Metabolism of Resistant Mutants of *Streptococcus faecalis*

I. Isolation and Characterization of the Mutants*

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The currently unavoidable phenomenon of the appearance of drug-refractory leukemic cells in children whose disease was at one time responsive to that drug has served as a stimulus for the study of strains of microorganisms resistant to such agents. A series of mutants of *Streptococcus faecalis* has been selected, and these mutants exhibit various degrees of resistance to A-methopterin, 6-mercaptopurine, and 2,6-diaminopurine—all of which have been shown to be temporarily effective against certain mouse leukemias and human leukemias (2, 3, 9, 15, 16, 21). The general procedures for selection of these resistant *S. faecalis* mutants as well as maintenance and growth characteristics of all cultures will be discussed.

MATERIALS AND METHODS

The growth media used throughout these experiments are various modifications of the folic acid assay medium described by Flynn et al. (10): this folic acid-free medium will be referred to as the F medium. The omission of the purines, adenine, guanine, and xanthine, and of the pyrimidine, thymine, results in a medium which will be designated as F-PP; supplements of folic acid, 1 mg/ml, or thymine at 1 μg/ml will be indicated as F-PP + PGA and F-PP + T, respectively.

*S. faecalis* ATCC 8043 (SF/O, which indicates a lack of resistance to the antimetabolite studied), which requires folic acid, 1 mg/ml, or thymine at 1 μg/ml will be indicated as F-PP + PGA and F-PP + T, respectively.

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The method, which has been described in detail (5, 18), used for the selection of the resistant mutants was that of serial transfer through an appropriate basal medium that had been supplemented with increasing concentrations of an antimetabolite. The solubility of the antimetabolite controlled, to some extent, the degree of resistance of the mutant. In general, when maximum resistance was attained, the culture was transferred daily or every other day in the medium which contained the antimetabolite until there was as much growth of the resistant mutant in 18 hours in the antimetabolite medium as there was of the parent in 18 hours in the absence of the antimetabolite. At this time the mutant was transferred to agar stabs which contained the same concentration of the antagonist as the selection medium, and it was then maintained and handled for experimental work according to the recommended procedures for lactic acid bacteria (20).

Conventional microbiological vitamin assay technics were employed, i.e., at the beginning of each week broth cultures (5 ml.) were made from the agar stabs and incubated at 35° C. for 24 hours. Subsequent cultures for the following day were made from this broth culture, which in turn served as the inoculum for the following day. The seed culture for each experiment was incubated for 18–20 hours at 35° C., after which interval it was spun down and washed with 5 ml. sterile saline. The washed suspension was then diluted so that it contained approximately 1.8 X 10^7 cells per milliliter. This suspension was used as the inoculum, 0.1 ml. of which was pipetted to sterile 13 X 100-mm. assay tubes which contained 2 ml. of medium. The rack of tubes was shaken vigorously to assure an even distribution of inoculum and was then placed in a 35° C. incubator for growth. Optical density measurements were made on a Coleman Junior Spectrophotometer at various times which will be indicated in each experiment.

The names of all the cultures will be appropriately abbreviated to the initial letters of the generic and specific names, and then these initials are followed by an abbreviation of the antimetabolite to which primary resistance was attained.*

RESULTS

The selection of the A-methopterin-resistant mutant, *S. faecalis*/A with a 20,000-fold increase in resistance over the parent SF/O strain has been reported (4). This mutant was subsequently transferred 18 more times in the F medium supplemented with A-methopterin, and an additional 15-fold increase in resistance was attained. This SF/A mutant, which has a 300,000 increase in A-methopterin tolerance over the parent strain in the F medium, has been used in these and subsequent studies. The procedure used in the selection of *S. faecalis*/MP has been described (11), and a second 6-mercaptopurine-resistant mutant *S.

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1 The strains studied have been designated as follows:

SF/O = parent (ATCC No. 8043).

SF/MP = resistant to 6-mercaptopurine.

SF/MP/A = double mutant, resistant to 6- and A-methopterin.

SF/MP/C = resistant to 6-MP, isolated independently of SF/MP.

SF/DAP = resistant to 2,6-diaminopurine.

SF/A = resistant to A-methopterin.

SF/A/O = partial revert of SF/A.

SF/A/MP = double mutant, resistant to A-methopterin and 6-MP.

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faecalis/MPcc was selected according to the same regimen. Both organisms show a 50-fold increase in resistance to 6-mercaptopurine in comparison with SF/O in the F-PP + PGA medium.

S. faecalis/A/O is a subculture of SF/A which was obtained after 350 transfers in F + PGA medium. The mutant has a 50-fold increase in A-methopterin resistance over SF/O and is thus MP/A, had the same quantitative requirement as SF/O; and two mutants, SF/A and SF/A/MP, required more folic acid and an additional 28 hours of incubation before maximum growth was obtained; the other three mutants had a lower quantitative requirement than SF/O, which observation suggests that they are more efficient in the de novo pathway of total nucleic acid biosynthesis.

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tbody>
<tr>
<td><strong>MAINTENANCE MEDIA FOR Streptococcus faecalis</strong></td>
</tr>
<tr>
<td><strong>SUPPLEMENTS (μg/ml) TO F-PP MEDIUM</strong></td>
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<tr>
<td><strong>Adenine (μg)</strong></td>
</tr>
<tr>
<td>S. faecalis ATCC 8043 (SF/O)</td>
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<tr>
<td>S. faecalis/A (SF/A)</td>
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<tr>
<td>S. faecalis/MP (SF/MP)</td>
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<tr>
<td>S. faecalis/MPcc (SF/MPcc)</td>
</tr>
<tr>
<td>S. faecalis/DAP (SF/DAP)</td>
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<tr>
<td>S. faecalis/A/O (SF/A/O)</td>
</tr>
<tr>
<td>S. faecalis/A/MP (SF/A/MP)</td>
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<tr>
<td>S. faecalis/MP/A (SF/MP/A)</td>
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</tbody>
</table>

The following abbreviations have been used: PGA, pteroylglutamic acid; A-meth., A-methopterin; 6-MP, 6-mercaptopurine; DAP, 2,6-diaminopurine.

* The amount of each supplement (in μg/ml) is given in parentheses. F-PP medium = folic acid-free medium without purines (adenine, guanine, and xanthine) and without the pyrimidine, uracil.

1/6000 as resistant as its immediate parent, SF/A. Mutant S. faecalis/DAP, which has more than a 300-fold increase in resistance to 2,6-diaminopurine in the F-PP + PGA medium, was isolated after 84 transfers in F-PP + PGA medium supplemented with 2,6-diaminopurine.

S. faecalis/A/MP is an isolate from SF/A, which was selected after 49 transfers on the F-PP + PGA medium containing 6-MP. S. faecalis/MP/A was selected from SF/MP after 70 transfers in an A-methopterin-supplemented F-PP + PGA medium.

The maintenance media for SF/O and the seven mutants are summarized in Table 1. The basal medium in all cases was the F-PP medium; all media were completely formulated and then sterilized at 121°C. for 10 minutes. Daily transfers of each organism were made on the above liquid media, and stock cultures were maintained in stab on the appropriate medium to which 1.5 percent agar had been added.

The quantitative requirements of the eight organisms for folic acid are compared in Table 2. Three media (F-PP, F-PP + T, and F) were compared. These amounts of folic acid in μg/ml for half-maximum growth were determined after an incubation period of 18–20 hours, except in the case of SF/A and SF/A/MP, in the F-PP and F-PP + T media, both of which were incubated for 48 hours. Two mutants, SF/MP and SF/MPcc, had the same quantitative requirement as SF/O; and two mutants, SF/A and SF/A/MP, required more folic acid and an additional 28 hours of incubation before maximum growth was obtained; the other three mutants had a lower quantitative requirement than SF/O, which observation suggests that they are more efficient in the de novo pathway of total nucleic acid biosynthesis.

<table>
<thead>
<tr>
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<tbody>
<tr>
<td><strong>QUANTITATIVE FOLIC ACID REQUIREMENTS (μg/ml) FOR HALF-MAXIMUM GROWTH</strong></td>
</tr>
<tr>
<td><strong>Incubation period: 18–20 hours</strong></td>
</tr>
<tr>
<td><strong>ORGANISM</strong></td>
</tr>
<tr>
<td>SF/O</td>
</tr>
<tr>
<td>SF/A</td>
</tr>
<tr>
<td>SF/MP</td>
</tr>
<tr>
<td>SF/MPcc</td>
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<tr>
<td>SF/DAP</td>
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<tr>
<td>SF/A/O</td>
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<tr>
<td>SF/A/MP</td>
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<td>SF/MP/A</td>
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</table>

* F-PP medium = see footnote *, Table 1.
† F-PP + T medium = same medium as above, except supplemented with thymine, 1 μg/ml.
‡ F medium = folic acid-free medium.
§ Incubation period, 48 hours.

The addition of thymine to the F-PP medium spares the folic acid requirement of SF/O (22) but has no sparing effect for any of the mutants except SF/A/MP. The results obtained in F-PP + T medium suggest that SF/A/MP is most similar to the parent strain in that it used preformed thymine in preference to de novo synthesis of thymine; the other mutants apparently utilized the de novo route preferentially. Quantitative thymine requirements in the F medium are also given in Table 2 and all mutants required less thymine than SF/O.
The addition of adenine, guanine, xanthine, and uracil to the F-PP medium, which results in the F medium, spared the folic acid requirement of all organisms except SF/MPcc, SF/DAP, and SF/A/O.

The purine-sparing effect on the folic acid requirement of SF/O was marked, and about the same effect was observed with SF/MP and SF/MP/A. A more pronounced effect was noted with SF/A, which required one-third the amount of folic acid that the parent strain needed for half-maximum growth (14).

TABLE 3

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>Norm</th>
<th>A</th>
<th>ADS</th>
<th>G</th>
<th>GUS</th>
<th>H</th>
<th>INS</th>
<th>X</th>
<th>XNS</th>
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</thead>
<tbody>
<tr>
<td>SF/O</td>
<td>0.38</td>
<td>.47</td>
<td>.43</td>
<td>.44</td>
<td>.45</td>
<td>.42</td>
<td>.44</td>
<td>.47</td>
<td>.45</td>
</tr>
<tr>
<td>SF/A</td>
<td>0.30</td>
<td>.40</td>
<td>.34</td>
<td>.33</td>
<td>.34</td>
<td>.31</td>
<td>.33</td>
<td>.34</td>
<td>.32</td>
</tr>
<tr>
<td>SF/MP</td>
<td>0.39</td>
<td>.41</td>
<td>.39</td>
<td>.39</td>
<td>.39</td>
<td>.36</td>
<td>.38</td>
<td>.40</td>
<td>.46</td>
</tr>
<tr>
<td>SF/MPcc</td>
<td>.34</td>
<td>.43</td>
<td>.39</td>
<td>.31</td>
<td>.32</td>
<td>.37</td>
<td>.38</td>
<td>.41</td>
<td>.40</td>
</tr>
<tr>
<td>SF/DAP</td>
<td>.35</td>
<td>.40</td>
<td>.37</td>
<td>.39</td>
<td>.37</td>
<td>.37</td>
<td>.38</td>
<td>.39</td>
<td>.40</td>
</tr>
<tr>
<td>SF/A/O</td>
<td>.40</td>
<td>.41</td>
<td>.39</td>
<td>.40</td>
<td>.39</td>
<td>.40</td>
<td>.41</td>
<td>.42</td>
<td>.40</td>
</tr>
<tr>
<td>SF/A/MP</td>
<td>0</td>
<td>.41</td>
<td>.34</td>
<td>.36</td>
<td>.36</td>
<td>.43</td>
<td>.41</td>
<td>.41</td>
<td>.41</td>
</tr>
<tr>
<td>SF/MP/A</td>
<td>.36</td>
<td>.37</td>
<td>.40</td>
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<td>.37</td>
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</tr>
</tbody>
</table>

- F-PP + PGA medium = folic acid-free medium without purines (adenine, guanine, and xanthine) and without the purine ribosides. Growth of SF/A and SF/A/MP was similar to SF/A; the ribosides, with the exception of adenosine, were equal to the folic acid in stimulating growth. However, when 42-hour incubation periods of similar cultures were carried out, the growth on all purine derivatives was essentially the same.

The growth of SF/A was slightly less on each riboside than on the corresponding free base, and at the concentration used in these experiments xanthine was the least effective base. When higher concentrations of xanthine were added to the medium, greater growth was obtained; but the growth was always less than that with an equivalent concentration of any of the other purines.

The quantitative folic acid requirement of certain cultures as shown in Table 2 was affected by the purine supplementation; therefore, experiments were carried out to determine the stimulatory effect of purines and purine ribosides in the F-PP + PGA medium and the growth-supporting effect they might have in the F-PP + T medium. The results (Table 3) show that during an incubation period of 18–20 hours in the folic acid medium (F-PP + PGA), the growth of only two organisms (SF/A and SF/A/MP) was stimulated by the addition of the purine derivatives to this medium. The results with SF/A indicate that there was little difference among the purine derivatives but that the growth on adenine and adenosine was somewhat better than on the other purines and purine ribosides. Growth of SF/A/MP was similar to SF/A; the ribosides, with the exception of adenosine, were equal to the folic acid in stimulating growth. However, when 42-hour incubation periods of similar cultures were carried out, the growth on all purine derivatives was essentially the same.

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The results obtained in the F-PP + T medium were essentially the same for all organisms, with the notable exception of SF/MP. As we had previously reported (11, 12), this mutant grows well only on xanthine and xanthosine, and a trace of growth was noted on isoguanine. The lack of such a marked change in SF/MPcc, which was selected under conditions identical to those used for the isolation of SF/MP, was surprising; however, the alterations in the double mutants which allow them to grow on all purines suggest that there may be several pathways which are responsible for resistance to 6-MP. In the F-PP + T medium...
the concentration of purine derivative (8 × 10⁻⁴ moles/liter) was the growth-limiting factor; in all cases when the concentration of purine was increased threefold there was an increase in growth of about 50 per cent.

Some additional observations (Table 4) on quantitative alterations in mutants were noted in a study of the effects of the antimetabolites, A-methopterin, 6-mercaptopurine, and 2,6-diaminopurine, on the inhibition of growth. These values (Table 4) represent the amounts of antagonist in μg/ml that permitted half-maximum growth. The results on A-methopterin inhibition in the F + PGA medium show that SF/A, SF/A/MP, and SF/MP/A had a 300,000-fold increase over the parent strain in A-methopterin resistance. SF/A/O was next in increased resistance and had a 50-fold increase. SF/DAP had the same tolerance as SF/O, while the two 6-MP-resistant mutants had a fourfold increase in resistance. In the F-PP + PGA medium somewhat different results were obtained. The inhibitory effect of A-methopterin was essentially the same as it was in the previous medium, with the exception of SF/A and SF/A/MP, both of which tolerated less A-methopterin in this medium than they did in the F medium; presumably this also may be a reflection of the decreased de novo biosynthetic capacities of the mutants.

The effects of 6-mercaptopurine and 2,6-diaminopurine are also enumerated in Table 4; SF/A was more sensitive to 6-MP than was the parent SF/O, while all others showed a marked increase in 6-MP resistance. There was an increase in resistance to 2,6-diaminopurine in all organisms except SF/A/MP.

**DISCUSSION**

These studies on the selection, cultivation, and maintenance of resistant mutants of *S. faecalis* indicate the facility with which such microorganisms can be obtained. If the ease of the selection of such assorted mutants in a biological system such as *S. faecalis* is an indication of the distribution of resistant mutants in a population of cells of mouse leukemia or cells of human leukemia, the problems of chemotherapy are extremely acute.

Among the seven mutants, SF/A and SF/A/MP are unique in that they are inefficient in the de novo synthesis of purines but have an increased efficiency in thymine synthesis. The decreased ability to make purines is evidenced in the increased folic acid requirements in the F-PP and F-PP + T media; the preferential utilization of preformed purines as shown by the marked stimulation of growth when purines such as adenine, guanine, hypoxanthine and xanthine are added to the F-PP + PGA medium; and the rather marked alteration in A-methopterin sensitivity in F-PP + PGA medium in contrast to the F + PGA medium. The increased efficiency in thymine synthesis may be observed in the data which show a decrease in the amount of thymine required for the growth of SF/A and SF/A/MP in the F medium. A further example is the decrease in the folic acid requirement of SF/A in the F medium in contrast to its folic acid requirement in the F-PP + T medium (0.1 μg/ml to 5.0 μg/ml).

Another profound difference was observed with SF/MP. It has been reported that the mutant cannot grow on purine derivatives other than xanthine and isoguanine (11, 12); thus an attempt was made to determine whether the alteration would be manifested in all mutants resistant to 6-mercaptopurine. Indeed this was not the case, as can be seen when three additional mutants of equal resistance, SF/MPcc, SF/MP/A, and SF/A/MP, are compared with SF/MP. Even though the three mutants are equally resistant to 6-mercaptopurine, they grow on adenine, guanine, hypoxanthine, and their respective ribosides as well as on xanthine; therefore their ability to use preformed purines for growth is identical with the nonresistant parent strain.

These 6-mercaptopurine-resistant organisms, except for SF/A/MP, whose immediate parent was SF/A, show no difference in quantitative folic acid requirements in the presence or absence of thymine; this suggests that they are slightly more efficient in de novo purine biosynthesis than is
SF/O, SF/MP and SF/MP/A are similar to the wild strain in folic acid requirement in the F medium. Since SF/MPcc needs the same amount regardless of thymine or purine supplementation, it would appear that SF/MPcc has an equal capacity to carry out de novo synthesis or to utilize preformed nucleic acid precursors.

SF/MP and SF/MPcc show an increase in resistance to A-methopterin, which, in the case of the first mutant, may be attributed to its slight decrease in folic acid requirement; however, this explanation certainly cannot be ascribed to the second mutant, since no such decrease was observed in the F medium even though it was noted in the F-PP medium.

SF/DAP is of interest in that it requires the same amount of folic acid in all the media studied; and this observation is further corroborated, since A-methopterin was equally effective in the F-PP and F media.

The mutant SF/A/O, which is a partial revert from SF/A, exhibits some characteristics that are identical with those of SF/A/MP and others like those of SF/MPcc. Therefore, one is left with the conclusion that resistance to one drug does not result in a set of unique modifications in the mutants.

Many biochemical mechanisms have been postulated as explanations of antibiotic and drug resistance in bacteria (6), and examples of each have been demonstrated by several investigators (7, 8, 17–19, 23). It is necessary to reiterate at this point that these mechanisms have been postulated on the basis of information gained, in most cases, from one resistant mutant. It is important that one should not conclude that all organisms resistant to a particular drug will have identical modifications—indeed, the results obtained with these antimetabolite-resistant mutants of *S. faecalis* indicate myriad alterations. In the present studies, in which emphasis has been placed on the growth processes relative to nucleic acid biosynthesis, with particular observations on both the route of de novo purine formation and the route concerned with the utilization of preformed purines and purine derivatives, it is obvious that the mutations which were responsible for these resistant strains were not the same for any two resistant organisms studied.

The multiplicity of alterations in these *S. faecalis* mutants suggests that in nature there are many mechanisms whereby an originally sensitive bacterial population can become refractory to an inhibitory agent. The interrelationships among the observations made here make it impossible to devise an unequivocal explanation for the resistance to A-methopterin, 6-mercaptopurine, or 2,6-diaminopurine in *S. faecalis*.

These growth data will be further elucidated by additional experiments dealing with the utilization of a variety of C14-labeled nucleic acid precursors (1).

**SUMMARY**

The selection and maintenance of seven antimetabolite-resistant strains of *Streptococcus faecalis* have been described. These mutants were compared with the parent strains in regard to quantitative folic acid utilization, and two A-methopterin-resistant mutants were significantly different from the other cultures. In media that did not contain purines, they required high concentrations of folic acid and an excessively long incubation time for growth. The other mutants in which a difference was noted (SF/MPcc, SF/DAP and SF/A/O) showed a shift toward a more efficient utilization of folic acid for de novo purine, or purine and thymine, synthesis.

SF/A and SF/A/MP were the only mutants whose growth on folic acid was stimulated by the addition of purines to the media; and SF/MP was unique in its lack of ability to grow on adenine, guanine, and hypoxanthine and their respective ribosides in a thymine medium.

Some degree of resistance to A-methopterin was observed with all mutants. Similar increases in resistance were observed toward 6-MP, with the exception of SF/A which, in short-term (18-hour) experiments, appeared to be more sensitive to the antimetabolite. Increased resistance to 2,6-diaminopurine was seen in all cases except SF/A/MP.

The multiplicity of changes observed in these mutants has been compared with the parent strain in an attempt to elucidate some of the pathways involved in nucleic acid biosynthesis.

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**REFERENCES**


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5. ———. Development of Resistance to 2,4-Diamino-5-(S',4'-dichlorophenyl)-6-methylpyrimidine (SK 5955) by Streptococcus faecalis. Ibid., 82:836-38, 1953.


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Dorris J. Hutchison


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