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JOSHUA LEDERBERG AND ESTHER M. LEDERBERG

*Department of Genetics, College of Agriculture, University of Wisconsin, Madison, Wisconsin*

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*Department of Genetics,[1] College of Agriculture, University of Wisconsin, Madison, Wisconsin*

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Elective enrichment is an indispensable technique in bacterial physiology and genetics (van Niel, 1949). Specific biotypes are most readily isolated by the establishment of cultural conditions that favor their growth or survival. It has been repeatedly questioned, however, whether a selective environment may not only select but also direct adaptive heritable changes. In accord with similar discussions in evolutionary biology (Huxley, 1942), we may denote the concepts of spontaneous mutation and natural selection in contrast to specific induction as "preadaptation" and "directed mutation", respectively. Many lines of evidence have been adduced in support of preadaptation in a variety of systems (Luria and Delbrück, 1943; Lea and Coulson, 1949; Burnet, 1929; Newcombe, 1949; Lewis, 1934; Kristensen, 1944; Novick and Szilard, 1950; Ryan and Schneider, 1949; Demerec, 1948; Welsch, 1950; also reviewed: Braun, 1947; Luria, 1947; Lederberg, 1948, 1949). This paper concerns an approach to this problem that makes use of a replica plating technique which facilitates the handling of large numbers of bacterial clones for classification on a variety of media.

## METHODS

*Replica plating.* A frequent chore in bacteriological work is the transfer of isolates from one substrate to other selective or indicator agar media. In place of an inoculating needle, one might imagine a device consisting of many needle tips in fixed array, so that one operation would substitute for repeated transfers with a single needle. The requirements of this design are met by pile fabrics such as velvet or velveteen. The pile provides space in a vertical plane for moisture that might otherwise cause lateral smearing of any impression. (According to Dr. N. Visconti, in a private communication, dampened filter paper may be applicable to some replication problems considered by him independently of the present work.)

In our practice, twelve cm squares were cut from velveteen yardage, packed in large petri dishes, and sterilized in the autoclave. A square is placed, nap up, on a cylindrical wood or cork support of nine cm diameter and held firmly in place with a metal flange or hoop pushed over the fabric and around the rim of the support. The agar plate carrying the initial colonies is inverted onto the

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fabric with slight digital pressure to transfer the growth. The imprinted fabric then provides the pattern for transferring replica-inocula to subsequent plates impressed in the same way.

Replica plating is used to facilitate routine tests involving repetitive inoculations of many isolates on different media. Such tests are frequently required in

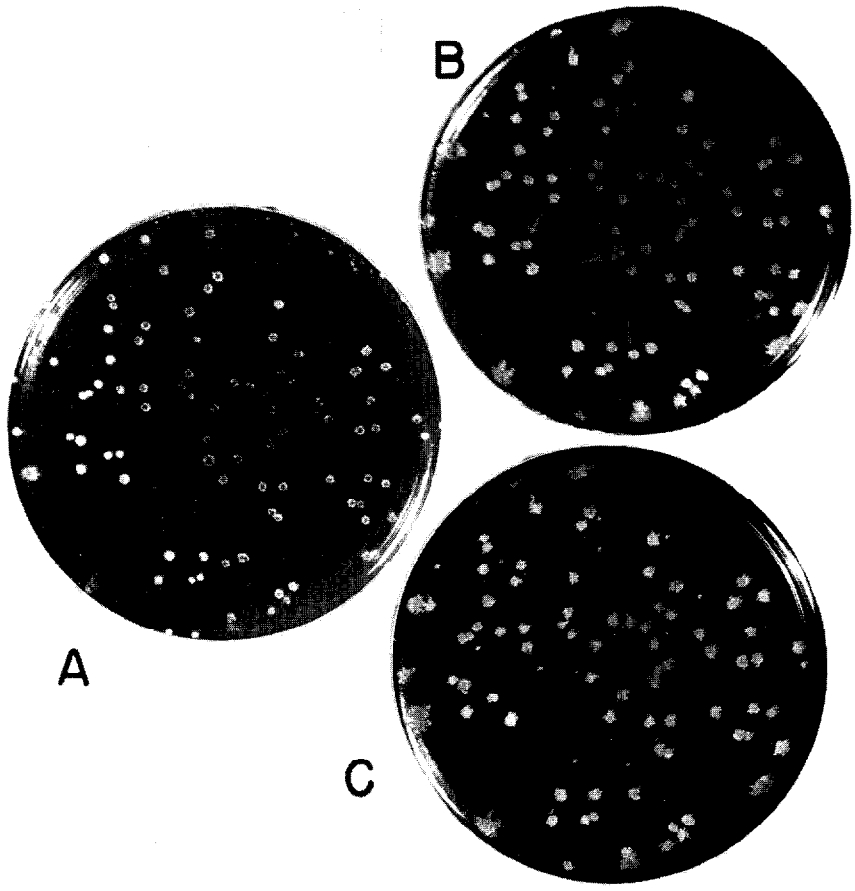


*Figure 1.* Replica plating for the isolation of auxotrophic colonies. *A*, Initial plate; *B*, Replica; both on complete agar medium. *C*, Second replica to minimal agar. The arrows designate the auxotrophic colonies which fail to grow on minimal medium. The resolution of these replicas is of fair to average quality.

genetic work, but the method should be applicable to other routine practice. Traits which lend themselves to classification by replica plating include antibiotic-sensitivity spectra, responses to bacteriophages (as in phage typing), fermentation characters, nutritional requirements, or any characteristic for which a selective or indicator agar medium can be devised. An application of replica plating to the detection of auxoheterotrophic mutants is illustrated in

figure 1, which also demonstrates the precision of the replicas. However, more faithful reproductions than those shown in the figure can be obtained with the use of dry, hard (2 or 2.5 per cent) agar.

The type of initial growth to be replicated may be varied according to specific needs. It may consist of surface colonies, localized growths from stab or spot inocula, or, as in the latter part of this paper, confluent growth from dense inocula previously spread over the agar and incubated. Freshly seeded sites will yield replicas of restricted inoculum size. A single initial plate may be used to imprint more than one fabric if carryover from one replica plate to another vitiates serial transfer. A fabric square may be washed, sterilized, and used repeatedly.

Replica platings may be quantitatively variable and influenced by many physical factors in common with some conventional methods of repetitive inoculation. There is no practical limitation on the number of serial replicas available, except for the accumulation of moisture that may exude from the agar surfaces. The resolution depends on the texture of the agar, colonies, and fabric. Unless the initial colonies are very plump, the distortion in size and shape is minimal and usually less than illustrated in figure 1. A crude estimate was made of the efficiency of transfer from initial plates spread with measured numbers of *Escherichia coli* cells. Approximately 10 to 30 per cent of the initial cells were transferred to the fabric, and an equal proportion again of these was found to be deposited on the replica plates.

## RESULTS

*Clonal occurrence of phage resistant mutants.* Preadaptive mutation as the basis of bacterial resistance to phages has been supported by two types of evidence. Burnet (1929) succeeded in isolating phage resistant mutants of *Salmonella* by observing the colonial morphology of the R and S phases. The other evidence is biometric: Luria and Delbrück (1943) working with *E. coli*, strain B and phage T-1, showed that the numbers of mutants selected from parallel broth cultures followed a clonal rather than a random sampling distribution. This was substantiated by more direct evidence of clonal occurrence of the mutants. Newcombe (1949) sprayed phage on films of growth on agar to assay them for their count of resistant mutants. The counts were greatly augmented by redistributing the growth at the time the phage was sprayed. The increase was believed to result from the (preadaptive) occurrence of the mutants in coherent clones. On the undisturbed plates, the assay would give the count of clones; the redistribution would give the total count of resistant cells.

The replica plating method allows a more direct demonstration of the clonal occurrence of the mutants: clones on an initial plate would be detected by the recurrence of resistant colonies at superimposable sites on serial replica-plates containing the phage. If the resistant cells did not exist already in clones on the initial plate, they should occur in only a random distribution in serial replicas from a confluent film of growth.

For this test, a culture (W-1), derived from *E. coli*, strain K-12, and the phage

T-1 were used. The culture is fully sensitive to the phage T-1, as well as to streptomycin, and like most *E. coli* strains gives rise to resistant mutants at rates of approximately $10^{-7}$ and $10^{-10}$ per division, respectively.

The media used included "Difco penassay" broth in 5 or 10 ml volumes (referred to as "broth") and EMB lactose agar ("plain agar"). The replicas were made on EMB agar previously coated with ca $10^9$ particles of T-1 per plate ("phage agar").

In a typical experiment, a dense broth culture was grown from a single colony on plain agar. One-tenth ml was spread on plain agar, and the plate was incubated 4 to 6 hours at 37 C. Serial replicas then were transferred, as described previously, to two or more phage agar plates which then were incubated overnight. The plates were marked either with a glass marking pencil or, for greater precision, by means of pins inserted into the velvet, which indented the agar. Figure 2 shows a typical result, except that a 0.01 ml inoculum was used to restrict the number of clones. In several experiments, at least half and often nearly all of the resistant mutants on the replica-plates recurred at congruent sites. The preoccurrence of the resistant cells in coherent families or clones within the confluent film on plain agar is inferred from this result.

*Indirect selection of phage resistant mutants.* The hypothesis of preadaptation would be further strengthened if adapted mutants could be isolated in pure culture without direct exposure of the bacteria to the selective agent. Replica plating has made this possible.

In the experiments of the previous section, the sites of preadapted mutants in the initial film of growth on plain agar are discernable from the replicas. If the initial inocula are made sufficiently dilute, there may be only one or a few clones on a single plate. If the congruent sites are chosen for the inoculum of a second broth tube, the mutants will be concentrated or enriched in about the same proportion as the cells per plate to the cells included in the inoculum. An enrichment of a hundredfold was anticipated and confirmed for each stage and this sufficed for our experiments.

After incubation, the enriched broth is treated in the same way, except that a more diluted inoculum is spread on plain agar to give again but one or a few mutant clones per plate. After about four stages of indirect selection, the resistant clones appear as discrete colonies which can then be characterized, purified, and maintained by conventional methods.

The indirect selection for phage resistance was applied in two independent runs, both quite successful. The mutants showed the same indifference to the phage as did mutants previously isolated by direct selection. Their stability was verified by making ten serial loopful to broth transfers, for a total of about 100 bacterial generations in each series. The terminal cultures then were diluted and plated on plain agar. A total of 836 and 447 colonies tested, respectively, were all resistant to the phage as determined by replica platings, and by a few tests of cross-streaking colonies against the phage.

It should be reemphasized that the indirect selection line itself has not been exposed to the phage at any time. Its history consists of the transfer cycle:

broth to plain agar to broth, with side transfers from agar to velvet discs used to imprint phage agar. Each broth was tested for any stray phage that might lead to fallacious conclusions with no indication of its presence. Replica plating

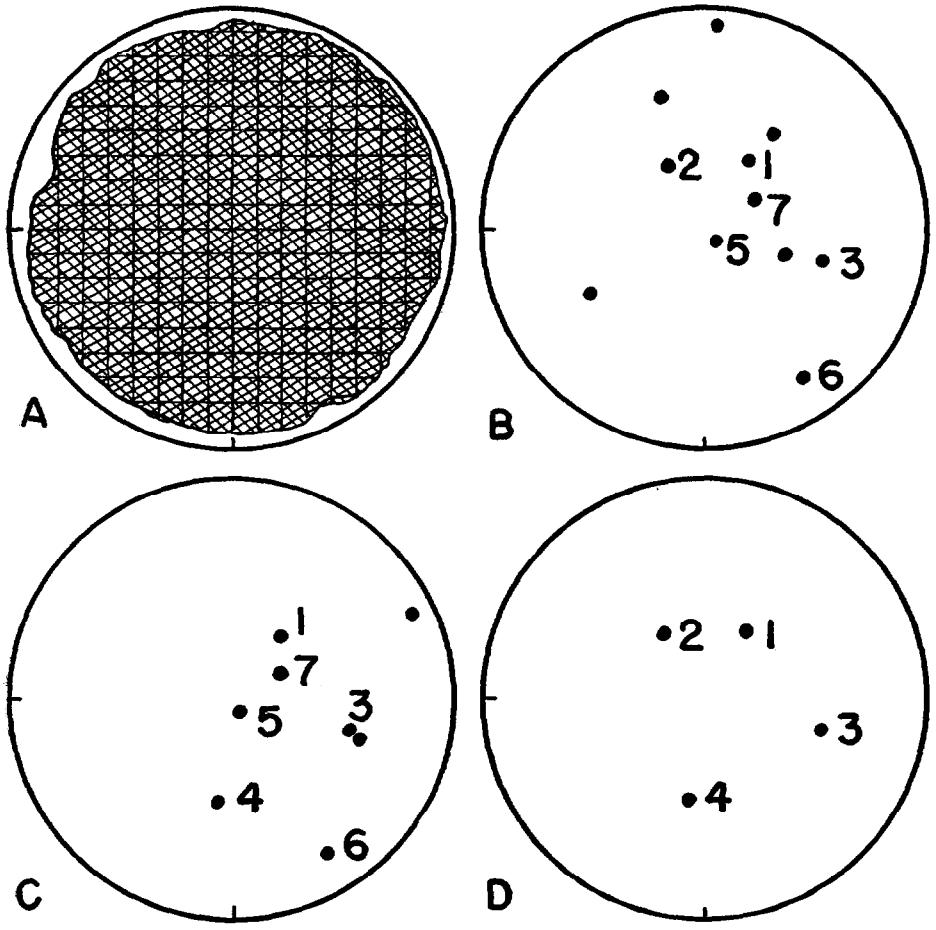


*Figure 2.* Clonal occurrence of mutants resistant to phage T-1. *A*, Initial or replica plate on plain agar with diffuse, confluent growth, (semidiagrammatic). *B*, *C*, and *D*, Successive replicas from *A* to agar coated with phage, from tracing of a typical experiment. Superimposable colonies of resistant cells are numbered. These are concluded to be derived from small clones of resistant mutants already present at corresponding sites on the plain agar plate, *A*.

thus provides a technique for isolating resistant or otherwise adapted mutants without altering the media in which the bacteria are grown.

*Streptomycin resistance.* In order to verify the general applicability of indirect selection, the procedure was also applied to streptomycin resistance. Instead of "phage agar", EMB agar containing 200 μg of streptomycin per ml ("sm agar") was used for the replica plates. The only deviation of these experiments from

those with phage results from the extremely low rate of mutation to sm-resistance. In order to obtain any resistant mutants at all, the W-1 culture was transferred several times in large volumes of broth. Concentrated inocula, about $3 \times 10^9$ cells, were used on the initial plates. Only two or three resistants were seen in experiments with 20 or 30 initial plates. One mutant clone was found by recurrence on serial sm replicas, and its site on plain agar was used to initiate the selection. After six stages of enrichment, the sm-resistant was obtained in pure culture. Two hundred and thirty-nine colonies were tested after 100 additional generations of growth in broth, and all retained resistance to sm. The sequence of transfers was identical with that exercised in indirect selection for phage resistance. The transfer operation itself could not have been responsible for resistance, for the sm selections remained sensitive to the phage and *vice versa*.

The infrequency of sm-resistant mutants hinders the tests for clonal occurrence. The culture 58-278, derived from *E. coli*, strain K-12, has been found to exhibit a much higher rate for this mutation, about $10^{-7}$ per division (H. P. Treffers, personal communication). Replicas of films of this culture on plain agar to sm agar plates repeatedly gave patterns similar to those illustrated in figure 1 for phage resistance. Indirect selection was also exerted successfully on this culture with results similar to those already described.

We conclude that resistance to streptomycin, as to phage, is a spontaneous mutation that occurs independently of the presence of the selective agent.

## DISCUSSION

Indirect selection is experimentally but not logically dependent on the clonal occurrence of the mutants. The latter had been established inferentially by Luria and Delbrück (1943) and by Newcombe (1949). It shows that the adapted cells are not randomly distributed in space. The success of indirect selection provides a sound basis for this nonrandom clustering in hereditary transmission. In particular, neither the adaptive change nor its inheritance depends upon a specific environment, which is what we mean by spontaneous mutation or preadaptation.

This demonstration does not conflict with reversible adaptive responses to a specific environment which disappear after some generations of growth in an indifferent medium. Directed, but nonheritable, responses have been clearly demonstrated in adaptive enzyme formation (Monod, 1947) and may be involved in the resistance phenomena investigated by Eagle (1951). However, no unequivocal case of a mutation specifically directed by and adapting cells to a chemical agent has yet been defended, despite numerous attempts of varying clarity (e.g., Barer, 1951). The concept of the "genotype as the norm of reaction" is pertinent to this discussion. The status of a microorganism's reaction as realized at any time, i.e., its phenotype, will reflect its immediate history, but its competence to react is an intrinsic quality subject for the most part only to sporadic, indeterminate mutations.

Indirect selection and tests for clonal occurrence should be applicable to other

adaptive systems, but some difficulties may be anticipated. With low mutation rates, sufficient numbers of cells must be used to allow a reasonable number of mutants to appear. Since the proportion of mutants should also increase in time (Novick and Szilard, 1950), the serial transfer of large volumes of cultures is also indicated. A very high mutation rate may also cause difficulties if preexisting clones are outnumbered by new mutations during the growth of the initial plates. This can be compensated for again by serial transfer in broth to pre accumulate mutants and by restricting the inoculum size and time of incubation of the initial plates. The presumably indifferent "plain medium" may prove to be adverse to the mutants. For example, it is not likely that sm-dependent mutants will be detected as clones in plain agar films. However, any applications of replica plating that fail to demonstrate the clonal occurrence of a mutant type may be controlled by suitably designed reconstruction experiments. These would involve the addition of known numbers of directly selected mutants to the original cultures. If the intruded mutants are detected in clones, and new occurrences are not, this would support the conclusion that the latter is not of spontaneous origin.

Some adaptations may be less amenable to these approaches, depending on the availability of suitable selective media for the replica plates. An example may be the development of lactose-positive papillae in cultures of *E. coli "mutabile"* on lactose-peptone agar. It would be necessary to devise a medium that would detect mutants in the replica inoculum without losing them in an avalanche of new mutations occurring on the indicator plate itself.

The indirect selection procedure is paralleled by improvement methods which depend upon the performance of the kinships of a plant or animal rather than its own phenotype, as for example in the selection of roosters for egg production breeding stock (Lush, 1945).

## SUMMARY

A method, replica plating, was developed to permit the copying of a pattern of microbial growth from one initial agar plate to a series of others. The method uses velveteen or other fabrics to make the transfer without disturbing spatial relationships. It may be useful in the detection of biochemical mutants, classification of fermentation reactions, determination of antibiotic sensitivity spectra, and other routines requiring repetitive inoculation of several media.

Replica plates were used in an approach to the problem of the preexistence of adaptive mutants prior to their selection by specific environments. Replicas to agar containing bacteriophage or streptomycin showed that mutants of *Escherichia coli* resistant to these agents existed in clones on the initial plates of indifferent agar medium. In addition, concentration or enrichment for such mutants was accomplished by taking inocula from bacterial films at sites demonstrated to contain mutants by replica plates. After several stages of enrichment, each type of resistant mutant was isolated in pure culture. The procedure at no time exposes the indirectly selected populations to the specific agent. These observations, therefore, are cited as confirmation of previous evidence for the

participation of spontaneous mutation and populational selection in the heritable adaptation of bacteria to new environments.

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