GENETIC STUDIES OF ACRIDINE-INDUCED MUTANTS IN *STREPTOCOCCUS PNEUMONIAE*

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ABSTRACT

The mutagenic properties of acridines on pneumococcus are described. All seven acridines tested were mutagenic at the *amiA* locus conferring a resistance to 10^{-5} M aminopterin. The effects of quinacrine were more specifically investigated. It was observed that: mutants can be obtained only by treatment of exponentially growing cells; a sharp maximum mutagenic effect occurs at a concentration slightly lower than the bacteriostatic value; and the amount of quinacrine required to yield the maximum mutagenic effect decreases with the pH of the medium. Moreover, the number of mutants detected after quinacrine treatment varies from locus to locus. The majority of quinacrine-induced mutants are readily reverted by quinacrine, but not by nitrosoguanidine treatment. This suggests that in pneumococcus quinacrine induces mainly frameshift mutations. A further study of the revertants obtained by quinacrine treatment of quinacrine-induced mutants strengthens this interpretation: most of the revertants result from a mutation at the same site; some partial revertants exhibiting an intermediate resistance to aminopterin were found to contain two very closely linked mutated sites, each mutation conferring the maximum level of resistance to aminopterin. Thus, the majority of quinacrine-induced mutants at the *amiA* locus of pneumococcus consists of frameshift mutations. Nearly all of the isolated mutants induced by quinacrine as well as other acridines belong to the low efficiency class of transformation. It was concluded that the mismatch resulting from the pairing between the wild type and the frameshift-containing sequence is recognized by the excision-repair system involved in the discrimination function in a way similar to that in which it recognizes mismatched base pairs between a transition mutation and the wild-type sequence.

GENETIC markers used in pneumococcal transformation differ in the characteristic efficiency with which they are integrated into the recipient genome (LACKS and HOTCHKISS 1960). EPHRUSSI-TAYLOR, SICARD and KAMEN (1965) showed that single-site markers fall in two classes: high efficiency (HE) and low efficiency (LE). LE markers transform with a six- to ten-fold reduced efficiency as compared to HE markers. A few markers that are integrated with an efficiency 1.5 to 2 times higher than HE markers have been described (LACKS 1966; SIROTNAK and HACHTEL 1969; TIRABY and SICARD 1973). These are referred to as very high efficiency markers (VHE) (TIRABY and SICARD 1973; ROGER 1977). Strik-

ingly, all mutations induced by chemical mutagens tested so far belong to the LE class. For example, mutants isolated after nitrous acid (SICARD and EPHRUSSI-TAYLOR 1965) or hydroxylamine treatment on transforming DNA are LE markers. It has been suggested that LE markers result from transitions, whereas HE markers could result from transversions (LACKS 1966; GASC and SICARD 1972; TIRABY and FOX 1974). Therefore, the efficiency of transformation might depend upon the nature of the mismatch created by the mutation during the pairing process between donor and recipient DNA. In the course of a systematic survey of the efficiency distribution of mutations produced by different mutagens, we have used mutagens known to induce frameshift mutations. The present article reports the characterization of mutants induced by acridines, already known to be mutagenic in other systems (DRAKE 1970). Acridine-induced mutations in pneumococcus appear to belong mostly to the LE class, and reversion tests suggest that a majority of them are frameshift mutations.

MATERIALS AND METHODS

Strains: The properties of strains and markers used in this study are indicated in Table 1. The recipient bacteria for transformation experiments were the discriminating strain Cl3 or the nondiscriminating strain 801. The mutants were isolated in strain Cl3 carrying the *str-41* marker.

Media: P medium used for the growth of Cl3 has been described in an earlier publication (SICARD 1964). The transformation medium (CSA) was prepared as follows: 10 g of vitamin-free casein hydrolysate (Nutritional Biochemicals) and 8.5 g NaCl were dissolved in a liter of distilled water. The pH of the solution was adjusted to 7.8 by addition of 1 N NaOH. After sterilization, 10 ml aliquots of this medium were supplemented with 0.01 ml of 25% glucose, 2.4 ml of 8% yeast extract solution (SICARD 1964), 1 ml of 4% albumin, 1 ml of a solution of asparagine (0.5 mg/ml) and 0.2 ml of a solution of glutamine (5 mg/ml).

TABLE 1

Properties of markers and strains

Markers	Phenotype	Reference
hex$^-$	No discrimination between markers which are all transformed as very high efficiency markers	TIRABY and SICARD (1973)
str-41	Resistance to 2 mg/ml of streptomycin	HOTCHKISS and MARMUR (1954)
amiA	Resistance to 2×10^{-5} M aminopterin and sensitivity to 5×10^{-3} M isoleucine	SICARD (1964)
opt	Resistance to 1.5×10^{-5} M optochin	EPHRUSSI-TAYLOR (1960)

Strains	Genotype	Reference
Cl3	*hex*+ *str*^{s} *amiA*^{s} *opt*^{s}	EPHRUSSI-TAYLOR and LATARJET (1955)
Cl3 *str-41*	*hex*+ *str*^{r} *amiA*^{s} *opt*^{s}	This work
801	*hex*$^-$ *str*^{s} *amiA*^{s} *opt*^{s}	TIRABY and FOX (1973) CLAVERYS, personal communication

Transformation procedure: The basic transformation procedure for Cl3 is a modification of the method described previously (SICARD 1964). Frozen cultures in P medium were inoculated (at a 1:20 dilution) into CSA medium. Competence appeared after 80 to 120 min of incubation at 37°. All samples from a given batch of frozen cultures reached competence at the same time. Cells were mixed with DNA and left in contact for 20 min at 37°. Transformants were selected by plating on complete agar medium. After two hr incubation at 37° an overlayer of agar containing the required concentration of antibiotic was poured onto the plates. The transformation procedure for strain 801 has been described previously by TIRABY, FOX and BERNHEIMER (1975).

Source of mutagens: 1-methyl-3-nitro-1-nitrosoguanidine was obtained from Aldrich Chemical Co. Inc. Proflavine (2–8 diaminoacridine) was purchased from National Aniline Division. Acridine orange, 3–9-diamino acridine, 9-amino-1-methyl acridine and euflavine were a generous gift from Dr. ALBERTS. Quinacrine was obtained from Specia. ICR-170 was a gift from DR. ROSSIGNOL (University of Orsay, France).

The proposals of the International Union of Pure and Applied Chemistry for the nomenclature of acridines have been adopted in this publication.

Mutagenesis: Nitrosoguanidine-induced mutants were obtained by the following procedure: exponentially growing cells, cultivated in the standard medium, were centrifuged, resuspended in the same volume of 0.1 M acetate buffer at pH 5.5 and treated for 30 min at 37° with 50 or 75 μg/ml of nitrosoguanidine. The cells were centrifuged, washed once and diluted 50-fold in the growth medium. After incubation six to eight hr at 37° to re-establish a normal growth rate, the number of aminopterine- or optochin-resistant bacteria was determined by plating on media containing 10^{-5} M aminopterin or 0.6×10^{-5} M optochin.

Acridine-induced mutants were isolated in the following way: exponentially growing cells were treated with different concentrations of acridine in the growth medium, then incubated at 37° in the dark for one hr. To score the mutants, aliquots, of the culture were plated on complete agar medium. After two to three hr of incubation at 37°, an overlayer of agar containing the required concentration of the selective agent was poured onto the plate. The number of viable cells was determined after overnight incubation at 37°.

The mutagenic effect is measured by a mutagenic index defined as the ratio of the frequency of mutants after treatment over the frequency of spontaneous mutants.

Selection of revertants at the amiA *locus*: Aminopterin-sensitive revertants were selected by taking advantage of a special property of *amiA* mutants; in a synthetic medium, mutations at this locus confer sensitivity to an unbalance in the relative concentrations of isoleucine, leucine and valine (SICARD 1964). To select either aminopterin-sensitive transformants or revertants, cells were plated on synthetic agar medium containing 5×10^{-3} M isoleucine, the concentration of the other two branched aminoacids being kept at 5×10^{-5} M. After incubation overnight at 37°, aminopterin sensitive bacteria appeared as small colonies. Reconstruction experiments indicated that no more than 10^7 cells should be plated to recover individual wild-type colonies. It is likely that under the selective condition the residual growth of the aminopterin-resistant bacteria accounts for such a limitation. A direct test of resistance to aminopterin was routinely performed as a control to check the phenotype of isolated colonies.

RESULTS

Mutagenic effect of acridines at the amiA *locus*: A variety of acridines was tested. The cells were incubated for one hr in growth medium in the presence of various concentrations of the drug and then plated. Results observed with quinacrine are shown in Figure 1. The bacteriostatic concentration for this drug is 70 μg/ml. At higher concentrations, the ability to form colonies slowly decreases. Mutants were efficiently induced. The optimal mutagenic concentration was always slightly lower than the bacteriostatic concentration. A similar pattern

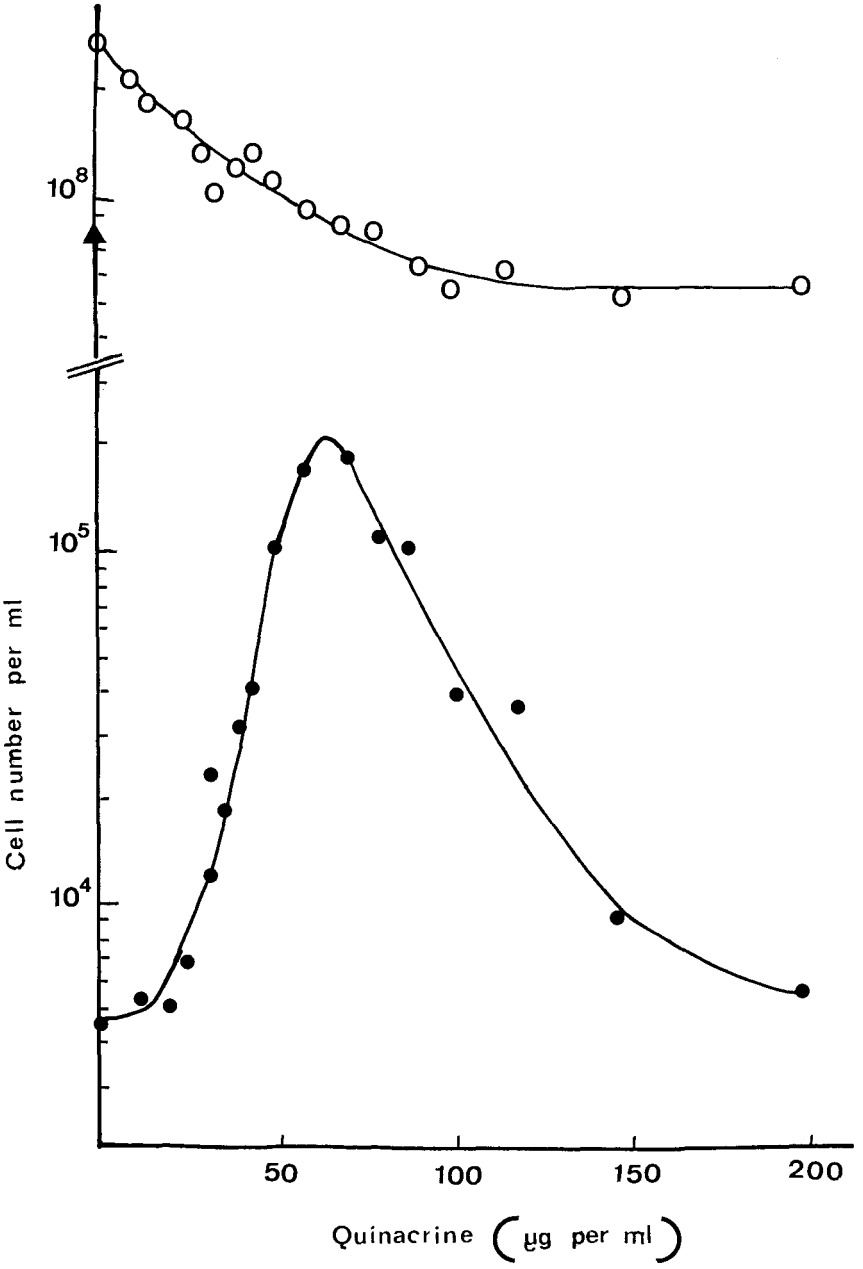

FIGURE 1.—Mutagenic effect of quinacrine at the *amiA* locus. An exponentially growing culture of pneumococcus was treated for one hr with different concentrations of quinacrine (abscissa); ▲ viable cells before treatment, ○ viable cells after treatment, ● *amiA* mutants after treatment.

TABLE 2

Mutagenic effect of acridines at the amiA *locus*

Mutagen	Optimal mutagenic concentration ($\times 10^{-5}$ M)	Bacteriostatic concentration ($\times 10^{-5}$ M)	Optimal mutagenic index
Proflavine	1	1.5	40
Quinacrine	13	16	140
ICR 170	0.37	0.74	10
3,9-diamino-acridine	1.9	4.3	50
9-amino-1-methyl-acridine	1.1	4.8	73
acridine orange	4	5	8
euflavine	0.23	0.38	23

The mutagenic index is the ratio of the frequency of acridine-induced mutants over the frequency of spontaneous mutants.

was observed for all tested acridines. The results of these experiments are summarized in Table 2.

A high mutagenic index was obtained by treatment with proflavine, 3-9-diaminoacridine, 9-amino-1-methyl acridine, euflavine and quinacrine, whereas this index was low using acridine orange and ICR-170. These results may be compared with some other observations (DRAKE 1970). In Salmonella it has been reported that under certain conditions 3-9-diaminoacridine and proflavine are more potent mutagens than acridine orange and ICR-170 (AMES and WHITFIELD 1966). In contrast, the very strong mutagenic effect of quinacrine seems to be specific to pneumococcus. Therefore, the remainder of this paper will be devoted more specifically to the study of this mutagen.

Optimal conditions for the mutagenic effect of quinacrine: Mutations could not be induced if the bacteria had reached the stationary phase when the treatment was initiated. On the other hand, when cells collected for the treatment, were in full exponential growth, mutagenic indexes higher than 100 were routinely obtained. By the end of the exponential phase of growth, mutagenicity was barely detectable. This suggests that full metabolic activity is required to obtain the maximum yield of mutants.

An effect of the pH of the culture at the time of treatment has also been observed (Figure 2). The amount of quinacrine required to yield the maximum mutagent effect decreases markedly as the pH of the medium increases. Although no experiments have been performed to analyze this pH effect further, it may be related to an increase in uptake of the acridine when the pH rises, as has been observed in *E. coli* (SILVER, LEVINE and SPIELMAN 1968).

Gene specificity of mutation induction by quinacrine: The ability of quinacrine to induce mutations in other antibiotic resistance genes has been checked. It can be seen in Table 3 that quinacrine is mutagenic for three other loci, but the efficiency of mutagenesis depends upon the gene. There is little or no induction of mutations in the genes conferring resistance to 200 μg/ml of streptomycin, 4 μg/ml of novobiocin, 0.6×10^{-5} M optochin and 0.1 μg/ml of erythromycin.

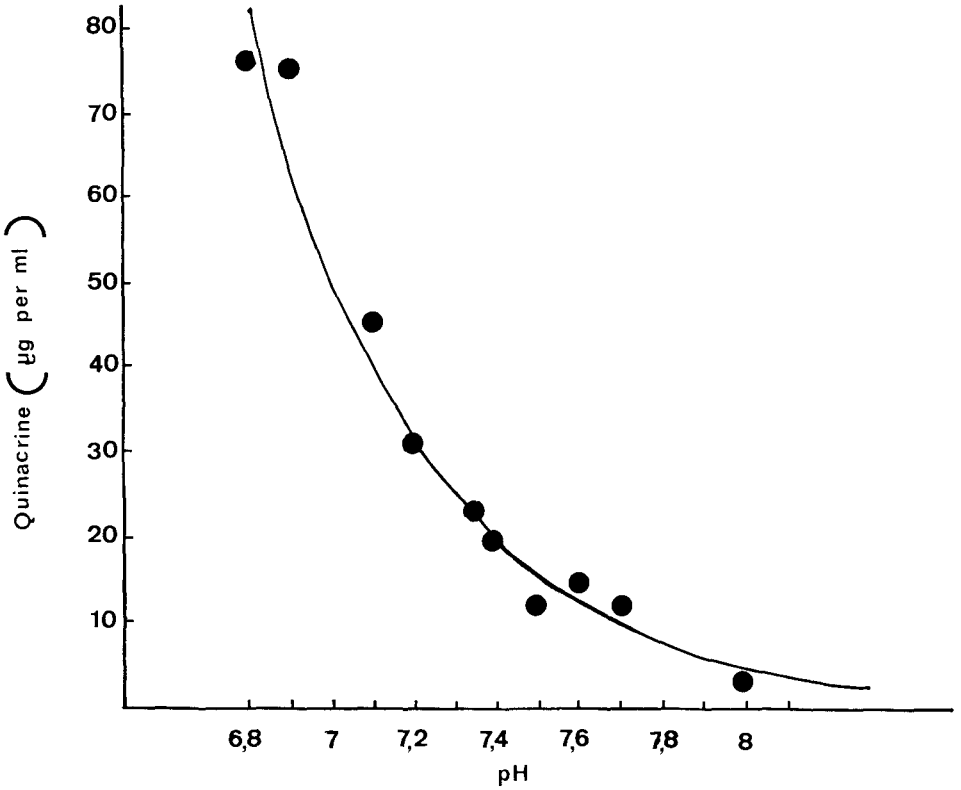

FIGURE 2.—Effect of the pH of the culture on the amount of quinacrine required to obtain the maximum mutation index.

TABLE 3

Mutagenic effect of quinacrine on different loci

Locus	Mutation frequency Without treatment	With treatment	Mutagenic index
amiA	2.6×10^{-5}	1.2×10^{-2}	460
fus	5.3×10^{-7}	1.4×10^{-5}	26
rif	5.3×10^{-7}	9.1×10^{-6}	17
stg	2.6×10^{-7}	3.0×10^{-6}	11
ery	1.1×10^{-6}	4.6×10^{-6}	4
opt	1.9×10^{-6}	4.6×10^{-6}	2
nov	$< 10^{-8}$	$< 10^{-8}$	—
str	$< 10^{-8}$	$< 10^{-8}$	—

The mutagenic index is defined in Table 1.

The optimal conditions of mutagenesis were controlled by measurement of the mutation index for aminopterin resistance. Mutants were scored at a concentration where spontaneous mutants have already been selected : aminopterin 10^{-5} M, fusidic acid 50 µg/ml, rifampicin 1 µg/ml, streptolidigin 5 µg/ml, erythromycin 0.1 µg/ml, optochin 0.6×10^{-5} M, novobiocin 4 µg/ml, streptomycin 0.2 mg/ml.

Efficiency of transformation of markers induced by acridines: At the *amiA* locus most spontaneous mutants are found in one of the two main efficiency classes, HE and LE. To determine the efficiency distribution of acridine-induced mutants, we transformed strain C13 by donor DNA extracted from independently isolated acridine-induced mutants carrying the *str-41* marker as reference. Their relative efficiency, determined by the ratio of aminopterin to streptomycin-resistant transformants, is presented in Table 4. It can be seen that 20 mutants induced by quinacrine mutagenesis fall into the LE class. Likewise most of the proflavine and ICR-170-induced mutants belong to LE class. The very few HE

TABLE 4

Transformation efficiencies of acridine-induced mutations resistant to aminopterin

Proflavine mutation number	Transformation efficiency	Quinacrine mutation number	Transformation efficiency	ICR 170 mutation number	Transformation efficiency
1	0.12			1	0.15
2	0.09			2	0.11
3	0.21			3	0.10
4	0.11			4	0.14
5	0.10			5	0.13
10	0.19			6	0.15
13	0.08			8	0.12
17	0.18	1	0.15	9	0.16
18	0.10	2	0.15	10	0.18
21	0.17	3	0.14	11	0.22
24	0.15	4	0.16	12	0.15
25	1.80	5	0.13	13	0.16
26	0.26	6	0.12	14	0.11
101	0.12	7	0.08	15	0.16
102	0.18	8	0.10	16	0.12
103	0.19	9	0.16	17	0.10
104	0.10	10	0.14	18	0.16
105	0.12	11	0.08	19	0.27
106	0.11	12	0.16	20	0.77
107	0.15	13	0.12	21	0.15
111	0.29	14	0.13	22	0.10
113	0.07	15	0.10	23	0.12
116	0.17	16	0.12	25	0.20
118	0.15	17	0.14	26	0.14
119	0.48	18	0.18	27	0.97
120	0.84	28	0.14	28	1.3
121	1.10	31	0.09	29	0.17
122	0.47			30	0.70
124	0.54			31	0.25
125	0.12			32	0.16
127	0.20			33	0.17
128	0.19				

Transformation efficiency is the ratio of the number of transformants resistant to aminopterin to the number of transformants resistant to streptomycin.

markers observed could have been truly induced by these drugs, but they could also have been spontaneous mutants. Indeed, the ICR-170 mutants were isolated in an experiment where the mutation index was ten. Thus, a few spontaneous HE markers are expected, which could account for the results shown in Table 4. Therefore, it is likely that the acridines induce almost exclusively LE markers in pneumococcus.

Efficiency of quinacrine-induced markers in a nondiscriminating hex⁻ *strain*: A characteristic property of most LE markers is that they are integrated with very high efficiency when used in a nondiscriminating strain (*hex*⁻). The behavior of quinacrine induced mutations has been tested. The 801 strain, which does not discriminate, was transformed by DNAs extracted from a sample of quinacrine induced *amiA* mutants carrying the *str-41* reference marker. The results are presented in Table 5; the efficiency of each individual marker is higher than 1. Thus it is clear that the *hex* function recognizes the mismatches between wild type and quinacrine induced base changes in the DNA.

Location of the mutations in the amiA *gene*: A further characteristic of the quinacrine induced mutations is their location in the *amiA* locus. DNA's extracted from a sample of quinacrine-induced mutants (5, 7, 9, 12, 13, 17, 18 and 31) were used to transform three recipient strains carrying the sites 9, 17 or 1, respectively located in the left, the right, and the middle parts of the genetic map of the *amiA* locus (SICARD and EPHRUSSI-TAYLOR 1965). All markers appear to belong to this locus, as each one exhibits linkage to at least one of the sites taken as reference (data not shown). Although no fine-structure mapping has been performed, this recombination analysis indicates that the eight markers involved at least six different sites spread along the full length of the locus.

Characterization of the frameshift nature of the mutations induced by quinacrine: reversion studies: Information on the nature of a mutation can be obtained

TABLE 5

Transformation efficiencies of quinacrine-induced mutations resistant to aminopterin in the 801 hex⁻ *strain*

Quinacrine mutation number	Number of *str* cells per ml × 10^2	Number of *amiA* cells per ml × 10^2	Transformation efficiency
4	305	334	1.09
5	107	118	1.10
7	515	620	1.20
9	232	280	1.21
12	279	336	1.20
13	279	366	1.31
17	135	163	1.21
18	658	766	1.16
31	109	146	1.34

Transformant efficiency is the ratio of the number of transformants resistant to aminopterin to the number of transformants resistant to streptomycin.

through reversion studies; for instance, if a mutation results from a base substitution, it should revert after a treatment by nitrosoguanidine, a chemical which induces a variety of base substitutions (WHITFIELD, MARTIN and AMES 1966). On the other hand, acridines are known to revert mutations resulting from the addition or deletions of bases (WHITFIELD, MARTIN and AMES 1966), although exceptions to these rules have been reported (FINK, KLOPOTOWSKI and AMES 1967; BERGER, BRAMMAR and YANOFSKY 1968). To ascertain the validity of these criteria, a preliminary study was carried out to show that nitrosoguanidine can revert base-substitution mutations under our experimental conditions. A sample of seven *amiA* mutants obtained by nitrous acid treatment of DNA (EPHRUSSI-TAYLOR, SICARD and KAMEN 1965) was mutagenized by nitrosoguanidine, and wild-type revertants were scored as indicated in MATERIALS AND METHODS. Revertants appeared from all treated strains, except one, the number of wild type revertants varying from 20- to 200-fold over the number of spontaneous revertants, depending upon the site tested. It is concluded that nitrosoguanidine generally reverts base-change mutations such as those produced by nitrous acid.

Quinacrine-induced mutations can thus be classified according to their ability to revert after treatment by quinacrine itself or by nitrosoguanidine. Quinacrine-induced mutants belonging to the *amiA* locus were treated either by nitrosoguanidine or by quinacrine. Revertants were detected on synthetic medium containing 5×10^{-3} M isoleucine. The results of such experiments are summarized in Table

TABLE 6

Classification of quinacrine-induced aminopterin resistant mutations according to their ability to revert after a treatment with quinacrine or with nitrosoguanidine (NTG)

	Mutations	Frequency of spontaneous revertants	Mutagenic index NTG	Mutagenic index Quinacrine
Class I	2	$< 0.4 \times 10^{-7}$	—	—
	4	$< 0.2 \times 10^{-7}$	—	—
	6	10^{-6}	< 2	< 3
Class II	1	$< 0.2 \times 10^{-7}$	—	> 1300
	5	$< 0.1 \times 10^{-7}$	—	> 40
	7	$< 0.1 \times 10^{-7}$	—	> 20
	9	2.3×10^{-6}	1	830
	12	0.3×10^{-7}	1	1100
	13	$< 0.1 \times 10^{-7}$	—	> 3000
	18	3.5×10^{-7}	1	1200
	28	3.7×10^{-6}	1.5	270
	17	0.3×10^{-7}	3	1500
Class III	8	5×10^{-5}	50	28
	11	3×10^{-5}	8	6
	14	5×10^{-5}	14	2
	30	3×10^{-5}	43	4

Mutagenic index is defined in Table 1. When no mutants could be detected either spontaneously or after treatment, this index could not be calculated.

6. Among the 16 mutants tested, several different categories are found. Three mutations do not revert significantly after either nitrosoguanidine or quinacrine treatment (class I) and have not been studied further. These mutations could represent complex structural changes such as the addition or deletion of many bases. Nine mutations are very effectively reverted by quinacrine, but not by nitrosoguanidine (class II). These are properties characteristic of frameshift mutations (DRAKE and BALTZ 1976). The other four mutations (class III) are characterized by a high spontaneous rate of reversions and a slight response to either quinacrine or nitrosoguanidine action. Although it is difficult to be sure of their nature, they could be base substitutions. Quinacrine could thus cause some base substitutions in pneumococcal DNA as it does in other systems; it has been reported, for instance, that nonsense mutations in Salmonella can be reverted by acridines (FINK, KLOPOTOWSKI and AMES 1967). In conclusion, our analysis suggests that in pneumococcus, quinacrine induces different kinds of mutations. However, a large proportion of these (class II) seem to be frameshift mutations.

Genetic analysis of quinacrine-induced revertants of class II mutants: As already found in a variety of organisms, if a mutation is due to an addition of a nucleotide, for example, quinacrine can revert it through an induced deletion event. One expects to find two kinds of revertants: (1) deletion of the additional base at the same site; transformation of the wild-type strain Cl3 by DNA extracted from these revertants should result in the absence of aminopterine-resistant recombinants, or (2) deletion at a closely linked site so that the nearby second mutation restores the correct reading frame except in the region between them; the phenotype expected may be leaky and by recombination it should be possible to recover mutants exhibiting complete resistance to aminopterin. This kind of investigation was performed on five mutant strains, 5, 7, 13, 18 and 28, which revert frequently after quinacrine treatment. Quinacrine-induced revertants from these strains were selected on synthetic agar medium supplemented with isoleucine. In their sensitivity to an excess of branched amino acids and aminopterin, they were identical to the wild type strain Cl3. This phenotypic observation suggests that these revertants are true revertants, *i.e.*, a return to the original wild-type DNA sequence. To confirm this prediction, a genetic analysis of some of these revertants was performed.

The recipient strain Cl3 was transformed by DNA extracted from a variety of revertants of each strain. Each DNA carried the reference marker *str-41* to check the transforming activity. The number of bacteria resistant to streptomycin and aminopterin was measured after transformation. The results are presented in Table 7. The number of aminopterin-resistant colonies is not different from the number of spontaneous mutants. This suggests that the reversion events occurred at the site of the mutation or, if a suppressor mutation was involved, it was very closely linked to the original mutation site.

Genetic analysis of a quinacrine-induced suppressor: To seek examples of intragenic suppression, we looked for "partial revertants" showing an intermediate sensitivity to an unbalanced concentration of branched amino acids. The

TABLE 7

Transformation of strain Cl3 by DNA from aminopterin-sensitive revertant strains

Revertant strains	Number of *str* transformants per ml ($\times 10^6$)	Number of *amiA* cells per ml ($\times 10^3$)	Number of spontaneous *amiA* cells per ml ($\times 10^3$)
5 rev1	1.5	2	1.9
5 rev2	0.67	2	1.9
5 rev3	3	3	2.6
5 rev4	2.3	2.6	2.6
7 rev1	1.4	2.9	2.6
7 rev2	1.8	1.9	2.6
9 rev1	0.4	2.9	4
9 rev2	0.37	3.4	4
13 rev1	0.16	4.1	4
13 rev2	0.99	3.6	4
13 rev3	0.70	3.4	4
18 rev1	0.11	2.4	4
18 rev2	0.33	3.7	4
28 rev1	0.97	3.6	4
28 rev2	0.55	3.6	4

The revertant strains were selected after quinacrine treatment of *amiA* mutants induced by the same acridine (5, 7, 9, 13, 18 and 28).

The control number of spontaneous *amiA* mutants is measured in the competent culture without DNA.

five acridine-induced mutants already studied (5, 7, 13, 18 and 28) were treated with quinacrine, and cells were plated on a synthetic medium containing 2×10^{-3} M isoleucine. This concentration of isoleucine is 2.5 times lower than the one used to select *amiA*$^+$ bacteria. It is not possible to lower it further since the residual growth of aminopterin-resistant cells will compete with the growth of revertants (SICARD 1964). Under these conditions small colonies were observed. Some of these clones were isolated. Most of them were phenotypically wild type and were not further studied. In the case of mutant 18, however, we observed a clone (18rev3) having a sensitivity to isoleucine and to aminopterin intermediate between that of wild-type cells and of *amiA* mutant 18 (Table 8).

TABLE 8

Level of resistance to aminopterin and to an excess of isoleucine of strains 18 and 18rev3

Strains	Aminopterin concentration ($\times 10^{-5}$M)	Isoleucine concentration ($\times 10^{-3}$M)
18	2	1
18rev3	1.5	2

The drug concentrations are the maximum doses allowing optimal growth of colonies on solid medium.

A genetic analysis of this clone was performed. The wild-type strain was transformed by DNA extracted from this partial revertant carrying the reference marker *str-41*. Transformants selected for streptomycin resistance or resistance to 0.25×10^{-5} M aminopterin were scored to check the level of competence. Clones more resistant to aminopterin (2×10^{-5} M) than the parent strain, 18rev3, were selected. Despite the use of a highly competent culture, the number of such clones was only twice the background of spontaneous mutants occurring in the competent culture without transforming DNA. This difference between transformants and spontaneous mutants is then at the limit of significance. Such clones could, therefore, either be spontaneous mutants or result from recombination between two very closely linked sites carried by the strain 18rev3. Another genetic analysis was required, therefore, to discriminate between recombinants at the 18rev3 sites and spontaneous mutations. Spontaneous mutations should occur at any site in the *amiA* locus, and the probability of their being homoallelic to 18rev3 should be extremely low, whereas recombinants from the 18rev3 marker should be homoallelic to the 18rev3 marker. Discrimination between the two types of fully resistant clones was accomplished by transforming the 18rev3 strain with DNA prepared from individual cultures of those clones. Wild-type transformants were sought using synthetic medium containing 5×10^{-3} M isoleucine. Of the aminopterin-resistant strains, 50% yielded wild-type transformants, suggesting that they were spontaneous mutants. For the others, no wild-type transformants were detected, showing that they were homoallelic to 18rev3. Therefore, fully resistant clones have been dissected out of a partial revertant obtained by quinacrine treatment of quinacrine-induced mutations. This suggests that a very close intragenic suppressor was induced by quinacrine, and supports the hypothesis that quinacrine induces frameshift mutations. However, such intragenic suppression was observed for only one quinacrine-induced mutant. The failure to select partial revertants of other mutated sites suggests that only a few sites belonging to the *amiA* locus can be suppressed by a closely linked second mutation. To obtain other examples of intragenic suppression, we tried to select directly for double mutants induced by quinacrine.

Genetic analysis of a quinacrine-induced mutant exhibiting a partial resistance to aminopterin: Assuming that quinacrine induces the addition or deletion of bases, the reading frame will not be grossly upset in certain mutants if the addition is a multiple of three or the addition is followed by a deletion. Such mutants, if any, could show a phenotype intermediate between the wild-type and the *amiA* phenotype. Hence, we tried to isolate directly quinacrine-induced mutants resistant to an intermediate concentration of aminopterin. A culture of strain Cl3 *str-41* was treated with quinacrine, and cells resistant to a concentration of 0.3×10^{-5} M aminopterin were selected. Among 42 clones tested, 37 could grow in the presence of 2×10^{-5} M aminopterin, but five clones exhibited only a partial resistance to aminopterin. The least resistant mutant, strain 31, which would grow in the presence of 0.75×10^{-5} M aminopterin but was inhibited by a higher concentration, was chosen for more intensive investigation.

Analysis of this strain was performed as described in the previous section for strain 18rev3. Wild-type bacteria were transformed by the DNA extracted from strain 31. The number of bacteria resistant to streptomycin or to 0.25×10^{-5} M aminopterin after transformation was 1.4×10^{6} per ml. The number of bacteria resistant to 2×10^{-5} M aminopterin was three times higher than the number of spontaneous mutants. As already described in the preceding section, such a difference, although at the limit of significance, suggests that fully resistant recombinants appeared along with spontaneous mutants. Such fully resistant colonies were isolated and crossed with the original strain 31. Two-thirds of them were allelic to mutant 31, suggesting that they resulted from a recombination event between two very closely linked sites harbored by the 31 strain. In other words, the leaky strain 31 can be dissociated into closely linked mutations conferring independently the highest level of resistance to aminopterin.

The pronounced leakiness of strain 31 suggests that it should be possible to reconstruct it from the dissociated mutants through selection in synthetic medium supplemented with a limiting amount of isoleucine. A concentration of 2×10^{-3} M isoleucine was found to be optimal since, under these conditions, full mutants do not grow, wild-type cells show a normal growth and strain 31 bacteria form small colonies after a prolonged incubation. However, the efficiency of this selection procedure is poor; a large number of bacteria has to be plated because very few recombinants are expected. Moreover, the levels of resistance of the fully resistant and the leaky strains are so similar that the selection is difficult. Individual colonies suspected to be leaky recombinants should be further tested. Reconstruction of strain 31 was undertaken by performing all the possible crosses between five fully resistant mutants A, B. C, D and E, homoallelic to strain 31. Small colonies, if any, growing at a concentration of 2×10^{-3} M isoleucine in synthetic medium were isolated and subcultured. The resistance to aminopterin was determined on solid complete medium containing increasing concentration of this drug. Clones showing a resistance indentical to the leaky strain 31 were kept. An additional test was made to discard clones resulting from spontaneous mutations. DNA was prepared from each clone and used to transform strain 31. No wild-type transformed cells could be detected, indicating that all the clones were homoallelic to leaky strain 31. After such a screening procedure, respectively three, four and one leaky recombinants were isolated in the crosses E × A, E × C and E × D; likewise in the crosses B × A, B × C and B × D one, two and four leaky recombinants were isolated. In all other crosses, no such recombinants could be detected although repeated attempts were made. These results show that it was possible to reconstruct the leaky phenotype by recombining the fully resistant mutations that were orginially present in the strain 31. The small number of recombinants obtained suggests that the mutational sites are adjacent or very close. One mutated site acts as an intragenic suppressor for the other one. Similar results with acridine-induced mutants of bacteriophages were interpreted as frameshift mutations (CRICK *et al.* 1961). We, therefore, think that in pneumococcus quina-

crine is able to induce the deletion or the addition of one or more bases, an interpretation already proposed for acridine-induced mutants by CRICK *et al.* (1961).

DISCUSSION

The purpose of this investigation was to isolate and study the genetic properties of frameshif mutations in pneumococcus. Such mutants have been obtained following treatment with acridines. The seven acridines tested were mutagenic at the *amiA* locus, conferring resistance to 10^{-5} M aminopterin. The mutagenic proficiency of acridine has been already noticed in pneumococcus, inducing mutations at a capsular gene (BERNHEIMER and WERMUNDSEN 1972).

The kinetics of mutation induction exhibits interesting properties. (1) Mutagenesis can be obtained only during exponential growth. (2) When the drug concentration is increased, the maximal mutagenic effect is observed at a concentration slightly lower than the bacteriostatic value. The ability of acridines to be inserted between DNA bases has been shown in other systems (LERMAN 1961 and 1963; LUZZATI, MASSON and LERMAN 1961; PRITCHARD, BLAKE and PEACOCKE 1966), and acridine-induced mutations could result from errors in DNA replication or repair due to these insertions (STREISINGER *et al.* 1966; NEWTON *et al.* 1972). The inability to obtain mutants during the stationary phase could then result from the arrest of DNA synthesis or repair: mutants will appear as long as residual DNA synthesis persists. The absence of a mutagenic effect when the acridine dose is higher than the bacteriostatic level could be due to a release of the drug from the DNA before the resumption of either DNA replication or repair when the cells are washed and transferred to a new medium after treatment.

Another possible interpretation of the kinetics of mutant induction can be afforded by the demonstration that in Salmonella derepression is required to obtain mutants after proflavine treatment (KOHNO and ROTH 1974). At a higher drug concentration, transcription would be stopped. Similarly, genes could be repressed in the stationary phase. However, as no further experiments have been done to investigate the mechanism of mutagenesis by these compounds, either hypothesis can account for the observed results.

More intensive studies were performed on the mode of action of quinacrine, one of the most potent mutagens in our system. The optimal concentration of this drug for mutagenesis was found to depend upon the pH of the medium. Moreover, the efficiency of mutagenesis is strongly locus specific. Two possible explanations might be proposed. First, this phenomenon could be related to the function of the genes involved. Most frameshift mutations occurring in genes coding for nondispensable proteins should be lethal. For instance, our failure to obtain acridine-induced mutants for streptomycin or erythromycin genes can be explained by the fact that they code for ribosomal proteins (STUART and RAVIN 1968). Second, the high frequency of mutations for some loci might result from a selective binding of quinacrine to specific DNA sequences. Although there is no general agreement on that point (CHAN and VAN WINKLE 1969),

it has been proposed (RAMSTEIN, DOURLENT and LENG 1972) that acridines such as quinacrine or proflavine bind more specifically to AT-rich sequences. Although the precise nature of the mutagenic action of these intercalating drugs is still controversial (DRAKE and BALTZ 1976), the base composition of the DNA may account for preferential induction of mutation by acridines. Whatever the interpretation, our observation that acridine-induced mutants can be selected in only a few genes and that very strict conditions of concentration and growth are required for the mutagenesis effect may explain why these drugs were not mutagenic in some bacterial systems (LERMAN 1963). The fact that KOHNO and ROTH (1974) were unable to induce leucine or tryptophan auxotrophic mutations in Salmonella by proflavine, although they obtained high mutation rates for lactose or histidine genes, may be related to our observations of locus specificity in the effect of acridines.

The majority of quinacrine-induced mutants are readily reverted by quinacrine and not by nitrosoguanidine treatment. This suggests that the nature of the mutations induced by the two agents is different. Evidence from other systems (DRAKE 1970) strongly suggests that quinacrine may induce frameshift mutations. However, some mutants selected after quinacrine treatment might belong to the base substitution category, although their identification will remain doubtful due to their very high rate of spontaneous reversion.

A further study of the revertants obtained by quinacrine treatment of quinacrine-induced mutants was performed to ascertain whether they could result from suppressor mutations. We found no evidence of any extracistronic suppressor. Most of the revertants were genetically and phenotypically true revertants, *i.e.*, identical to the original wild type. Although the resolving power for the detection of a closely linked suppressor is fairly high in our recombination system (SICARD and EPHRUSSI-TAYLOR 1965), one cannot exclude the possibility that some revertants result from the mutation of an adjacent or nearby base.

To further identify mutations induced by quinacrine as frameshift alterations, we looked for intracistronic suppressor mutations. Some strains exhibiting partial resistance to aminopterin were found to contain two very closely linked mutated sites that could be dissected out by recombination. Each mutation conferred the maximum level of resistance to aminopterin, and an intermediate resistance was seen when the two were associated. Such strains could, therefore, contain two frameshift mutations such as a base addition and deletion that can suppress each other. Although only a few examples of such mutations have been studied in this report, due to the absence of an efficient test for the selection of leaky aminopterin-resistant mutants, this conclusion was strengthened by the results of the comparative analysis of the reversions by quinacrine and nitrosoguanidine. We propose, in consequence, that the majority of quinacrine-induced mutants at the *amiA* locus of pneumococcus consists of frameshift mutations. These mutations as well as other LE markers seem to occur anywhere on the genetic map of the *amiA* locus.

Nearly all of the isolated mutants induced by quinacrine, as well as the other acridines, belong to the LE class of transformation. It is unlikely that they are

short deletion of bases since well defined short deletions generally transform with a high efficiency (LACKS 1966; EPHRUSSI-TAYLOR, SICARD and KAMEN 1965).

These LE mutations could be either long chromosomal aberrations, such as the deletions leading to a poor efficiency of transformation as described by LACKS (1966, 1970), or single site mutations. The first possibility is excluded for at least those quinacrine-induced mutations that exhibit a high reversion rate by quinacrine and have been classified as frameshift mutations (class II). Moreover, in a nondiscriminating strain their efficiencies became similar to the reference marker, as LE single site mutations do. On the contrary, the efficiency of well-characterized deletions (LACKS 1970) does not increase in a nondiscriminating strain.

The mismatch resulting from the pairing between the wild type and the frameshift-containing sequence is recognized by the discriminating function. A small loop including the extra base may stick out of the paired structure of complementary DNA. Such a structure should be a substrate for the excision and repair system involved in the discriminating function, in a way similar to the process that recognizes mismatched base pairs between a sequence containing a transition mutation and the wild-type sequence. It appears that the discriminating function is specific for some transition and frameshift mutations.

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