STEPWISE INTRODUCTION OF TRANSFORMABLE PENICILLIN RESISTANCE IN PNEUMOCOCCUS[1]

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WITH the introduction of drug resistances into Pneumococcus by DNA transformation (HOTCHKISS 1951), it became clear that heritable traits not presumably connected to the classic capsular antigens (AVERY, MACLEOD and MCCARTY 1944; AUSTRIAN and MACLEOD 1949) could also be carried by DNA. Certain practical and conceptual developments were derived from this finding. Selective resistance markers allowed quantitative estimation of the number of cells converted, the quantitative improvement of transformation recipes, and the determination of the degree of independence of various marker pairs. Independence of penicillin and streptomycin resistance markers from capsule-type transforming agents was interpreted as indicating the existence of differently marked regions of DNA (considered then to be separate molecules) within the total DNA of a single cell line (HOTCHKISS 1951). The specificity of resistance-transforming units for each kind of drug, and the correspondence of each unit quantitatively to the spontaneous mutational steps of cumulative penicillin resistance, indicated the close parallelism of DNA-carried factors and the units of mutational history present in the cell lines.

The original data on penicillin-resistant Pneumococci were briefly reported in 1951 and summarized thereafter at various times. Other demonstrations of transferable drug resistance were soon reported (ALEXANDER and LEIDY 1953) and served, as have later developments in bacterial, and eventually bacteriophage, genetics to demonstrate that DNA can bear a wide variety of traits besides surface antigens. Meanwhile, modifications in the Pneumococcal strains, markers, and techniques used have made it unfeasible to reproduce precisely the conditions of the early experiments. The present article reports a reexamination in the light of modern techniques, of the system which played a considerable part in fashioning our early views on the molecular basis of genetics. In it we describe four identifiable units of penicillin resistance in currently available material, their transfer by DNA to recipient cells, their cooperative phenotypic effects in multiply marked cells, and the indications of certain linkages exhibited by some of them.

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MATERIALS AND METHODS

Strains: Commencing with nonencapsulated wild-type Pneumococcal strain R6, spontaneous mutants resistant to penicillin were derived in three successive selection steps. Ten ml of a culture (10^9 to 10^{10} colony-forming units) were mixed with 90 ml of melted neopeptone agar medium containing 2 percent defibrinated rabbit blood and appropriate concentrations of the potassium salt of penicillin G. The mixture was poured into plates and incubated at 37°C for 2–6 days. Surviving colonies were inoculated individually into tubes of drug-free neopeptone broth and grown for several generations. Progeny of single-cell isolates from these cultures were employed for the preparation of DNA. Following an established principle of this laboratory, the stock culture lines were maintained in absence of drug, and exposed to penicillin only once in their entire history for each mutation step.

Transformation and assay procedure: In initial experiments, purified DNA prepared by a procedure described by HOTCHKISS (1957) based on the method of McCARTY and AVERY (1946) was used. In some later experiments, less purified DNA preparations were employed. Transformations were performed in the semisynthetic medium described by LACKS and HOTCHKISS (1960). Recipient cells were grown to competence (usually 2×10^7 per ml) and maintained as such by storage at −20°C after the addition of glycerol to 10% concentration. For a transformation experiment, the frozen cultures were thawed at 0°C, diluted 1:50 to 1:100 in the transforming medium and DNA added to a final concentration of 1–2 μg/ml. After 30 min incubation at 30°C excess DNase (about 2 μg/ml of Worthington Chemicals Corp. bovine pancreatic deoxyribonuclease) was added. The cells were then incubated an additional 120–180 min at 37°C to allow for phenotypic expression. The transformants were assayed in liquid medium of the following composition: 1 liter of freshly prepared neopeptone-meat infusion broth, 25 ml 0.5M K_2HPO_4, 25 ml 4% albumin, 10 ml fresh yeast extract, 0.5 g glucose and sufficient anti-Pneumococcal antiserum globulin to ensure clonal colony formation. The transformed cells were serially diluted into media containing the desired concentration of penicillin. After 16 hr incubation at 37°C, each transformant had given rise to a colony visible at the bottom of the tube. In later portions of this investigation, an improved medium was employed similar to that used for the sulfonamide resistance system (MINDICH and HOTCHKISS 1964) in which the yeast extract was replaced by a mixture of shikimic acid, adenosine, thymine, enzymatic casein hydrolysate, and sucrose.

In some instances it was desirable to compare the frequency of transformation of various recipients by penicillin markers with that obtained on the same day with a standard reference marker upon the same batch of cells. The two reference markers were used, the streptomycin and the sulfonamide *d* (*sul*-d) resistances (HOTCHKISS and EVANS 1958). It should be noted that, contrary to the assumption made by several authors, this specific high streptomycin-resistance marker used in this laboratory is not identical with an earlier one. The former one was used until about 1962, was supplied to Dr. H. EPHRUSSI-TAYLOR about 1952 and a passage of it was later designated by ROTHEIM and RAVIN (1964) as $str\text{-}r_{41}$. We designate our currently used marker as *str*-rvii to distinguish it from their numerical series.

Because of the relatively slow expression of penicillin resistance, overall transformation yields are expressed as "net yields"–i.e., percent of total colonies at time of scoring, rather than the larger but uncertain "actual yield" of transformants per 10^2 colony-forming units at the time of exposure to DNA.

RESULTS

Mutant selection: Summarized in Table 1 are the penicillin-resistance levels in liquid media of wild type and first-, second- and third-step mutants selected initially on penicillin-agar plates. Slight fluctuations in the values can occur in different experiments; however, the relative sensitivities of the various strains remain the same. Exposure of wild-type cultures to 0.05 units/ml of penicillin

TABLE 1

Penicillin resistance levels of wild-type and mutant strains—small inoculum

Strain	Maximum concentration of penicillin permitting 75 to 100 percent of colonies to develop	Minimum concentration of penicillin completely preventing colony formation
Wild-type R6	0.01	0.018–0.02
First-step mutant RP1	0.025	0.035
Second-step mutant RP2	0.05	0.06
Third-step mutant RP3	0.12	0.13–0.15

regularly permitted survival of about 4 to 8 colonies per 10^9 cells. Single-colony strains of such first-step mutants had about twice the resistance of wild strains; reinoculated upon this same (0.05 units/ml) concentration of penicillin, they did not grow massively but gave rise to a second class of mutants. These second-step mutants were approximately twice as resistant as first-step mutants. Finally, a third class of mutants, showing a further two-fold increase in resistance, was obtained when second step mutants were exposed to 0.05–0.07 units/ml of penicillin. The emergent colonies at any one selection step, when several were more closely examined, showed no significant differences in their resistance levels. The occurrence of penicillin-resistant mutants was found to be about 1 to 10 per 10^9 cells for each of these mutation steps.

It is to be noted that the third-step mutants withstand a considerably higher concentration of penicillin than that used in the selection procedure. Several attempts to recover mutants at higher drug concentrations were unsuccessful. The survival of rare resistant cells upon solid media can be prolonged by the presence of a large population of more sensitive cells dying in the presence of penicillin. Reconstruction tests performed in liquid media indicated that at a ratio of 1 mutant to 10^5 or more sensitive cells, growth of the mutant is suppressed at a concentration of penicillin normally sublethal for the mutant. These findings are similar to certain of those reported by SAZ and EAGLE (1953) for what they designated a co-killing phenomenon.

It was observed that the first-step mutant colonies appeared about 2 days after exposure of wild cells to penicillin. Second- and third-step mutant colonies appeared 4 and 6 days, respectively, after the selecting agent had been imposed on the appropriate populations.

Pattern of appearance of resistant transformants during expression: Transformations of wild type with DNA from a first-step mutant and a third-step mutant are described in Tables 2 and 3, respectively. The pattern obtained using DNA from second-step mutants is similar to that obtained with that of third-step mutants, but will be discussed in more detail below. The recipient cells employed were from frozen aliquots of the same culture and, therefore, in each instance were of equivalent competence. Thus, the yields of transformants at a given level of antibiotic presumably express genetic differences in the two DNA preparations.

TABLE 2

Rate of appearance of transformants after transformation of wild type with DNA from first-step mutant RP1

Concentration of penicillin units/ml	Number of resistant colonies at indicated times after exposure to DNA				Apparent yield at 180 minutes percent
	0 min	60 min	120 min	180 min	
0	130,000	160,000	800,000	3,200,000	...
0.020	0	460	1,550	1,240	0.04
0.025	0	30	360	1,550	0.05
0.030	0	0	100	690	0.02

The apparent yield at 180 min, multiplied by the selective factor of increase (20) for wild/transformants during 180 min, indicates that nearly one percent of the exposed cells were transformed. The correction factor for a difference in colony-forming units or chains—calculated as: (number of resistant cells per average resistant chain)/(number of total cells per average chain)—although less than 1 at early stages of expression, becomes approximately 1 by 180 min and can be ignored.

Transformation of wild type with DNA from the first-step mutant RP1 (and also from three independent transformants obtained from this interaction) yielded one class of transformants, with a uniform resistance level equal to that of the donor strain. The net yield of transformants was at first low (see Table 2) because of difficulties of selection in the earlier medium. In later studies employing the improved medium and scoring at 0.015 units/ml of penicillin net yields of 0.5 to 2 percent were routinely obtained (indicating perhaps 3 to 10 percent transformation at the time of treatment with DNA).

Transformation of wild type with DNA of third-step mutant RP3 produced two classes of transformants (Table 3). The small number of transformants which grew at 0.04 and 0.05 units/ml of penicillin were phenotypically similar to the second-step mutants. At lower drug concentrations two phenotypes were found. These types were established by testing of several single-colony lines in a series of concentrations of penicillin. In all cases the two types that could be recognized conformed phenotypically to first- and second-step mutant classes. More resistant transformants were not detected.

TABLE 3

Rate of appearance of transformants after transformation of wild type with DNA from third-step mutant RP3

Concentration of penicillin units/ml	Number of resistant colonies at indicated times after exposure to DNA				Apparent yield at 180 minutes percent
	0 min	45 min	90 min	180 min	
0	230,000	260,000	550,000	7,600,000	...
0.02	1,550	13,900	27,900	186,000	2.4
0.03	1,320	500	42,000	110,000	1.5
0.04	30	250	50	2,000	0.025
0.05	0	0	30	2,600	0.035

In this experiment there is at each level of penicillin a rather uneven course of increase (or of chain scission), but each gives at one time an abrupt increase in survivors. From smoothed growth curves, the actual yields indicated here seem to be about 4, 5, 0.85 and 1.0 percent at the respective levels, when based on colonies present at time of DNA exposure.

Although resistance to 0.02 units/ml of drug could often be detected at 0 min in a small number of cells, it appears clear (Table 2) that, in keeping with the results of HOTCHKISS (1951), at least 120–180 min are required for substantial expression of penicillin resistance markers.

Identification of unit markers from RP2: Transformation of R6 with DNA prepared from the second-step mutant, RP2, produced a large class of low-resistance transformants surviving 0.025 to 0.030 units/ml of penicillin. Since RP2 was derived from RP1 in a single selection step, it was expected that this population of transformants might contain two resistant types, exhibiting separately a marker from the first-step (*pen*-r1) and another (*pen*-r2) acquired in the second-step mutation. The separation of these was accomplished as follows. Several multiple colony tubes were chosen containing from six to twenty transformants resistant to only a low level of penicillin (0.020 units/ml), rejecting mixtures which contained any transformants resistant at the level of the second-step donor. Both competent cultures and DNA were prepared from these mixed lines. Genotypic heterogeneity in cultures was tested by self crosses; DNA derived from each culture was used to transform that culture. A number of these mixtures gave clear evidence of self-transformation to a resistance level of 0.050 to 0.060 units/ml of penicillin. Single-colony strains were isolated from these mixtures and tested for their phenotypes and genotypes. One class was phenotypically indistinguishable from RP1 (marker *pen*-r1). A second class, *pen*-r2, also phenotypically similar to the first, was distinguishable on the basis of genotype. A doubly marked transformant type, closely similar to RP2, was produced by reciprocal transformations from either class as recipient to DNA from the other (see Table 4).

Studies of the third-step mutant: The recognition of two independent mutations r1 and r2 in the second-step mutant led us to postulate an additional mutation in the third-step mutant. It was reasonable to suppose that this marker might transfer a resistance to wild type or, when added to r1 or r2, produce a double mutant with phenotypic properties distinct from those of the double mutant RP2. The second possibility was tested first. Transformants bearing single marker *pen*-r2 were treated with DNA derived from the third-step mutant and transformants selected at concentrations of penicillin varying from 0.04 to 0.1 units/ml. At 0.04 to 0.06 units/ml of penicillin, transformants were obtained in a net yield of approximately 0.1 to 0.3 percent. At 0.08 units/ml of penicillin, transformants resistant to 0.09 units/ml appeared at a frequency of about 0.002 to 0.003 percent. No transformants were observed at higher levels.

The production of this intermediate high resistance indicated at least one new genetic unit in the donor RP3. The low frequency of transformation to this level of resistance suggested that two units might have been transferred. In any case, the intermediate does not have as great a resistance as does RP3, so it must be less complex than this third-step mutant. A typical strain of the high resistance transformant, X27-A, was backcrossed to an r2 recipient. One class of transformants was produced having the phenotype of RP2 and able to transfer to wild type (DNA from several strains of this class was tested) the markers

TABLE 4

Analyses of penicillin-resistant Pneumococcal genotypes

Genotype	Transformants produced		Maximal penicillin resistance units/ml	Genotypes in cross	Routes of formation		Relative yield
	Strain designation	Number of isolates tested			Strain of origin or recipient	DNA donor in cross	
pen-r1	RP1	1	0.025	mutation	R6	....	....
	T-1	3	0.025	r1 × W	R6	RP1	high
	T-2	5	0.025	r1r2 × W	R6	RP2	high
	T-3	3	0.025	r1r2 × W	R6	T-6	high
	T-4	P	0.025	r1r2 × W	R6	T-12	high
	T-5	7	0.025	r1r2r3r4 × W	R6	RP3	high
pen-r1r2	RP2	1	0.05	mutation	RP1	....	....
	T-6	7	0.05	r1r2 × W	R6	RP2	low
	T-7	P	0.05	r1r2 × W	R6	T-12	low
	T-8	3	0.05	r1r2r3 × W	R6	X27-A	low
	T-9	10	0.05	r1r2r3r4 × W	R6	RP3	low
	T-10	5	0.05	r1 × r2	X7-B	RP1	high
	T-11	10	0.05	r2 × r1	RP1	X7-B	high
	T-12	10	0.05	r1r2r3 × r2	X7-B	X27-A	high
pen-r2	X7-B	3	0.025	r1r2 × W	R6	RP2	high
	T-13	P	0.025	r1r2 × W	R6	T-12	high
	T-14	P	0.025	r2r3 × W	R6	T-28	high

pen-r1r2r3r4	RP3	1	0.12	mutation	RP2	....	....
	T-15	2	0.12	r1r2r3r4 × r1r2r3	X27-A	RP3	mod.high
pen-r1r2r3	X27-A	3	0.09	r1r2r3r4 × r2	X7-B	RP3	low
	T-16	2	0.09	r1r2r3 × W	R6	X27-A	very low
	T-17	P	0.09	r2r3 × r1	T-1	X8-8	very low
	T-18	3	0.09	r1r2r3 × r1	RP1	X27-A	low
	T-19	2	0.09	r1r2r3 × r2	X7-B	X27-A	low
	T-20	P	0.09	r2r3 × r1r2	T-8	X8-8	high
	T-21	P	0.09	r1r2r3 × r1r2	T-8	X27-A	mod.high
	T-22	P	0.09	r1r2r3r4 × r1r2	T-8	RP3	mod.high
	T-23	2	0.09	r1 × r2r3	X8-8	RP1	high
	T-24	P	0.09	r1r2 × r2r3	X8-8	RP2	high
	T-25	P	0.09	r1r2r3 × r2r3	X8-8	X27-A	high
	T-26	P	0.09	r1r2r3r4 × r2r3	X8-8	RP3	high
pen-r2r3	X8-8	2	0.05	r1r2r3 × r2	X7-B	X27-A	high
	T-27	P	0.05	r2r3 × r2	X7-B	X8-8	low
	T-28	P	0.05	r2r3 × W	R6	X8-8	low

W: wild genotype; T: transformant; X: transformants used to define new units
P: indicates that the appropriate phenotype was observed in several (5–20) colonies, but genotype tests were not made.
Note: other transformations failing to change resistance (allelism tests) are mentioned only in text. Other tests not shown: transfer of donor types r1 and r2 unchanged to wild type, also transformation of r1 and r2 recipients by donor r1r2.

pen-r1, *pen*-r2 and *pen*-r1r2 at the same frequencies as did RP2 donor. These strains were transformed to a higher resistance level by DNA prepared from strains X27-A or RP3, but not by DNA prepared from RP1 or RP2. Therefore, it would appear that the strain X27-A has been able to donate the markers *pen*-r1 and *pen*-r2, and contains, besides these, another which can be designated *pen*-r3. It is also apparent that its genotype, *pen*-r1r2r3, does not provide the full resistance shown by third-step mutant, RP3.

A second class of transformants coming from the action of X27-A DNA on r2 strains, selected at 0.04 units/ml of penicillin, was also similar to the second-step mutant in phenotype, but on genetic analysis proved to be different. One strain, X8–8, was identified as *pen*-r2r3. Its DNA transformed wild-type R6 to give a low frequency of the donor type, and a large number surviving a low penicillin concentration and resistant to about 0.025 units/ml.

When strain X8-8 was transformed by DNA from RP1 or any strain containing *pen*-r1, the resistance level was increased to about 0.09 units/ml of penicillin. These transformants so produced could be transformed to a higher level of resistance by DNA from RP3 only. This result indicates the existence of a fourth factor, *pen*-r4, in RP3. These transformations and some of the others supporting this analysis are summarized in Table 4.

Thus, RP3 can be assigned the genotype *pen*-r1r2r3r4, and when its DNA is used to transform wild type, it disseminates to produce the types r1 and r1r2 and probably others. When acting upon r2 strains, it produces the genotypes *pen*-r1r2r3 (X27-A class) and it can transform this to something resembling itself (adding r4). The genotype of the r1r2r3 class is demonstrated by its production of r1r2 (like RP2) and r2r3 (like X8-8) when transforming r2 strains. The constitution of the r2r3 class is indicated by its being converted back to the r1r2r3 type by the single marker, *pen*-r1. These experimental observations support the adequacy of the definitions by which *pen*-r1 was taken as a marker defining RP1 resistance, *pen*-r2 was the one added in the second-step mutant RP2, while *pen*-r3 and *pen*-r4 both appeared in the third-step mutation, to RP3. Of this pair, *pen*-r3 was taken as that one which was easily introduced into r2 strains along with *pen*-r1 by the action of DNA from RP3. The marker *pen*-r4 is the remaining mutant portion of the genome of RP3, but it has not been obtained separately and not enough experiments have been done to demonstrate whether it is a single resistance factor, a modifier, or somewhat more complex.

The four markers developed and tested with regard to penicillin resistance in most cases also lead to similar small increases in resistance of Pneumococci toward the analogs oxacillin and methicillin. It is noteworthy that the factors r3 and r4, however, make little or no contribution to methicillin resistance (SHOCKLEY, unpublished).

Indicated association of the markers: In transformations in various combinations, it is observed that markers *pen*-r1 and *pen*-r2 are probably slightly linked, being cotransferred at two or three times random frequencies. The marker *pen*-r3 is a low efficiency marker, being incorporated 0.07 as frequently as the first two and 0.04 as often as *sul*-d. It is, therefore, possible to demonstrate co-transfer to-

gether with r1 and also r2, the doubles being about 20% and 25% of the total r3 transformants. Thus far, the co-transfer frequencies clearly support the indicated order of markers, r1–r3–r2, but not all of the relevant three-point marker tests have been available to completely test this sequence. Inasmuch as *pen*-r3 and *pen*-r4 are singly incorporated at only moderate efficiencies, co-transfer of this pair should have been detected, had it been appreciable.

DISCUSSION

The stepwise pattern of mutations toward high levels of penicillin resistance as described for Staphylococcus (Demerec 1945) was exhibited by Pneumococcus both now and in the earlier work. As reported then (Hotchkiss 1951), the levels of resistance represented in the successive mutants are identifiable with those transferred by DNA into individual transformants. Thus, the markers introduced stepwise by DNA transformation are those which formed part of the previous mutational experience of the strain from which the DNA was separated. As earlier indicated, the ability of transformants to grow at new levels of penicillin resistance requires the relatively long phenotypic expression period of 2 to 3 hr. This interval might nowadays be construed as necessary to "dilute out" the penicillin-sensitive components of the cell wall or of the wall-forming system. It may be recalled that Ephrussi-Taylor (1962) has reported that survivorship is attained before viability for streptomycin-resistant transformants.

This reexamination of penicillin resistance in Pneumococcus suggests somewhat more complexity than first reported. The apparently obligate *sequence* of resistance steps, though it had been questioned (Hotchkiss 1952) seemed specific until now. We now see that first-step transformants produced by more resistant donors may be of at least two types (r1 or r2), whereas when investigated formerly, they all seemed to be phenotypically alike and genotypically indistinguishable. On the other hand, there is no evidence as yet whether the r3 or r4 markers pass into wild-type recipients, and if so, whether they produce penicillin-resistant transformants. It may be, therefore, that they are modifiers of resistance rather than simple resistance factors.

We have not, in the present work, explored other spontaneous mutations to penicillin resistance, so we have no information about the possible obligateness of mutational sequences. It is possible to assume the identity of the present mutational steps with those previously reported, although it is not feasible, and probably not profitable, to investigate this question. Earlier high resistance strains were different from RP3 and since they were many times multiply passed in penicillin, they may have had additional modifiers of resistance not observed in recent easily accessible mutations. Surviving stock strains of lower resistance levels from the earlier period seemed quite comparable to the types now designated r1 and r1r2, when they were last available. Although they had been described as somewhat more resistant originally, in a day when penicillin was still conserved as an expensive and perhaps variable commodity, we probably overestimated resistance levels by using (besides different test media) drug solutions of low potency.

Besides separating *pen*-r1 and *pen*-r2, we can now describe the additional resistance conferred upon either of them or their combination by *pen*-r3. Thus far, the r4 factor has been detected only by its effect upon the triply resistant intermediate. The two units, r3 and r4, which appeared in RP3 after a single selection step in penicillin are transferable as units separately, and co-transfer was not observed. Nevertheless, it remains true that the stepwise mutations are identifiable with, and imitated by, transformable steps carried by DNA. As in the case of the high sulfonamide resistance (HOTCHKISS and EVANS 1958), the number of transferable units is larger than the number of selection steps in which they were obtained.

Of the factors identified, *pen*-r3 seems closely linked to the r1 and r2 factors which appear weakly linked, so we may assign the map order r1-r3-r2. No relation to any other marker has been established. The factors r3 and r4, derived together in selection, have not appeared to be linked in co-transfer.

Transferable penicillin resistances that have been described since the earlier work with penumococcus are generally and perhaps exclusively of the variety attributable to penicillinase. BOMAN and associates (NORDSTRÖM, ERIKSSON-GRENNBERG and BOMAN 1968; BURMAN, NORDSTRÖM and BOMAN 1968) have analyzed several loci in *Escherichia coli* K12 affecting a penicillinase, including a modifier for nonchromosomal resistance factors. Penicillinase systems mediated by an R factor in *E. coli* (DATTA and RICHMOND 1966) and by a plasmid in Staphylococcus (HARMON and BALDWIN 1964; NOVICK and RICHMOND 1965) have been reported. A borderline penicillin resistance was claimed to be induced in Staphylococci by a DNase-insensitive factor by GILLISSEN (1958), but he reported a considerably greater transfer of resistance mediated by specific DNase-sensitive components. The penicillin resistance reported by NESTER (1964) in *Bacillus subtilis* is a transient physiological resistance associated with transformability but not of a heritable type.

The low, quantitatively discrete, levels of penicillin resistance reported in this paper could hardly be attributable to a penicillin-destroying system such as penicillinase whether extra- or intracellular. They remain quantitatively identifiable in mixed populations, and not affected by inoculum size, except insofar as, under certain conditions, a 10^5-fold or greater excess of sensitive cells may contribute to survival as already mentioned. On the other hand, the relatively massive co-killing effect alluded to above (SAZ and EAGLE 1953) may be an indication that in large populations, the dying sensitive cells can, under appropriate conditions, convey by lysate transformation (HOTCHKISS 1951) sensitivity to a small resistant subpopulation.

SUMMARY

The spontaneous stepwise mutation of Pneumococci to low levels of penicillin resistance has been correlated to DNA transformation of resistance. Confirming earlier work, the DNA appears to transfer identifiable units of the mutational experience. A mutational step may however consist of one, or more than one transformable step. Contrary to previous suggestions, the order of acquisition is

not obligatory in transformation, although some later steps appear to be modifiers of the early steps. Linkage between the four loci detected leads to appreciable co-transfer for at least two of the pairs. Physiological development of newly introduced ability to grow in penicillin is slow, requiring two to three hours. It is accompanied for some of the mutant loci by increased resistance to penicillin analogs, oxacillin and methicillin.

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