Evaluation of the capacity of oral streptococci to produce hydrogen peroxide

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Summary. The capacity of 11 strains of oral streptococcal species (Streptococcus sanguis, S. oralis, S. mitis and S. sobrinus) to produce hydrogen peroxide (H₂O₂) was studied in vitro. Detection of this property in solid media, particularly with trypticase soy agar-benzidine-peroxidase, was more sensitive than in liquid media. The addition of carbohydrates (arabinose, xylose, mannose, sorbose and lactose), sorbitol and saccharin to buffered trypticase soy broth increased H₂O₂ production in S. oralis NCTC 11427, although the concentrations obtained with some substrates (glucose, galactose, mannitol and xylitol) were lower than those obtained in controls. In S. sanguis NCTC 7863, H₂O₂ production was detected only with galactose, sorbitol, lactose and saccharin.

Introduction

Some members of the genus Streptococcus are able to produce substances, such as H₂O₂, that inhibit the growth of other micro-organisms.1-4 McLeod and Gordon5 were the first to study its production by S. pneumoniae. Thompson and Johnson6 reported that the inhibitory properties of saliva were due to H₂O₂ produced by oral streptococci and Hamon and Klebanoff7, Holmberg and Hallander8, LeBien and Bromel9 also investigated the inhibitory capacity of H₂O₂ produced by various strains of oral streptococci. Other studies have investigated the relationship between the capacity of oral streptococci to produce H₂O₂ in relation to available substrates such as glucose.8-10

The metabolism of facultatively anaerobic streptococci is anaerobic, depending mainly on glycolysis to generate adenosine triphosphate (ATP), and producing lactic acid as the main end-product.11 Because these streptococci are unable to form haemic groups, they cannot synthesise most of the enzymes required for oxygen metabolism (cytochromes) or catalase.12,13 Moreover, these micro-organisms can take up oxygen in amounts similar to those used by aerobic bacteria, due to their flavoproteins such as NADH-oxidase,12 which are able to reduce oxygen by one, two or four electrons to superoxide anion (O₂⁻), H₂O₂ or water, respectively. Superoxide anion and H₂O₂ are highly toxic to the micro-organisms, and are removed from the medium by superoxide dismutase (SOD)β,14 and NADH-peroxidaseβ,15 respectively.

This qualitative and quantitative study of H₂O₂ production by 11 strains of oral streptococci was designed to investigate the influence of 11 carbohydrates, three polyalcohols and saccharin on H₂O₂ production.

Materials and methods

Micro-organisms

S. oralis NCTC 11427, S. mitis NCTC 3165, and S. sanguis JENA 2697 and NCTC 7863 were obtained from international collections, and S. mitis strains OGS 218, 232, 420 and 628 and S. sobrinus strains OGS, 415, 324 and 529 were from the Microbiology Laboratory collection of the University of Granada Hospital (OGS = Odontología Granada Streptococcus). These strains were identified according to the criteria described by Hardie16 and Loesche.17

Qualitative study of H₂O₂ production

Qualitative studies of H₂O₂ production were performed with two media—trypticase soy agar-orthodianisidine (TSA-o-D) and TSA-benzidine-peroxidase (TSA-B-P).

TSA-o-D was prepared by adding to 1 L of TSA (Difco) 10 ml / L of a solution of o-dianisidine (Sigma; 5 mg/ml in methanol) at 45°C. The medium was prepared with double-distilled water and was sterilised by autoclaving.

TSA-B-P was prepared by adding 10 ml of benzidine 5 mg/ml (Sigma) to 1 L of TSA and horseradish...
peroxidase (EC 1.11.1.7; Sigma) solution (HRP) to a final concentration 1 mg/ml, at 45°C.

The 11 strains were inoculated on to plates of TSA-o-D and TSA-B-P and incubated under anaerobic conditions at 36°C ± 1° for 24 h. For the TSA-o-D medium, a second incubation for 4 h at 36°C ± 1° aerobically was used, after which the areas of growth were covered with 5 µl of HRP and left until a colour change to orange (a positive result) was observed (table I). For the TSA-B-P medium, the plates were exposed to atmospheric oxygen at room temperature after anaerobic incubation, and the appearance a few minutes later of a dark brown colour (a positive result) in the growth zone was noted (table I).

Quantitative study of H₂O₂ production

Standard curve. Buffered trypticase soy broth (B-TSB) containing (/L) tryptone (Difco) 17 g, soytone (Difco) 3 g, PO₄HNa₂ (Merck, Darmstadt, Germany) 9 g and PO₄H₂O₂ (Merck) 5.9 g (pH 7.0) was prepared for standard curve assays. The medium was sterilised by the autoclaving.

Solutions (30% w/v) in distilled water of ribose (Sigma), arabinose (Sigma), xylose (Sigma), glucose (Panreac, Barcelona, Spain), mannose (Sigma), galactose (Sigma), fructose (Panreac), sorbose (Sigma), mannitol (Sigma), sorbitol (Sigma), xylitol (Sigma), saccharose (Merck), lactose (Merck), maltose (Panreac) and saccharin (Sigma) were prepared and autoclaved.

Substrate solutions were added to B-TSB to bring the final concentration to 55.5 mM. In addition to individual assays of all substrates, a combination of glucose-galactose solutions (27.7 mM each) was also tested.

Commercial H₂O₂ (Panreac) 30% w/v, was titrated with potassium permanganate. Dilutions in B-TSB alone and B-TSB with the respective substrate were made with H₂O₂ at concentrations of 10, 7, 6, 5, 4, 3, 2, 1 and 0.5 µg/ml. To 3-ml volumes of these dilutions were added 30 µl benzidine solution (5 mg/ml) and 30 µl of HRP. The mixtures were incubated for 10 min at 36°C ± 1° and the optical density (OD) was measured spectrophotometrically at 360 nm. Controls consisted of 3 ml of B-TSB alone or B-TSB with respective substrate to which were added 30 µl of benzidine solution and 30 µl of HRP. On the basis of these results, the molar extinction coefficient (E) was calculated. This coefficient relates OD at a given wavelength and concentration, and in this case coincided with the slope of the resulting standard curve (fig. 1). The slope was calculated by the least squares linear regression method. Pearson’s correlation coefficient (r), indicating the correlation between the OD₃₆₀ data and [H₂O₂], was also calculated. The following data were obtained: B-TSB, E (ml/µg/cm) = 2.15 ± 0.016 and r = 0.97 ± 0.03; B-TSB + substrate (55.5 mM), E (ml/µg/cm) = 0.291 ± 0.019 and r = 0.94 ± 0.03.

Preliminary experiment. Tubes containing 5 ml of B-TSB, which had been deoxygenated by boiling, were inoculated with 0.2 ml of bacterial suspension in saline solution (NaCl 0.9% in double-distilled water, turbidity 0.5 on the McFarland scale) and incubated at 36°C ± 1° in anaerobic conditions for 24 h. The cultures were then aerated by shaking (Pfizer Autobac I, Incubator/Shaker 102U) at 37°C for 3 h. To each tube were added 50 µl of benzidine solution 5 mg/ml and 20 µl of HRP, and the tubes were centrifuged at 8000 g for 10 min at 4°C. The OD₃₆₀ of the supernate was measured when the OD₃₆₀ was > 0.010 (table I).
Table I. Qualitative study of \( H_2O_2 \) production in TSA-o-D and TSA-B-P, and preliminary quantitative study in B-TSB

<table>
<thead>
<tr>
<th>Medium</th>
<th>( H_2O_2 ) production by strain no.</th>
<th>( H_2O_2 ) production by strain no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>TSA-o-D</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TSA-B-P</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B-TSB</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>


Table II. Maximum concentration, aeration time until maximum production, and rates of excretion of \( H_2O_2 \) by \textit{S. oralis} NCTC 11427 and \textit{S. sanguis} NCTC 7863 in B-TSB alone or in combination with the substrate(s)

<table>
<thead>
<tr>
<th>Medium</th>
<th>( [H_2O_2] ) at maximum growth (( \mu g/10^6 ) cfu)</th>
<th>Aeration time (h) from maximum growth</th>
<th>Rate of ( H_2O_2 ) excretion (ng/min/( 10^6 ) cfu)</th>
<th>( [H_2O_2] ) at maximum growth (( \mu g/10^6 ) cfu)</th>
<th>Aeration time (h) from maximum growth</th>
<th>Rate of ( H_2O_2 ) excretion (ng/min/( 10^6 ) cfu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-TSB alone</td>
<td>1.32 (0.13)*</td>
<td>3</td>
<td>5.79</td>
<td>0.31 (0.08)</td>
<td>4.5</td>
<td>1.83</td>
</tr>
<tr>
<td>Ribose</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Arabinose</td>
<td>3.02 (0.24)</td>
<td>8</td>
<td>5.04</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Xylose</td>
<td>2.08 (0.19)</td>
<td>12</td>
<td>3.05</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.17 (0.05)</td>
<td>4.5</td>
<td>0.71</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Mannose</td>
<td>1.74 (0.17)</td>
<td>8</td>
<td>1.46</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.30 (0.07)</td>
<td>6</td>
<td>1.04</td>
<td>0.23 (0.06)</td>
<td>6</td>
<td>1.05</td>
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<tr>
<td>Fructose</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Sorbose</td>
<td>1.96 (0.22)</td>
<td>6</td>
<td>5.30</td>
<td>...</td>
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<td>...</td>
</tr>
<tr>
<td>Mannitol</td>
<td>0.68 (0.11)</td>
<td>6</td>
<td>1.06</td>
<td>...</td>
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<td>...</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>1.79 (0.29)</td>
<td>8</td>
<td>5.90</td>
<td>1.19 (0.15)</td>
<td>3</td>
<td>2.78</td>
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<td>Xylitol</td>
<td>0.60 (0.06)</td>
<td>6</td>
<td>1.02</td>
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<td>...</td>
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</tr>
<tr>
<td>Saccharose</td>
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<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Lactose</td>
<td>2.00 (0.32)</td>
<td>8</td>
<td>2.24</td>
<td>0.21 (0.05)</td>
<td>4.5</td>
<td>0.65</td>
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<tr>
<td>Maltose</td>
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<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Glucose +</td>
<td>0.70 (0.13)</td>
<td>8</td>
<td>2.00</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>galactose</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Saccharin</td>
<td>2.21 (0.35)</td>
<td>4.5</td>
<td>5.08</td>
<td>0.37 (0.07)</td>
<td>6</td>
<td>1.33</td>
</tr>
</tbody>
</table>

All experiments were performed in triplicate.
* ( ), Standard deviation.

\( H_2O_2 \) production. Erlenmeyer flasks containing 200 ml of B-TSB alone or with one of the substrates, from which oxygen had been eliminated by boiling, were inoculated with 1 ml of bacterial suspension in saline solution. The suspension was prepared from log-phase cultures in B-TSB or B-TSB plus substrate with a turbidity of 0.5 on the McFarland scale. The flasks were incubated anaerobically at 36°C ± 1°C for 24 h, placed in a water bath at 37°C and aerated by shaking in a circular orbital motion at 900 rpm; 3-ml samples were removed periodically and replaced with 3 ml of B-TSB or B-TSB with substrate that had been pre-incubated at 37°C. To each sample were added 30 \( \mu l \) of benzidine solution 5 mg/ml and 30 \( \mu l \) of HRP, and the mixture was centrifuged at 6000 \( g \) for 10 min at 4°C. The OD \( 660 \) of the supernate was measured, compared with the control, and the concentration of \( H_2O_2 \) was calculated as a function of the corresponding molar extinction coefficient (\( E \)).

Viable counts. At the same intervals as in the determinations of \( H_2O_2 \) concentration, viable counts as cfu/ml were determined on Mitis Salivarius Agar (Difco). Serial dilutions of \( 10^3-10^{12} \), depending on the period of aeration and the strain, were prepared from 0.2 ml of the culture, and the plates were incubated anaerobically at 36°C ± 1°C for 48 h.

Results

In qualitative studies with both (TSA-o-D and TSA-B-P (table I), all strains produced \( H_2O_2 \) when the cultures were exposed to air, as indicated by a colour change; the greater change was produced with TSA-B-P.

Previous quantitative studies with B-TSB (table I) detected \( H_2O_2 \) production only in strains \textit{S. oralis} NCTC 11427 and \textit{S. sanguis} NCTC 7863. Therefore,
the quantitative determinations were performed only with these strains. Table II summarises the findings with these two strains grown in B-TSB alone or in combination with the substrate(s), B-TSB alone acting as the control. The growth curves and evolution of H\textsubscript{2}O\textsubscript{2} production by *S. oralis* NCTC 11427 in B-TSB alone, with glucose, and with glucose plus galactose are shown in fig. 2, and fig. 3 shows the growth curve and curve of H\textsubscript{2}O\textsubscript{2} production for *S. sanguis* NCTC 7863 in B-TSB alone, with sorbitol and with lactose.

After aeration for 3 h, H\textsubscript{2}O\textsubscript{2} production by *S. oralis* NCTC 11427 from peptones in B-TSB was 1.32 \( \mu \)g/10\textsuperscript{6} cfu. No H\textsubscript{2}O\textsubscript{2} was detected in cultures containing ribose, fructose or saccharose, and < 1.32 \( \mu \)g/10\textsuperscript{6} cfu from glucose, galactose, glucose plus galactose, mannitol and xylitol. The H\textsubscript{2}O\textsubscript{2} concentrations were > 1.32 \( \mu \)g/10\textsuperscript{6} cfu in cultures containing arabinose, xylose, mannose, sorbose, sorbitol, lactose and saccharin. The rate of H\textsubscript{2}O\textsubscript{2} excretion, with the exception of cultures with sorbitol, was slower in all these cases than in control cultures, indicating that these substances reduced the rate of production. Although greater amounts of H\textsubscript{2}O\textsubscript{2} were produced in total, peak production took longer.
With *S. sanguis* NCTC 7863, **H**\textsubscript{2}O\textsubscript{2} production in B-TSB alone was 0.31 µg/10⁶ cfu after aeration for 4.5 h. **H**\textsubscript{2}O\textsubscript{2} was detected only in cultures containing galactose, lactose, sorbitol and saccharin; the accumulation of **H**\textsubscript{2}O\textsubscript{2} was greater than in control cultures only when sorbitol was added, whereas cultures with saccharin produced slightly more **H**\textsubscript{2}O\textsubscript{2} than did the control medium. All substrates except sorbitol slowed or stopped **H**\textsubscript{2}O\textsubscript{2} generation compared with the controls.

**Discussion**

Production of **H**\textsubscript{2}O\textsubscript{2} by oral streptococci has often been used to distinguish species,⁵,¹⁶,²²,²³ usually by the use of qualitative methods.²⁴ However, results are not always clear, depending on the culture medium, reagents and method used to read them. TSA-B-P offers some advantages over TSA-o-D, in so far as the reaction develops within minutes and no additional reagents were required to detect **H**\textsubscript{2}O\textsubscript{2}. Moreover, the former medium is more sensitive and minimal amounts of **H**\textsubscript{2}O\textsubscript{2} are readily detected.

Of the 11 strains analysed, representing four different species that produced **H**\textsubscript{2}O\textsubscript{2}, only two produced a detectable amount of **H**\textsubscript{2}O\textsubscript{2} in broth. This strongly suggests that tests with solid media (TSA-B-P) are more sensitive than those with liquid media, although this advantage is diminished by the fact that the former are unsuited to quantitative studies. In liquid media (B-TSB), a proportion of the **H**\textsubscript{2}O\textsubscript{2} is probably inactivated by oxidising organic compounds,¹⁹ and by NADH-peroxidase further interfering with detection of **H**\textsubscript{2}O\textsubscript{2}. In solid media, **H**\textsubscript{2}O\textsubscript{2} excreted by bacteria immediately oxidises benidine, a reaction catalysed by the peroxidase incorporated in the medium.

Quantitative studies of the capacity of oral streptococci to produce **H**\textsubscript{2}O\textsubscript{2} should be performed with buffered media, to avoid the decrease in pH caused by the excretion of lactic acid into the medium, since a change in pH affects the molar extinction coefficient.¹⁹,²⁰ Furthermore, B-TSB and the substrates used should be sterilised separately to avoid undesirable reactions between aldoses and phosphate groups which can decrease the available concentration of aldose.²⁵ Although sterilisation is not required for polyalcohols and saccharin, this step was included in all experiments to standardise the procedure.

Several authors have stated that oral streptococci require glucose, metabolised by means of the glucose oxidase pathway, for **H**\textsubscript{2}O\textsubscript{2} production.⁵,⁶,¹¹,¹² Our work with *S. sanguis* NCTC 7863 and *S. oralis* NCTC 11427 contradicts this assertion; the addition of glucose to the medium inhibited **H**\textsubscript{2}O\textsubscript{2} production. With very few exceptions, such as sorbitol for *S. sanguis* NCTC 7863, the addition of substrates to the control medium slowed **H**\textsubscript{2}O\textsubscript{2} generation, although the final concentration of **H**\textsubscript{2}O\textsubscript{2} in the medium was higher than that in B-TSB alone.

In substrates metabolised by means of the glycolytic pathway, oxidation-reduction is well-balanced, with the subsequent formation of lactate, and only the utilisation of the pentose phosphate pathway would give rise to the reduction equivalents (NADPH) required to reduce oxygen to **H**\textsubscript{2}O\textsubscript{2}. This would account for the low **H**\textsubscript{2}O\textsubscript{2} production by *S. oralis* NCTC 11427 in the presence of glucose (0.17 µg/10⁶ cfu) or galactose (0.30 µg/10⁶ cfu).

In contrast, arabinose metabolism gives rise directly to pyruvate. Because of the lack of production of NADH, pyruvate is not metabolised to lactate, thus favouring the pyruvate oxidase pathway for **H**\textsubscript{2}O\textsubscript{2} production. This would explain why arabinose is the most highly peroxidogenic substrate (3.02 µg/10⁶ cfu) for *S. oralis* NCTC 11427.

Sorbitol is metabolised by means of the glycolytic pathway, entering as fructose-6-phosphate and generating an extra molecule of NADH; in comparison with other substrates metabolised by the glycolytic pathway this mechanism would produce **H**\textsubscript{2}O\textsubscript{2} by means of NADH-oxidase activity.¹²,²⁸

As saccharose and fructose are readily metabolised, amino-acid degradation is completely inhibited. In addition, these substrates may be able to activate NADH-peroxidase, thus destroying **H**\textsubscript{2}O\textsubscript{2}.

Unmetabolised saccharin remained unaltered, but nonetheless gave rise to a notable increase in **H**\textsubscript{2}O\textsubscript{2} production by *S. oralis* NCTC 11427, and a slight increase by *S. sanguis* NCTC 7863. These results may have been due to interaction between saccharin and oxidases, causing increased activity of the latter; alternatively, saccharin may have acted as an NADH-peroxidase inhibitor. Arabinose and sorbitol were the most strongly peroxidogenic substrates; this raises the possibility that arabinose and sorbitol may have advantages as saccharose substitutes.

One can postulate that **H**\textsubscript{2}O\textsubscript{2} production may be a selective factor in the different habitats of the oral cavity, whereby bacteria producing **H**\textsubscript{2}O\textsubscript{2} are favoured in the competition for colonisation. In this study, two species of oral streptococci excreted high levels of **H**\textsubscript{2}O\textsubscript{2} into the medium; in quantitative terms, this feature appears to be strain-dependent. Although *S. sanguis* NCTC 7863 and *S. oralis* NCTC 11427 were peroxidogenic in both solid and liquid media, *S. sanguis* JENA 2697, *S. mitis* OGS 218 and others produced detectable amounts of **H**\textsubscript{2}O\textsubscript{2} only in solid media, which are more sensitive indicators of **H**\textsubscript{2}O\textsubscript{2} than liquid media. These findings are of potential importance for studies of peroxide production in vitro.

In conclusion, **H**\textsubscript{2}O\textsubscript{2} production by oral streptococci appears to be species- and strain-dependent, and varies according to the substrate used. The accurate detection of **H**\textsubscript{2}O\textsubscript{2} in vitro depends on the method used, regardless of whether solid or liquid media are used, although the former are more sensitive.
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References