A NEW SYNTHETIC MEDIUM FOR DIPLOCOCCUS PNEUMONIAE, AND ITS USE FOR THE STUDY OF RECIPROCAL TRANSFORMATIONS AT THE *amiA* LOCUS¹

A. M. SICARD

*Laboratoire de Génétique Physiologique du C.N.R.S., Gif-sur-Yvette, France,² and
Developmental Biology Center, Western Reserve University, Cleveland, Ohio*

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THE investigation of genetic fine structure has progressed rapidly during the past 15 years owing to the elaboration of systems of selection with which infrequent genetic events can be quantitatively investigated. With rare exceptions, such studies have involved scoring the frequency of a unidirectional genetic change such as mutation or recombination leading to prototrophy, or to resistance to a toxic substance. Clearly, it would be very informative to be able to investigate rare genetic changes both to and from wild type at one and the same genetic site.

DNA-induced transformation provides the experimenter one of the most important known means for study of the structure of hereditary material in bacteria. Investigators have been reluctant to work with the first bacterial species shown to be transformable, *Diplococcus pneumoniae*, owing to our ignorance of its nutritional requirements, for this ignorance has limited the types of selective systems which could be developed for genetic work. Semi-synthetic or truly synthetic media have been devised (ADAMS and ROE 1945; RAPPAPORT and GUILD 1959), but have proven inadequate for the culture of strains used in this laboratory. The present report describes a synthetic medium in which our lines of *D. pneumoniae*, descended from AVERY's standard transformable rough strain, not only grow but also survive for relatively long periods of time following cessation of growth. With the development of this defined medium, it has proven possible to elaborate a selection system which specifically represses the growth of pneumococci bearing a mutation in the *amiA* locus. Since these mutations confer resistance to 1×10^{-5} M aminopterin, we have the possibility of scoring transformations of wild-type cells with a mutant DNA by selecting for resistance, and transformations of mutant cells with wild-type DNA by plating in the appropriate defined medium.

MATERIALS AND METHODS

Strains: The standard wild-type strain in this laboratory, referred to as Clone 3, was used both as recipient in certain transformation experiments, and as the parental strain from which

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² Actual address.

all mutants were derived. This strain is descended from AVERY's strain R36A (AVERY, MACLEOD and McCARTY 1944). Eleven spontaneous mutants were isolated from rare single colonies appearing when wild-type cells are incorporated in blood-agar plates containing 1×10^{-5} M aminopterin. HNO_2 -induced mutants were isolated in the following way: wild-type DNA was treated for 4 minutes in 1 N NaNO_2 , buffered at pH 4.3 with acetate buffer. The reaction was stopped by dilution into peptone-yeast extract medium at pH 7.4. After further dilution the HNO_2 -treated DNA was mixed with wild-type cells competent to transform, and the cultures incubated for two hours at 37° to allow for DNA uptake and phenotypic expression of aminopterin-resistant mutants. Samples of cells exposed to the HNO_2 -treated DNA as well as of control cells, were plated in 1×10^{-5} M aminopterin blood-agar, and the plates incubated for 36 hours. On the average, ten times more resistant colonies appear on plates made from treated cells than on plates of control cells. A colony picked from the plates made with treated cells thus stands one chance in ten of being a spontaneous mutant rather than HNO_2 -induced.

Culture methods: Stocks are maintained in medium containing 1 percent Difco Neopeptone, 0.8 percent charcoal-adsorbed Difco yeast extract, 0.85 percent NaCl and 0.025 percent glucose. Usually 0.1 ml of sterile defibrinated horse blood is added to each 5 ml culture tube of medium, to improve storage of strains at 2 to 3°C . Strains bearing *amiA* mutations grow and survive better if a small amount of sterile fresh yeast autolysate is added to the medium, and this was usually done.

The yeast autolysate was prepared by making a slurry of 250 g of commercial baker's yeast in 200 ml of distilled water, and pouring it into 800 ml of boiling distilled water. The suspension was rapidly brought again to a boil, then cooled by immersing the beaker in running tap-water. After sedimenting the cells in a Servall RC-2 centrifuge at 6,000 rev/min, the supernatant liquid was decanted and sterilized by passage through a Millipore membrane filter.

As work proceeded, the technique of freezing stocks was introduced. One tenth of a volume of glycerine was added to the cultures, which were then placed at -70°C for storage. Cells appear to survive in this state for an indefinite time.

To obtain precultures of *amiA* strains for transformation experiments, stock cultures were thawed rapidly and diluted 1:20 into 10 ml of the peptone-yeast extract medium described above, containing 0.1 ml of fresh yeast autolysate, 0.2 ml of 0.1 M glutamine solution, and 0.5 ml of 0.1 percent of asparagine solution, but no blood. After 2 hours incubation, one tenth volume of glycerine was added, the culture distributed in 0.5 ml amounts in small screw-cap tubes, and the aliquots placed at -70°C . When competent cells were desired, an aliquot of frozen preculture was thawed and transferred to 10 ml of special transformation medium. The same procedure is followed to obtain competent wild-type cells, except that the yeast autolysate must be omitted.

The special transforming medium, essentially that of EPHRUSI-TAYLOR (1951), is prepared as follows: 30 g Difco Neopeptone, 9 g KCl, 14.2 g Na_2HPO_4 and 0.1 g CaCl_2 are dissolved in 2.5 liters distilled water to which 9 ml of concentrated HCl is added. The pH of the solution should be about 3. 30 g of charcoal (MATHESON, COLEMAN and BELL "Bone Black") are added in three portions, stirring well after each addition. Adsorption is allowed to take place during 2 hours in the cold. The charcoal is removed by filtration on Whatman #12 paper. The pH is then brought to 7.8, and the heavy flocculent precipitate filtered off. The filtrate is divided into two portions, in large erlenmeyer flasks, and heated to 120°C for 20 minutes. To obtain a good transforming medium the heavy precipitate of phosphates must be filtered off *immediately upon removal from the autoclave*. The filtrate is completed to a volume of 3 liters and distributed in convenient amounts, in screw-cap erlenmeyer flasks, and sterilized. This basal medium is stable for months, and is referred to as medium NS. Prior to use, the basal medium is completed with glucose at a final concentration of 0.025 percent, and, for each 100 ml, with 8 ml of the following charcoal-adsorbed yeast extract preparation.

Twenty grams of Difco Yeast Extract are dissolved in 180 ml of distilled water, and the pH brought to 3 by the addition of approximately 12 ml of 4 N HCl. Eight grams of charcoal are added with vigorous stirring. The charcoal is then filtered off on a large buchner funnel, through Whatman #3 paper covered with a thin layer of Filter-Cel. The pH of the solution is then brought to 7.4 to 7.6 with 10 N NaOH (about 5 ml) and the heavy precipitate removed by

filtration. The filtrate is completed to a volume of 200 ml and sterilized by passage through a membrane filter. This solution is stable for several months. Occasionally a lot number of Difco Yeast Extract fails to give a precipitate following neutralization to pH 7.4, and if the resulting yeast extract solution is used to complete medium NS, it will not give rise to competent cells. It is preferable to discard such batches, though a partial correction can be obtained by adding CaCl_2 when the solution is acid, just prior to adding charcoal.

Preculture cells inoculated into the completed NS medium will give a low-level competence reaction some 80 to 120 minutes after placing the culture at 37°C . Competence will develop in an acute, highly synchronized fashion only when 0.2 percent fraction V bovine serum albumin is added. Albumin solutions are prepared at a concentration of 4 percent and at pH 2.5. They are sterilized at 100°C for 15 minutes, and stored acid, in the cold. Prior to use, they are neutralized with sterile 1 N NaOH.

Transformation was always performed in NS medium, the transformants being scored either in complete or synthetic medium, depending on their nature.

Other procedures: Techniques of preparation of DNA are described elsewhere (MARTIN and EPHRUSSI-TAYLOR 1964).

Transformation to aminopterin resistance was scored in complete agar medium (1 percent Neopeptone, 0.4 percent Difco Yeast Extract, 0.85 percent NaCl, 1.5 percent Bacto Agar, pH 7.2) into which is incorporated at the time of plating a suitable aliquot of transformed culture and 0.2 ml of defibrinated horse blood. After $2\frac{1}{2}$ hours incubation at 37°C , plates received an overlay of agar containing 2×10^{-5} M aminopterin. The two agar layers were measured, to avoid errors of concentration of aminopterin. Colonies were counted after 24 to 36 hours incubation. Transformation of *amiA* mutants to wild type were scored in synthetic agar medium. The selection procedure will be described below. These colonies were counted using a binocular microscope at a magnification of seven, since they are rather small, and colorless.

Platings of both types were made in Falcon plastic petri dishes of tissue culture quality. Determinations of growth rate in synthetic liquid medium were made in 1×7 cm culture tubes containing 2 ml of medium at 37°C , and always without agitation. All glassware was washed in dichromate-sulfuric cleaning solution followed by extensive washing, first in tap-water, and finally in glass-distilled water.

Source of chemicals: All inorganic chemicals were Mallinckrodt Analytical Reagent grade. All organic chemicals were California Biochemical Corporation quality A. Bovine serum albumin, obtained from Armour, was of two qualities: fraction V, and crystalline. When solid medium was prepared from the synthetic liquid medium, Difco Purified Bacto Agar was employed. (Standard Bacto-Agar was used to solidify complete medium.) Pyrex-distilled water was used for all solutions entering into the synthetic medium.

EXPERIMENTS

The synthetic medium: The synthetic medium, whose composition is shown in Table 1, was derived by introducing a number of modifications into the one synthetic medium published for pneumococcus (RAPPAPOORT and GUILD 1959).

The various modifications were tested in the following way: cells growing exponentially in complete medium (peptone and yeast extract) were centrifuged, washed twice in balanced salt solution (Solution I, Table 1), and suspended in synthetic medium. A 10^{-2} dilution of the final suspension was inoculated into the growth tubes.

After a latent period of about 2 hours, exponential growth is initiated at a rate of one generation per hour. The maximal density obtained in the medium is 10^8 cells/ml. The limitation in growth is not due to the concentration of glucose or to a drop in pH, but the nature of the limitation is not established. Chains of cocci

TABLE 1
Synthetic medium for Diplococcus pneumoniae

I. Minerals and buffer		II. Amino acids	
NaCl	5 g	L-Arginine	200 mg
NH ₄ Cl	2 g	L-Asparagine	10 mg
KCl	0.4 g	L-Cysteine-HCl	100 mg
Na ₂ HPO ₄	0.12 g	L-Glutamine	20 mg
MgSO ₄	0.024 g	Glycine	120 mg
CaCl ₂	0.010 g	L-Histidine	150 mg
FeSO ₄ 7H ₂ O	0.00055 g	L-Isoleucine	6.55 mg
Tris-(hydroxymethyl) aminomethane	4.84 g	L-Leucine	6.55 mg
Glass distilled H ₂ O	1 liter	L-Lysine	420 mg
pH	7.55	L-Methionine	180 mg
III. Vitamins		L-Threonine	175 mg
Biotin	0.015 mg	L-Valine	5.85 mg
Choline	5.0 mg	IV. Other	
Nicotinamide	0.6 mg	Glucose	4 g
Pantothenate, Calcium	2.4 mg	Pyruvate, Sodium	0.8 g
Pyridoxal HCl	0.6 mg	Uracil	1 mg
Riboflavine	0.3 mg	*Linoleic Acid	10 ⁻⁷ g
Thiamine	0.6 mg	*Spermidine, Phosphate	10 ⁻⁵ g

* Can be replaced by 0.8 g of fraction V Armour Bovine Albumin.

In practice, balanced salt solution is prepared, and all amino acids and uracil dissolved in it except glutamine and cysteine. This is distributed and stored in 200 ml amounts and sterilized by autoclaving. Just prior to use, a flask is completed by adding 3.2 ml of 25 percent glucose, 4 ml of 4 percent albumin solution, and 2 ml of a concentrated solution containing vitamins, glutamine, cysteine and pyruvate. The latter solution is made at 100× final concentration, sterilized by filtration and stored at -70°C.

of variable length appear toward the end of growth. A culture can be initiated in this medium with fewer than 10 cells per ml as inoculum.

When the synthetic medium is solidified with purified agar, and cells incorporated in the agar medium, visible colonies are formed by 24 hours at 37°C. In practice, 3 ml of molten 2 percent agar (in distilled water) is mixed with 7 ml of synthetic medium, and poured immediately onto the sample of washed cells to be plated, yielding a final agar concentration of 0.67 percent. Plating efficiency, compared to that in complete blood agar, is 100 percent, but colonies lying near the surface may be very small.

The synthetic medium appears to support very adequately growth of the wild-type strain, Clone 3, for after serial transfer in synthetic medium for 100 generations no detectable change has been observed in its properties. In particular, when the cells were returned to complete medium and tested under standard conditions for their transformability, their behavior was entirely typical.

The following facts have been ascertained, concerning the required nutrients:

Asparagine and uracil must be present at a level of at least 0.5 µg/ml, and glutamine at a level of 10 µg/ml. Neither glutamic nor aspartic acids replace the corresponding amides. While uridine can be utilized in place of uracil, orotic acid cannot. Serine and glycine are interchangeable, as are proline and arginine. Lysine is required at a level of at least 1 µg/ml, but is spared if histidine is added. Biotin, riboflavin and threonine, added individually, improve growth somewhat, without, however, being indispensable. If bovine serum albumin (either Fraction V or

crystalline) is added in the place of linoleic acid and spermidine, growth is more rapid, the generation time falling to 36 minutes.

In order to have good growth, L-valine, L-leucine and L-isoleucine must be present in approximately equal amounts. An excess of any one or two of these branched amino acids can inhibit growth. Furthermore, in experiments in which one of the three is varied and the others are held constant, inhibition has been found to be competitive. The minimum concentration of these amino acids, which seem to be required, is 10^{-6} M. Experiments were also performed which showed that neither the D-amino acids nor the glycyl dipeptides containing the L-forms supported growth of the strains examined. Furthermore, none of these latter substances enter into competition with the L-amino acids in question.

Table 2 shows the ability of a variety of strains to grow in the synthetic medium. Strains of distinct origin are able to grow; i.e., Type I strain SV-1, Type II strain D39 and its rough derivative R36A (from which Clone 3 was isolated), as well as strain M3R, a rough derivative from Type III strain A66. The requirement for uracil is also shown in Table 2. While Clone 3 requires this pyrimidine, strains from which it arose do not. Clone 3 appears to have become uracil⁻ sometime during 1960, since most of the mutants selected from it during 1960 and thereafter, are also ura⁻. Mutants selected within Clone 3 prior to 1960 proved all to be ura⁺ (strains ami-r1 and 2, for example). Table 2 also summarizes data on the level of resistance of the aminopterin-resistant mutants selected within Clone 3. All except two (1 and 2) show identical resistance levels. Although aminopterin resistance did not prove to be associated with increased auxotrophy, all resistant strains are particularly sensitive to an imbalance in concentrations of branched amino acids. While wild-type cells will grow when isoleucine concentration is raised to 20×10^{-4} M, aminopterin-resistant cells will not. This observation has served as a basis for the elaboration of selective screening for aminopterin-sensitive cells, as will be described below. Finally, it will be noted that strain 14 is unable to grow in synthetic medium. When the *ami-r* gene of this

TABLE 2

Nutritional characteristics of various strains

	Strain								
	SV-1	M3R	D39	R36A	Clone 3	1, 2	3-13 15-16 18-29	14	17
Molarity $\times 10^{-5}$ of aminopterin resisted.					0.04	4	1	1	1
Growth in synthetic medium.	+	+	+	+	+	+	+	—	+
Growth in synthetic medium + 20×10^{-4} M isoleucine.					+	—	—	—	—
Growth without uracil	—	+	+	+	—	+	—		+

SV-1, Type 1 encapsulated strain. M3R, strain with reduced capsule, derived by mutation from Type III strain A66. D39, Type II encapsulated strain. R36A, rough strain derived from D39. Clone 3 and all *ami-r* strains are derived from R36A. Strains 1, 2 and 21 to 29 are spontaneous mutants, while strains 3 to 20 are HNO₂-induced.

strain is transferred to Clone 3, the resulting resistant cells do grow in synthetic medium. Strain 14 contains, therefore, a second mutant gene unrelated to aminopterin resistance, responsible for increased auxotrophy. The nature of this requirement is under investigation.

Selective recognition of aminopterin-sensitive cells: The observation that aminopterin resistant strains are more sensitive to an imbalance of branched amino acids has been exploited to develop a system for selecting aminopterin-sensitive cells, either mutants or transformants, in resistant populations. Although an excess of any one of the three amino acids will lead to suppression of growth of resistant cells, isoleucine has been chosen as the amino acid in excess because, on a molar basis, it is about five times more effective than the two others, in the albumin-containing medium described above.

The validity of the selection method has been evaluated in the following way: a known number of wild-type cells was mixed with an excess of bacteria from strain 5. Suitable dilutions of the mixed culture were pour-plated in a layer of synthetic agar medium. As soon as the agar had solidified, a second, equal layer was added, containing 20×10^{-4} M isoleucine. After incubation for 24 to 36 hours, colonies appear, and their number corresponds to the number of wild-type cells in the mixed inoculum. Upon isolation, these colonies prove to be composed of aminopterin-sensitive cells. Selection operates equally well if the second layer is added as late as 3 hours after the first has been made. The range of isoleucine concentrations permitting growth of wild type and suppressing growth of aminopterin-resistant cells is relatively large (5 to 25×10^{-4} M). Similar results are obtained when strain 5 is replaced by another aminopterin-resistant line. With this system, therefore, it is possible to select with 100 percent efficiency aminopterin-sensitive cells, and these alone, in populations of aminopterin-resistant cells.

Genetic applications of the new selective system: Since quantitative recovery of ami-s cells among ami-r cells proved possible, two applications of this selective system have been explored:

(1) *The selection of cells bearing reverse mutations, in ami-r populations.* Since reversions are rare, large numbers of cells must be plated in the synthetic medium. In practice, cultures which have been grown to a density of about 2×10^8 cells in complete medium are employed. The cells are spun down, the complete medium decanted, and the cells are then washed once with 5 ml of the salt solution shown in Table 1. The cells are suspended in a final volume of mineral solution equal to the volume of the original culture, and 0.01 ml of this suspension is plated as described, in synthetic medium. As soon as the agar is firm, a second layer containing 40×10^{-4} M isoleucine is added, and the plates are incubated for 36 hours before counting. Using this technique, isoleucine-resistant reverse mutations have been detected in ami-r strains 1, 6, 10, and 22.

(2) *The selection of transformants in crosses of ami-r recipient cells by various DNA preparation:* In these experiments, competence being high, it proved possible to perform the transformations in complex media, and plate dilutions of the cell suspensions directly in the synthetic medium without washing the cells. Dilutions were made in the salt mixture of the synthetic medium to which serum albumin

was added at a concentration of 0.02 percent. Cells remain viable for hours in this diluent. Small volumes (0.1 to 0.4 ml) of the appropriate dilution were plated in 5 ml of synthetic agar-medium, in small plastic dishes, and after an incubation period appropriate for phenotypic expression (see experiment below) an overlayer is made with 0.6 percent agar containing isoleucine. The concentration of isoleucine in the overlayer was varied from 40 to 20×10^{-4} M depending on the final concentration of NS medium carried over with the inoculum (0.1×10^{-1} to 0.1×10^{-3}). The range of volumes of the transformed culture plated in synthetic medium depended on the cross, i.e., the expected frequency of wild-type transformants. The details of these crosses will be discussed below, and in a second publication which is in preparation.

Genetic analysis of ami-r strains 1 and 2: These two strains are able to survive and grow at higher aminopterin concentrations than are tolerated by the other resistant strains.

When strain 1 is cultivated serially for a considerable period, revertants always appear, in sufficient numbers to be detectable by replica plating. The revertants can also be recognized in the selective, synthetic medium, and the numbers found are concordant by the two methods. However, although these revertants are wild-type with respect to resistance to isoleucine, and no longer resist 4×10^{-5} aminopterin, they prove to resist 0.15×10^{-5} M of the analogue, while wild-type cells will not tolerate more than 0.04×10^{-5} M.

This result would be explained if strain 1 were to contain two resistance genes *amiA* and *amiB*. Gene *B* would confer resistance to 0.15×10^{-5} M, and have no altered nutritional requirement, while gene *A* would be responsible for higher resistance to aminopterin, and sensitivity to unequal amounts of the three branched amino acids.

This hypothesis has been established by the following transformation experiments: (1) Clone 3 has been transformed by DNA from strain 1, and the transformants resisting at different levels of aminopterin scored. Figure 1 shows that the number of cells growing in aminopterin remains constant until the level of 1×10^{-5} is exceeded, dropping rapidly to a value which remains constant through 4×10^{-5} M. This result showed the genetic complexity of strain 1, but failed to reveal the three phenotypes expected from two genes (*A*, *B* and *AB*). Clearly, a class of transformants resisting at 0.15×10^{-5} M, corresponding to *B* transformants, was expected on the basis of the hypothesis. The simplest explanation of these results would be that gene *B* is a low-efficiency gene which transforms with far less frequency than gene *A*. The following experiments demonstrate that this is in fact so.

(2) From the transformants in the preceding experiment a clone was set up which resists 1×10^{-5} M aminopterin, but not 4×10^{-5} M. These cells were transformed with DNA from strain 1, yielding transformants resistant to 4×10^{-5} M. The efficiency of this transformation can be measured relative to a reference gene, by introducing gene *str-r41* into strain 1, and preparing a DNA from it containing the *ami-r* and *str-r41* genes. The efficiency of transformation of *A*-bearing cells to *AB* proves, indeed, to be 0.1 with respect to the reference gene *str-r41*.

FIGURE 1.—Resistance levels of transformants appearing in cross of Clone 3 by DNA of strain *ami-r1*.

The same DNA introduced into Clone 3 yields *ami-r* transformants resistant to 1×10^{-5} M aminopterin at a frequency equal to that of *str-r* transformants. These results are consistent with the hypothesis that strain 1 contains the two aminopterin resistant genes, *A* and *B*, but that *B* transforms with one tenth the frequency of *A*, and of *str-r41*.

(3) Using the synthetic medium, it is possible to score directly *amiB* transformants in Clone 3. Wild-type recipient cells are prevented from growing by a low level of aminopterin in the synthetic medium, while *amiA* transformants are inhibited by isoleucine (MICHEL, SICARD and EPHRUSSI-TAYLOR 1964). The relative frequency of *amiB* to *str-r41* transformants proves also to be 0.1 in this experimental system.

We recognize, thus, two resistance genes in strain 1: *amiA* (1×10^{-5} M), *amiB* (0.15×10^{-5} M) which, when present together confer resistance to 4×10^{-5} M.

The *A* and *B* genes are not closely linked, since the frequency of their simultaneous transfer is very much lower than the frequency of the transfer of either to a recombinant chromosome. Nonetheless, they are linked, though distant, as has been shown in appropriate experiments (MICHEL, SICARD and EPHRUSSI-TAYLOR 1964).

Strain 2 has shown no reversion as yet. However, through transformation experiments it has been found that it resembles strain 1 in that it contains an *A* and a *B* gene, conferring resistance levels, both singly and in combination, identical to the *A* and *B* genes in strain 1. Since the two strains are of independent origin, we shall refer to the genes in strain 1 as *amiA-r1* and *amiB-r1*, and those of strain 2 as *amiA-r2* and *amiB-r2*.

Genetic analysis of the remaining aminopterin-resistant strains: When Clone 3 is transformed by DNA from any one of the remaining aminopterin-resistant strains, a single type of resistant transformant is produced: resistant to 1×10^{-5} M aminopterin, and sensitive to an imbalance of branched amino acids.

In order to be sure that these two characteristics arise from mutation at a single locus, the following experiment was performed. Clone 3 was transformed by DNA from strains 1, 5, 13 and 18. A single transformant, resistant to 1×10^{-5} M aminopterin, was isolated from each cross, DNA prepared from each of these lines, and Clone 3 transformed now by these DNAs. Then, five clones were isolated, and their properties examined. In every instance, the resistance level was 1×10^{-5} , and sensitivity to isoleucine was unchanged. These two characteristics seem to be a unit character, due in every instance to a single genetic change—mutation in the *amiA* gene.

It has been noted that differences exist in the growth requirements of some of the strains under study (strain 14, an unknown requirement; strains 1 and 2 and 17 are *ura*⁺). Each of these nutritional differences is separable by recombination, from the *amiA* gene.

Transformation of resistant strains to wild type: In the preceding experiment, the *amiA* functional gene has been defined by showing that every cell resistant to 1×10^{-5} M aminopterin is also sensitive to isoleucine. Other nutritional differences between *ami-r* strains and wild-type Clone 3 can be recombined away from the aminopterin resistance gene, but isoleucine-sensitivity cannot. The unity of these two genetic properties can be shown by the reverse transformation: the transformation of isoleucine-sensitive cells to isoleucine resistant ones, with the concomitant loss of aminopterin resistance. When a strain resistant to 1×10^{-5} M aminopterin is treated with DNA from an aminopterin sensitive strain, and isoleucine-resistant transformants recovered in the selective synthetic medium, every transformant proves to be not only isoleucine resistant, but also aminopterin sensitive. Again, the two properties can be considered determined by unitary change in the *amiA* gene.

The quantitative nature of the selection method of *amiA-s* (isoleucine resistant) transformants has been investigated further by examining the characteristics of phenotypic expression of the *amiA* wild-type gene.

A competent culture of strain *amiA-r1* was given a 4 minute exposure to *amiA-s* DNA. Suitable dilutions were plated in synthetic agar medium, and the plates were incubated for various time intervals before receiving an overlay of isoleucine-containing agar. The number of colonies appearing in these plates after further incubation proved to be constant, even when as long as 6 hours elapsed between plating and overlaying. One can study phenotypic expression in complete liquid medium by plating the DNA-bacterial complexes, at later and later times, directly into synthetic medium containing an excess of isoleucine. In this instance the number of isoleucine-resistant colonies recovered remains constant for an hour, and then increases because of transmission of the acquired character to new colony-forming units in the course of growth of the population. Transformation incidence measured by either technique proves to be the same. One can

thus be confident that all of the wild-type transformants are recovered by the agar overlay selection technique. Figure 2 presents data of an experiment on phenotypic expression. The apparent early resistance of transformants to isoleucine means either the *amiA-s* gene is very rapidly expressed, or isoleucine is a sufficiently mild poison so that phenotypic expression can occur in its presence.

Testing for allelism between amiA-r mutants: The occurrence of wild-type transformants in crosses of mutant cells by mutant DNA is proof of recombination between genetic sites. Furthermore, as has been stressed by LACKS and HOTCHKISS (1960), if in any such cross, the frequency of formation of wild-type cells is less than that observed in crosses of mutant cells by wild-type DNA, we can consider the recombining sites as linked.

A series of crosses of mutant cells by mutant DNA was performed, the details of which will be discussed in a publication now in preparation. The *A* mutations of the 29 *ami-r* strains were found to be located in 20 different genetic sites. It should be stressed here, that all appear to be linked, and that it has been impossible to find in these crosses transformants resistant to more than 1×10^{-5} M aminopterin. There is no additivity of two such mutant sites at the phenotypic level. However, the transformation of any one strain by DNA containing an *amiB-r* gene leads in all cases to cells which resist as do strains 1 and 2. Further, in every instance the efficiency of the *amiB-r* gene proves to be 0.1 with respect to both *amiA-r1* and *str-r41*.

These results are further evidence that all of the *amiA-r* genes belong to a single functional genetic unit, as already stressed above. The unit will be referred to, henceforth, as the *ami-A* cistron, for brevity, although a cis-trans test is not yet possible in transformation systems.

Test for complexity of amiA-r1 mutations: A system for testing for recombination at a particular genetic site by transformations both to and from wild type makes it possible to determine whether a particular mutant has a simple or com-

FIGURE 2.—Phenotypic expression of transformation of *amiA-r* cells to *amiA-s*. Competent *amiA-r1* cells are given *amiA-s1* DNA for 15 minutes. The reaction is stopped by the addition of DNase for 1 minute and the culture is diluted $100 \times$ in fresh complete medium. At the indicated times, samples are withdrawn and plated in synthetic medium containing isoleucine. *amiA-s* colonies were scored after 36 hours incubation. Generation time in complete medium is 25 minutes.

plex recombination behavior. The utility of such crosses (one-point reciprocal crosses) has been proposed by EPHRUSSI-TAYLOR (1963). The *amiA-r1* mutation has been most thoroughly examined in this respect (see Table 3). A first test consists of comparing the frequency of transformation in reciprocal crosses; i.e., the frequency with which an *r* mutation replaces its wild-type (*s*) allele in a wild-type genome, as opposed to the frequency with which the *s* allele replaces the *r* allele in a resistant genome. Were recombination occurring within a multi-site mutation, one might expect the crosses to yield nonequivalent numbers of transformants in the two directions. Neither *amiA-r1* nor any of the 30 mutants examined do, in fact, show such behavior, when transformation in the pairs of reciprocal crosses is compared with respect to the nonlinked reference gene *str-r41*, present in all DNAs. (The ratio of the number of *ami-r* or *ami-s* to *str-r41* transformants measures the efficiency of the particular transformation.)

Five clones of cells independently transformed by the *amiA-r1* gene were set up. Sublines of these clones were marked with the *str-r41* genes and DNA isolated from them. The efficiency of the five *ami-r* genes was again measured on Clone 3, and found to be like that of the parental *amiA-r1* gene (see Table 3, transformation 3). The five clones were also tested by transforming them back to wild type (transformation 4), where the *s* gene was found to have the same efficiency as either the original *r*, or the derived *r* genes. Finally, an *amiA-s* clone was set up from transformation 2 of Table 3, and found to be transformable back to resistance with the same efficiency as Clone 3.

No evidence for recombination within the *amiA-r1* gene could be found.

DISCUSSION

The elaboration of a synthetic medium for the pneumococcal strains currently employed in transformation work in this laboratory has established the nutritional requirements of these strains. It is interesting to note that already in 1950, requirements for certain substances had been demonstrated in closely related strains (ADAMS and ROE 1945; BADGER 1944; BERNHEIMER, GILLMAN, HOTTLE, and PAPPENHEIMER 1942; GILBERT 1944; HOEPRICH 1957; RANE and SUBBAROW 1940, 1940a). This is true for glutamine and asparagine. However, it is certain that appreciable differences are now to be found between pneumococcal strains of common origin, cultivated in different laboratories. Indeed, the medium described by RAPPAPORT and GUILD (1959), which differs in many ways from the present one, will not permit growth of our strains. Furthermore, two of our strains, R36A and Clone 3 derived therefrom, cultivated separately since 1956, differ with respect to uracil requirements.

Knowledge of the amino acid requirements of pneumococcus reveals several interesting facts. Since arginine and proline are interchangeable in the medium, it appears that pneumococcus possesses an interconversion system for these amino acids, as does *E. coli* (VOGEL, ABELSON, and BOLTON 1953). The ability to use glycine or serine indicates the presence of a one-carbon transfer system and serine hydroxymethylase (HATCH, TAKEYAMA and BUCHANAN 1959). On the

other hand, how histidine and lysine can exert a sparing effect on each other is not evident from published work.

The effectiveness of albumin in improving growth can be explained by its affinity for fatty acids, long-chain fatty acids being required by our strains (DAVIS and DUBOS 1947). Presumably, the required acids are released in small amounts into the medium without reaching a toxic level.

Competition between branched amino acids could be taking place at one or more levels of cell function: at the level of active transport across the cell membrane (COHEN and RICKENBERG 1956), or at the level of incorporation into protein, for example. The constant associated between resistance to a certain level of aminopterin and sensitivity to an imbalance of these amino acids could possibly be explained if we were in possession of a detailed biochemical analysis of mutant and wild-type strains. Although the biosynthetic pathways of the branched amino acids are not known to involve enzymes with tetrahydrofolic cofactors, our knowledge of these pathways is incomplete. Such a cofactor-dependent step may in fact exist. These amino acids have, however, been implicated in studies of inhibition of cellular functions by aminopterin (WEBB 1958) and sulfanilamides (WINKLER and DE HAAN 1948). In particular, WEBB (1958) has observed the accumulation of valine when *Acetobacter aerogenes* is cultivated in the presence of sublethal concentrations of aminopterin. It is, thus, conceivable that resistant strains utilize greater amounts of branched amino acids following an alteration of regulation of their biosynthesis, in which a folic acid derivative seems to be operating.

The genotypes of all the strains examined in the present study have been determined. Two units, distinct both genetically and functionally, have been defined. One, the *amiB* locus, increases aminopterin resistance by a factor of 4 to 5, while the other, *amiA*, increases resistance by a factor of 20. Together, they increase resistance 100-fold, as would be expected from functional independence.

The application of the synthetic medium for selection of *ami-s* cells has rendered possible a quantitative study of transformation both to and from resistance, in one-point crosses. Each of the 30 mutants examined exhibits the same efficiency of transformation in either direction: efficiency is clearly determined by the site of the selected recombination event, not the direction of the cross. Indirect methods which depend upon selection of a linked gene have led to the same conclusion (GOODGAL 1961). In the experiments described in Table 3, the efficiency of the *amiA-r1* or *s1* gene was 1.1 with respect to the reference gene *str-r41*. However, the majority of the 29 *amiA* mutants studied show, in fact, an efficiency of 0.1 (SICARD and EPHRUSSI-TAYLOR, in preparation). Yet the efficiency is the same in either direction of transformation. The implication of this result, insofar as fine structure transformation studies are concerned, is of some importance. A mutation can be either (a) point, by alteration or loss of a nucleotide pair, or (b) multisite, through alteration of several contiguous or noncontiguous nucleotide pairs, or (c) a deletion, if a sequence has been lost. Equivalent transformation frequencies in reciprocal crosses are expected only if the modification of the genome is limited to a small number of nucleotides. Such seems to be the

TABLE 3

Summary of results of various crosses designed to test for complex recombination behavior of mutant site *amiA-r1*. See text for explanation

Transformation number	Recipient	Donor	$\frac{\text{amiA-s or r}}{\text{str-r41}}$
1	<i>amiA-s1</i>	<i>amiA-r1 str-r41</i>	$\frac{\text{amiA-r}}{\text{str-r}} = 1.18$
2	<i>amiA-r1</i>	<i>amiA-s1 str-r41</i>	$\frac{\text{amiA-s}}{\text{str-r}} = 1.03$
3	<i>amiA-s1</i>	<i>amiA-r1 str-r41</i> transformed from 1	$\frac{\text{amiA-r}}{\text{str-r}} = 1.07$
4	<i>amiA-r1</i> transformed from 1	<i>amiA-s1 str-r41</i>	$\frac{\text{amiA-s}}{\text{str-r}} = 1.18$
5	<i>amiA-r1</i>	<i>amiA-s1 str-r41</i> transformed from 2	$\frac{\text{amiA-s}}{\text{str-r}} = 1.1$

case for the 29 mutants under study. The possibility remains that some of them may represent the alteration of a very small number of base pairs, which might escape detection by the reciprocal crossing method. This question will be considered in another publication (SICARD and EPHRUSSI-TAYLOR, in preparation).

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SUMMARY

A completely synthetic medium suitable for cultivation of a number of strains of pneumococci has been developed. The medium has been used in a modified form, in which bovine serum albumin replaces the requirement for linoleic acid and spermidine, for the study of genetic changes to and from wild type in the *amiA* gene. Mutations of this gene confer a moderately high level of resistance to aminopterin, and, concomitantly, sensitivity to an imbalance in the relative concentrations of isoleucine, leucine and valine. In practice, the growth of aminopterin resistant cells was selectively inhibited by increasing the amount of isoleucine, and holding the amounts of the other two amino acids constant. The two properties, present in 29 independent mutants, have not been separated either by reverse mutation or by recombination during DNA-induced transformation. It is concluded that the two properties of each of the mutants are manifestations of a unitary change in a single functional gene, the *amiA* gene. Since growth of cells mutant at this locus is selectively inhibited by isoleucine, it is possible to score quantitatively transformations to both the mutant and wild-type state. One-point reciprocal transformations have been performed with the majority of the mutants and have shown the same recombination frequency in both directions of

crossing. A second gene, *amiB*, conferring low-level resistance to aminopterin, has been identified by the use of the synthetic medium. Mutations at this locus are not associated with any detectable nutritional alteration.

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