Isolation of Penicillin-Susceptible Mutants from Penicillinase-Producing Strains of *M. pyogenes.** (20405)

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Recent reports(3,5) indicate that the incidence of penicillin-resistant strains of *Micrococcus pyogenes* causing infections of man is increasing. Many of the factors responsible for the emergence of such a resistant population of micrococci are as yet unknown. It is a well established fact(1,2,4), however, that the ability to produce the enzyme, penicillinase, is the primary factor in the natural resistance of these cultures of *M. pyogenes* to penicillin. Of considerable interest in this respect is the report of Barber(6) indicating that penicillin-sensitive mutants may be isolated from cultures of penicillinase-producing micrococci. The sensitive mutants described by this worker did not produce the enzyme. The study reported in this paper was undertaken to investigate this phenomenon further in an attempt to determine its possible relationship to the problem of the increasing incidence of penicillin-resistant micrococci.

Materials and methods. The strains of micrococci studied consisted of a group of penicillinase-producing cultures isolated from infections over a period of the past 5 years. Since there is no correlation between penicillinase-production and type of pigment(7), we have not classified these strains as *Micrococcus pyogenes* var. *aureus* or *Micrococcus pyogenes* var. *albus*. All cultures were maintained on brain heart infusion (B.H.I.) agar slants covered with sterile mineral oil. During the period of investigation, stock cultures of each strain were transferred monthly on B.H.I. agar slants and stored in the refrigerator. Unless otherwise noted, nutrient agar and broth were used exclusively throughout the study. During the early phases of this study, the method for detecting susceptible mutants was similar to that of Barber(6) with minor modifications. Cultures of penicillinase-producing micrococci to be tested were inoculated into large tubes of broth, 30 ml per tube, and incubated at 37°C for 24 hours, and subsequently stored at room temperature. These cultures were plated out on the surface of agar plates at various intervals, ranging from one day to several months. After suitable incubation, 50 or more colonies were picked and single streaks of each made across the surface of an agar plate. As many as 13 colonies could be tested conveniently on a single plate. Strips of sterile filter paper approximately ¼″ x 3″ saturated with penicillin, 50 units/ml, were placed across the plate perpendicular to the lines of inocula. Staphylococcus H, a known susceptible strain of *M. pyogenes* var. *aureus*, was likewise included as a control. The inocula from resistant wild-type colonies grew luxuriantly adjacent to the penicillin-saturated strip of filter paper; the inocula from sensitive mutant colonies showed zones of inhibition similar to that of the control strain. Although this method, designated as the "stripping procedure", is highly accurate as a method for detecting susceptible colonies, only a small number of cells could be tested at one time. During the course of this study, a more selective method was developed, therefore, for the detection of non-penicillinase-producing mutants. In this procedure the penicillinase-producing culture to be tested was plated on tetrazolium agar plates of the following composition: .5% tryptose, .5% proteose peptone, .4% beef extract, 1.0% dextrose, .5%NaCl and 2% agar. The medium was adjusted to pH 7.5 and sterilized in the autoclave at 15 pounds pressure for 15 minutes. Triphenyltetrazolium, .0025%, and penicillin, .025 unit/ml were added aseptically to the melted and cooled agar. On such plates, the parent wild type colonies in 48 hours are pink, glistening and convex; colonies of the sensitive mutants are paler pink, flattened, and have one or two raised concentric rings and small central dark spots. The two types of colonies

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TABLE I. Incidence of Penicillinase-Negative Mutants Isolated from Penicillinase-Producing Strains of *M. Pyogenes*.

Strain	Stripping procedure — Colonies tested	S mutants — No.	S mutants — %	Tetrazolium method — Colonies tested	S mutants — No.	S mutants — %
7170	133	6	4.5			
163	291	1	.3			
7739	154	4	2.6			
" SM resist.	1109	6	.5	39127	126	.3
7701	100	2	2.0			
7729	256	192	75.0	1797	1410	78.0
2255	150	1	.7			
161	482	0	0	13625	0	0
446	181	20	11.0	3147	317	10.1
D-3	185	3	1.6	1183	23	1.9
1215	261	17	6.5			
1175*	169	14	8.3			
7970	224	39	17.4	3489	631	18.1
7791	243	1	.4	2519	21	.8

* This strain was completely inhibited on the tetrazolium agar plates.

are readily differentiated making possible the evaluation of 200 to 400 colonies on a single plate. The mechanism of the reaction which makes possible the separation of the two types of colonies is presently under investigation. The susceptibility to penicillin of a suspected mutant colony of *M. pyogenes* was confirmed by the disk test, and when indicated by the tube dilution method. This was sufficient evidence of this organism's inability to produce penicillinase in view of the direct correlation between its susceptibility to penicillin and its inability to produce the enzyme (1,2,4). In this paper, colonies from penicillinase-producing cultures which do not produce the enzyme are designated S mutants.

Experimental results. Thirteen strains of penicillinase-producing micrococci were studied to determine their ability to give rise to non-penicillinase-producing mutants. Cultures of the strains were tested after different periods of incubation. On each trial a minimum of 50 colonies was selected at random and tested by the "stripping procedure." For comparison certain of the cultures were likewise tested by the tetrazolium agar plate method in which case only 48 hr broth cultures were tested. As shown in Table I, 12 of the 13 cultures tested produced S mutants. In general the two methods compare rather favorably as indicated by the mutation rates for those cultures tested simultaneously by both methods. The tetrazolium agar plate method, however, has the advantage of permitting the testing of much larger populations of cells. The results indicate that the mutation in question occurs at a relatively high rate of frequency although the rate varies considerably from one strain to another.

To eliminate the possibility that S mutants were contaminants, a streptomycin resistant (SMR) mutant of *M. pyogenes*, strain 7739, was included as an additional control. As indicated in Table I, penicillinase-negative colonies were also isolated from this SMR strain. These mutants were likewise streptomycin resistant. The improbability of isolating spontaneously such a double mutant (non-penicillinase-producing and streptomycin-resistant) makes less likely the possibility that the mutants isolated were contaminants. In all further work, whenever possible, the SMR strain of *M. pyogenes* 7739 was used. Further evidence that the mutants isolated were true mutants was obtained by phage typing.† Similar patterns of lysis were given by wild type and mutant cultures. Repeated passage of S mutants always yielded 100% sensitive colonies. Evidence of reverse mutation has not been detected following repeated subculture over a period of two years. The susceptibility of the mutant strains to penicillin is quite comparable to that of the known sensitive cultures of *M. pyogenes*, namely strains 209 P and H as shown in Table II.

† We are indebted to Dr. John Blair, New York City for the typing of these strains.

TABLE II. Penicillin Susceptibility of Penicillinase-Negative Mutants of *M. pyogenes* 7739 SMR.

Strain	Penicillinase	Inoculum, 0.1 ml	M.I.C.,* units/ml
7739	+	10^{-1}	25.0
		10^{-4}	.4
7739 S_1	—	10^{-1}	.1
		10^{-4}	.05
7739 S_2	—	10^{-1}	.1
		10^{-4}	.03
7739 S_3	—	10^{-1}	.1
		10^{-4}	.05
H	—	10^{-1}	.05
		10^{-4}	.013
209 P	—	10^{-1}	.1
		10^{-4}	.05

* M.I.C. = Minimal inhibiting concentration.

TABLE III. Incidence of S Mutants Isolated from *M. pyogenes* 7739 SMR Grown under Various Cultural Conditions.

Cultural condition	No. colonies evaluated	S mutants	
		No.	%
Aerobic control	5345	55	1
Anaerobic*	2498	19	.8
5% dextrose	1364	4	.3
.6% HCOH†	974	11	1.1
.04% $MnCl_2$†	1842	20	1.1
5% Na desoxycholate†	1597	36	2.2
.001% hydroxyquinoline	2504	8	.3
.08% NaN_3	3752	99	2.6
Erythromycin‡	2173	4	.2
Magnamycin‡	3092	8	.26
Terramycin‡	1724	5	.3
Chloromycetin‡	4617	200	4.3
Aureomycin‡	1906	14	.7
Growth at °C			
23	2965	36	1.2
30	1445	23	1.6
37	910	17	1.9
45	4385	1081	24.6
pH 5.85	3323	43	1.3
7.56	3365	22	.6
8.10	2836	30	1.1
Ultraviolet irradiation:			
Zero point	1426	26	1.8
End "	1227	35	2.8

* Method of Spaulding and Goode(10).
† Tested against resting cells.
‡ The maximum sub-inhibiting concentration of each.

Studies were carried out to determine factors which might influence the rate of mutation. Cultures of *M. pyogenes* 7739 SMR were grown under a variety of conditions for 48 hours and tested for S mutants by the tetrazolium agar method. Many of the cultural conditions were selected because they were known to induce various types of mutations. Others were chosen on the basis of preliminary evidence that the mutation rate might be higher under certain conditions(11) or that certain agents(8,9) might influence penicillinase production. The results of these experiments are tabulated in Table III. Most of the cultural conditions studied did not significantly influence the mutation rate. The outstanding exception, however, is cultivation of the strain tested at 45°C. At this temperature, the mutation rate was increased 20 fold. Preliminary studies indicate that this increased rate is not due to a selective action of the higher temperature.

Discussion. The sensitive mutants of penicillin-resistant strains of *M. pyogenes* as described in this report appear to be identical with those described by Barber(6). We were successful in isolating sensitive mutants from 12 of 13 (92%) strains tested whereas Barber found only 17 of 32 (53%) cultures to be positive. Our success in this respect may be attributed to the testing of larger populations of cells. Barber(6) likewise reported a high rate of mutation which varied considerably from one strain to another. Our failure to detect S mutants in strain 161 does not necessarily indicate that they are not produced by this strain. The mutation rate could be extremely low or the S mutants of this strain might be inhibited on the tetrazolium agar plates. Such inhibition was observed (Table I) with strain 1175 which produced S mutants when tested by the "stripping procedure".

The high incidence of S mutants in strain 7729 is not surprising. Barber(6) showed that certain of her strains had similar high rates of mutation. Of particular interest in regard to strain 7729, however, is the fact that when tested two years previously it had a much lower incidence of S mutants. This observation would indicate that in the case of this strain certain factors as yet unknown influence the mutation rate.

Further study is necessary before one can evaluate the significance of these susceptible mutants of *M. pyogenes,* or determine their relationship to the increasing incidence of resistant strains. It is interesting to speculate

as to the possibility of this mutation occurring *in vivo*. Preliminary attempts to isolate non-penicillinase-producing mutants from other penicillinase-producing organisms such as *E. coli* and *Shig. paradysenteriae* were unsuccessful. If non-enzyme-producing mutants are produced by these gram-negative bacilli, the frequency with which they occur is considerably lower than that of *M. pyogenes*.

Summary. Twelve of 13 strains of *M. pyogenes* which produce penicillinase yielded non-penicillinase-producing mutants which were susceptible to penicillin. This apparent spontaneous type of mutation occurs at a high rate of frequency. Cultivation of a strain of this organism at 45°C significantly increased the number of mutants. No evidence of reversibility of the mutation was obtained.

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Effect of Diet on Rate of Alcohol Oxidation by the Liver.* (20406)

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Many studies on the effects of alcohol have been conducted with little attention being given to other details of the diet. This is particularly true when relatively large amounts of alcohol are administered to animals who are otherwise consuming an adequate diet, for under these circumstances the total caloric intake is not increased, the amount of food eaten is decreased, and the consumption of essential nutrients may be reduced to a deficiency level. Effects which are attributed to alcohol may in fact be due to a concomitant nutritional deficiency (such as protein) produced by the substitution of alcohol for food. The present studies were conducted to determine how much the oxidation of alcohol itself by the liver might be influenced by protein depletion or inanition.

Experimental. The oxidation of alcohol was measured manometrically.† Livers were removed from decapitated rats, and weighed portions were homogenized with 4 volumes of 0.04M phosphate buffer, pH 7.4. Two pairs of Warburg flasks each contained 1 ml of the homogenate and 0.2 ml of 0.1M semicarbazide hydrochloride; one pair of flasks contained 0.2 ml of 15% ethyl alcohol (by volume) in the side arm. Water was added to all flasks to give a total volume of 2.0 ml, and an additional 0.2 ml of 10%KOH plus a filter paper were placed in the center wells. The flasks were equilibrated at 37° for 10 minutes, the zero reading was taken, and the alcohol was tipped in from the side arm. Readings were taken thereafter at 10-minute intervals for 80 minutes. The increased oxygen consumption in the alcohol-containing flasks was obtained by subtracting the endogenous respiration in the control flasks, and the net oxygen

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† This method is similar to the one used by Dr. Victor A. Najjar, to whom we are indebted for supplying the details prior to their publication.