Sequential Mutagenesis of Drug Resistance in *Streptococcus mutans* during Synchronous Replication

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The species *Streptococcus* (*S.*) *mutans* has a cariogenic property thought to be related to the production of a capsular glucan that facilitates colonization of the tooth (Gibbons and Nygaard 1968). A role of plasmids (Higuchi et al. 1973) and genetic exchange (Freedman and Tanzer 1974) which might be responsible for the glucan synthesis has been discussed with regard to the pathogenicity of *S. mutans* (Tanzer et al. 1974). However, no direct evidence for the existence of intra- or extra-chromosomal elements responsible for the synthesis of glucan has been reported. One of the possible reasons for this may be the lack of methods to analyze the gene action in *S. mutans*, although some mutation researches of the organisms by using transformation (Westergren and Emilson 1977), or conjugation (LeBlanc et al. 1978) have been reported. Jyssum (1969) described a method for mapping of several genes of *Neisseria* (*N.*) *meningitidis*, which is based on the enumeration of mutants induced by nitrosoguanidine during synchronous replication of chromosomes after release from prolonged chloramphenicol inhibition. The results showed that the treatment with nitrosoguanidine of successive samples from

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synchronized cultures of *N. meningitidis* produces a maximum of a given type of mutants at the time the corresponding gene is being replicated, in way that has been described for *Escherichia (E.) coli* (Cerdà-Olmedo et al. 1968) or *Mycobacterium tuberculosis* (Woodley et al. 1981). This method permitted the construction of replication maps which were in general agreement with mapping based on marker frequency analysis by use of transformation system (Jysum 1965). Thus, the methods may be applied to study the genomes of *S. mutans* that might control the glucan synthesis or other biochemical properties related to their cariogenicity.

As one approach to explore such fields, in the present paper we describe that the method can determine the replication order of loci for resistance to streptomycin, bacitracin and rifampicin in *S. mutans*.

**MATERIALS AND METHODS**

*Bacterial strains and media.* The strains 102, originally isolated from the dental plaque of a patient with dental caries (Usui 1977), and GS5 (Gibbons et al. 1966) of *S. mutans* were used. The strain 102 produced both soluble and insoluble glucans in heart infusion broth (Difco Laboratories, Detroit, Mich.) containing 5 per cent sucrose, showed a mucoid form on mitis salivarius agar (Difco), and utilized mannitol as a source of carbon. Both strains were maintained on brain heart infusion agar (Difco) at 4°C and transferred by culturing in this medium every two months.

*Agents.* N-methyl-N'-nitro-N-nitrosoguanidine (nitrosoguanidine) was obtained from Wako Pure Chemical Co., Osaka. It was dissolved in sterile, distilled water at 2 mg/ml, and kept frozen until use. Chloramphenicol, streptomycin sulfate, bacitracin and rifampicin were purchased from Sankyo Co., Tokyo, Meiji Seika Co., Tokyo, Sigma Chemical Co., St. Louis, Mo., and Daiichi Seiyaku Co., Tokyo, respectively.

*Synchronization and mutagenesis.* The cells were synchronized by the procedure of Jysum (1969) with slight modifications. One ml of an exponentially growing culture was added to 25 ml of brain heart infusion broth (Difco) and then incubated for 2 hr at 37°C in an atmosphere of 5 per cent CO₂/95 per cent air using a CO₂ incubator for tissue culture (Usui 1977). The culture to which chloramphenicol was added in a final concentration of 4 µg/ml was incubated for another 2 hr. Subsequently, the cells were harvested by centrifugation at 2,500 × g for 20 min and suspended in 1 ml of phosphate-buffered saline (PBS). The cells were then suspended in 70 ml of brain heart infusion broth and incubated at 37°C. Five ml of aliquote was taken from the culture every 5 min, quickly cooled in ice water and centrifuged at 1,500 × g for 10 min at 4°C. The cells thus obtained were re-suspended in 1 ml of PBS containing nitrosoguanidine and incubated at 37°C for 20 min (Adelberg et al. 1965). After incubation the suspensions were diluted with 5 ml of PBS. The cells were recovered by centrifugation, washed once with 5 ml of PBS, and finally suspended in 10 ml of brain heart infusion broth.

Selection of mutants resistant to streptomycin, bacitracin and rifampicin was carried out as follows: The test cell suspensions were incubated for 3 hr or longer at 37°C before exposure to drugs for phenotypic expression. The cells were then plated on brain heart infusion agar containing 60 µg/ml of streptomycin, 5 units/ml of bacitracin or 5 µg/ml of rifampicin, incubated at 37°C for 48 hr and counted number of colonies appeared on each selective plate. The inoculates during platings were chosen according to the mutation frequencies observed in preliminary experiments, so that the calculation should be based on counting no less than 100 colonies. Viable cells were counted by the plating of appropriate dilutions on brain heart infusion agar plates. Each point in the graphs represents the frequency of mutants obtained with one plate of mutants and the average of two to three plates of viable count.
RESULTS

Mutagenic effect of nitrosoguanidine on drug resistance

First, the induction of streptomycin-resistance (Str-r) by nitrosoguanidine was examined in nonsynchronized populations of strains 102 and GS5 at an exponentially growing phase (Table 1). Strain 102 tolerated more nitrosoguanidine than strain GS5. The treatment of strain 102 with nitrosoguanidine at 200 µg/ml resulted in a 26 per cent survival and induced Str-r mutants with a mutation rate of $765.7 \times 10^{-5}$/survivor. In strain GS5, at 50 µg/ml of nitrosoguanidine a 25 per cent survival and a mutation rate of $958.3 \times 10^{-5}$/survivor were obtained.

Table 2 shows the mutation rates of streptomycin-, bacitracin-(Bac) and rifampicin-(Rif) resistance induced by nitrosoguanidine at 200 µg/ml in nonsynchronized cultures of strain 102. The mutation rates by nitrosoguanidine were high in order to bacitracin ($5,862.1 \times 10^{-5}$/survivor), streptomycin ($765.7 \times 10^{-5}$/survivor), and rifampicin ($5.8 \times 10^{-6}$/survivor).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dose (µg/ml)</th>
<th>Surviving cells</th>
<th>Str-r mutants (10⁻⁵ survivors)</th>
</tr>
</thead>
<tbody>
<tr>
<td>102</td>
<td>0</td>
<td>$3.7 \times 10^7$</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>$2.1 \times 10^7$</td>
<td>489.2</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>$9.5 \times 10^6$</td>
<td>765.7</td>
</tr>
<tr>
<td>GS5</td>
<td>0</td>
<td>$7.7 \times 10^6$</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>$1.9 \times 10^6$</td>
<td>958.3</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>$3.0 \times 10^5$</td>
<td>1,043.5</td>
</tr>
</tbody>
</table>

Nonsynchronized cultures at a growing phase were mutagenized with nitrosoguanidine for 20 min and plated on brain heart infusion agar containing 60 µg/ml of streptomycin (Str). Numbers of Str-r mutants were counted after 48 hr of incubation.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (µg/ml)</th>
<th>Resistant mutants (10⁻⁵ survivors)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomycin</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>765.7</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>0</td>
<td>49.5</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>5,862.1</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>5.8</td>
</tr>
</tbody>
</table>

Nonsynchronized cultures at a growing phase were mutagenized and plated on brain heart infusion agar containing 60 µg/ml of streptomycin, 5 units/ml of bacitracin or 5 µg/ml of rifampicin, respectively.
survivor) and rifampicin (\(5.8 \times 10^{-5}/\text{survivor}\)). In either case, nitrosoguanidine-induced mutation rate was 100 times or more as high as its spontaneous mutation rate.

**Synchronous growth by chloramphenicol inhibition**

Fig. 1 shows the viable counts of strain 102 in culture after release from chloramphenicol inhibition demonstrating a stepwise growth with a doubling time of approximately 50 min in three cycles during a 150 min period. With strain GS5, a stepwise growth in three cycles with a doubling time of approximately 50 min was also observed.

![Fig. 1. Synchronous growth of *S. mutans* strain 102 by chloramphenicol treatment.](image)

After release from 2 hr of incubation with chloramphenicol (4 \(\mu\)g/ml), viable cells of successive samples were counted on brain heart infusion agar every 10 min.

**Mutagenesis of drug resistance in synchronized cultures**

Samples of synchronized cultures of strain 102 which had been released from chloramphenicol inhibition were mutagenized with 200 \(\mu\)g/ml of nitrosoguanidine every 5 min and the mutation frequencies to streptomycin, bacitracin and rifampicin were measured (Fig. 2). To either drug, an increase in the mutation frequency could be observed at certain times after release from chloramphenicol inhibition. Between the peaks corresponding to individual markers, time differences were observed; the times of peaks for Str-, Bac- and Rif-r were 13, 22 and 12 min, respectively. The distances between the first and second peaks in each case were approximately 50 min, indicating that the peak in the second step of growth occurred at the same time as that in the first step. A similar experiment recording the appearance of Str-r mutants in strain GS5 shows that the two peaks of Str-r mutation were observed at 7 and 60 min (Fig. 3).

Table 3 depicts the results of repeated experiments in which the replication times and the times of the first peak in mutation frequency for three markers of strain 102 are shown. The times of peaks in mutation frequency are also represented as the quotients to average replication time. The results indicate that the mutations of Rif-, Str- and Bac-r may occur sequentially in *S. mutans* during synchronous replication and that the markers for Rif- and Str-r may be near the replication origin.
Fig. 2. Sequential mutagenesis for resistance to various drugs in synchronized populations of *S. mutans* strain 102. Successive samples were taken every 5 min from synchronized cultures, mutagenized sequentially with nitrosoguanidine (200 µg/ml) and assayed for resistance mutation to streptomycin (A), bacitracin (B) or rifampicin (C).

Fig. 3. Sequential mutagenesis for streptomycin resistance in synchronized populations of *S. mutans* strain GS5.

<table>
<thead>
<tr>
<th>Expt</th>
<th>Rif</th>
<th>Str</th>
<th>Bac</th>
<th>Replication time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12*</td>
<td>13</td>
<td></td>
<td>49</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>15</td>
<td>22</td>
<td>54</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td></td>
<td>20</td>
<td>52</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>21</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Average</td>
<td>(0.25)†</td>
<td>14</td>
<td>21</td>
<td>51</td>
</tr>
</tbody>
</table>

* The time (min) of appearance of the first peak.
† The quotients of the average time of appearance of first peaks to average replication time.

Samples of the synchronized culture were taken every 5 min and mutagenized with nitrosoguanidine for the 20 min.
DISCUSSION

Cerdá-Olmedo et al. (1968) described that the treatment with nitrosoguanidine of successive samples from a culture synchronized by amino acid starvation followed by thymine deprivation in *E. coli* produces a maximum of a given type of mutants at the time the corresponding gene is being replicated. They noted that nitrosoguanidine mutagenizes the replication point of the chromosome with higher efficiency than other parts of the chromosome and used this property to describe the sequence of gene replication in *E. coli*. Later, Jyssum (1969) found that the determination of nitrosoguanidine-induced mutation frequencies in *Neisseria* species during synchronous replication after release from chloramphenicol inhibition permitted the construction of replication maps. He also showed that this replication map was in general agreement with mapping based on marker frequency analysis by use of a transformation system (Jyssum, 1965). Thus, it is found that the model to describe the sequence of gene replication presented by Cerdá-Olmedo et al. (1968) in *E. coli* may be valid also for *N. meningitidis*.

The present results demonstrate that the treatment with nitrosoguanidine of successive samples from synchronized cultures after release from chloramphenicol inhibition of *S. mutans* produces a clear maximum of Str-, Bac- and Rif-r mutants at certain times in the stepwise growth in at least two cycles. These times were different for Str-, Bac- and Rif-r. At a definite time after the first maximum, there was a second one. The intervals between successive maxima were identical for all markers in each experiment. An increase in the frequency of Str-r in strain GS5 was also observed at the same time after release from chloramphenicol inhibition of strain 102. The intervals between two peaks were identical between strain GS5 and strain 102. Repeated experiments showed that the time of appearance of a maximum for Str-r mutants is nearly constant in different experiments, showing a quotient to average replication time of 0.27 near the value for Rif-r (0.25). These results suggested that both markers for Str-r and Rif-r are near the replication origin. Using transformation analysis, Harford and Sueoka (1970) demonstrated that the markers for Rif-r and Str-r in *Bacillus subtilis* are clustered near the replication origin. Sarubbi, Blackman and Sparling (1974) also showed that independent genes for Rif-r and Str-r in strains of *Neisseria gonorrhoeae* occurred in a linked cluster. The position of replication order for Str-r in *S. mutans* (0.27) demonstrated in the present study corresponds well to that in *E. coli* as revealed by synchronized culture treated with nitrosoguanidine (Cerdá-Olmedo et al. 1968). Although the resistance to bacitracin is one of the important markers used for typing of *S. mutans* subgroups (Shklair and Keene 1974), no information is available on the position of replication order either in *S. mutans* or other species. The present results suggest the possibility that the position of Bac-r gene in the chromosomes is far from the replication origin in *S. mutans*.

Of the various *Streptococcus* species examined for competence in transformation system, none of the *S. mutans* isolates exhibited a transforming ability (Westergren and Emilson 1977). We have found that the present methods also can detect the
sequence of replication of genes responsible for the fermentation of certain carbohydrates in *S. mutans* (in preparation). Therefore, the method may provide a useful tool for determining the sequence of gene replication for the extracellular polysaccharide production or for other biochemical properties related to a cariogenic potential of *S. mutans*.

**References**


