Evaluation of the capacity of oral streptococci to produce hydrogen peroxide

A. GARCÍA-MENDOZA, J. LIÉBANA*, ANA M. CASTILLO, ANGUSTIAS DE LA HIGUERA
and G. PIÉDROLA

Department of Microbiology, School of Medicine and *School of Odontology, University of Granada,
Avda. de Madrid n.11. E-18071 Granada, Spain

Summary. The capacity of 11 strains of oral streptococcal species (*Streptococcus sanguis*, *S. oralis*, *S. mitis* and *S. sobrinus*) to produce hydrogen peroxide (H_2O_2) 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 saccharine to buffered trypticase soy broth increased H_2O_2 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, H_2O_2 production was detected only with galactose, sorbitol, lactose and saccharin.

Introduction

Some members of the genus *Streptococcus* are able to produce substances, such as H_2O_2 , 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 H_2O_2 produced by oral streptococci and Hamon and Klebanoff⁷, Holmberg and Hallander¹, LeBien and Bromel² and Willcox and Drucker⁴ also investigated the inhibitory capacity of H_2O_2 produced by various strains of oral streptococci. Other studies have investigated the relationship between the capacity of oral streptococci to produce H_2O_2 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 (O_2^-), H_2O_2 or water, respectively. Superoxide anion and H_2O_2 are highly toxic to the micro-organisms, and are removed from the medium by superoxide dismutase (SOD)^{8,14} and NADH-peroxidase¹⁵ respectively.

This qualitative and quantitative study of H_2O_2 production by 11 strains of oral streptococci was designed to investigate the influence of 11 carbohydrates, three polyalcohols and saccharin on H_2O_2 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 H_2O_2 production

Qualitative studies of H_2O_2 production were performed with two media—trypticase soy agar-orthodiansidine (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*-diansidine (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

Fig. 1. Standard curve for H_2O_2 production in B-TSB (—▲—) and B-TSB plus substrate (—■—).

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 $36^\circ\text{C} \pm 1^\circ$ for 24 h. For the TSA-o-D medium, a second incubation for 4 h at $36^\circ\text{C} \pm 1^\circ$ 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 H_2O_2 production

Standard curve. Buffered trypticase soy broth (B-TSB) containing (/L) tryptone (Difco) 17 g, soytone (Difco) 3 g, PO_4HNa_2 (Merck, Darmstadt, Germany) 9 g and $\text{PO}_4\text{H}_2\text{Na} \cdot 2\text{H}_2\text{O}$ (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 H_2O_2 (Panreac) 30% w/v, was titrated with potassium permanganate.¹⁸ Dilutions in B-TSB alone and B-TSB with the respective substrate were made with H_2O_2 at concentrations of 10, 7, 6, 5, 4, 3, 2, 1 and 0.5 $\mu\text{g/ml}$. 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 $36^\circ\text{C} \pm 1^\circ$ 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.^{19,20} 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.²¹ Pearson's correlation coefficient (r),²¹ indicating the correlation between the OD₃₆₀ data and $[H_2O_2]$, was also calculated. The following data were obtained: B-TSB, E (ml/ $\mu\text{g/cm}$) = 2.15 ± 0.016 and $r = 0.97 \pm 0.03$; B-TSB + substrate (55.5 mM), E (ml/ $\mu\text{g/cm}$) = 0.291 ± 0.019 and $r = 0.94 \pm 0.03$.

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 $36^\circ\text{C} \pm 1^\circ$ in anaerobic conditions for 24 h. The cultures were then aerated by shaking (Pfizer Autobac 1, Incubator/Shaker 102U) at 37°C for 3 h. To each tube were added 50 μl of benzidine solution 5 mg/ml and 50 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 H₂O₂ production in TSA-o-D and TSA-B-P, and preliminary quantitative study in B-TSB

Medium	H ₂ O ₂ production by strain no.										
	1	2	3	4	5	6	7	8	9	10	11
TSA-o-D	+↓	+↓	+↓	+↓	+↓	+↓	+↓	+↓	+↓	+↓	+↓
TSA-B-P	+	+	+	+	+	+	+↓	+↓	+	+↓	+
B-TSB	+	—	—	+	—	—	—	—	—	—	—

1, *S. oralis* NCTC 11427; 2, *S. mitis* NCTC 3165; 3, *S. sanguis* JENA 2697; 4, *S. sanguis* NCTC 7863; 5, *S. oralis* OGS 218; 6, *S. mitis* OGS 232; 7, *S. mitis* OGS 628; 8, *S. sobrinus* OGS 324; 9, *S. sobrinus* OGS 415; 10, *S. sobrinus* OGS 529; 11, *S. mitis* OGS 420; +, positive; +↓, slight positive; —, negative.

Table II. Maximum concentration, aeration time until maximum production, and rates of excretion of H₂O₂ by *S. oralis* NCTC 11427 and *S. sanguis* NCTC 7863 in B-TSB alone or in combination with the substrate(s)

Medium	<i>S. oralis</i> NCTC 11427			<i>S. sanguis</i> NCTC 7863		
	[H ₂ O ₂] at maximum growth (µg/10 ⁶ cfu)	Aeration time (h) from maximum growth	Rate of H ₂ O ₂ excretion (ng/min/10 ⁶ cfu)	[H ₂ O ₂] at maximum growth (µg/10 ⁶ cfu)	Aeration time (h) from maximum growth	Rate of H ₂ O ₂ excretion (ng/min/10 ⁶ cfu)
B-TSB alone	1.32 (0.13)*	3	5.79	0.31 (0.08)	4.5	1.83
Ribose	...	...	...	...	...	...
Arabinose	3.02 (0.24)	8	5.04	...	...	...
Xylose	2.08 (0.19)	12	3.05	...	...	...
Glucose	0.17 (0.05)	4.5	0.71	...	...	...
Mannose	1.74 (0.17)	8	1.46	...	...	...
Galactose	0.30 (0.07)	6	1.04	0.23 (0.06)	6	1.05
Fructose	...	...	...	...	...	...
Sorbose	1.96 (0.22)	6	5.30	...	...	...
Mannitol	0.68 (0.11)	6	1.06	...	...	...
Sorbitol	1.79 (0.29)	8	5.90	1.19 (0.15)	3	2.78
Xylitol	0.60 (0.06)	6	1.02	...	...	...
Saccharose	...	...	...	...	...	...
Lactose	2.00 (0.32)	8	2.24	0.21 (0.05)	4.5	0.65
Maltose	...	...	...	...	...	...
Glucose + galactose	0.70 (0.13)	8	2.00	...	...	...
Saccharin	2.21 (0.35)	4.5	5.08	0.37 (0.07)	6	1.33

All experiments were performed in triplicate.

* (), Standard deviation.

H₂O₂ 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° 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 µl of benzidine solution 5 mg/ml and 30 µl of HRP, and the mixture was centrifuged at 8000 g for 10 min at 4°C. The OD₃₆₀ of the supernate was measured, compared with the control, and the concentration of H₂O₂ was calculated as a function of the corresponding molar extinction coefficient (*E*).

Viable counts. At the same intervals as in the determinations of H₂O₂ concentration, viable counts as cfu/ml were determined on Mitis Salivarius Agar (Difco). Serial dilutions of 10¹–10¹², 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° for 48 h.

Results

In qualitative studies with both (TSA-o-D and TSA-B-P (table I), all strains produced H₂O₂ 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 H₂O₂ production only in strains *S. oralis* NCTC 11427 and *S. sanguis* NCTC 7863. Therefore,

Fig. 2. *S. oralis* NCTC 11427 in B-TSB alone: growth curve (—▲—), H_2O_2 production (—△—); with glucose: growth curve (—●—), H_2O_2 production (—○—); with glucose plus galactose: growth curve (—■—), H_2O_2 production (—□—).

Fig. 3. *S. sanguis* NCTC 7863 in B-TSB alone: growth curve (—▲—), H_2O_2 production (—△—); with sorbitol: growth curve (—●—), H_2O_2 production (—○—); with lactose: growth curve (—■—), H_2O_2 production (—□—).

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_2O_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_2O_2 production for *S. sanguis* NCTC 7863 in B-TSB alone, with sorbitol and with lactose.

After aeration for 3 h, H_2O_2 production by *S. oralis* NCTC 11427 from peptones in B-TSB was $1.32 \mu\text{g}/$

10^6 cfu. No H_2O_2 was detected in cultures containing ribose, fructose or saccharose, and $< 1.32 \mu\text{g}/10^6$ cfu from glucose, galactose, glucose plus galactose, mannitol and xylitol. The H_2O_2 concentrations were $> 1.32 \mu\text{g}/10^6$ cfu in cultures containing arabinose, xylose, mannose, sorbose, sorbitol, lactose and saccharin. The rate of H_2O_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_2O_2 were produced in total, peak production took longer.

With *S. sanguis* NCTC 7863, H_2O_2 production in B-TSB alone was $0.31 \mu\text{g}/10^6$ cfu after aeration for 4.5 h. H_2O_2 was detected only in cultures containing galactose, lactose, sorbitol and saccharin; the accumulation of H_2O_2 was greater than in control cultures only when sorbitol was added, whereas cultures with saccharin produced slightly more H_2O_2 than did the control medium. All substrates except sorbitol slowed or stopped H_2O_2 generation compared with the controls.

Discussion

Production of H_2O_2 by oral streptococci has often been used to distinguish species,^{5, 16, 22, 23} usually by the use of qualitative methods.^{23, 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_2O_2 . Moreover, the former medium is more sensitive and minimal amounts of H_2O_2 are readily detected.

Of the 11 strains analysed, representing four different species that produced H_2O_2 , only two produced a detectable amount of H_2O_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_2O_2 is probably inactivated by oxidising organic compounds,¹⁹ and by NADH-peroxidase further interfering with detection of H_2O_2 . In solid media, H_2O_2 excreted by bacteria immediately oxidises benzidine, a reaction catalysed by the peroxidase incorporated in the medium.

Quantitative studies of the capacity of oral streptococci to produce H_2O_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_2O_2 production.^{2, 3, 13} Our work with *S. sanguis* NCTC 7863 and *S. oralis* NCTC 11427 contradicts this assertion; the addition of glucose to the medium inhibited H_2O_2 production. With very few exceptions, such as sorbitol for *S. sanguis* NCTC 7863, the addition of substrates to the control medium slowed H_2O_2 generation, although the final concentration of H_2O_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_2O_2 . This would account for the low H_2O_2 production by *S. oralis* NCTC 11427 in the presence of glucose ($0.17 \mu\text{g}/10^6$ cfu) or galactose ($0.30 \mu\text{g}/10^6$ 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^{26, 27} for H_2O_2 production. This would explain why arabinose is the most highly peroxidogenic substrate ($3.02 \mu\text{g}/10^6$ 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_2O_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_2O_2 .

Unmetabolised saccharin remained unaltered, but nonetheless gave rise to a notable increase in H_2O_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_2O_2 production may be a selective factor in the different habitats of the oral cavity, whereby bacteria producing H_2O_2 are favoured in the competition for colonisation. In this study, two species of oral streptococci excreted high levels of H_2O_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_2O_2 only in solid media, which are more sensitive indicators of H_2O_2 than liquid media. These findings are of potential importance for studies of peroxide production *in vivo*.

In conclusion, H_2O_2 production by oral streptococci appears to be species- and strain-dependent, and varies according to the substrate used. The accurate detection of H_2O_2 *in vitro* depends on the method used, regardless of whether solid or liquid media are used, although the former are more sensitive.

This study was supported partly by the Education Council of the Andalusian Regional Government through a project titled "Microbiology, immunology and epidemiology of oral diseases". We thank

Dr M. Hayes of the Department of Biochemistry, Medical School, University of Bristol, for providing reference strains and valuable suggestions regarding the study.

References

- Holmberg K, Hallander HO. Production of bactericidal concentrations of hydrogen peroxide by *Streptococcus sanguis*. *Arch Oral Biol* 1973; **18**: 423-434.
- LeBien TW, Bromel MC. Antibacterial properties of a peroxidogenic strain of *Streptococcus mitis* (mitis). *Can J Microbiol* 1975; **21**: 101-103.
- Vernazza TR, Melville TH. Inhibitory activity of *Streptococcus mitis* against oral bacteria. *Microbios* 1979; **26**: 95-101.
- Willcox MDP, Drucker DB. Partial characterisation of the inhibitory substances produced by *Streptococcus oralis* and related species. *Microbios* 1988; **55**: 135-145.
- McLeod JW, Gordon J. Production of hydrogen peroxide by bacteria. *Biochem J* 1922; **16**: 499-504.
- Thompson R, Johnson A. The inhibitory action of saliva on the diphtheria bacillus: hydrogen peroxide, the inhibitory agent produced by salivary streptococci. *J Infect Dis* 1951; **88**: 81-85.
- Hamon CB, Klebanoff SJ. A peroxidase-mediated, *Streptococcus mitis*-dependent antimicrobial system in saliva. *J Exp Med* 1973; **137**: 438-450.
- Fridovich I. Superoxide dismutases. *Annu Rev Biochem* 1975; **44**: 147-159.
- Dingman DW, Stalhy DP. Protection of *Bacillus* larvae from oxygen toxicity with emphasis on the role of catalase. *Appl Environ Microbiol* 1984; **47**: 1228-1237.
- Edwardsson S. Microorganismos asociados a la caries dental. In: Thylstrup A, Fejerskov O (eds) Caries. Barcelona, Ediciones Doyma. 1988: 85.
- Cole JA. A biochemical approach to the control of dental caries. *Biochem Soc Trans* 1977; **5**: 1232-1239.
- Dolin MI. Cytochrome-independent electron transport enzymes of bacteria. In: Gunsalus IC, Stanier RY (eds) The bacteria, vol 2. New York, Academic Press. 1961: 425.
- Thomas EL, Pera KA. Oxygen metabolism of *Streptococcus mutans*: uptake of oxygen and release of superoxide and hydrogen peroxide. *J Bacteriol* 1983; **154**: 1236-1244.
- DiGiuseppi J, Fridovich I. Oxygen toxicity in *Streptococcus sanguis*. The relative importance of superoxide and hydroxyl radicals. *J Biol Chem* 1982; **257**: 4046-4051.
- Dolin MI. The DPNH-oxidizing enzymes of *Streptococcus faecalis*. II. The enzymes utilizing oxygen, cytochrome c, peroxide and 2,6-dichlorophenol-indophenol or ferricyanide as oxidants. *Arch Biochem Biophys* 1955; **55**: 415-435.
- Hardie JM. Oral streptococci. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG (eds) Bergey's Manual of systematic bacteriology, 9th ed, vol 2. Baltimore, Williams and Wilkins Co. 1986: 1054-1063.
- Loesche WJ. Role of *Streptococcus mutans* in human dental decay. *Microbiol Rev* 1986; **50**: 353-380.
- García-Villanova R (ed). Volumetrías de óxido-reducción. In: Análisis químico. Principios y métodos, 2nd edn. Granada, Universidad de Granada. 1980: 347.
- Lehninger AL. Enzimas de oxido-reducción y transporte electrónico. In: Lehninger AL (ed) Bioquímica, 2nd edn. Barcelona, Ediciones Omega. 1978: 487.
- Levine IN (ed). Espectroscopia y fotoquímica. In: Physicochemistry. Bogotá, McGraw Hill. 1981: 472.
- Calvo F (ed). Estadística aplicada. Bilbao, Deusto. 1978: 596.
- Hamada S, Slade HD. Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiol Rev* 1980; **44**: 331-384.
- Whiley RA, Fraser H, Hardie JM, Beighton D. Phenotypic differentiation of *Streptococcus intermedius*, *Streptococcus constellatus*, and *Streptococcus anginosus* strain within the "Streptococcus milleri Group". *J Clin Microbiol* 1990; **28**: 1497-1501.
- Whittenbury R. Hydrogen peroxide formation and catalase activity in lactic acid bacteria. *J Gen Microbiol* 1964; **35**: 13-26.
- Carlsson J, Iwami Y, Yamada T. Hydrogen peroxide excretion by oral streptococci and effect of lactoperoxidase-thiocyanate-hydrogen peroxide. *Infect Immun* 1983; **40**: 70-80.
- Thylstrup A, Fejerskov O (eds). Actividades metabólicas de las bacterias de la cavidad oral. In: Caries. Barcelona, Ediciones Doyma. 1988: 56.
- Carlsson J, Edlund M-BK, Lundmark SKE. Characteristics of a hydrogen peroxide-forming pyruvate oxidase from *Streptococcus sanguis*. *Oral Microbiol Immunol* 1987; **2**: 15-20.
- Low IE, Zimkus SM. Reduced nicotinamide adenine dinucleotide oxidase and H₂O₂ formation of *Mycoplasma pneumoniae*. *J Bacteriol* 1973; **116**: 346-354.