THE USE OF MICROBIAL MEMBRANES TO ACHIEVE ANAEROBIOSIS

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ABSTRACT

Several years ago, it was observed that sterile microbial membrane preparations stimulated recovery of certain radiation-injured bacteria. Later it was noted that these same preparations reduce dissolved oxygen to water in a variety of environments, including bacteriological media. This reduction of oxygen is an enzymatic process and is influenced by parameters such as temperature, pH, and the availability of specific oxidizable substrates. Oxygen-reducing membrane preparations can be made from several different bacterial species. When added to liquid or solid bacteriological media, membrane preparations rapidly produce and maintain anaerobic conditions favorable for the growth of a wide variety of oxygen-sensitive microorganisms. When used with a specifically designed disposable dish, membrane preparations allow the development of colonies of many anaerobic microorganisms on the surface of agar without the use of anaerobic hoods or other devices. In addition to providing conditions suitable for the growth of anaerobes, membrane preparations stimulate recovery of heat and cold injured bacteria of several different genera including facultative organisms. These results are reminiscent of the early observations regarding the recovery of radiation-injured bacteria. In addition to their usefulness in microbiology, oxygen-reducing membrane preparations have the potential for protecting a wide variety of oxygen-sensitive organic compounds.

INTRODUCTION

Historical Perspective

The use of microbial membranes for producing anaerobiosis has its roots in basic radiation microbiology studies initiated at the Oak Ridge National

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Laboratory in the early 1960s. At that time, we were studying the effects of ionizing radiation on the bacterium *Escherichia coli*. The work was being done with mutant strains that, after exposure to radiation, retained the ability to grow but could no longer divide. We were trying to explain the curious observation that such cells recovered the ability to divide if they were incubated in the presence of certain other bacteria (Adler and Hardigree 1996). The phenomenon did not seem to require direct contact between the irradiated cells and the "neighbor" cells. For a long time, it was thought that some low-molecular-weight material must be leaking from the neighbor cells and stimulating the division process in the irradiated cells, but solid evidence for this postulated material could never be produced.

The problem became even more difficult when, in the early 1970s, we found that a particulate fraction derived from the neighbor cells was responsible for stimulating division. The particulate material, while clearly too large to migrate through the solid bacteriological media in which the bacterial cells were growing, somehow induced division in these radiation-injured cells. The bacteria could then produce normal appearing macro-colonies.

By the late 1970s, it was shown that the active particulate fraction was derived from the cytoplasmic membrane. Initially, it was not clear how any of the activities known to be present in the cytoplasmic membrane could possibly account for the ability of the membrane fraction to promote cell division.

An insight came in 1979 when it was observed that the amount of particulate fraction required to promote division was directly related to the amount of oxygen present in the medium in which the irradiated cells and the fraction were incubated. In other words, if most of the oxygen was flushed out of the system by an inert gas, a very small amount of the particulate fraction could promote division. This was the first observation suggesting the possibility that the particles themselves might have the ability to reduce the oxygen content of bacteriological media. This was subsequently shown to be the case and it was established that the cytochrome-based electron transport system located in the cytoplasmic membrane was the responsible enzyme system (Adler *et al.* 1981). About this time, we began to consider the possibility that membrane fragments might be a valuable way of removing oxygen from bacteriological media designed to support the growth of anaerobes. This approach to providing anaerobic conditions appeared to have several potentially advantageous features when compared to currently used methods.

Many current methods employ strong nonspecific chemical reducing agents or oxygen-free gases to sweep oxygen out of bacteriological media. These techniques are not problem-free. Because chemical agents are often toxic, they can be added in only limited amounts. Their toxicity probably results from the tendency of these agents to react with components of the medium other than oxygen and even to penetrate the bacteria and react with important cell

constituents. Treatment with oxygen-free gases also has limitations. When liquid media are sparged with such agents, foaming and evaporation can present problems. If solid media are exposed to oxygen-free gases, the diffusion of oxygen from the solid into the gas phase is usually a slow and inefficient process.

Other current methods rely on airtight bags, jars and chambers from which oxygen is removed by the action of inorganic catalysts or gas flushing. Manipulating these containers can be cumbersome. Furthermore, there is a delay between the time at which the atmosphere within a container becomes oxygen-free and the time at which bacteriological media within the device becomes oxygen free. In contrast to these agents and devices, we anticipated that membrane fractions added directly to media would be nontoxic and remove oxygen rapidly and efficiently. The expectation that membrane fractions would be nontoxic was based on our observation that they specifically reduce oxygen to water in the medium and that the membrane particles are too large to penetrate the bacterial cells. Furthermore, in natural environments, anaerobes are accustomed to growing in the presence of membranes, both their own and those of surrounding cells.

If membrane fragments were to be used for this purpose, they would, of course, have to be sterile. This was achieved by the development of a filtration technique that separated the membrane fragments from any contaminating bacteria.

The first report establishing the use of sterile membrane fragments and their associated enzymes as effective reagents for the reduction of dissolved oxygen appeared in 1981 (Adler and Crow 1981). This publication demonstrated that several species of anaerobic bacteria grew very well in liquid media containing small amounts of the membrane preparation. In the intervening years, several reports have appeared confirming and extending these initial observations (Adler 1990; Spangler *et al.* 1995). Exploration of the usefulness of membrane fragments in microbiology was significantly enhanced by the introduction of the standardized consistent product now known as the Oxyrase® Enzyme System (Oxyrase, Inc., Mansfield, OH). Although oxygen-reducing membrane fragments have been made from a variety of microorganisms, EC Oxyrase®, made from *E. coli* remains the most widely used and best characterized material. The discussion that follows will first summarize some of the basic features of EC Oxyrase® and then deal with some recent observations from several laboratories.

Basic Properties of EC Oxyrase®

EC Oxyrase® is isolated from a selected strain of *E. coli*. The bacterial cells are first disrupted in a pressure cell. Unbroken cells and large debris are

removed by low-speed centrifugation. The supernatant is then subjected to high-speed centrifugation or tangential flow filtration designed to separate the small particles of cell wall and membrane from soluble proteins, deoxyribonucleic acid, etc. Sterility is achieved by passing the suspension of membrane fragments and associated particles through a 0.22-μm filter.

In the presence of suitable hydrogen donors, the enzymes of the electron transport system associated with the fragments reduce dissolved oxygen to water. The hydrogen donor must be a substrate for one of the dehydrogenase enzymes bound to the membrane particles. In organic environments such as complex bacteriological media, the appropriate compounds are often readily available, but in many chemically defined environments they must be added. Sodium lactate and sodium succinate have been determined to be among the suitable hydrogen donors for membrane fragments derived from *E. coli* because both lactate and succinate dehydrogenases are firmly bound to the membrane fragments.

Because the concentration of oxygen dissolved in liquids is always less than 1m*M*, the concentration of hydrogen-donating substrate required for complete and rapid oxygen reduction is correspondingly low. In practice, 5 to 50 m*M* concentrations of a hydrogen donor are sufficient to produce and maintain complete anaerobiosis. If extremely low concentrations of hydrogen donors are present, the membrane fragments can be used to produce microaerophilic environments (Crow *et al.* 1985).

During the course of oxygen reduction, the hydrogen-donating substrate is oxidized. For example, lactate is converted to pyruvate, and succinate is converted to fumarate. Because of the enzymatic nature of the system, repeated challenges with O_2 can be tolerated. The oxygen will be reduced to water as long as the enzymes remain active and substrates are present.

The oxygen-reducing ability of EC Oxyrase® depends on the interaction of several enzymes and at least one nonenzymatic component (ubiquinone). This complex system is, as would be expected, responsive to many factors in the environment. Nonetheless, the activity of EC Oxyrase® is remarkably stable, probably because the necessary components are protected by being imbedded in a protein-lipid matrix. These membrane fragments may be stored for more than 1 year at -20C without losing activity. They can be frozen and thawed repeatedly. They will survive at least 5 h at 45C.

The pH optimum for oxygen reduction by membrane fragments undoubtedly reflects a consensus of the pH optima of the individual components in the system. The oxygen reducing system from *E. coli* operates at a maximum rate at pH 8.4 when lactate is used as a substrate. At the extremes of the pH range, 6 or 9, approximately 30% of the maximum rate is retained. Although the rate of oxygen reduction depends on pH, the final level of anaerobiosis achieved does not.

Applications of EC Oxyrase® for Growing Anaerobes and Microaerophiles

EC Oxyrase® can be used to produce and maintain anaerobic conditions in both liquid and solid media. It can also be used to produce and maintain an anaerobic headspace in a specially designed agar-containing dish (Oxydish™). When used in liquid media contained in tubes, fermenters, etc., it rapidly produces anaerobic conditions suitable for the growth of a wide variety of anaerobes representing more than 12 genera and 30 species (Table 1). Included in this group are most of the industrially and clinically significant anaerobes. When used in agar pour plates, EC Oxyrase® can produce anaerobic conditions suitable for the development of colonies from single cells (Adler *et al.* 1984). When used in connection with the Oxydish™, EC Oxyrase® permits development of colonies with characteristic morphologies on the surface of agar (Gannon and Thurston 1996).

TABLE 1.
ALL OF THESE ORGANISMS HAVE BEEN GROWN IN LIQUID MEDIA IN TEST TUBES WITH LOOSE FITTING CAPS. MANY OF THEM HAVE ALSO BEEN GROWN AS ISOLATED SURFACE COLONIES IN OXYPLATES™. THE INFORMATION COMES FROM SEVERAL PUBLISHED SOURCES AND PERSONAL COMMUNICATIONS.

Microorganisms Grown with Oxyrase®		
· *Actinomyces*	· *Clostridium*	· *Desulfovibrio*
- *meyeri*	- *acetobutylicum*	- *vulgaris*
- *naeslundii*	- *bifermentans*	· *Eubacterium*
- *odontolyticus*	- *butyricum*	- *lentum*
· *Bacteriodes*	- *difficile*	- *limosum*
- *caccae*	- *histolyticum*	· *Fusobacterium*
- *capillosus*	- *kluyveri*	- *mortiferum*
- *distasonis*	- *pasteuranium*	- *necrophorum*
- *fragilis*	- *perfringens*	- *varium*
- *nodosus*	- *ramosum*	· *Peptostreptococcus*
- *ovatus*	- *sordellii*	- *asaccharolyticus*
- *thetaiotaomicron*	- *sporogenes*	- *anaerobius*
- *ureolyticus*	- *tetani*	- *magnus*
- *vulgatus*	- *tertium*	- *micros*
· *Veillonella*	- *thermoautotrophicum*	- *intermedia*
- *parvula*		
· *Porphorymonas*	· *Propionibacterium*	- *melaninogenica*
- *levii*	- *acnes*	

Because methods based on EC Oxyrase® do not depend on the use of anaerobic chambers, inert gas cylinders, or other devices, they are beginning to be applied in research, clinical, and quality control laboratories. They are also

beginning to have an impact on the microbiology teaching laboratory (Van Demark and Batzing 1986).

EC Oxyrase® has also been used for producing the microaerophilic conditions required by organisms such as *Campylobacter* (Abeyta *et al.* 1995; Niroomand and Fung 1994; Raben and Slavik 1994; Tran 1995). When used for this purpose, the rate at which EC Oxyrase® reduces oxygen must be carefully balanced against the rate at which oxygen infiltrates the system so as to assure a low but significant dissolved oxygen concentration. This can be done by manipulating the oxyrase concentration, the level of hydrogen donating substrates, and parameters such as shape, size, and amount of agitation in the vessels being used to contain the growth medium.

One of the limitations of EC Oxyrase® is the fact that it functions most effectively at pH 8.4. At the initial pH of many bacteriological media (pH 6.8-7.0), its rate of reaction is reduced by approximately 40%. This, in practice has not been a significant drawback. However, at pH 5 and below, EC Oxyrase® operates very slowly. Media designed for the isolation and cultivation of acidophilic and aciduric microorganisms are often poised at pH values in this range. For this reason, an oxyrase has recently been developed (AC Oxyrase®) which has its optimum at pH 5.5 and remains effective at pH 4 and below (Fig. 1). The methods for preparing this in large quantities as a standardized product have been worked out but, at this time, the product has not yet been released as

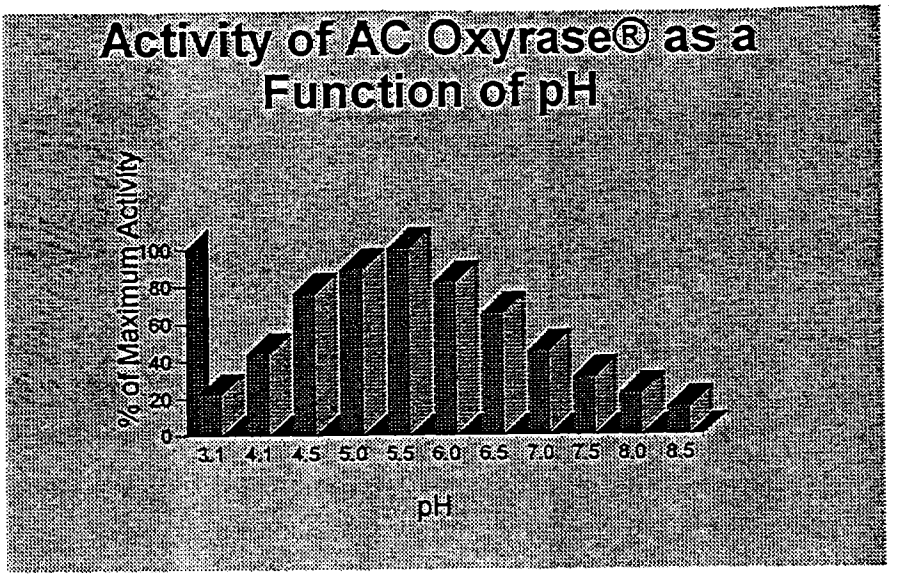

FIG. 1. ACTIVITY WAS DETERMINED ON SAMPLES IN 0.04M PHOSPHATE BUFFER USING DL LACTIC ACID AS A SUBSTRATE
Oxygen reduction rates were determined by the use of a Clark electrode in a Gilson Oxygraph maintained at 37C.

a free-standing item. It is, however, being used in an Oxyplate™ configuration with a selective medium (Beerens 1990) for the isolation of *Bifidobacterium spp.* from stool samples and other sources. We believe that this new oxyrase will find several interesting applications. When mixed with EC Oxyrase®, it can be used to produce a hybrid oxyrase that remains uniformly effective over a wide pH range (Table 2).

TABLE 2.
A MIXTURE OF EQUAL VOLUMES OF EC AND AC OXYRASES® WAS MADE AND THE ACTIVITY DETERMINED AT THE INDICATED pHs USING A MIXTURE OF DL LACTATE (50m*M*) AND D-GLUCOSE (50m*M*). OXYGEN REDUCTION RATES WERE DETERMINED USING A CLARK ELECTRODE IN A GILSON OXYGRAPH MAINTAINED AT 37C.

	Activity (μ/mL) of a Mixture of Oxyrases*				
	pH 5.5	pH 6.1	pH 6.5	pH 6.9	pH 7.9
EC Oxyrase	3.3	8.1	13.0	18.9	23.5
AC Oxyrase	24.4	22.3	18.3	14.4	6.5
Mixture	18.6	18.6	18.9	18.3	17.1

Enhancement of Cell Recovery by EC Oxyrase®

Several laboratories have now reported that the recovery of colony-forming units, particularly from injured populations of bacteria, can be enhanced by growth in the presence of EC Oxyrase. This phenomenon has been observed in both anaerobic and facultative organisms. The mechanism(s) is still not understood. It should be recalled that the original reason for studying the material now known as EC Oxyrase® was that it stimulated colony formation in *E. coli* injured by ionizing radiation. It was suggested that this recovery resulted from the interplay of two properties associated with EC Oxyrase®. One of these was the establishment of anaerobic conditions and the other involved the modification of substances found in the yeast extract and peptone components of the post-irradiation plating medium (Gill and Adler 1985). Several other early reports of EC Oxyrase®-stimulated recovery of various *E. coli* mutants have previously been reviewed (Adler 1990). Some of these seem to share the characteristics noted above for recovery of irradiated *E. coli,* other cases do not seem to require the establishment of anaerobic conditions. Several studies, pertinent to injured organisms associated with food spoilage, also report enhanced recovery of colony-forming units in the presence of EC Oxyrase®.

Heat and cold stressed *Clostridium perfringens* are recovered in greater numbers in the presence of EC Oxyrase® (Hoskins and Davidson 1988). Likewise, it has been observed that heat injured *Listeria monocytogenes, E. coli* O157:H7, and *Yersinia enterocolitica* recover in the presence of EC Oxyrase® (Yu and Fung 1991a; Thippareddi *et al.* 1995; Patel and Beuchat 1995). Doyle *et al.* 1996 reported that sodium hypochlorite-injured *Salmonella typhimurium* can be recovered using EC Oxyrase® and other agents. In all of these studies, EC Oxyrase® was added to a liquid medium in which the injured cells were incubated prior to plating in the absence of EC Oxyrase®. None of the studies shed much light on the mechanism(s) by which EC Oxyrase® stimulates repair of the injured cells, but a consideration of the incubation conditions used suggests that, at least in some of these studies, anaerobiosis may not be an important determinant of the observed results. If this is true, then one must consider the possibility the EC Oxyrase® is providing some essential ingredient required by injured cells or that it is producing some desirable change in a component of the post-injury growth medium similar to that which was observed in the early radiation studies.

EC Oxyrase®-Induced Stimulation of Growth in Uninjured Cells

Several recent papers have reported that the addition of EC Oxyrase®to liquid media containing cells that have not been intentionally injured results in shorter lag phases, enhanced rate of growth in log phase or enhanced maximum titer. This has been observed for *L. monocytogenes, L. spp., E. coli O157:H7, S. typhimurium, S. paratyphi, S. arizonae, Streptococcus faecalis, C. jejuni, C. coli,* and *Arcobacter butzleri* (Yu and Fung 1991a; Niroomand and Fung 1992; Niroomand and Fung 1994; Liu *et al.* 1995). The improvements in growth rates and maximum titers are relatively small but can lead to significant improvements in rapid detection of microorganisms. The mechanism(s) accounting for these effects in liquid media is unknown, but it appears likely that, at least for some of them, anaerobiosis is not a key feature.

A limited number of similar observations have been made when certain uninjured anaerobic organisms are plated in or on the surface of solid media with and without EC Oxyrase®. For example, it has been reported for *Cl. perfringens* that the number of colony-forming units observed in tryptose sulfite cycloserine agar was generally greater when the medium contained EC Oxyrase® than when the medium was incubated anaerobically in its absence (Hoskins and Davidson 1988). Likewise, we have recently observed that the colony counts of *Bifidobacterium adolescentis* on the surface of a highly selective medium (Beerens 1990) are generally greater in the presence of Oxyrase than in its absence (Table 3).

TABLE 3.
SEVEN EXPERIMENTS ARE REPRESENTED IN WHICH THE ORGANISM WAS PLATED ON A SELECTIVE AGAR MEDIUM (BEERENS 1990) ± AC OXYRASE® IN PETRI DISHES WHICH WERE THEN INCUBATED IN A NITROGEN 5% CO_2 ATMOSPHERE FOR 48 H AT 37C

	Effect of Oxyrase on Plating Efficiency *Bifidobacterium adolescentis*						
	1	2	3	4	5	6	7
Without Oxyrase*	0.5	0.3	0.2	0.7	0.4	0.9	0.2
With Oxyrase*	1.2	0.9	0.4	1.8	0.9	1.0	0.8
Ratio	2.3	3.0	2.3	2.5	2.3	1.1	3.3

*CFU/mL × 10^8 after 48 h in N_2 + 5% CO_2 @ 37C

Nonmicrobiological Uses of EC Oxyrase®

EC Oxyrase® and similar enzyme systems are useful in a variety of nonmicrobial situations in which an effective and specific means of oxygen reduction is desired. For example, EC Oxyrase® has been used in a biochemical study to protect tetrahydrobiopterin, a coenzyme involved in the oxidation of aromatic amino acids (Jacobson *et al.* 1987). It has also been used to establish the oxygen dependence of the ecdysone 20-monooxygenase isolated from land crabs (Soumoff and Skinner 1988). More recently, EC Oxyrase® has been used in a study of the effects of anoxia on intracellular calcium concentrations in isolated rat ventricular cardiomycetes (Rose *et al.* 1994). In our own laboratory we have recently demonstrated that EC Oxyrase® can protect aqueous solutions of bilirubin from oxidizing to biliverdin (Fig. 2). It appears likely that enzymatic reduction of oxygen will, in the future, be useful in several systems in addition to the microbiological ones emphasized here.

CONCLUSION

EC Oxyrase® is a sterile, partially purified preparation of *E. coli* cytoplasmic membrane fragments. It is useful in promoting the growth of anaerobes because it can be incorporated directly into bacteriological media where it rapidly and specifically reduces dissolved oxygen to water by an

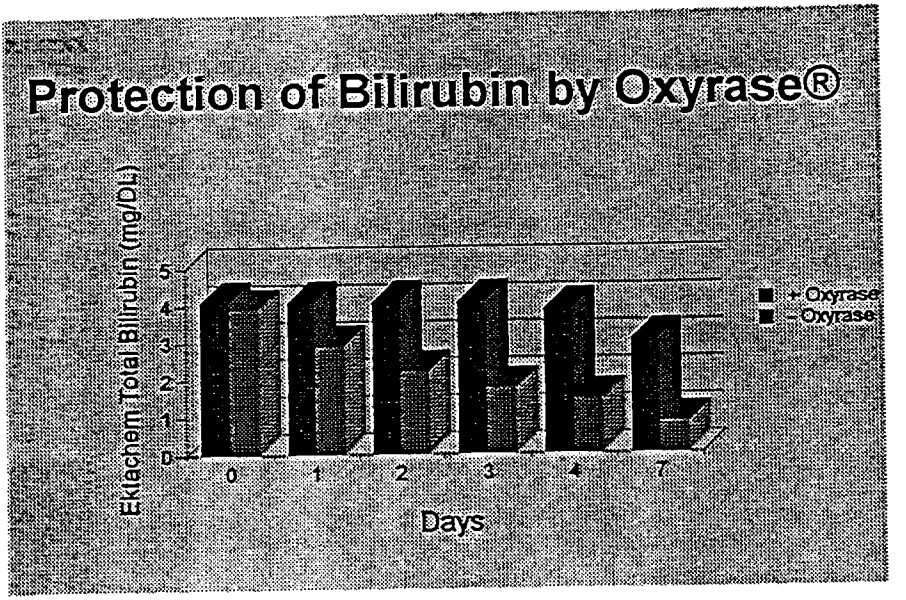

FIG. 2. SAMPLES OF AN AQUEOUS SOLUTION OF BILIRUBIN WERE SATURATED WITH AIR AND THEN INCUBATED IN OPEN TUBES ± EC OXYRASE AT 37C FOR THE INDICATED PERIODS

enzymatic process. The enzymes involved are those of the cytochrome-based electron transport system. Variations of this system are found in most microorganisms and are potential sources of oxyrase-like materials. In addition to its usefulness in producing anaerobic conditions, EC Oxyrase® has been found to stimulate the growth of injured and uninjured, facultative and anaerobic organisms. The mechanism(s) for these latter effects is yet to be resolved but it may be possible to use the phenomenon as an aid in the rapid detection of microorganisms in foods and other environments.

EC Oxyrase® has also been investigated in several nonmicrobial environments. It is beginning to prove useful for removing oxygen from a variety of aqueous biochemical solutions where a nonspecific reducing agent is undesirable.

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