on *B/1,trp* produced by the action of penicillin on the wild type "B" organisms. Survival of *B/1,trp* in salt-glucose (SG) medium with penicillin (SG + Pen) and after penicillin (500 units per ml) had been in contact with the wild type bacteria (SG + Pen + "B"). "B" bacteria were removed by filtration (●) or by heat (×).

The results depicted in figure 1 show an apparent marked enhancement of the killing effect of penicillin by the previous action with wild type cells. After 2 hr, 85 per cent of the original *B/1,trp* population was recovered from the untreated synthetic medium; 22 per cent from the same medium containing penicillin; but only 0.18 per cent from the medium in which penicillin had been allowed to act on the wild type organisms. No difference was found between the filtered and

the boiled preparations, indicating that the lethal action is unaffected by heat.

At this point several questions can be considered. Does the penicillin action on the wild type bacteria cause the accumulation of a substance which increases the sensitivity of *B/1,trp* to penicillin? This could be either a substrate of a penicillin-inhibited enzyme system or an unknown cellular constituent which would allow the growth of *B/1,trp*, thus exposing it to the bactericidal action of penicillin. Or, is the factor produced in the penicillin-inhibited system lethal without the further intervention of penicillin? That the latter is indeed the case was shown by the simple expedient of adding penicillinase to the medium after penicillin had acted on the wild type.

As in the previous experiment, the medium was prepared by inoculating wild type bacteria into synthetic medium containing penicillin. This time, the concentration of penicillin was that which proved to be just on the threshold of 100 per cent inhibition as measured turbidimetrically (62.5 units per ml). Surviving organisms were removed by filtration and penicillinase was added to one half of the filtrate and incubated for 4 hours before adding *B/1,trp*. Previously uninoculated medium was similarly treated. Penicillinase was also incorporated into the plating medium. Figure 2 shows that the penicillin effect in synthetic medium is completely removed by penicillinase, but the lethal action in the penicillin medium previously inoculated with wild type organisms was only slightly reduced by penicillinase.

That an inhibitory substance is present in the wild type bacteria which is liberated by autolysis has been ruled out by the addition of a sonically prepared cell-free extract of wild type cells to synthetic medium. After an exposure for 2 hr in this environment, recovery of *B/1,trp* was almost 100 per cent. This was also true when *B/1,trp* was added to the cell-free culture fluid obtained from a 24 hr culture of wild type bacteria grown in penicillin-free synthetic medium.

A phage-sensitive tryptophan auxotroph, *B-82*, which is nutritionally similar to *B/1, trp*, but differs by its indole accumulation (Gots *et al.*, 1954) was compared with *B/1,trp*. As with *B/1,trp*, penicillin alone reduced the number of survivors of *B-82* to 7.4 per cent at 2 hr, but unlike *B/1,trp* the survival rate was unaffected by the preliminary inoculation of wild type bacteria. In both cases, penicillinase relieved the inhibition of the *B-82* mutant. Thus, the tryptophan requirement of *B/1,trp* is, *per se*, not an essential property for its susceptibility.

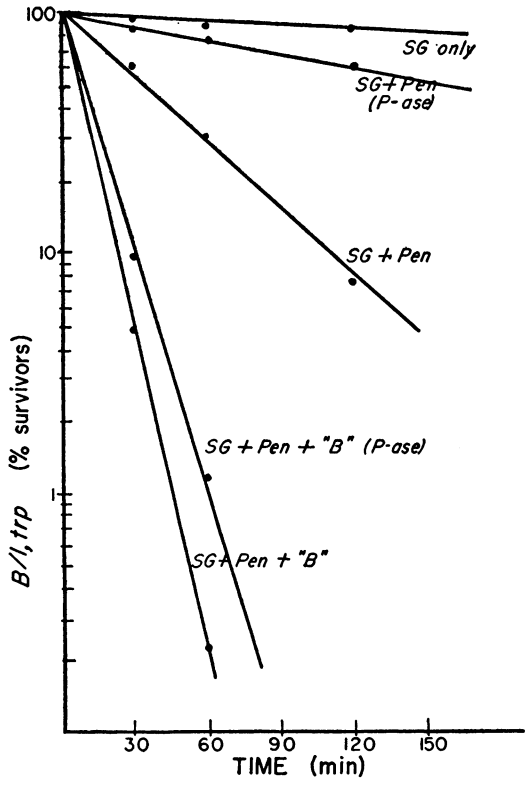

Figure 2. Killing effect on *B/1,trp*, after removal of penicillin by penicillinase (P-ase). See figure 1 for explanation of symbols.

Not only is the lethal factor heat stabile but it is also unaffected by treatment with trypsin. These properties would make it very unlikely that it is colicinelike in nature.

DISCUSSION

The experiments described here show that penicillin can act on a population of *E. coli* strain B to create an environment which is lethal for a particular mutant derived from the same population. Hence, it is impossible to isolate such a mutant by the popular penicillin method for auxotrophic selection. There is no way of knowing how many other types of mutants may go unrecognized because of a similar killing action imposed by the presence of penicillin. Without the alternate method of selection for its phage

resistant character the mutant *B/1,trp* would have remained undiscovered. Phage action might be supposed to elicit a mechanism of "induction." However, such a mechanism is very unlikely, particularly since Brenner (1954) has demonstrated that these mutants are present in the population before the addition of phage. He showed that *B/1,trp* mutants could be isolated only from cultures grown up in the presence of tryptophan and not from cultures previously grown in tryptophan-free media.

The killing effect on *B/1,trp* produced by the action of penicillin on the wild type progenitor remains after penicillin has been removed. Penicillin need no longer be present. This suggests the production or accumulation of a metabolite, normally not present, by the action of penicillin. Such a substance could be the substrate, or alternate product of the substrate, of an enzymatic reaction blocked by penicillin. The acute sensitivity of *B/1,trp* to such a metabolite might be another manifestation of an apparent pleiotropic mutation. Many instances are known in which an auxotrophic mutation results in the ability to be inhibited by normally occurring metabolites.

Saz and Eagle (1953) found that when penicillin resistant bacteria were mixed with penicillin sensitive bacteria in the presence of penicillin, the resistant types could no longer be recovered. This "co-killing" effect of penicillin is somewhat similar to the effect reported here. It differs in that it could be demonstrated only with gram positive cocci and not with *E. coli*, and occurred in a solid rather than a liquid medium. They, too, postulated a mechanism involving the release of a species-specific toxic factor from penicillin-killed sensitive cells which killed the resistant cells directly without the further intervention of penicillin.

The isolation and identification of the lethal factor described in these studies would be a significant contribution to our knowledge of penicillin action. The *B/1,trp* mutant offers a convenient method of assay for such a task.

SUMMARY

Reconstruction experiments have shown that the tryptophan-requiring T_1-resistant mutant of *Escherichia coli* strain B (*B/1,trp*) cannot be isolated by selection for its auxotrophic character with penicillin. An investigation into the nature of this phenomenon has revealed that the action of penicillin on the wild type population creates an environment which is deadly for *B/1,trp* mutants. This killing effect requires penicillin for its production but its action is not dependent on the presence of penicillin. The properties and significance of this factor are discussed.

REFERENCES

ANDERSON, E. H. 1946 Growth requirements of virus-resistant mutants of *Escherichia coli* strain B. Proc. Natl. Acad. Sci. U. S., **32**, 120–128.

BRENNER, S. 1954 A study of bacteriophage resistance in *Escherichia coli* strain B. D.Phil. Thesis, Oxford University, England.

DAVIS, B. D. 1949 The isolation of biochemically deficient mutants of bacteria by means of penicillin. Proc. Natl. Acad. Sci. U. S., **35**, 1–10.

DEMEREC, M. AND FANO, U. 1945 Bacteriophage-resistant mutants in *Escherichia coli*. Genetics, **30**, 119–136.

GOTS, J. S., KOH, W. Y. AND HUNT, G. R. JR. 1954 Tryptophan metabolism and its relation to phage resistance in *Escherichia coli*. J. Gen. Microbiol., **11**, 7–16.

SAZ, A. K. AND EAGLE, H. 1953 The co-killing of penicillin sensitive and penicillin resistant bacteria at low concentrations of the antibiotic. J. Bacteriol., **66**, 347–352.