Role of unsaturated fatty acid biosynthesis in virulence of *Streptococcus mutans*

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Running title: fatty acid synthesis and virulence in *S. mutans*

Key words: acid adaptation, oral streptococci, virulence, membrane fatty acids
An insertionally inactivated \textit{fabM} strain of \textit{S. mutans} does not produce unsaturated membrane fatty acids and is acid sensitive (E.M. Fozo and R. G. Quivey, Jr., J. Bacteriol. 186: 4152-4158, 2004). In this study, the strain was shown to be poorly transmissible from host to host. Animals directly infected with the \textit{fabM} strain exhibited fewer and less severe carious lesions than observed with the wild-type strain.
Streptococcus mutans, a major etiologic agent of dental caries in humans, produces organic acids during fermentation and must survive the resulting acidic milieu. One result of decreasing environmental pH is an increase in the proportion of monounsaturated membrane fatty acids (UFAs) (4, 9). We have shown, via insertional inactivation of fabM in S. mutans, that the gene is solely responsible for unsaturated fatty acid production (3). Moreover, the fabM mutant strain exhibited several markedly acid-sensitive attributes that differed from the wild-type, S. mutans UA159: it was approx. 3.5-log orders more sensitive to extreme acid stress; it produced approx. 1.5-log orders less acid; and, it was able to maintain approx. half of the trans-membrane ΔpH (3). In addition, the construct was highly stable through at least 20 generations in our chemostats (data not shown). The ability to survive low pH conditions is thought to be critical for S. mutans to persist in the oral cavity and cause disease. The availability of a defined strain, defective in unsaturated fatty acid biosynthesis and acid-resistance, provided an opportunity to investigate whether acid-sensitivity influences transmissibility from host to host and pathogenesis in a rodent model of dental caries (1, 7).

Transmission of a fabM strain (UR117StR) was measured by the ability of the organism to pass from dams to pups and from infected cagemate to uninfected cagemate as outlined in Figure 1. For the transmission experiment, 8 litters of Sprague Dawley rats, aged 15 days, and their dams were obtained from Harlan Laboratories. Dams were determined to be mutans streptococci-free and sialoacroadenitis virus free by as described previously (1, 8). Mid-logarithmic cultures of either wild-type UA159StR (2) or UR117StR, a spontaneous streptomycin-resistant strain of UR117, were used to infect 2 dams on three consecutive days while the remaining dams were uninfected. It should be noted here, that while the streptomycin-
resistance genotype of the two test strains may not be identical, growth of the UR117St\textsuperscript{R} strain was virtually identical to that of the UR117 strain, in vitro; and, that streptomycin-resistance has not previously been shown to effect results from rodent infection studies (2, 11, 12). In the present study, in all cases, infected animals received Diet-2000 (5) and 5% (w/v) sucrose drinking water \textit{ad libitum}; uninfected animals were fed lab chow and water \textit{ad libitum}. On day 6, pups (aged 21 days) associated with infected dams were screened for successful infection of either strain by oral swabbing and plating on MSS agar. None of the pups caged with infected dams became infected by day 6 (Table 1a).

After we determined that pups had not become infected with detectable numbers of bacteria, (Group I and Group II animals, Fig. 1), the animals were directly infected by oral swabbing with UA159St\textsuperscript{R} or UR117St\textsuperscript{R}, respectively. Following oral swabbing, all pups became infected by experimental day 8 (data not shown), demonstrating that both strains, UA159St\textsuperscript{R} and UR117St\textsuperscript{R}, were capable of productive infection. The infected pups from both groups were paired with uninfected cagemates on experimental day 10. We observed that within one day of being paired with pups infected with the wild-type, UA159St\textsuperscript{R}, 6 out of 16 recipient pups had become infected (TABLE 1a). By day 17, all of the recipients had become infected with the wild-type strain (Group IV). In contrast, only four of the uninfected recipient pups became infected with the \textit{fabM} defective strain (UR117St\textsuperscript{R}, Group V).

The experiment was concluded on experimental day 19, at which time the rat pups were aged 34 days. The average number of bacteria recovered from wild-type donors, expressed as CFUs/ml of jaw sonicate, was higher than that determined for mutant strain donors (Table 1b). The average recovered CFU from the 16 recipient animals successfully infected with UA159 St\textsuperscript{R}
was approximately three log-orders higher than the average recovered CFU from the four animals that had detectable infection by UR117 StR (Table 1b).

A separate caries study was undertaken to test the hypothesis that the fabM mutant strain, UR117StR, would be less cariogenic than the wild-type strain, UA159StR. Four litters of pups, aged 15 days, and their dams were obtained from Harlan Laboratories. Dams were screened as in the transmission study. Two dams were infected with actively growing UA159 StR or with UR117StR on days 1 and 2 (pups were aged 16 and 17 days) and infection confirmed by oral swabbing. On day 6 (pups aged 21 days), pups were weaned and infected with either UA159StR or UR117StR (based on their initial exposure) for 2 consecutive days. Infection was confirmed via oral swabbing. Rats were fed Diet-2000 and 5% sucrose (w/v) water ad libitum for 5 weeks. Animals were killed by CO2 asphyxiation and bacterial counts were determined from lower left jaw sonicates. Similar to what was observed in the transmission experiment, the average recovered CFU was significantly higher in animals infected with UA159StR than those infected with UR117StR (Table 2).

Caries were scored by the method of Keyes as modified by Larson (6) and data were evaluated following arcsine transformation (10). Animals infected with UR117StR experienced far fewer smooth-surface carious lesions than animals infected with UA159StR. As the severity index increased (from moderate, D_m, to extensive, D_x), the severity score (D_x) was approximately 90% reduced in animals infected with the fabM deficient strain (Table 2a). The smooth surface caries scores in animals infected with UR117StR were striking, in that they were even lower than scores reported previously from experiments involving the well-established virulence factor, gtfB (12).
Typically, sulcal caries scores do not as readily reveal strong differences between infecting strains because the bacteria compact into the fissures of teeth. Nevertheless, we recorded sulcal caries scores from the animals infected with the wild-type or fabM strains. Not surprisingly, the differences were not as pronounced as those seen with smooth-surface caries. However, the sulcal scores were significantly different (at the P < 0.05 level), when severity of the lesions was taken into account (Dₙ to Dₓ, Table 2b).

From these studies, it is clear that membrane fatty acid composition plays a significant role in the acid-resistance phenotype of S. mutans, and has a significant role in the virulence of the organism. We attribute the differences in infectivity and caries-forming ability of the wild-type and the fabM strains to the inability of the mutant to produce monounsaturated membrane fatty acids (UFAs). Our previous reports provided strong physiological evidence that production of UFAs, during growth at low pH, directly impacts the ability of the organism to withstand acid stress. We have shown here that disrupting the ability of S. mutans to produce UFAs also leads to the inability of the organism to be transmitted from infected to uninfected animals, thereby not fulfilling a key component of Koch’s postulates. Importantly, severity of caries in animals infected with the fabM strain was clearly less than those infected with a wild-type strain. The results of this study provide a link between the ability of S. mutans to produce acid, survive acidic environments, and cause severe disease to a single gene product, FabM.
ACKNOWLEDGEMENTS

We thank Ms. Sylvia Pearson and Mrs. Jennifer Scantlin for assistance with the animals during this work. We thank Ms. Roberta Faustoferri for technical assistance and editorial comments. We also thank W. H. Bowen and R. E. Marquis for helpful discussion throughout. This work was supported by grants from the NIH/NIDCR DE-017157 and DE-01627. E.M.F. was supported by the Rochester Training Program in Oral Infectious Diseases, NIH/NIDCR T32-DE07165.
References


Figure Legend.

Fig.1. Transmission experiment outline.
1a. Transmission of UA159 St<sup>R</sup> and UR117 St<sup>R</sup> infected to uninfected cagemates<sup>a</sup>.

<table>
<thead>
<tr>
<th>Experimental Day</th>
<th>Age of the animals (days)</th>
<th>Infected animals&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>UA159 St&lt;sup&gt;R&lt;/sup&gt; (n=16)</td>
</tr>
<tr>
<td>6</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>26</td>
<td>6</td>
</tr>
<tr>
<td>15</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>17</td>
<td>32</td>
<td>16</td>
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<tr>
<td>19</td>
<td>34</td>
<td>16</td>
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</table>

Table 1b. UA159 St<sup>R</sup> and UR117 St<sup>R</sup> colony-forming units recovered from Day 19 pups.

<table>
<thead>
<tr>
<th>Source&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Average CFU of recovered bacteria&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected donor pups</td>
<td>2.1 x 10&lt;sup&gt;8&lt;/sup&gt; ± 1.0 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td>Recipient pups</td>
<td>1.0 x 10&lt;sup&gt;8&lt;/sup&gt; ± 1.0 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
</tbody>
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<sup>a</sup> On day 6, pups from dams infected with either UA159 St<sup>R</sup> or UR117 St<sup>R</sup> were directly infected with their dam’s respective bacterial strain by oral swabbing. Cotton swabs were wetted using mid-log phase cultures of each strain, as performed previously (1). Infection was monitored from day 11 to day 19 of the experiment.

<sup>b</sup> Determined by oral swabbing and plating on MSS agar plates.

<sup>c</sup> Determined by serial dilutions of jaw sonicates of day 19 animals, as previously described (12). Average CFU were determined by total CFU/# of animals harboring selectable strain ± standard deviation.

<sup>d</sup> Transmission rates of UA159 St<sup>R</sup> and UR117 St<sup>R</sup> were statistically significant using Chi-square analysis, χ = 19.2, p < 0.01.
TABLE 2. Incidence and Severity of Dental Caries in Rats Infected with *S. mutans* UA159 St<sup>R</sup> (wild-type) and *S. mutans* UR117 St<sup>R</sup> (∆*fabM*).

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Total Caries (E)</th>
<th>Smooth Severity (D&lt;sub&gt;s&lt;/sub&gt;)</th>
<th>Smooth Severity (D&lt;sub&gt;m&lt;/sub&gt;)</th>
<th>Smooth Severity (D&lt;sub&gt;x&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UA159 St&lt;sup&gt;R&lt;/sup&gt; (n = 16)</td>
<td>30.2 ± 10.5</td>
<td>16.2 ± 10.7</td>
<td>10.0 ± 9.2</td>
<td>5.4 ± 6.4</td>
</tr>
<tr>
<td>UR117 St&lt;sup&gt;R&lt;/sup&gt; (n = 16)</td>
<td>19.2 ± 9.3</td>
<td>4.1 ± 4.2</td>
<td>1.2 ± 0.9</td>
<td>0.6 ± 0.9</td>
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b. Sulcal-Surfaces

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Total Caries (E)</th>
<th>Sulcal Severity (D&lt;sub&gt;s&lt;/sub&gt;)</th>
<th>Sulcal Severity (D&lt;sub&gt;m&lt;/sub&gt;)</th>
<th>Sulcal Severity (D&lt;sub&gt;x&lt;/sub&gt;)</th>
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<tbody>
<tr>
<td>UA159 St&lt;sup&gt;R&lt;/sup&gt; (n = 16)</td>
<td>46.7 ± 5.4</td>
<td>39.3 ± 5.1</td>
<td>27.8 ± 8.2</td>
<td>6.5 ± 5.2</td>
</tr>
<tr>
<td>UR117 St&lt;sup&gt;R&lt;/sup&gt; (n = 16)</td>
<td>42.8 ± 3.8</td>
<td>35.3 ± 3.4</td>
<td>19.1 ± 6.0</td>
<td>2.6 ± 2.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Average recovered CFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>UA159 St&lt;sup&gt;R&lt;/sup&gt;</td>
<td>3.1 X 10&lt;sup&gt;8&lt;/sup&gt; ± 1.2 X 10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td>UR117 St&lt;sup&gt;R&lt;/sup&gt;</td>
<td>7.7 X 10&lt;sup&gt;7&lt;/sup&gt; ± 4.0 X 10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
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</table>

* Average cfu between UA159 St<sup>R</sup> and UR117 St<sup>R</sup> were statistically significant as determined by Student’s *t*-test, *p* < 0.01.

Animals infected with the *fabM* mutant strain developed lower scores for caries (incidence and severity) when compared with those infected with the wild-type strain, and the differences were statistically significant (*p* < 0.05, comparison for all pairs using Tukey-Kramer HSD; as well as Student’s *t*-test).
8 litters of Sprague Dawley pups (aged 15 days) + dams
screen animals for mutans streptococci and SDV

4 litters infected

Experimental day 1, 2, 3 (pups aged 16, 17, and 18 days)
Infect 2 dams with UA159 StrR; 2 with UR117 StrR

Day 6 (pups aged 21 days)
Wean pups and screen for absence of infection
Create two groups of animals, caged in pairs
Group I (n = 16 pups)
Group II (n = 16 pups)

Day 6, 7, 8 (pups aged 21, 22, and 23 days)
Directly infect pups by oral swabbing

Day 10 (pups aged 25 days)
Pair an infected pup with an uninfected pup
Two animals per cage
Group IV (n = 16 pairs): (one Group I animal (donor) paired with one Group III animal (recipient))

Group V (n =16 pairs): (one Group II animal (donor) paired with one Group III animal (recipient))

Day 11, 15, 17 (pups aged 26, 30, 32 days)
Screen for infection in the recipient animals

Day 19 (pups aged 34 days)
Experiment ends; plate jaw sonicates for bacterial enumeration

Transmission from dams to pups

Transmission from infected pups to uninfected pups

Fig. 1. Fozo et al.