TERTIARY LEVEL BIOLOGY

Anaerobic Bacteria

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Preface

This book is appropriate for advanced undergraduate students of microbiology and biological sciences in universities and colleges, as well as for research workers entering the field and requiring a broad contemporary view of anaerobic bacteria and associated concepts.

Obligate anaerobes, together with microaerophils, are characterized by their sensitivity to oxygen. This dictates specialized laboratory methods—a fact which has led to many students being less familiar with anaerobes than their distribution and importance would warrant. The metabolic strategies such as methanogenesis, anoxygenic photosynthesis and diverse fermentative pathways which do not have equivalents in aerobic bacteria also make anaerobes worthy of attention. In these limited pages an attempt has been made to cover the varied aspects of anaerobic bacteria, and a bibliography has been included, which will allow individual topics to be pursued in greater detail.

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INTRODUCTION

The surface of the world is in contact with the oxygenated atmosphere and all readily apparent life forms are oxygen dependent, but there are many situations where oxygen is deficient. Organisms able to utilize the available nutrients in such environments must be capable of an anaerobic way of life. Such anaerobic life is almost entirely bacterial and indeed most prokaryotic species, unlike most eukaryotes, are capable of prolonged growth in the complete absence of oxygen. Many of these organisms, such as *Escherichia coli*, grow more efficiently in the presence of oxygen and are *facultative anaerobes*. Even types often regarded as obligate aerobes, such as pseudomonads, behave as facultative anaerobes if they can obtain energy from anaerobic respiration, commonly by the reduction of nitrate.

There are, however, large numbers of bacteria, such as the clostridia, which readily grow anaerobically, but are unable to utilize oxygen productively, and in addition are inhibited or even killed by oxygen. These are the *obligate anaerobes*, with which this book is mainly concerned.

In addition, there is increasing awareness of the prevalence of *microaerophilic* bacteria. These are more difficult to recognize, since they are inhibited by atmospheric oxygen concentrations and therefore resemble obligate anaerobes, but their growth is also stimulated by low oxygen concentrations. *Campylobacter* is a microaerophilic genus, some of which grow poorly if at all in the complete absence of oxygen, but will not grow in atmospheric oxygen.

To define obligate anaerobes adequately is not quite as simple as might at first appear. A simple practical definition might state that obligate anaerobes are those for which anaerobic cultivation methods are necessary, but this would include the microaerophilic types and exclude bacteria such as *Clostridium histolyticum*, which may grow poorly in air. A physiologically based definition might involve the inability of the obligate anaerobe to metabolize oxygen usefully with consequent increased growth, and the term *anoxybiontic* has been used to describe this situation. This criterion correctly describes obligate anaerobes, but its application would need a

precise examination of the relationship between growth and oxygen consumption which is not easy to carry out.

The *obligate anaerobe* is therefore best defined as an organism for which anaerobic cultivation methods give optimum growth and for which oxygen is inhibitory. The truly microaerophilic species are thereby excluded and so are those bacteria with deficient anaerobic respiration, such as many streptococci which grow equally well aerobically or anaerobically. On the other hand, the more oxygen-tolerant obligate anaerobes are included.

The importance of the obligately anaerobic, or anoxybiontic, bacteria lies in their ubiquity and consequent ecological roles. The human intestinal tract provides one example. Food residues and debridement of the gut wall provide a complex range of nutrients with consequent extensive bacterial growth and removal of oxygen. Prolonged growth must therefore be anaerobic and it is found that the obligate anaerobes have an apparent advantage over the facultative bacteria such as *Escherichia coli*. The complex mixed flora totals about 10^{12} bacteria in each gram of faecal material, but the obligate anaerobes outnumber the facultative anaerobes about a thousandfold. This complex population is usually surprisingly stable, major changes being associated with pathological disorder. Disturbance of gut anatomy by disease or surgery may lead to colonization of the upper intestine and interfere with nutrient absorption; disturbance of the normal flora by injudicious antibacterial therapy may lead to overgrowth by minority populations such as *Clostridium difficile* and associated pseudomembraneous enterocolitis; exposure to primary pathogens such as the cholera vibrio may lead to the pathogen becoming a major component of the flora in the profuse exudate caused by the effects of the cholera enterotoxin.

In the case of the intestine it is not clear what properties of the species involved gives them their particular selective advantage, but in other habitats many anaerobes have metabolism more clearly related to available nutrients. In the gut of herbivores many anaerobes are cellulolytic or stimulated by the fatty acids formed by fermentation in other bacteria. The fermentation of pairs of amino acids by the Stickland reaction is likely to be of advantage in an ecological niche provided by the putrefaction of protein. The ability of anaerobic phototrophs to couple photosynthesis with sulphide oxidation gives the characteristic flora of sulphide-containing aquatic environments, which in turn may be created by the activities of sulphate-reducing anaerobes. The specialized and archaic ability of the methanogens to utilize carbon dioxide and hydrogen is a unique and

satisfactory way to take advantage of what are normally the final products of bacterial fermentation by other organisms.

Methanogenesis is a component of what is probably the most widespread anaerobic industrial process—anaerobic sewage sludge digestion. In this, methane is a by-product usable as an energy source but the overall properties of this mixed culture fermentation demonstrate the ability of anaerobes to degrade a wide variety of materials. This ability, together with the cellulose digestion and production of low-molecular-weight compounds by fermentation in the rumen, has stimulated interest in future uses of anaerobes as industrial organisms and producers of organic chemicals as an alternative to oil-based products. This interest includes reappraisals of perhaps the first deliberately devised microbial process, the clostridial acetone–butanol fermentation.

Anaerobic bacteria therefore include many with features not found elsewhere, but this in itself does not provide justification for separate study of anaerobes. This really lies in their relationship to oxygen. Although oxygen restricts the growth of anaerobes to oxygen-deficient environments, where other life forms are at a disadvantage, such environments are not extreme in the same way as those at high temperatures or high salinity, where only restricted ranges of organisms grow. For microbes anaerobic niches are commonplace and nutritionally diverse, and the variety of anaerobes is great. Anoxia is therefore an extreme environment only to the laboratory worker, who must use special methods for the cultivation of anaerobes and thus regards them as set apart from other bacteria.

CHAPTER ONE

ANAEROBES AND OXYGEN

The ability to grow in the absence of oxygen, combined with readily apparent sensitivity to its presence, was the characteristic feature of obligate anaerobes noted by Pasteur when he first described them. This feature differentiates them from other organisms, but it is now realized that the oxygen intolerance is relative. Oxygen is potentially toxic to all living cells.

1.1 Oxygen toxicity

Oxygen toxicity might be regarded as the ultimate consequence of exergonic reactions with reduced components of biological materials, but even with hydrogen highly exergonic reactions do not normally occur without initiation by high temperatures or catalysis. The normal triplet electronic state of oxygen is stable, and a high activation energy is required for reactions to occur. Only singlet oxygen ($^1O_2$), activated to a higher energy state, possibly by absorption of a photon, combines rapidly with a wide range of organic material. This may be of relevance in photo-oxidation.

It is therefore likely that the toxic effects of oxygen do not result from a general oxidation of structural and functional components. Oxygen will, however, react with some highly reduced compounds and many of these occur as a result of metabolism, accompanied by other substances, such as cations of copper and iron which may act as catalysts. In the case of thiol-containing enzymes, direct oxygen inactivation and consequent interference with metabolism and growth may occur, but toxic effects of oxygen are found to include major changes to cell function and rapid loss of viability. Such damage is presumably mediated by less direct means.

Attention was concentrated on this means by the discovery by Fridovich of superoxide dismutase (SOD) and the awareness that the primary reduction product of oxygen is usually the superoxide anion (O_2^-), a radical formed by the addition of an electron. This radical is the anion of the hydroperoxide radical ($HO_2\cdot$), and in aqueous solution at biological pH values both will be present ($pK_a = 4.69$). The radicals are normally

dispersed rapidly by dismutation to hydrogen peroxide and oxygen, the most rapid reaction at pH 7 involving them both (Draganic and Draganic, 1971).

$$HO_2\cdot + O_2^- + H^+ \longrightarrow O_2 + H_2O_2 \; (k = 10^8\ M^{-1}\,s^{-1})$$

In much of normal aerobic metabolism this single electron reduction does not occur. For many oxygenases and cytochrome chains where oxidation is being used productively, systems have evolved to reduce the oxygen to water or incorporate it directly into larger molecular structures. It will be seen, however, that in other circumstances both O_2^- and hydrogen peroxide will inevitably be formed and these oxygen reduction products appear to be the origin of toxic effects. The products are removed in most aerobic organisms by catalase and peroxidases for hydrogen peroxide and SOD for O_2^-, the last enzyme increasing the spontaneous dismutation rate of O_2^- over a thousandfold.

There is much evidence that either hydrogen peroxide or O_2^- alone is of limited reactivity and toxicity, but that their simultaneous presence is most undesirable. The derivative likely to be responsible is the hydroxyl radical ($\cdot OH$) which is so highly reactive with other radicals and organic molecules ($k = 10^7$–$10^{10}\ M^{-1}\,s^{-1}$) that the normal concentration of reactant molecules in both growth media and cytoplasm will ensure that the reaction is diffusion controlled, with $\cdot OH$ disappearing in a fraction of 1 μm from the site of its origin. Typical of these extremely fast reactions are:

(i) Hydrogen abstraction:

$$H-\overset{|}{\underset{|}{C}}-OH + \cdot OH \rightarrow \cdot\overset{|}{\underset{|}{C}}- OH + H_2O$$

(ii) Addition to double bonds:

$$-CH=CH- + \cdot OH \rightarrow -\dot{C}H-CHOH-$$

The organic radicals formed may then combine with each other or with oxygen to form peroxyradicals. These in turn may combine with each other or may further react providing an additional route to structural change, perhaps involving a propagation chain as in lipid peroxidation:

$$R_1\cdot + O_2 \longrightarrow R_1OO\cdot$$
$$R_1OO\cdot + R_2 \longrightarrow R_1OOH + R_2\cdot, \text{ etc.}$$

The hydroxyl radical is therefore an oxygen reduction product readily capable of disrupting macromolecules such as DNA or membrane components which are crucial for cell viability. Hydrogen abstraction reactions of type (i) often have reaction rate constants of $10^{10}\ M^{-1}\,s^{-1}$ and are rather more likely than type (ii) on the basis of distribution of

susceptible hydrogen atoms and also in terms of reaction rates. Compounds such as mannitol are therefore often used to 'quench' ·OH reactions. It should be noted, however, that in complex biological systems with high concentrations of susceptible molecules, added quenching agents may not be in sufficient concentration to have an apparent effect, even when ·OH is present. In laboratory studies, formate and thiourea are also used to quench ·OH, catalase to remove hydrogen peroxide and superoxide dismutase (SOD) to remove O_2^-. Experiments using these and other approaches have shown that ·OH, hydrogen peroxide and O_2^- are all involved in toxic effects when O_2^- is generated. This led to a proposition that the reaction

$$H_2O_2 + O_2^- \longrightarrow \cdot OH + OH^- + O_2$$

might explain the effects. However, this reaction, the so-called Haber–Weiss reaction, does not occur, as has been shown by examination of the radiolysis products of water where ·OH, O_2^- and hydrogen peroxide can be generated under defined conditions. The production of ·OH from O_2^- and hydrogen peroxide can be explained by indirect reactions involving the reductive capability of O_2^- for metallic ions (Hill and Okolow-Zubkowska, 1981). A possible reaction sequence would be:

$$O_2^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$
$$Fe^{2+} + H_2O_2 \longrightarrow \cdot OH + OH^- + Fe^{3+}$$

the second reaction being the Fenton reaction. This concept is supported by experimental systems in which certain chelating agents inhibit the reaction. The reactions are outlined in Figure 1.1.

The complete details unfortunately remain topics for speculation. This is partly because, although there is good evidence for reactions of the type described in experimental systems in which O_2^- is exogenously produced, by using xanthine oxidase, for example, direct oxygen toxicity is more difficult to examine. There are also other possibilities. These include for example additional 'incestuous relationships' of reduced oxygen products, e.g.

$$2 \cdot OH \longrightarrow H_2O_2, \cdot OH + H_2O_2 \longrightarrow H_2O + HO_2 \cdot$$

and the possibilities that different reactions may occur in non-aqueous environments such as membranes and that cations may react as complexes, and also the concept that superoxide is not essential for the formation of intracellular ·OH. There are reducing agents in the cytoplasm other than O_2^- which could maintain the concentration of Fe^{2+} and so maintain the Fenton-type production of ·OH. Because of this Fee (1981), for example, has considered that the superoxide theory is not proven. One aspect of this

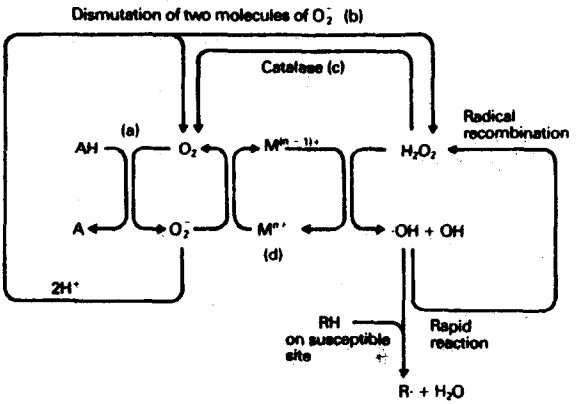

Figure 1.1 Reactions of O_2^-, hydrogen peroxide and the formation of ·OH. (*a*) Absence of O_2 or reducing system capable of reaction with O_2 will avoid O_2^- formation. (*b*) SOD increases the rate of spontaneous dismutation to prevent reactions other than dismutation. (*c*) Catalase removes hydrogen peroxide. (*d*) The cations M^{n+} and $M^{(n-1)+}$ may be, for example, Fe^{3+} and Fe^{2+} or Cu^{2+} and Cu^+.

possibility is that key enzymes with oxygen-labile sites could be directly inactivated, thus initiating a series of ultimately lethal disturbances in metabolism.

The superoxide theory does not therefore have undisputed dominance, but on the whole the direct and circumstantial evidence for the toxic consequences of oxygen formation is considerable. The field of interest is wider than obvious oxygen toxicity and includes the bactericidal mechanisms of leucocytes, mutagenesis and chronic inflammatory processes; the problems of the obligate anaerobe may be directly obvious, but aerobic organisms have still not yet evolved the means of reducing oxygen with complete impunity.

1.2 The intolerance of anaerobes

Oxygen toxicity is a general phenomenon, but in aerobic organisms it is a deviation from normal metabolism and the evolved defence mechanisms are usually effective. In obligate anaerobic bacteria the underlying mechanisms are probably the same, but oxygen is always inhibitory and may be lethal. Quantitative details are sparse, but common experience among anaerobic bacteriologists shows that lethal effects are more likely to be observed when handling fresh isolates from natural environments, especi-

ally when these are newly inoculated on to growth media. On the other hand, quantitative survival measurements carried out by a number of workers using laboratory strains have shown that most lose viability in air only slowly. The methods used included exposure of inocula to air on the surface of nutrient media and aeration of suspensions in buffer; conflation of the results from 137 strains showed that 129 of these showed little loss of viability in 30 minutes, indicating that in many cases subculture in air is unlikely to cause death by oxygen toxicity (Shoesmith and Worsley, 1984). It is possible that strains undergo some form of adaptation on transfer from the growth conditions of natural environments to the laboratory conditions, because there are undoubtedly bacteria in natural anaerobic populations which do not grow after exposure to air. Counts of viable bacteria from the intestine, for example, are higher if all manipulations are carried out in the oxygen-free conditions of the anaerobic cabinet or Hungate roll-tube methods. On the other hand, rigorous methods have given little improvement in recovery from clinical specimens, possibly because sensitive bacteria from the endogenous origins of infection would have been killed by tissue oxygen levels before the specimen was taken. Viewed in this way, oxygen tolerance becomes a virulence factor in anaerobic bacteria; the fact that the intracellular bactericidal mechanisms of neutrophils also involve oxygen reduction products lends added weight to this idea.

In some bacteria there is evidence that oxygen sensitivity depends on the phase of growth. It has been found that exposure of agar surface cultures to air after one day's incubation and before slow growing colonies are visible causes decreased recovery, even though exposure to air on initial inoculation did not affect recovery (Wren, 1980). A greater oxygen sensitivity of young cultures, and possibly interaction with the growth medium, are implied.

The basis of the wide range of oxygen resistance in anaerobic bacteria is not clear. The wide variation applies both to bactericidal effects, where tolerance extends to several hours in pure oxygen with some *Bacteroides fragilis* strains, and to bacteriostatic effects where tolerance extends from, for example, *Clostridium histolyticum*, able to grow slowly in air, to those anaerobes apparently inhibited by oxygen concentrations below the limits of measurement. These divergencies have led to the terms 'oxygen-tolerant anaerobes' and 'extremely oxygen-sensitive (EOS) anaerobes' being adopted, but there are, unfortunately, no widely accepted definitions; the context of such usage usually needs to be examined to determine not only the tolerance levels but also whether the tolerance refers to the practicality of

handling an organism in air or to the level of oxygen in the growth environment. This is especially the case with 'oxygen-tolerant anaerobes'. In various circumstances this might indicate those tolerant of aerobic manipulation, those able to grow in contact with measurable oxygen in the atmosphere (e.g. *Clostridium perfringens*), or even microaerophiles.

At the moment, no single factor can be defined as responsible for these variations in tolerance between different anaerobes or even between aerobes and anaerobes (Morris, 1979). Historically, McLeod and Gordon expounded the hypothesis by which hydrogen peroxide sensitivity was regarded as crucial, in association with the absence of catalase from anaerobic bacteria. Inconsistencies were soon noted, catalase being absent from many aerobes which were yet peroxide tolerant, and catalase also occurred in some obligate anaerobes, but this theory was dominant until 1971. A similar postulate was then made by McCord, Keele and Fridovich regarding superoxide dismutase (SOD). In the bacteria they examined, with the exception of *Lactobacillus plantarum*, aerobes and facultative anaerobes were found to have SOD, while obligate anaerobes did not. In due course *L. plantarum* was found to have a manganese-catalysed method of accelerated O_2^- dismutation, but on the other hand, further study has shown SOD in a number of obligate anaerobes, including species of *Bacteroides, Clostridium, Peptococcus, Selenomonas* and *Veillonella* as well as anoxygenic photosynthetic bacteria and sulphate reducers, and is probably commoner in obligate anaerobes than is catalase.

There is some, but far from perfect, correlation between protective enzyme activity and oxygen tolerance (Rolfe *et al.*, 1978). More of the oxygen-tolerant strains will be found to have SOD and catalase than the oxygen-sensitive strains, but it is not possible for SOD activity to be correlated with oxygen tolerance in any quantitative way. A range of other factors must also be considered:

(i) *Rate of oxygen reduction.* Anaerobic cultures are likely to have considerable powers of oxygen reduction. If O_2^- is the reduction product, toxic processes are initiated. On the other hand, low oxygen consumption reduces this risk. Extreme examples of this are spores of clostridia, whose dormancy makes them oxygen stable, even without the overall mechanisms of spore resistance.

(ii) *Mode of oxygen reduction.* In aerobes the main productive pathways of oxygen reduction yield water. Some anaerobes, such as *Clostridium acetobutylicum* (O'Brien and Morris, 1971), other clostridia

and some peptostreptococci have been shown to reduce molecular oxygen to water by NADH oxidases, but this may be regarded as a detoxification mechanism rather than productive, because the NADH thus consumed is no longer available for reduction of metabolites.

(iii) *Protective enzymes.* In addition to SOD and catalase, bacteria may also possess peroxidases thus maintaining low levels of O_2^- and hydrogen peroxide.

(iv) *Cell composition.* The macromolecules involved in bacterial oxygen damage are not known, but DNA, cell membranes or proteins such as ferredoxins are most probable. With any of these, differences in composition or concentration of reactive sites might explain degrees of oxygen sensitivity which appear to be in contradiction to the indications given by other properties such as protective enzymes.

(v) *Repair mechanisms.* Evidence from the examination of a series of strains of *Escherichia coli* has indicated that decreased resistance to hydrogen peroxide correlates better with loss of DNA repair systems than loss of SOD or catalase (Carlsson and Carpenter, 1980) and this might be extended to oxygenated anaerobes, where DNA damage has been demonstrated. It is possible that other macromolecular sites could be repaired, but this has not been examined.

Of all these factors, the most important is the primary reduction of oxygen. Modulations of the other effects leading to ultimate significant molecular damage can be used to provide a general explanation of variations in oxygen tolerance, but as yet cannot provide quantitative predictions.

1.3 Oxygen and redox potential

It has long been known that a low oxidation–reduction potential in growth media favours the growth of obligate anaerobic bacteria and that their growth often results in a lower potential than found in facultative anaerobes. This potential, also termed the *redox potential* or E_h, is given by:

$$E_h = E_o' + \frac{RT}{nF} \ln \frac{[\text{oxidized state}]}{[\text{reduced state}]}$$

where E_h is the potential developed at an electrode of appropriate inert

composition, such as platinum, E'_o is the standard electrode potential of the redox couple involved at the pH under consideration, usually pH 7, n is the number of electrons transferred for each molecule reduced, R, T and F are the gas constant, the temperature (Kelvin) and the Faraday constant respectively. E'_o is therefore the expected E_h when the concentrations of oxidized and reduced states of the compound concerned are equal. Although there is a value of E'_o corresponding to the reduction of oxygen, this cannot be used to calculate oxygen concentrations in either uninoculated media or growing cultures because such systems contain many redox couples and many of these are not rapidly reversible or able to react directly with oxygen. This also implies that similar E_h values recorded in different cultures are likely to have been the result of different combinations of redox couples. The redox potential, unlike pH which gives a measure of hydrogen-ion concentration, cannot therefore be used as a measure of any component in most culture situations.

The concept has, however, long been used as an indication of the deoxygenation of growth media. This is done by the use of indicator dyes, methylene blue and resazurin being the most used. With methylene blue, for example, at pH 7 and 37 °C an E_h value of -28 mV corresponds to a 95% reduction of the blue oxidized form; the absence of any apparent colour should ensure that a value below this has been attained, and in normal circumstances this indicates successful removal of oxygen.

Investigation has shown that the relevant parameter controlling anaerobic growth is oxygen concentration and not the electrode potential *per se*.

Experiments have been carried out combining variation of oxygen concentration with changing the redox potential independently. Growth usually takes place only when oxygen is removed, irrespective of the electrode potential created by added substances. For example, if a positive electrode potential is produced by the addition of ferricyanide, growth will not be inhibited providing oxygen has been removed (Marounek and Wallace, 1984). On the other hand, the variety of redox couples present in media and growing cultures makes it impossible to relate E_h and dissolved oxygen concentration except that in general terms oxygenation will cause the E_h to increase.

1.4 Oxygen and growth media

Oxygen in a growth medium may affect bacteria directly, but there is much evidence to show that the medium itself may be made inhibitory by