APPLICATION OF THE MEMBRANE FILTER FOR THE QUANTITATIVE STUDY OF TRANSFORMATIONS WITH PARTICULAR REFERENCE TO PHENOTYPIC EXPRESSION OF AN ERYTHROMYCIN-RESISTANCE MUTATION

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ABSTRACT

IYER, V. N. (University of Rochester, Rochester, N.Y.). Application of the membrane filter for the quantitative study of transformations with particular reference to phenotypic expression of an erythromycin-resistance mutation. J. Bacteriol. **84**:326-330. 1962.—A technique using membrane filters for quantitative studies of transformation of pneumococci is described and some possible applications discussed. This method has been found amenable to the study of the integration and phenotypic expression of an erythromycin-resistance mutation of pneumococci which is characterized by a low frequency of transformation and delayed expression. The expression of survival occurs prior to the expression of colony-forming ability in the presence of erythromycin.

It has been recognized in recent years that a satisfactory measurement of genetic transformations mediated by deoxyribonucleic acid (DNA) may be accomplished by (i) limiting the period of exposure of a competent population of cells to high molecular weight deoxyribonucleate, (ii) maintaining potential transformants in an environment that is not selective until expression of the transformed character has occurred, and (iii) exposing the mixture of untransformed cells and transformed-expressed cells to a colony-forming situation that is selective for the transformants. These requirements have been successfully met in several quantitative transformation studies (Hotchkiss and Marmur, 1954; Hotchkiss and Evans, 1958; Lacks and Hotchkiss, 1960; Ravin and Iyer, 1961). Two situations may, however, arise making measurement technically less feasible than it would otherwise appear to be: (i) certain genetic markers borne by a transforming molecule may yield relatively low frequencies of transformants (e.g., large deficiencies, multisite mutants, mutants with markers located terminally on the transforming molecule, and transforming DNA that has undergone partial biological denaturation); and (ii) there may be an appreciable delay between the integration of a marker into the bacterial genome and the full expression of its character, a period during which population changes must not be allowed to mar the quantitative aspects of an observation. A combination of both these situations was encountered during studies on the transformation of pneumococcus to erythromycin resistance by a mutation designated *ery-r7* (Ravin and Iyer, 1961). The need to overcome the technical difficulties posed led to the development of a relatively simple procedure that is described here and should find wider application. Another specific use to which the method has been put in this laboratory is also described and discussed in this paper. It concerns the question as to whether, after the integration of the *ery-r7* mutation by sensitive pneumococci during transformation, the expression of survival in the presence of erythromycin occurs prior to the expression of colony-forming ability.

MATERIALS AND METHODS

In principle, the method consists of exposing a large population of competent recipient cells of pneumococci to transforming DNA for a limited period, inactivating the extracellular DNA, and immediately layering an adequate number of cells on the surface of a sterile membrane-filter disc. Such a disc, holding a large number of cells including potential transformants, is placed on a nonselective solid medium to permit the phenotypic expression of the transformed character.

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Thereafter, the membrane is transferred to a selective medium and further incubated to permit only the growth of colonies containing transformed bacteria. Since each recipient bacterium is localized on the membrane and colonizes *in situ*, colony counts on the membrane when related to the total number of cells layered on the disc provide a direct measure of the frequency of recipient bacteria from which transformed progeny eventually arise. This method is similar in principle to the agar-overlaying technique employed by Ephrussi-Taylor (1960), in that it permits recipient bacteria to reproduce for a controllable number of generations before conditions selecting for the transformed phenotype are applied. However, the membrane-filter method has the additional advantage of permitting the experimenter to vary conditions at any time, simply by moving the membrane disc from one agar medium to a different one, as will be illustrated below.

Strains and genetic markers. A capsule-deficient mutant strain of a Type III pneumococcus (Ravin, 1959) was used as the recipient in these studies. This strain, designated Rx, is sensitive to erythromycin and streptomycin at concentrations above 0.01 and 10.0 μg per ml, respectively. Discretely transferable factors determining higher levels of resistance to these antibiotics were introduced into this strain by transformation. Five markers determining resistance to erythromycin and one marker determining resistance to streptomycin were utilized in these studies (*see* Ravin and Iyer, 1961).

Procedure. Competent cells of the recipient strain were obtained by following a method essentially similar to that outlined by Fox and Hotchkiss (1957). At 15-min intervals during the exponential phase of growth in Medium 3 of Ephrussi-Taylor (1951), when the viable population was of the order of 10^6 to 10^7 per ml, samples were withdrawn and rapidly frozen at −80 C in a glycerol-peptone medium (Ravin and Iyer, 1961). The frozen batches were subsequently thawed in the cold and tested for competence. Only batches showing a frequency of transformation of the order of 10^{-3} or higher were retained.

The transformation reaction was carried out by adding a saturating concentration of transforming DNA to the competent recipient cells suspended in Medium 3 plus 0.2% (w/v) beef serum albumin at 32 C. The reaction was allowed to proceed for 15 min, after which the extracellular DNA was inactivated with Mg^{++}-activated pancreatic deoxyribonuclease (Worthington) and the mixture transferred to an ice bath. Equal samples of the cold mixture, diluted where necessary to provide a larger volume of fluid for filtration, were next passed through sterile cellulose filter membranes (Millipore Filter Corp., Type HA grid; pore size, 0.45 μ). The amount of the transformation mixture filtered and, therefore, the number of cells on each membrane could be varied within wide limits and was determined by the expected frequency of transformation for the particular genetic marker(s) being studied. After filtration, the membranes, with the bacteria supported on them, were transferred to a nonselective solid medium and incubated at 37 C for a predetermined time (sufficient to permit the expression of the transformed character). Thereafter, the membranes were transferred to a selective medium containing streptomycin or erythromycin and further incubated for 48 hr to permit the development of resistant colonies only. The concentration of agar in the solid media could be varied within limits (0.5 to 2.5%), and this had an influence of the size of colonies obtained. Transformant colonies could be observed on the membrane under low-power magnification (× 30). More conveniently, the resistant colonies on the membrane were stained with a 1:4 dilution of Loeffler's Methylene Blue, after which they could be macroscopically observed and conveniently counted. An adaptation of this procedure will be described with reference to a particular type of experiment in which it was used.

RESULTS

Evaluation of the method. Transformant colonies, secured and stained in the manner described, are shown in Fig. 1. A few thousand colonies could be conveniently counted on each filter disc of a diameter of 47 mm. If they were picked out before staining, regrown on a nonselective medium, and tested, they displayed a resistance that was characteristic of the marker or markers in the donor-transforming molecule.

The method was evaluated by comparing it to a standard procedure used in this laboratory. In a series of brief exposures, competent sensitive cells were exposed to transforming DNA bearing each of a number of genetic markers determining resistance to erythromycin or streptomycin. The

frequency of transformants in each case was determined by the membrane-filter method described above and by a conventional method in which the cells exposed to DNA were first allowed to multiply in a nonselective liquid culture medium before spreading samples of suitable dilutions directly upon antibiotic-containing media. The time allowed to elapse before applying selective conditions was identical in the two methods. The time actually allowed to elapse depended on the marker studied and was that previously determined by the "spreading" method (Ravin and Iyer, 1961) as necessary for phenotypic expression. The results obtained by both methods in each such exposure were similar (Table 1). It is evident from these results and from a more extensive analysis (Ravin and Iyer, *to be published*) that the mutation *ery-r7* has a relatively low probability of being integrated during the transformation of a recipient bacterium.

Expression of survival and of colony-forming ability in the presence of erythromycin. In most transformation studies utilizing drug resistance as a genetic marker, resistance is considered to be the ability of a cell to reproduce and to give rise to a colony of cells in the presence of the drug in question. For convenience, this was the operational definition of resistance utilized in evaluating the membrane-filter method. It is possible, however, that a cell which, during transformation, has integrated a drug-resistance

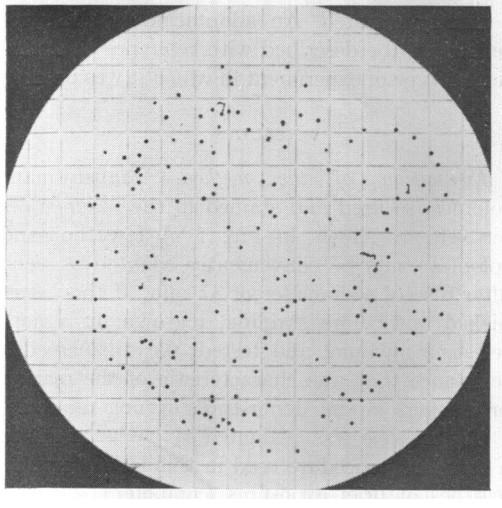

FIG. 1. *Streptomycin-resistant transformants of pneumococcus secured and stained by the membrane-filter technique* (× 2).

TABLE 1. *Comparison by two methods of the frequency of transformants forming colonies on media containing 0.1 μg erythromycin per ml or 100.0 μg streptomycin per ml after the transformation of sensitive pneumococci (Rx strain) by DNA from resistant strains*

Resistance marker in donor trans-forming DNA	Time allowed to elapse before selection applied	Frequency of resistant colony-forming transformants determined by	
		Spreading	Membrane-filter technique
ery-r2	1	0.94×10^{-3}	1.01×10^{-3}
ery-r3	1	0.87×10^{-3}	1.2×10^{-3}
ery-r5	1	3.4×10^{-5}	2.5×10^{-5}
ery-r6	1	3.7×10^{-4}	1.7×10^{-4}
ery-r7	3	4.6×10^{-5}	4.5×10^{-5}
str-r1	1½	0.92×10^{-3}	1.3×10^{-3}

mutation may express the ability to survive in the presence of the drug in question before it expresses the ability to reproduce in the presence of that drug (Ephrussi-Taylor, 1960).

That this in fact occurs in the case of erythromycin resistance was shown as follows. A competent batch of sensitive cells was exposed briefly to transforming DNA bearing the mutation *ery-r7*. After the inactivation of extracellular DNA, equal samples of the culture were filtered through sterile membrane filters. The filters were placed on the agar medium, without antibiotic, on which expression occurred. At desired intervals, two filters were transferred to media containing erythromycin. One of the filters was left in contact with the erythromycin-containing agar and incubated for a period of 48 hr at 37 C. The colonies arising on this filter, as a function of the time interval before exposure to erythromycin, provide a measure of the kinetics of expression of colony-forming ability in the presence of erythromycin. To determine the expression of survival, the second filter placed on erythromycin agar was again transferred to erythromycin-free agar after an incubation period of 1 hr. It was known from earlier observations that exposure for 1 hr to 5.0 μg per ml of erythromycin in the solid medium suffices to eliminate all sensitive cells. Cells that are able to give rise to colonies, on the subsequent transfer of the filters to erythromycin-free agar, are therefore surviving cells.

Although the expression of survival of all potential transformants at a concentration of 5 μg per ml of erythromycin was complete by 4.75 hr (Table 2), the detectable expression of colony-

TABLE 2. *Expression of survival and of colony-forming ability after transformation of sensitive cells by DNA bearing the mutation ery-r7*

Period of expression	Expression of survival*	Expression of colony-forming ability†			
		5.0	1.0	0.5	0.1
min					
15					0
135					6
165					21
195				10	20
225				13	25
255			8	10	24
285	28	0	10	26	28
315	32	3	18	24	
345	29	5	21	27	
375	34	12	19		
405	31	10	28		
435	28	26			
465	30	23			

* Number of colonies on erythromycin-free agar after exposure of the transformed population to 5 μg/ml of erythromycin for 1 hr.

† Number of colonies on the agar medium containing the indicated concentrations of erythromycin (μg/ml).

forming ability at the same concentration of antibiotic did not appear until 5 hr and was not complete until 7.25 hr. Table 2 also shows the expression of colony-forming ability at several challenging concentrations of erythromycin. Although the maximal number of expressed transformants was similar at all challenging concentrations, it can be seen that the lower the concentration, the earlier the expression of colony-forming ability. A similar displacement of the expression curves on the time scale with alterations in the challenging concentration of antibiotic has been observed by Fox (1959) during transformations to streptomycin resistance. Perhaps it would be well to re-emphasize at this point that the transformants selected at low concentrations of the antibiotic always showed an ability to produce colonies in the presence of a much higher concentration, characteristic of the marker *ery-r7* (Ravin and Iyer, 1961).

DISCUSSION

Current attempts to alter deliberately the physical nature of the transforming DNA molecule and to correlate such alterations with the biological activity of the molecule may be expected, in most cases, to result in a reduction in the yield of transformants (Lerman and Tolmach, 1959; Marmur and Lane, 1960; Roger and Hotchkiss, 1961). With such trends in investigation, it may become increasingly necessary to examine relatively large numbers of cells for the transformed character. The method that has been described is amenable to the examination of such large populations. With precautions to distinguish transformants from possible spontaneous mutants, transformation occurring at a very low frequency and that might otherwise go undetected would become manifest.

In this laboratory, the method has been usefully applied to the study of phenotypic expression following the integration of an erythromycin-resistance mutation, *ery-r7*. In previous studies (Ravin and Iyer, 1961), it was found that the mutation *ery-r7* needed a relatively long time for expression of resistance. This being the case, the low transformation frequency associated with this mutation could have been ascribed to the more rapid multiplication of the untransformed cells in the nonselective environment, rather than to an inherently low probability of integration. The membrane-filter technique, however, rules out the possibility of the former explanation. The present studies have shown, moreover, that, after the integration of this mutation, a stage occurs during which transformed cells express their ability to survive without being able to reproduce and to form colonies in the presence of erythromycin. A similar observation has been made for streptomycin resistance by Ephrussi-Taylor (1960), who refers to the stage of survival alone as "pseudo-resistance."

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