Oxygen tolerance of anaerobic bacteria isolated from necrotic dental pulps

JAN CARLSSON, FRED FRÖLANDER &
GÖRAN SUNDQUIST

Department of Oral Microbiology, University of Umeå, Sweden

Carlsson, J., Frölander, F. & Sundquist, G. Oxygen tolerance of anaerobic bacteria isolated from nectrotic dental pulps. *Acta Odont. Scand.* 35, 139–145.

The oxygen tolerance of 43 anaerobic reference strains and 36 anaerobic strains from necrotic dental pulps was studied. All strains survived for two hours or more as colonies on the surface of a medium supplemented with blood, and as many as 26 of the 79 strains survived for more than seven days. The hemolysed blood in the medium significantly increased the survival time for many of the strains. Factors influencing the death rate were studied in one of the strains and it was found that the lysed red cells of the blood and not the serum had a protective effect and that catalase had the same protective effect as the hemolysed blood.

The finding that hemolysed blood significantly increased the oxygen tolerance of many anaerobes may explain some of the divergent results regarding the efficiency of various methods for the recovery of anaerobic bacteria from clinical specimens. The use of media supplemented with blood during various phases of processing a specimen might be more important for a high recovery of anaerobic bacteria from clinical sources than the measures taken to minimize exposure of the specimen to air.

Key-words: Anaerobic bacteriology; oxygen toxicity; endodontics

Jan Carlsson, Department of Oral microbiology, University of Umeå, S-901 87 Umeå, Sweden

Recent improvements in anaerobic bacteriological technique have led to recognition of the importance of anaerobic bacteria in many clinical infections (see *Balows et al.*, 1974). Infections of the necrotic pulps of intact teeth have attracted special interest because these teeth can be sampled without gross contaminations from the normal oral flora (*Brown & Rudolph*, 1957, *Macdonald, Hare & Wood*, 1957; *Engström & Frostell*, 1961; *Möller*, 1966; *Kantz & Henry*, 1974; *Bergenholtz*, 1974; *Wittgow & Sabiston*, 1975; *Sundqvist*, 1976). In the study of *Brown & Rudolph* (1957) anaerobic bacteria made up 24 per cent of the isolated strains. By improving the anaerobic technique *Möller* (1966) was able to isolate anaerobic bacteria from most of the infected necrotic dental pulps and in the study of *Sundqvist* (1976) more than 90 per cent of the isolated strains were anaerobic.

Although some anaerobic bacteria may be killed by a brief exposure to oxygen (*Loesche*, 1969), *Tally et al.* (1975) recently found that anaerobic bacteria freshly isolated from clinical specimens tolerated 8 hours or more of exposure to the oxygen in room air. They

Received for publication, December 8, 1976

concluded that the brief oxygen exposure in connection with bench techniques in clinical laboratories would not be deleterious to the anaerobic bacteria present in clinical specimens. The significant increase in the recovery of anaerobic bacteria from infected necrotic dental pulps by improvements in the anaerobic technique indicates that the bacteria recovered from nectrotic pulps are less tolerant to oxygen than bacteria isolated from other sites. The purpose of this study was to compare the oxygen tolerance of anaerobic strains isolated from the pulp chamber of intact teeth with nectrotic pulps with the oxygen tolerance of anaerobic reference strains.

MATERIAL AND METHODS

Micro-organisms

The micro-organisms included in this study are 36 anaerobic strains isolated from necrotic dental pulps (*Sundqvist*, 1976) and 43 anaerobic reference strains (Table 1). The anaerobic strains from dental pulps had been isolated and stored under anaerobic conditions (*Sundqvist*, 1976) until this study was performed.

Bacteriological media and culture conditions

Brain heart infusion and trypticase soy broth (TSB) were obtained from BBL, Cockeysville, Md. Bacto-agar and yeast extract were from Difco Laboratories, Detroit, Mich. Hemin, menadione and catalase were from Sigma Chemical Co., St. Louis, Mo. Catalase was purified before use by gelchromatography on Sephadex G 200 (Pharmacia, Uppsala, Sweden) in 0.05 M potassium phosphate buffer, pH 7.0, containing 0.1 M KCl. Superoxide dismutase was from Truett Laboratories, Biochem. Div., Dallas, Texas. Horse blood (Statens bakteriologiska laboratorium, Stockholm, Sweden) was hemolysed by freeze-thawing before used in BHIA medium (*Holdeman & Moore*, 1975). All agar media were prepared in room atmosphere and were stored for at least 24 hours in an anaerobic glove box. PY-glucose broth, BHIA medium and PRAS-dilution blanks were prepared as described by *Holdeman & Moore* (1975). The anaerobic box had an atmosphere of 85 per cent nitrogen, 10 per cent hydrogen and 5 per cent carbon dioxide and has previously been described (*Yamada & Carlsson*, 1975).

Assay of enzyme activity

Catalase activity was measured according to *Beers & Sizer* (1952). Superoxide dismutase activity was determined as described by *McCord & Fridovich* (1969).

Determination of oxygen tolerance

The bacterial strains were grown on the surface of BHIA medium and on BHIA medium with 4 per cent horse blood for three days at 37 °C in the anaerobic box. The plates were brought out of the box and the bacterial colonies on the plates were exposed to air for a week at 25 °C in the dark. The survival of the bacterial strains was determined by taking samples of the colonies from the plates after 2, 6, 24, 48, 96 and 168 hours and inoculating blood agar plates, which were then immediately put into the anaerobic box and incubated for 7 days at 37 °C.

Survival of single cells of anaerobic bacteria on the surface of agar plates

The organism was grown in PY-glucose broth overnight in the anaerobic box and then diluted in PRAS-dilution blanks in ten-fold steps. Aliquots of 0.1 ml from appropriate dilutions were spread on the surface of agar plates in duplicate and the plates were brought out of the box and exposed to air at 25 °C in the dark. One series of plates was kept in the box as control. After 1, 2, 3 and 4 hours the plates were brought back into the box again and they were incubated for 7 days at 37 °C.

Table I. *Survival of 46 anaerobic reference strains and 36 anaerobic strains from necrotic dental pulps as colonies on the surface of BHIA medium when exposed to atmospheric oxygen for a week at 25 °C*

SURVIVAL TIME ON BHIA MEDIUM WITH HEMOLYZED HORSE BLOOD

	TWO HOURS	SIX HOURS	ONE DAY	TWO DAYS	FOUR DAYS	SEVEN DAYS
LESS THAN TWO HOURS	Bacteroides melaninogenicus ss. asaccharolyticus H11a-e	Eubacterium alactolyticum Ac-a				
TWO HOURS	Eubacterium alactolyticum ATCC 19301 Actinomyces sp. (group 2) G9a-c1 Eubacterium sp. (group 1) P11a-e Eubacterium sp. (group 1) BN11a-a Peptostreptococcus anaerobius AB13a-c	Eubacterium sp. (group 1) H9a-b Fusobacterium nucleatum BH11a-d Fusobacterium sp. (group 1) AB13a-g Peptostreptococcus anaerobius C11b-a Peptostreptococcus anaerobius G11a-a	Peptostreptococcus anaerobius D13a-b	Lactobacillus sp. (group 1) BA11a-c		
SIX HOURS		Bacteroides melaninogenicus ss. asaccharolyticus BN11a-f Fusobacterium nucleatum NCTC 10562 Lactobacillus sp. (group 3) AB13a-e Eubacterium sp. (group 4) UJB13-d	Fusobacterium naviforme ATCC 25832 Fusobacterium necrophorum NCTC 10575 Fusobacterium nucleatum EL9a-a Fusobacterium sp. (group 2) UJB13-e2 Eubacterium pseudoramosum NCTC 10474 Bacteroides melaninogenicus ss. melaninogenicus ATCC 25845	Eubacterium alactolyticum AB13a-n Eubacterium sp. (group 4) P9a-h Lactobacillus sp. (group 3) BN11a-h	Lactobacillus sp. (group 1) AB13a-m Bacteroides melaninogenicus ss. intermedius AB13a-f	Lactobacillus catenaforme AB13a-b
ONE DAY			Lactobacillus catenaforme ATCC 25536 Bacteroides melaninogenicus ss. intermedius UJB13-c	Bacteroides oralis ATCC 15930 Fusobacterium gonidiaformans ATCC 25563 Bacteroides ochraceus VPI 9775	Fusobacterium russii ATCC 25533 Bacteroides hypermegas NCTC 10570	
TWO DAYS				Peptococcus sp. (group 1) AB13a-n Campylobacter sputorum AB13a-k	Peptococcus aerogenes ATCC 14963 Bacteroides melaninogenicus ss. intermedius NCTC 9336	Clostridium ramosum ATCC 25554 Eubacterium lentum HN11a-e
FOUR DAYS					Peptococcus sp. (group 1) BA11a-k Bifidobacterium dentium VPI 7511 Bacteroides melaninogenicus ss. asaccharolyticus VPI 4198	Campylobacter bubulus NCTC 10355 Peptostreptococcus micros BH11a-b Peptostreptococcus micros AC9a-c Peptostreptococcus micros AB13a-d
SEVEN DAYS				Arachnia propionica ATCC 14157 Actinomyces israelii ATCC 12102 Actinomyces sp. (group 2) D13a-a	Actinomyces odontolyticus NCTC 9935 Actinomyces naeslundii ATCC 12104 Actinomyces viscosus ATCC 15987 Peptococcus sp. (group 1) UJB13-e1 Veillonella parvula P11b-c Bacteroides corrodens VPI 7815 Eubacterium lentum ATCC 25559	Eubacterium limosum ATCC 8486 Bifidobacterium bifidum NCTC 10471 Bifidobacterium bifidum NCTC 10472 Propionibacterium acnes NCTC 737 Propionibacterium avidum ATCC 25577 Propionibacterium acidi-propionici ATCC 25562 Actinomyces naeslundii BA11a-h Clostridium perfringens NCTC 8237 Peptococcus prevotii ATCC 9321 Peptococcus saccharolyticus ATCC 14953 Peptococcus variabilis ATCC 14955 Peptococcus variabilis ATCC 14956 Peptostreptococcus anaerobius VPI 4330-1 Bacteroides fragilis ss. fragilis NCTC 10584 Bacteroides fragilis ss. vulgatus NCTC 10583 Bacteroides fragilis ss. thetaiotaomicron NCTC 10582 Fusobacterium varium NCTC 10560 Fusobacterium varium ATCC 8501 Streptococcus intermedius ATCC 27335

Table II. *Survival of anaerobic bacteria when exposed to air for two hours as single cells on the surface of BHIA medium*

Surviving cells:	
less than 0.1 %	
Eubacterium sp. (group 1)	P11a-e
Peptostreptococcus anaerobius	AB13a-c
Peptostreptococcus anaerobius	D13a-b
Peptostreptococcus anaerobius	C11b-a
less than 1 %	
Fusobacterium nucleatum	BN11a-d
Peptostreptococcus anaerobius	G11a-a
less than 10 %	
Eubacterium sp. (group 4)	P9a-h
less than 50 %	
Fusobacterium nucleatum	NCTC 10562
Eubacterium alactolyticum	AB13a-n
Peptostreptococcus micros	AC9a-c
more than 50 %	
Peptostreptococcus micros	AB13a-d
Actinomyces sp. (group 2)	D13a-a
Peptococcus variabilis	ATCC 14956
Bacteroides fragilis subsp. *fragilis*	NCTC 10584
Fusobacterium varium	NCTC 10560
Clostridium perfringens	NCTC 8237
Bifidobacterium bifidum	NCTC 10472

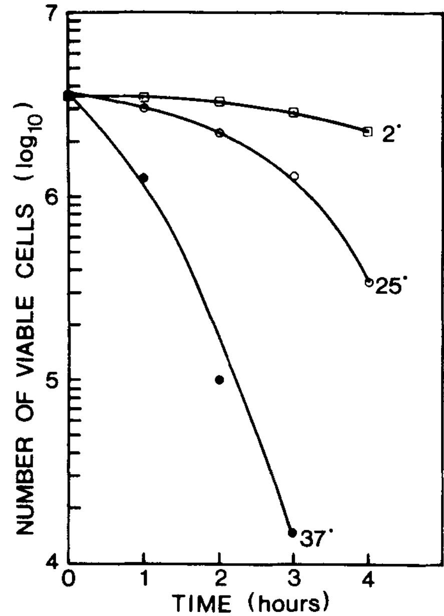

Fig. 1 The effect of temperature on the death-rate of *Peptostreptococcus anaerobius* C11 b–a, when exposed to atmospheric oxygen as single cells on the surface of BHIA medium supplemented with 4 per cent hemolysed horse blood.

RESULTS

When the anaerobic bacteria were exposed to air as colonies on the surface of agar plates, all strains survived for 2 hours or more on the BHIA medium supplemented with blood and only one strain was killed within 2 hours on the BHIA medium without blood. The blood in the medium increased the survival time for many of the strains (Table I). However, some of the strains, including all strains of the genus Actinomyces, survived for seven days on the BHIA medium but died after 2–4 days on the medium with blood (Table I).

In order to estimate the death-rate of the organisms, single cells of 17 of the strains were exposed to air for 2 hours on the surface of BHIA medium. Some of the tested strains died rapidly but many of them survived the 2-hours exposure to air (Table II). When *Peptostreptococcus anaerobius* C11b-a was further studied, it was found that the death-rate was dependent on the temperature (Fig. 1) and the concentration of blood in the medium (Fig. 2). The protective factors of blood were found to be in the lysed red cells and not in the serum. Catalase, added to the surface of the BHIA medium in an amount similar to that in the blood agar, had the same protective effect as hemolysed blood. Superoxide dismutase had no protecting effect.

DISCUSSION

The exploitation of the anaerobic technique in studies on the bacterial infections of dental pulps has been very successful and has resulted in an increase in recovery of anaerobic bacteria from infected pulps (*Möller*, 1966; *Berg & Nord*, 1973; *Kantz & Henry*, 1974; *Bergenholtz*, 1974; *Wittgow & Sabiston*, 1975; *Sundqvist*, 1976). This increase in the

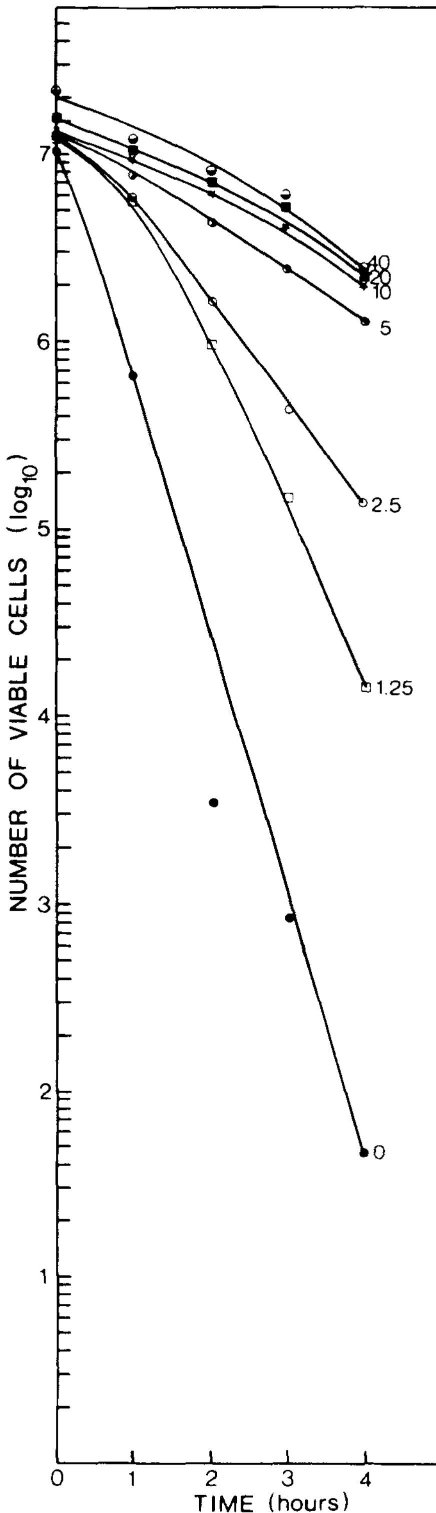

Fig. 2. The death-rate of *Peptostreptococcus anaerobius* C11b-a, when exposed to atmospheric oxygen as single cells on the surface of BHIA medium supplemented with various amounts of hemolysed horse blood. The figures indicates millilitres of blood added per litre of medium.

recovery of anaerobes with refinements in the technique should indicate that the isolated strains are very sensitive to oxygen exposure. The results of the present study, however, show that most strains recovered from infected pulps could tolerate substantial oxygen exposure. This is in agreement with the findings of *Tally et al.* (1975). They studied the oxygen tolerance of 57 freshly isolated anaerobic strains from various clinical sources and found that all the strains survived 8 hours or more of exposure to atmospheric oxygen. One reason for the high oxygen tolerance of the tested strains could be that the tolerance was evaluated with pure cultures obtained by subculturing the original isolates. However, *Bartlett et al.* (1976) recently demonstrated that most anaerobes survived in purulent exudate despite extended periods of exposure to atmospheric oxygen. From eleven purulent specimens from mixed aerobic-anaerobic infections, 26 anaerobes were isolated in the initial processing and 22 were still present after the specimens had been exposed to atmospheric oxygen for 24 hours. The four anaerobic organisms which were lost after exposure to air, were representatives of the genera Fusobacterium and Peptostreptococcus and this finding is in agreement with the present results where fusobacteria and peptostreptococci were among those strains that died most rapidly.

The recently increased awareness of the importance of anaerobic bacteria in human infections (*Balows et al.*, 1974) has rekindled the interest in various methods for the isolation of anaerobic bacteria from clinical sources. However, when the efficiency of various methods for such recovery are compared, conflicting results have been reported (*McMinn & Crawford*, 1970; *Rosenblatt, Fallon & Finegold*, 1974; *Spaulding et al.*, 1974; *Lambe, Vroon & Rietz*,

1974). The high oxygen tolerance of many anaerobic organisms indicates that the time of exposure to atmospheric oxygen during the processing of a specimen is not the ultimate determinant for the efficiency of a method. Our results and those of *Bartlett et al.* (1976) imply that the conditions under which the organisms are exposed to oxygen are more important. The finding that hemolysed blood significantly increased the oxygen tolerance of many anaerobes may explain some of the divergent results concerning the efficiency of various methods for the recovery of anaerobic bacteria from clinical specimens. The use of media supplemented with blood during various phases of processing a specimen might be more important than minimizing its exposure to air. The protective effect of catalase, single and as a component of blood, implies that hydrogen peroxide is involved in the toxic effect of oxygen. Catalase has previously also been reported to protect anaerobic bacteria from the toxic effect of hyperbaric oxygen (*Hill & Osterhout*, 1972).

It is obvious that all the problems concerned with oxygen toxicity could be overcome by protecting a specimen from any exposure to oxygen, but such measures are difficult and expensive to put into routine practice. Therefore, further studies on the mechanisms of oxygen toxicity in anaerobes might yield valuable information about the most efficient way of handling anaerobic bacteria in clinical practice.

Acknowledgements. This study was supported by the Swedish Medical Research Council (project no 4977).

REFERENCES

Balows, A., DeHaan, R.M., Dowell, J.R. & Guze, L.B. (eds.). 1974. *Anaerobic bacteria. Role in disease*. Charles C. Thomas, Springfield, Ill.

Beers, R.F. & Sizer, I. W., 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195, 133-140

Berg, J.-O. & Nord, C.-E. 1973. A method for isolation of anaerobic bacteria from endodontic specimens. Scand. J. Dent. Res. 81, 163-166

Bergenholtz, G. 1974. Micro-organisms from necrotic pulp of traumatized teeth. Odontol. Revy 25, 347-358

Bartlett, J.G., Sullivan-Sigler, N., Louie, T.J. & Gorbach, S.L. 1976. Anaerobes survive in clinical specimens despite delayed processing. J. Clin. Microbiol. 3, 133-136

Brown, L.R., Jr. & Rudolph, C.E., Jr. 1957. Isolation and identification of microorganisms from unexposed canals of pulp-involved teeth. Oral Surg. 10, 1094-1099

Engström, B. & Frostell, G. 1961. Bacteriological studies of the non-vital pulp in cases with intact pulp cavities. Acta Odont. Scand. 19, 23-39

Hill, G.B. & Osterhout, S. 1972. Experimental effects of hyperbaric oxygen on selected clostridial species. I. In vitro studies. J. Infect. Dis. 125, 17-25

Holdeman, L.V. & Moore, W.E.C. 1975. *Anaerobe laboratory manual*, 3rd ed., Virginia Polytechnic Institute and State University, Blacksburg, Va.

Kantz, W. E. & Henry, C. A. 1974. Isolation and classification of anaerobic bacteria from intact chambers of non-vital teeth in man. Arch. Oral Biol. 19, 91-96

Lambe, D. W., Vroon, D. H. & Rietz, C. W. 1974. Infections due to anaerobic cocci, p. 585-599. In: Balows et al. (eds.). *Anaerobic bacteria. Role in disease*. Charles C. Thomes, Springfield, Ill.

Loesche, W. J. 1969. Oxygen sensitivity of various anaerobic bacteria. Appl. Microbiol. 18, 723-727

McCord, J. M. & Fridovich, I. 1969. Superoxide dismutase: An enzymatic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244, 6049-6055

Macdonald, J. B., Hare, G. C. & Wood, A. W. S. 1957. The bacteriologic status of the pulp chambers in intact teeth found to be nonvital following trauma. Oral Surg. 10, 318-322

McMinn, M. T. & Crawford, J. J. 1970. Recovery of anaerobic microorganisms from clinical specimens in prereduced media versus recovery by routine clinical laboratory methods. Appl. Microbiol. 19, 207-213

Möller, Å, J. R. 1966. *Microbiological examination of root canals and periapical tissues of human teeth*. Akademiförlaget, Göteborg

Rosenblatt, J. E., Fallon, A. & Finegold, S. M. 1974. Comparison of methods for isolation of

anaerobic bacteria, p.21-36. In: Balows et al. (eds.), *Anaerobic bacteria. Role in Disease*. Charles C. Thomas. Springfield, Ill.

Spaulding, E. H., Vargo, V., Michaelson, T. C. & Swenson, R. M. 1974. A comparison of two procedures for isolation of anaerobic bacteria from clinical specimens, p.37-50. In: Balows et al. (eds.), *Anaerobic Bacteria. Role in Disease*. Charles C. Thomas, Springfield, Ill.

Sundqvist, G. 1976. *Bacteriological studies of necrotic dental pulps*. Umeå University Odontological Dissertations No. 7

Tally, F.P., Stewart, P.R., Sutter, V.L. & Rosenblatt, J.E. 1975. Oxygen tolerance of fresh clinical anaerobic bacteria. J. Clin. Microbiol. 1, 161–164

Wittgow, W.C., Jr. & Sabiston, C.B., Jr. 1975. Microorganisms from pulpal chambers of intact teeth with necrotic pulps. J. Endodont. 1, 168–171

Yamada, T. & Carlsson, J. 1975. Regulation of lactate dehydrogenase and change of fermentation products in streptococci. J. Bacteriol. 124, 55–61