EFFECTS OF *ESCHERICHIA COLI* B/r ORNL MEMBRANES ON THE GROWTH AND CHARACTERIZATION OF *PEPTOSTREPTOCOCCUS ANAEROBIUS* AND THE INDUCTION OF MUTANTS BY MEANS OF CESIUM-137 IONIZING RADIATION

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To the Provost of the Graduate School:

I am submitting herewith a dissertation written by Shellye Kay Ainsworth Gathings entitled "Effects of Escherichia coli B/r ORNL membranes on the growth and characterization of Peptostreptococcus anaerobius and the induction of mutants by means of Cesium-137 ionizing radiation." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Radiation Biology.

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Shellye Kay Ainsworth Gathings

Effects of Escherichia coli B/r ORNL membranes on the growth and characterization of Peptostreptococcus anaerobius and the induction of mutants by means of Cesium-137 ionizing radiation

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Current methodologies used in the production, screening and characterization of bacterial mutants poses inherent problems when these methods are applied to strict anaerobes. Inclusion of sterile stable Escherichia coli B/r ORNL membranes in the cell suspension fluid during irradiation resulted in the scavenging of oxygen radicals and peroxides produced during exposure. This decreases bacterial death caused by these factors and increases the possibility that the radiation will produce changes in the chromosome.

The P_2 membranes eliminate the need for cysteine-HCl which acts as a radioprotective agent and allow aerobic culturing techniques to be applied to strict anaerobes in mutation studies. P. anaerobius VPI# 4330 was exposed to Cesium-137 gamma radiation. Cell survival, biochemical activities and changes in antibiotic resistance as effected by the inclusion of the P_2 membranes were determined on the prototype and the isolated variants. Resistance parameters were established, with and without the presence of the P_2

membranes, using the Kirby-Bauer disk diffusion method, the Minimal Inhibitory Concentration (MIC) determination and Gas Liquid Chromotography (GLC). The P_2 membranes were also used to investigate changes in tyrosine degradation and cell sensitivity to sodium polyanethol sulfonate, both of which are used in P. anaerobius identification.

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CHAPTER I

INTRODUCTION

The strict anaerobic bacterial species Peptostreptococcus anaerobius, P. micros, P. parvulus and P. productus are found as part of the normal microbial flora in humans. They are often isolated from wounds and abscesses, pelvic inflammations, empyema, peritonitis, puerperal fever and subacute bacterial endocarditis (Pien et al. 1972; Smith 1975; Wong et al. 1980). Peptostreptococcus and other clinically important anaerobes are often found in polymicrobic populations where they tend to be protected by the various reductive enzymes produced by other microorganisms co-existing with them. Healthy tissue maintains an oxidation-reduction potential of approximately +120 mV, which is not a suitable environment for the growth of obligate and strict anaerobes. As necrosis in damaged tissue develops, there is a succession of microorganisms from facultative to strict anaerobes as the oxidation-reduction potential decreases (Harwood and Canale-Parola 1984).

P. anaerobius has been reported to be one of the 10 most numerous bacterial species obtained from 153 specimens of 90 patients (Holland et al. 1977). It is frequently isolated from the female genital tract, occurring in both the endogenous flora and in infections (Hill and Ayers 1985), besides being implicated in periodontal disease (Harwood and Canale-Parola 1984).

Antimicrobial susceptibility testing is not routinely indicated for anaerobes since patients with mixed intraabdominal and wound infections respond well to appropriate surgical measures and nonspecific antimicrobial therapy (Washington 1981). Antibiotic susceptibility testing is recommended in cases of anaerobic bacteremia, brain abscess, septic arthritis, osteomyelitis and endocarditis (Washington 1981). Data obtained by Sanders and Lewis (1980) indicate that anaerobic bacteria are now becoming more resistant to antibiotics than has been previously reported. Washington (1981) stated that the use of increased levels of penicillin to combat infections caused by Bacteroides and Clostridium strains proved to be beneficial to the patients. Decreased susceptibility of P. anaerobius to moxalactam (Hill and Ayers 1985) and of Haemophilus to rifampin (Yogev et al. 1982) has been

substantiated. Organisms seem to develop resistance to these antibiotics and others as a result of chromosomal mutations (Bryan 1984), as indicated by the initial occurrence of susceptibility loss to a single antibiotic. Multiple resistance appears to be a result of R plasmid transfer rather than changes in chromosomal DNA. Chromosomal-mediated antibiotic resistance remains a therapeutic problem associated with the use of methicillin, rifampin, nalidixic acid, isonazide and in some cases certain β -lactam antibacterial chemotherpeutic agents (Levy 1982).

Not only do some anaerobes cause life threatening diseases, but many are of practical industrial importance. For example, methanogens are capable of producing methane (Hamilton 1982) and clostridia produce organic acids and solvents (Méndez and Gómez 1982). Besides, there is ever increasing interest in studying genetic systems of bacteria, in which the anaerobes are a special group.

P. anaerobius is a strict anaerobe, meaning it cannot tolerate air and is inhibited or killed by oxygen and oxidized components in the medium (Fulghum 1983). Although mutations may arise spontaneously, their frequency can be increased drastically in the presence of mutagenic agents.

Bryan (1984) stated that the normal frequency of streptomycin resistance mutations of 10^{-10} per generation may increase to 10^{-6} or 10^{-5} when bacteria are exposed to X-rays or UV light. Since patients receiving radiation and/or chemotherapy often develop bacterial infections,this may result in an increase of antibiotic resistant anaerobic bacterial strains.

Cysteine-HCl, routinely added to anaerobic culture media, acts as a reducing agent. During exposure to radiation, bacterial cells are often suspended in water containing cysteine-HCl. Although this amino acid removes radiation produced oxygen radicals, these singlet oxygen quenchers are, themselves, toxic to P. anaerobius (Carlsson et al. 1979). Hydrogen peroxide and superoxide radicals are also produced as a result of radiation and exposure to atmospheric oxygen. They are very toxic to anaerobic bacteria (Carlsson et al. 1978). The removal of these radicals from radiation suspension medium requires the presence of enzymes such as horseradish peroxidase, catalase and/or superoxide dismutase. These additives would increase the variables related to the radiation of strict anaerobes (Carlsson et al. 1978; Carlsson et al. 1979; Nyberg and Carlsson 1981). The use of sterile membranes with an active

intact membrane bound cytochrome system has been proposed to remove atmospheric oxygen and radiation induced oxygen radicals from the bacterial environment (Adler et al. 1981). This method has been applied in the investigations reported in this dissertation. The membrane fraction of disrupted E. coli B/r ORNL cells made possible the use of radiation as a mutagenic agent for anaerobic bacteria. The membranes excluded oxygen from the medium and techniques normally used in aerobic cultures could then be used for the growth of anaerobes.

The purpose of this investigation was to produce antibiotic mutants in a strict anaerobe by the use of gamma irradiation while negating the effects of radiation produced oxygen and its reaction products. The major objectives of this research are: 1. to develop a suitable technique to culture the strict anaerobe P. anaerobius by providing an oxygenless environment with the use of E. coli B/r ORNL membranes, which were successfully employed to culture other anerobic organisms (Adler et al. 1981); 2. to determine the effectivness of E. coli B/r ORNL membranes in the removal of free oxygen radicals and autooxidized products formed in media and in the fluid used for radiation exposure; 3. to establish optimum growth conditions for membrane culture of

P. anaerobius by determining various pH values, temperature and nutritional requirements; 4. to develop techniques for the induction of mutations employing Cesium-137 gamma radiation in a membrane environment; 5. to formulate a method to be used to reclaim mutants of *P. anaerobius* produced by radiation; and 6. to modify existing aerobic and anaerobic techniques to be used in combination with membrane technology for the selection, differentiation and characterization of antibiotic mutants.

CHAPTER II

LITERATURE REVIEW

The distinction between aerobes and anaerobes was noted as far back as 1861 by Louis Pasteur who referred to bacteria that can live in air and those from whom air must be excluded (Willis 1977). Present classification schemes of bacteria in regard to their ability to tolerate oxygen, include such designations as strict, obligate, facultative and aerotolerant (Fuerst 1983). Definitions most applicable to the nomenclature were proposed by Fulghum (1983):

"a) Aerobic bacteria will grow on the surface of a simple solid medium exposed to air.

b) Anaerobic bacteria will not grow on the surface of a simple solid medium exposed to air.

c) Facultative anaerobic bacteria are aerobic bacteria which have the ability to grow anaerobically.

d) Obligate anaerobic bacteria are bacteria with no ability to synthesize an oxygen-linked respiratory chain and which therefore must ferment.

e) Strict anaerobic bacteria are anaerobic bacteria which cannot tolerate oxygen and are inhibited or killed by oxygen and oxidized components of media."

An environment free of molecular oxygen (O_2) is not enough to assure the growth of strict anaerobes since a low oxidation potential (E_h) must be maintained as well (Smith 1973; Morris 1976). Reddick et al. (1980) reminds us that oxidation is the loss of electrons and reduction is the gain of electrons. Compounds which have strong reducing properties are active electron donors and are often included in anaerobic media to make it a reducing environment for bacteria. Cysteine-HCl, sodium thioglycollate and other SH-compounds are often included in anaerobic medium to act as reducing agents (Holdeman et al. 1977). These compounds are oxidized by O_2 before this molecule can attack the organisms being grown in the media. The presence of strong reducing properties in the media may be expressed as a low E_h. The function of a low E_h environment for the bacteria is to minimize the loss of reducing power by the organism which may be used as a source of energy yield.

Morris (1976) proposed that the O_2 interaction with media components produces substances that are more toxic to

the organism than O_2. Obligate anaerobes, unlike aerobes, lack the means to detoxify these compounds. When nutrient media are exposed to oxygen, the results are the formation of products such as organic peroxides (Patel et al. 1984), aldehydes (Morris 1976) and free radicals (Halliwell and Gutteridge 1984; Oberley 1982b). Oxygen interacts with cell constituents, such as reduced iron sulfur proteins (i.e. ferredoxins, flavodoxin, tetrahydropteridines and flavoproteins). The superoxide anion, the hydroxyl radical and singlet oxygen are damaging to a bacterial cell.

Investigations concerning these highly toxic byproducts of oxidation have assumed new importance because of the similarities between oxygen toxicity in anaerobes and lethal oxygen effects on aerobes such as hyperbaric oxygen toxicity, oxygen enhancement of radiation damage, cryo-injury and photodynamic lethality. Morris (1976) found similar areas of attack in aerobes and anaerobes, such as unrepaired single-stranded breaks in DNA and damage to membranes, either in permeability or as impairment of transport. Oxygen effects on enzymes differ between air tolerant and intolerant microorganisms. Hydrogenase, nitrogenase and pyruvate dehydrogenase are rendered nonfunctional in anaerobes while analogous enzymes in

aerobes are protected from oxygen.

Molecular oxygen is relatively insoluble in aqueous media compared to the O_2 demands of aerobic organisms. However, these low concentrations in media exposed to air, circa 7 mg/l, is sufficient to have lethal effects on anaerobes. Two reactions of biological interest are those that produce water and hydrogen peroxide, respectively (Morris 1976):

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \qquad E_o' \text{ at pH 7} = +0.815 \text{ V}$$

$$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2 \qquad E_o' \text{ at pH 7} = +0.270 \text{ V}$$

The oxygen molecule has two unpaired electrons, each located in a different antibonding orbital, but with paralled spins. This confers the ability to the molecule to behave as a diradical and tends to make O_2 accept electrons one at a time (Halliwell and Gutteridge 1984). This is the lowest energy state and may be excited to the next energy level to yield singlet oxygen. Singlet oxygen, is produced during cellular metabolism and photochemical oxidation (Slade et al. 1981). It is implicated in the formation of lipid peroxides in membrane interiors (Oberley 1982b). Singlet

oxygen may affect other systems by acting as an oxidant in neutrophils (Matsuura 1977) and in the dismutation of the superoxide ion (Mayeda and Bard 1974). O_2 may rapidly combine with dienes such as those found in unsaturated fatty acids, aromatic compounds and sulfur compounds (Krinsky 1976).

A single electron accepted by the ground state O_2 results in the formation of the superoxide radical, O_2^-, which is formed in almost all aerobic cells (Halliwell and Gutteridge 1984). In aqueous solutions superoxide anions interact with each other and are removed by dismutation reactions producing hydrogen peroxide, H_2O_2 and O_2. In the Haber-Weiss reaction O_2 catalyses the homolytic splitting of H_2O_2 to form free hydroxyl radicals, OH, (Morris 1976).

The addition of a second electron to O_2^- gives the peroxide ion, O_2^{2-}. The O_2^{2-} formed at physiological pH will immediately accept protons to form H_2O_2 (Halliwell and Gutteridge 1984). Halliwell (1979) reported that pure H_2O_2 is somewhat unreactive but that in the presence of transition metal ions it can decompose to give the more reactive hydroxyl radical, OH. Homolytic fission of the O-O bond in H_2O_2, producing two hydroxyl radicals is caused by heat and by ionizing radiation. The OH radicals react with

many types of molecules found in living cells, such as amino acids (Halliwell and Gutteridge 1984), phospholipids (Bonura and Smith 1976), DNA bases (Bonura and Smith 1976; Hutterman et al. 1978) and organic acids (Halliwell and Gutteridge 1984).

The toxicity of oxygen on anaerobes must be considered in several ways when radiation is used as a mutagenic agent. Cell killing may be caused by autooxidized products forming in bacterial environments if there is air contamination during incubation. The death rate may be accelerated by oxygen exposure when organisms are transferred. The production of free radicals during radiation may lower the recovery rate of viable cells as much as the direct effect of the gamma rays on the organism.

Arena (1971) reported that irradiation of pure water yields atomic hydrogen, atomic oxygen, hydrogen peroxide and H radicals as products with the actual yield of each product being dependent on the nature of the ionizing radiation, experimental conditions and the purity of the water. The consequence of air contamination and radiation produced radicals is an increased killing rate which produces cell death and an inability to recover radiation induced mutations.

Gamma radiation, such as that produced by Cesium-137, constitutes the high energy portion of the electromagnetic spectrum and is ionizing (Arena 1971). For cell killing effects of ionizing radiation on bacteria, it is assumed that DNA is the main target molecule, and that primary lesions are single and double strand breaks in DNA (Town et al. 1973). Hütterman et al. (1978) defines these primary processes as collisions with energy transfer from ionizing radiation to the medium. Iwanami and Oda (1985) broadened the target theory of bacterial cell killing to include indirect actions of intermediates such as free radicals, induced by effective primary events in the medium surrounding target molecules. This theory of target volume depends on the presence of sensitizer (oxygen) of protectors (radical scavengers) at the time of radiation.

Many types of damage to cells and their DNA have been reported in the literature. Bonura and Smith (1976) and Skov (1984) proposed that a significant number of DNA double-stranded breaks occur because of the action of $OH^{\bullet}$ radicals produced as a result of radiation of H_2O molecules. The primary lesion in DNA is a single-stranded break with $OH^{\bullet}$ radicals reacting with the other strand causing the second break. Singh and Bishop (1984) implicated hydroxyl

radicals in the formation of chain breaks in the ribosomal RNA of E. coli. Increased proton permeablility of Streptococcus membranes resulted from treatment with ionizing radiation at doses of 5 to 100 krad (Fomenko and Akoev 1979). Toxic effects of oxygen on both aerobes and anaerobes during and after irradiation with X-ray (Hodgkins and Alper 1963; Rolfe et al. 1978), UV light (Jones et al. 1980; Jones and Woods 1981; Slade et al. 1981) and gamma rays (Hodgkins and Alper 1963; Rolfe et al. 1978) has been reported in the literature. Morris (1976) theorized that although the catalase theory and the superoxide dismutase theory answered some questions about the nontoxicity of oxygen in aerobes, much work remains to be done to identify primary oxygen targets.

Ionizing radiation can produce the following general types of alterations in biologic polymers, as well as changes in H_2O molecules in and surrounding bacterial cells (Arena 1971). Degradation or disruption of the chains of basic units, alteration of secondary, tertiary or quaternary structures and intermolecular or intramolecular cross-linking in polymers such as proteins, enzymes, nucleic acids and polysaccharides may be found in microorganisms and their environments following radiation. Hütterman et al. (1978)

confirmed many of these alterations and further denoted variations specific to DNA components. Damage to DNA can result in cell death or a reproducible change in the genetic configuration identifiable as a mutation. The ability of a cell to survive a strand break caused by UV or X-irradiation is often indicated by the presence of a genome designated as recA initiating the production of the associated recA protein in response to the radiations. E. coli has the recA genome but it has not been found in the anaerobe Bacteroides fragilis, which indicates caution in extrapolating theories from the E. coli systems to conditions in anaerobes (Strike and Roberts 1981; Howard-Flanders et al. 1981; Woods 1982).

Natural and induced mutations of many different types have been documented in anaerobes. Several investigators (Adler et al. 1980; Adler and Crow 1981; Adler et al. 1981; Gill and Adler 1982; Nagel et al. 1982) studied morphological mutants of E. coli, a facultative anaerobe, and their bacterial membranes, with respect to overcoming detrimental effects of radiation. Biochemical auxotrophs of Clostridium thermocellum (Méndez and Gómez 1982), B. fragilis (Van Tassell and Wilkins 1978), the archaebacteria Methanococcus (Woods 1982) and Halobacterium (Sapienza and Doolittle 1982) have been reported in literature. Three

types of changes in microbial sensitivity to antibiotics were recorded: 1) those naturally occurring; 2) those plasmid conferred; and 3) the induced mutations. All three are increasing such as in the resistance to the antibiotic rifampicin (Van Tassell and Wilkins 1978; Méndez and Gómez 1982; Levy 1982; Dass and Jayaraman 1985). Davies (1984) investigated resistance to antimicrobial agents not associated with plasmids and determined that the antibiotic rifampcin belongs in this catagory.

The rifampin group of compounds are semisynthetic antibiotics derived from the natural product rifamycin B, which is produced by a strain of Streptomyces mediterranei (Kenny and Strates 1981). The rifamycins exert their antibiotic effect by inhibiting DNA-dependent RNA polymerase at the beta subunit, preventing chain initiation but not elongation (Farr and Mandell 1982). This action is highly specific to bacterial RNA polymerase and does not inhibit the synthesis of RNA in mammalian mitochondria (Farr and Mandell 1982). Rifamicin S has been reported to inhibit the growth of Gram-positive bacteria at concentrations as low as 0.0025 μg/ml (Wehrli and Staehelin 1971). This antibiotic is clinically important because of effectiveness in the treatment of tuberculosis and leprosy (Braude 1976) and the

possibility of inhibition of growth of mammalian DNA viruses as well as RNA tumor viruses (Wehrli and Staehelin 1971).

RNA polymerase transcription with DNA serving as a template transfers genetic information from DNA into relatively small RNA molecules which are used by the cells in various ways (Wehrli and Staehelin 1971). The bacterial enzyme was purified initially from E. coli, Micrococcus luteus and Azotobacter vinelandii and was found in many other bacteria (Chamberlin 1976). E. coli polymerase was one of the most extensively studied enzymes with RNA transcriptase activity (Chamberlin 1976). E. coli transcriptase consists of at least four different subunits, α, β, β' and σ (Nene and Glass 1984). The parts of the enzyme are involved in three main steps of translation: 1) initiation, 2) elongation and 3) termination. Initiation is blocked by the naturally occurring rifamycins or the more effective semisynthetic derivatives rifampin and streptovaricin (Davis et al. 1973). These antibiotics bind to the same β subunit and the rpoB gene mutants coding for this subunit have been studied extensively (Ovchinnikov et al. 1981a; Ovchinnikov et al. 1981b; Nene and Glass 1984). A λ prophage rif^d 18 (Kirschbaum and Konrad 1973) and a plasmid pNA219 (pHR3) containing an 11 kb, HindIII fragment

of λ rif^{d} 18 (Newman and Haywood 1980) were used extensively to map the rpoBC (β and β ' subunits) of E. coli K12 RNA polymerase. Ovchinnikov et al. (1981a) found that in contrast with the wild type gene, the rpoB (rif^{d} 21) mutant gene was found to contain an AT to TA transversion at position 1547 from the start of the rpoB gene. This entails a substition of a valine residue in the mutant RNA polymerase for the aspartic acid residue of the normal subunit.

Haywood (1986) reported the possibility of a second mutation to be found in λ AJN261, a dilysogen containing genes rpoBC and rplAJL which encode for β , β ' and several ribosomal proteins. This prophage carries the same rif^{d} 18 mutation as proven by DNA sequencing, but it is not expressed as resistance to rifampin. The emergence of rifampin-resistant microorganisms is of major concern and has been reported for Neisseria meningitidis (Weidmer et al. 1971; Norden 1975), Haemophilus influenza (Sande and Mandell 1975; Nicolle et al. 1982) as well as Mycobacterium tuberculosis (Beam 1979).

P. anaerobius was chosen for use in the present study because of its association with human pathogenicity. These anaerobic streptococci have frequently been described as

causative agents in puerperal septicemia, pelvic abscesses, septic abortion, brain abscesses and many types of wound infections (Pien et al. 1972). Since they are also found in the normal female genital tract, the oropharynx and the gastrointestinal tract, their pathogenicity may be opportunistic in nature (Buchanan et al. 1974).

P. anaerobius is a Gram-positive coccus about 0.8 μm in diameter and typically arranged in chains (Buchanan et al. 1974). It grows in relatively simple fluid media such as peptone water and cooked meat broth (Willis 1977). Gas is produced as a product of fermentation, consisting of large amounts of CO_2 and some H_2 (Willis 1977). Media prepared from fresh beef and commercial media containing thioglycollate (0.1%), cysteine (0.1%), sodium thiosulfate (1%) or flowers of sulfur (1%) encouraged the production of H_2S and have a marked fetid odor (Buchanan et al. 1974). Washington (1981) reported the major metabolic products of P. anaerobius as determined by gas liquid chromatography (GLC) as acetic acid, propionic acid, isobutyric acid and isovaleric acid.

P. anaerobius are reported to be the most common bacteria in the oral cavity and they appear to play an important roll in the pathogenicity of oral disease (Berg

and Nord 1972). Harwood and Canale-Parola (1984) described a hyaluronidase-producing strain of Peptostreptococcus whose normal metabolic activities provide nutrients for pectiolytic spirochetes. This type of microbial interaction may enhance the breakdown of tissue polymers, and thus increase the rate of tissue distruction during periodontal disease.

Identification methods for the species P. anaerobius have not been accepted widely by many clinicians. Holdeman et al. (1977) depended on GLC tracings of volatile fatty acids to differentiate between anaerobic species. However, other researchers have reported difficulty in interpreting tracings of organisms in the genera Peptostreptococcus and Peptococcus (Lambert and Armfield 1979; Wells and Field 1976). The inhibition of growth of P. anaerobius surrounding a disk of sodium polyanethol sulfonate was proposed as a method of identification since this organism is the only anaerobic Gram-positive coccus that reacts to this salt (Wideman et al. 1976; Wilkins and West 1976; Tilton 1982). Babcock (1979) noted the disappearance of L-tyrosine crystals suspended in Schaedler agar while Lambert and Moss (1980) showed by GLC analysis that L-tyrosine was degraded to p-hydroxyhydro-cinnamic acid. P.

anaerobius was reported to be the only anaerobic Gram-positive coccus with L-tyrosine metabolizing ability (Babcock 1979).

Antibodies have been developed to P. anaerobius. As a result rapid identification and differentiation between P. anaerobius and other members of Peptostreptococcus and the Peptococcus genera (Graham and Falkler 1979; Wong et al. 1980) were made possible.

CHAPTER III

MATERIALS AND METHODS

Strains employed

A lyophilized culture of Peptostreptococcus anaerobius (ATCC# 27337, VPI# 4330; W. E. C. Morre strain) was purchased from the American Type Culture Collection, Rockville, MD. This organism was used in all experiments reported in this dissertation. The strain Escherichia coli B/r ORNL, was kindly supplied by Dr. Howard I. Adler (Oak Ridge National Laboratory, Oak Ridge, TN).

Standard culturing techniques - broth

The P. anaerobius stock culture was grown in Brucella broth (BBL, Cockeysville, MD), containing 10 μg/ml of vitamin K_1 (United States Biochemical, Cleveland, Ohio) and 50 μg/ml of hemin (United States Biochemical) (Holdeman et al. 1977). When standard anaerobic media and techniques (Holdeman et al. 1977) were used, cysteine-HCl (Sigma, St. Louis, MO.) was added to produce anaerobiosis. It was included in the media, such as Brucella broth, at a

concentration of 50 μ g/ml (Holdeman et al. 1977).

Liquid medium was processed in Hungate anaerobic tubes (Bellco Glass, Vineland, NJ). These tubes possess a screw cap and are sealed with a black butyl rubber stopper (Bellco Glass). Standard anaerobic media preparation requires a period of boiling before the addition of cysteine-HCl (Holdeman et al. 1977). This step was omitted in this procedure and cysteine-HCl was added to the medium before autoclaving. Each tube was gassed for about 1 min to remove excess oxygen before the tube was capped. The replacement gas consisted of an oxygen scrubbed (Deoxo Purifier, Englehard Industries, Union, NJ) mixture of 80% nitrogen, 10% hydrogen and 10% carbon dioxide. Liquid medium was sterilized by autoclaving in the fast exhaust mode. Fast exhaust is not generally acceptable for use with liquids since the sudden release of pressure causes extreme boiling and loss of liquids. Sealed tubes build up pressure and either blow the seal or explode. However, Hungate type tubes are thick walled and resist the pressure produced during fast exhaust. The violent boiling action causes a transfer of dissolved oxygen to atmospheric oxygen and increases the anaerobiosis of the liquid media (Holdeman et al. 1977).

Standard culturing techniques - chopped meat medium

Chopped meat medium prepared according to Holdeman et al. (1977) was used for long term storage of P. anaerobius cultures. This medium, employed in liquid form was prepared from lean fresh beef. Difco chopped meat medium (Detroit, Mich.) was used on occasion for culturing, but the storage capabilities of freshly prepared chopped meat was superior to the Difco commercial product. After incubation of the inoculated medium for 48 h, P. anaerobius could be recovered from these tubes for about a year. Storage temperature of these cultures was 20 - 30 C.

Standard culturing techniques - membranes in broth

Aerobic culture techniques were made possible by the addition of E. coli B/r ORNL membranes (Adler et al. 1981) to the appropriate liquid medium. The membranes of (approximately 20 to 30 mg dry weight per ml) were added to the liquid media, the dilution tubes and the radiation suspension fluid at a concentration of 0.01 ml of membranes per ml of liquid used.

Standard culturing techniques - solid medium

The solidification of broth was accomplished by the addition of Gibco agar (bacteriological grade; control number 01667, Gibco Co., Madison, WI.) at a concentration of 0.7%. This particular brand and concentration of agar was used since it was found that growth of P. anaerobius was inhibited by most commercial brands of solidifying agents. The agar was melted by heating on a hot plate and dispensed into heavy walled isolation roll streak tubes (Bellco Glass) measuring 25 by 142 mm.

The tubes were sterilized in a tube press (Bellco Glass) to maintain anaerobiosis since autoclaving was done in the fast exhaust mode to remove dissolved oxygen from the medium. Butyl rubber stoppers acted as closures for these tubes and were not held in place by screw caps. A tube press is made of two aluminum plates connected at the four corners with long screws. The test tube rack with stoppered roll streak tubes may be placed in the press and the screws tightened to secure the stoppers in the tubes. All Bellco Glass anaerobic glassware is thick-walled to resist presssure build-up during the fast exhaust mode.

Solid medium may be prepared in advance of use when it was stored in gassed roll tubes because agar in plates

absorb a great deal of oxygen and this acts as a killing agent for strict anaerobes. Plates may be stored in BBL GasPac jars but the gas generators are more costly than the gas used in gassing tubes. When Petri plates are required, tube stored medium was melted in a boiling water bath and dispensed into plates using a sterile pipet. Brucella agar was added to the plates in 10 ml quantities. Normal techniques recommend that 25 ml of medium be used per plate and this allowed for the drying effect of a 48 h incubation at 37 C. The anaerobe plates were incubated in an air tight sealed GasPac jar and evaporation was not a problem. A 0.7% agar concentration resulted in a very wet medium where the formation of condensation was a problem rather than evaporation. GasPac jars supplied incubation space for 12 plastic Petri plates. An anaerobic atmosphere was provided by a hydrogen-carbon dioxide generator (BBL). All culturing of P. anaerobius on solid medium was done in the streak plate manner, as this organism would not grow in agar as in the pour plate technique.

Standard culturing techniques - membrane strain

E. coli B/r ORNL was grown in nutrient broth (Difco)

supplemented with (Difco) yeast extract (0.5%) and (Sigma) sodium chloride (0.6%). Cultures were maintained on supplemented nutrient agar slants. Agar for solidification was purchased from Gibco and was used at a concentration of 2%. Cells of E. coli B/r ORNL used in the preparation of membranes were grown in a New Brunswick fermentor (Model FS-307) (New Brunswick Co., NJ). Two 5 l fermentation flasks with attachments were sterilized and filled with about 4 l of supplemented sterile nutrient broth. The flasks were placed in a 37 C waterbath and the temperature was allowed to equilibrate. The inoculum, a 24 h culture of E. coli B/r ORNL, was grown in 250 ml of supplemented nutrient broth at 37 C. The culture was grown at 250 rpm on a New Brunswick shaker. Each 5 l flask received the 250 ml of inoculum. The New Brunswick fermentor was operated at 250 rpm with an air flow of 10 psi. Tween 80 (10 ml) was added to each of the flasks to control foaming. After an overnight incubation, the cells were harvested by centrifugation in an International Centrifuge (Needham Hts., MA), operated at room temperature at 3,000 x g for 15 min. The harvested cells were washed once in HEPES buffer (Sigma) and stored frozen (5 C) until they were used to prepare P_2 membranes. HEPES is a buffer of 0.1 M

N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid at pH 7.5.

Salts solution

The salts solution (S_2) used in this experiment was prepared by dissolving the following chemicals in distilled water to a final volume of 1 liter: KH_2PO_4, 1.5 g; Na_2HPO_4, 13.5 g; NH_4Cl, 2 g; $MgSO_4$, 0.1 g; $CaCl_2$, 1 ml of a 1% solution and $FeSO_4 \cdot 7H_2O$, 0.5 ml of a freshly prepared 0.1% solution. Half-strength S_2 was prepared fresh for each experiment by diluting the salts solution 1:1 with distilled water.

Spent medium

Spent medium was used by Juni et al. (1984) to determine the various individual nutrients required by a microorganism which normally grows in a complex medium. Spent medium provides a minimum amount of needed nutrients and the effect of added chemicals may be seen against a control of spent medium alone. Spent broth was prepared by inoculating 10 ml of Brucella broth with 0.1 ml of a rapidly

growing culture of P. anaerobius. The broth was incubated at 37 C for 24 h. The cells of P. anaerobius were removed from the now spent broth by centrifugation. The broth was placed in gassed screw capped centrifuge tubes and spun at 5,000 x g for 15 min in an unrefrigerated centrifuge. The fluid was decanted and was sterilized by filtration through a 0.22 μm membrane filter (Millipore Corp.). The sterilized spent medium was stored frozen until use.

Membrane preparation

The cytoplasmic membrane preparation followed Adler's procedure (Adler et al. 1981) except for the omission of the RNase and DNase incubation steps (Table 1). The membrane fraction (P_2) obtained by ultracentrifugation at 175,000 x g was passed three times through a Sorval Ribi Cell Fractionator at 20,000 psi to completely separate the membrane particles. A suspension of extremely fine membrane pieces resulted which could be sterilized by flowing it through a 0.22 μm Sterivex-GS filter (Millipore Corp., Bedford, Mass.). Frozen 1 ml aliquots of the preparation were stored in 1 dram screw cap vials (Kimble Glass, Toledo, Ohio). The sterility of each batch of membranes was

Table 1--Preparation of membranes from whole cell suspensions of Escherichia coli B/r ORNL

Suspend 10 g of washed Escherichia coli B/r cells in 25 ml of HEPES*.

Centrifuge the suspension for 15 min at 5 C at 3,000 x g and discard the supernatant.

Resuspend the pellet in 50 ml cold HEPES and disrupt the cells by putting the suspension through a French Pressure cell 2 to 3 times at 20,000 psi.

Centrifuge the cell fragments for 15 min at 0 C at 12,000 x g and retain the supernatant.

Centrifuge the supernatant for 5 h at 5 C at 175,000 x g.

Resuspend the pellet in 25 ml cold HEPES and pass the suspension through the French Pressure cell 2 to 3 times to disperse the membrane fragments.

Filter the membranes (P_2) through a 0.22 µm Sterivex-GS Millipore filter to sterilize.

The sterile P_2 is dispensed into 1 dram vials and frozen for storage.

*HEPES is a 0.1 M buffer of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid at pH 7.5.

confirmed by incubating 0.1 ml of P_2 in nutrient broth for 24 h. Prior to use the activity of the membranes as oxygen removers was determined with an oxygen meter (Yellow Springs Instrument Co., Yellow Springs, AR).

Determination of growth curves

Growth curves were used to determine various aspects of this research. The basic methodology used to obtain data was the same for all situations. Each variance is described under separate topic headings following this section titled "Determination of growth curves". Nephlo side arm culture flasks (Bellco Glass, Inc.) were used to incubate cultures of P. anaerobius. These flasks have a total capacity of 500 ml and are shaped like Erlenmeyer flasks with a test tube horizontally attached at the top just before the neck narrows. The purpose of the large volume is to allow for splashing if culturing aerobes on a shaker. The Nephlo flasks were placed under a stream of oxygen scrubbed gas consisting of 80% nitrogen, 10% hydrogen and 10% carbon dioxide. The membranes should remove oxygen from the flask contents, but its large empty volume allows the possibility of a great deal of oxygen diffusion into the broth.

Replacement of air would decrease the lag phase of the growth curve caused by oxygen contamination. Following the period of gassing, 10 ml of anaerobically prepared Brucella broth was added to the flask. Membranes at a concentration of 0.1 ml per 10 ml of medium were placed in the flask and the bacteria were introduced.

The inoculum was prepared by pelleting a stationary phase culture by centrifugation at 5,000 x g for 10 min at room temperature. The pellet was resuspended in half-strength S_2 salts solution (Juni et al. 1984) prepared anaerobically and the turbidity was adjusted to equal a #1 MacFarland (Difco) standard. The inoculum was added to the broth-membrane solution at a concentration of 0.5 ml/10 ml medium. A black butyl rubber stopper limited the contamination of the system with air. The culture was incubated without shaking at 37 C until the atationary phase was attained and maintained.

Turbidity measurements were obtained by tipping the Nephlo flask on its side and allowing the flask contents to enter the horizontal test tube. The tube was then inserted in a Bausch and Lomb-Spectronic 20 spectrophotometer (Rochester, NY) and absorption readings at 650 nm were

taken. The observation times were variable and dependent on the individual parameter being tested.

Optimum temperature for growth

A range of temperatures that fostered the growth of P. anaerobius was delineated by this experiment. The method of inoculation, incubation and measurement were described under "Determination of growth curves". An actual growth curve was not prepared for each temperature, but an initial (time zero) and a final (24 h) absorbance reading was taken. The difference between these two readings was used in the graph representing this experiment. Data were obtained for temperature effects on the growth of P. anaerobius at incubator settings of 25, 30 and increments between 35 - 45 C.

Nutritional requirements - media supplements

Three supplements added to the Brucella medium used in the experiments described in this dissertation were tested to determine the adviseability of including them on a regular basis as growth factors. Finegold (1982) suggested

that 10 μg/ml of vitamin K_1 (United States Biochemical), 50 μg/ml of hemin (United States Biochemical) and 50 μg/ml cysteine-HCl (Sigma) were to be included in the complex media when culturing P. anaerobius. The methods used in inoculation, incubation and measurement of growth were described under "Determination of growth curves". Brucella broth minus the three supplements was prepared anaerobically as were solutions of the three additives. Ten ml of Brucella broth was added to each Nephlo flask as the flask was being gassed under the oxygen scrubbed mixture as described previously. Vitamin K_1, hemin and cysteine-HCl were included in proper concentration both individually and in combination with each other. The inoculum (previously described) was added, the flask were stoppered and incubated at 37 C until the stationary phase was reached and maintained. Measurements of absorbance were taken during the lag, log and stationary phases of the growth curve.

Nutritional requirements - carbon source

In an attempt to develop a chemically defined medium, Nephlo flasks were used to determine the effects of various carbon sources on the growth of P. anaerobius. Chemicals

that might be used by the anaerobe as both carbon and energy sources were tested to determine their applicability. Sodium glutamate at a concentration of 0.25% and sodium lactate at 0.3% were added to supplemented Brucella broth in the following manner. A stock solution of 30% sodium lactate was prepared and frozen in 1 ml quantities. The various stock solutions were not prepared or stored anaerobically since the membranes were responsible for oxygen removal. Sodium lactate (0.1 ml) was then added to the 10 ml of broth, resulting in a final concentration of 0.3% in the medium.

Other carbon sources were assayed for their effect on the growth of P. anaerobius. They included 0.25% sodium glutamate, 0.3% sodium lactate, 0.3% glucose, 0.3% D(-)arabinose and 0,3% D(+)xylose, all purchased from Sigma. Each of these chemicals was prepared and frozen as described under methods used for the preparation of sodium lactate. The inoculation, incubation and measurement of growth were described under "Determination of growth curves". Measurements were taken at various times during the lag, the log and the stationary phase of the growth curve. The incubation temperature was 37 C.

Nutritional requirements - spent medium and glucose

Spent broth provides a growth-limiting nutrient medium in which the effect of individual additives can be studied (Juni et al. 1984). When glucose was used as a primary carbon source in this experiment, the concentration of spent medium best suited for further investigations was also determined. This medium was diluted with half-strength S_2 salts solution to final concentrations of 12.5, 15 and 50%. Glucose at a concentration of 0.3% was added to some of the flasks as the primary carbon source. The inoculation, incubation and measurement of the growth curves has been described under "Determination of growth curves". Measurements were taken at various times during the lag, log and stationary phases.

Optimum pH

Brucella broth was used as growth medium in the determination of the optimum pH for P. anaerobius. The broth contained vitamin K_1, hemin and cysteine-HCl as additives at a pH of 7.0 - 7.5. Screw cap roll tubes (Bellco Glass) containing media at pH 4, 5, 6, 7, 8, 9 and 10 were prepared in triplicate. The pH was measured with an

Orion Analog pH Meter (Model 301) with an Orion Combination probe (Model 91-05) (Cambridge, MA). Adjustments of pH were made with additions of 1N HCl or 1N NaOH as necassary. Membranes were added at a concentration of 0.1 ml/10 ml of medium. Growth was determined after 24 h incubation at 37 C. The turbidities in the various pH tubes were compared to the growth in the pH 7 control. Results were stated as growth equal to the control, slight growth less than the control and no growth as compared to the uninoculated broth.

Agar inhibition

To determine the effect of Difco agar versus Gibco agar on the growth and development of P. anaerobius the original culture (A100) and two later acquired cultures (B100) and (C100) were tested. All three cultures of P. anaerobius were genetically identical. Triplicate plates of 0.7% Brucella agar were prepared. Three plates contained only 0.7% Gibco agar. In succeding plates the quantity of Gibco agar was decreased by 0.1%, but the amount of Difco agar was increased by 0.1% increments, until a total of 0.7% Difco agar was reached. All plates were inoculated with 0.1 ml of an actively growing culture of P. anaerobius. The

inoculated plates were placed in a BBL GasPac jar and incubated at 37 C for 48 h.

Paper disk diffusion antibiotic sensitivity test

General antibiotic resistance or sensitivity of the organism was determined by the paper disk diffusion technique (Kwok et al. 1975). Sterile disks of carbenicillin (100 μg), lincomycin (2 μg), gentamicin (10 μg) and clindamycin (2 μg) were purchased from BBL. Sulfadiazine (300 μg), cefmandole (30 μg), rifampin (5 μg), chloramphenicol (30 μg), erythromycin (15 g), penicillin G (10 units) and tetracycline (30 μg) were obtained from Difco. The paper disk diffusion test was performed on anaerobically prepared Brucella agar (0.7% agar) in divided quadrant plastic Petri plates. A disk was placed in each inoculated quadrant. The plates were seeded with a cotton swab that had been immersed in Brucella broth containing P. anaerobius at a turbidity equal to a #1 MacFarland. Any excess liquid was removed from the swab by rolling it on the inside of the test tube. The sterile antibiotic disk was placed in the center of the quadrant using the flammed forceps technique. The inoculated plates

were placed in a BBL GasPac jar and incubated for 48 h at 37 C. Zones of inhibition of growth around the antibiotic disks were measured in mm as reported. Standard designations of antibiotic sensitivity as resistant, intermediate or sensitive, are not applicable to P. anaerobius since the zones of inhibition surrounding most of the antibiotics tested exceeded those reported for other bacteria.

Modified broth - disk diffusion test

The modified broth - disk diffusion method reported by Wilkins and Thiel (1973), was tested for its applicability to the research reported in this dissertation. Brucella broth was prepared in 10 ml quantities and antibiotic disk(s) were added to the broth to produce a range of dilutions of antibiotics. The disk containing tubes were removed from the gassing jet, sealed and the contents were mixed. The tubes were replaced under the oxygen scrubbed gas mixture and inoculated with 0.1 ml of an actively growing culture of P. anaerobius. The tubes were sealed and incubated for 24 h at 37 C. The presence or absence of growth was noted. This technique takes advantage of the quality testing and standarization of antibiotics by the

companies producing the disks. A disk containing 10 units of penicillin G is like all other disks in the batch since each of the disks contains the same amount of antibiotic. The standardized antibiotic concentration in commercially prepared disks quantitates the amounts of antibiotic per ml in each tube. For example, a single penicillin G disk of 10 units in 10 ml of broth results in a final concentration of 1 unit/ml. The P. anaerobius culture used in this experiment exhibited such strong reactions to most of the antibiotics tested that this method for determining antibiotic sensitivity was not applicable. However, once antibiotic resistant mutants of the prototype were obtained it proved to be a very good method for culturing and maintaining the antibiotic variants.

Preparation of concentrated antibiotic stock solutions

A concentrated solution of carbenicillin (Sigma) was prepared by dissolving 400 mg of the powder in 10 ml distilled water resulting in 40,000 μg/ml. Rifampin (Sigma) was dissolved in 20 ml of absolute ethanol to which 80 ml of distilled water were added. A 100 mg quantity of the powdered antibiotic produced a concentration of 1,000

μ g/ml. Chloramphenicol and erythromycin (Sigma) stock solutions were prepared at a concentration of 4,000 μ g/ml by dissolving 40 mg in 10 ml ethanol. The solutions were filter sterilized through a 0.22 μ m membrane (Millipore Corp.) and frozen aliquotes were stored in 1 dram vials (Kimble Glass).

Minimal inhibitory concentration (MIC)

Antibiotic mutants differing from the prototype P. anaerobius, as reported in this research, were identified by a modified microdilution technique (Rotilie et al. 1975). MIC is defined as the lowest concentration of an antibiotic which inhibits visible growth after incubation for 24 h at 37 C. Microdilution plates were prepared by cutting flexible plastic multi-welled sheets to fit inside a plastic Petri dish. The plates had 16 cone shaped wells and were sterilized by exposure to UV light for two hours. The wells had a maximum capacity of 1.5 ml and were labeled with a letter vertically and a number horizontally. Final antibiotic concentrations employed depended on the stock solutions. Shown in Table 2 is the dilution scheme used for rifampin. Well A-1

Table 2--Dilution procedure for the antibiotic rifampin as used to determine Minimal Inhibitory Concentration (MIC).

Well #	Brucella broth ml	Antibiotic dilution* ml	Inoculum** ml	Final antibiotic conc. µg/0.5 ml
A-1	none	0.125	0.375	12.5000
A-2	0.125	0.125	0.375	6.2500
A-3	0.125	0.125 ml from A-2	0.375	3.1250
A-4	0.125	0.125 ml from A-3	0.375	1.5620
B-1	0.125	0.125 ml from A-4	0.375	0.7810
B-2	0.125	0.125 ml from B-1	0.375	0.3900
B-3	0.125	0.125 ml from B-2	0.375	0.1970
B-4	0.125	0.125 ml from B-3	0.375	0.0970
C-1	0.125	0.125 ml from B-4	0.375	0.0488
C-2	0.125	0.125 ml from C-1	0.375	0.0240
C-3	0.125	0.125 ml from C-2	0.375	0.0120
C-4	0.125	0.125 ml from C-3	0.375	0.0060
D-1	0.125	0.125 ml from C-4	0.375	0.0030
D-2	0.125	0.125 ml from D-1	0.375	0.0015
D-3	0.125	0.125 ml from D-2, discard 0.125 ml	0.375	0.0007
D-4	0.125	none	0.375	0

*A concentration of 100 µg/ml of rifampin was used in this example.

**A 24 h culture of P. anaerobius diluted 1:1,000 in Brucella broth was used as the inoculum.

contained the highest concentration of antibiotic while well D-4 had no antibiotic and served as a growth control. A 1:1,000 dilution of an early stationary phase culture of P. anaerobius was prepared in 10 ml Brucella broth containing 0.1 ml of P_2 membranes. This was added in 0.375 ml quantities to the serially diluted antibiotics as shown in Table 2. Triplicate plates were placed in BBL GasPac jars which were sealed, activated and incubated. The plates were read following a 24 h incubation at 37 C. Growth was determined by a comparison of turbidity and color between the A-1 antibiotic well and each other well containing growth. The presence of P_2 and the inoculum imparted a slight turbidity to even the antibiotic control.

Characterization of the prototype

To assure the purity of the experimental strains, the P. anaerobius prototype and the mutants were retested each month. A Gram stain, the sodium polyanethanol sulfonate test (Wideman et al. 1976), the tyrosine degradation test (Babcock 1979) and an oxygen tolerance test were performed on each strain.

The Gram stain was used to determine if contamination

was present and indicated any possible changes in morphology. The cells, as seen with a light microscope, were considerably smaller when grown in medium that did not support good growth. At times, a plate was streaked, isolated colonies were picked to obtain pure cultures and these were characterized to ascertain the properties of the strain.

Sterile paper disks of 1/4 inch diameter (BBL), containing 20 µl of 5% sodium salt of polyanetholesulfonic acid (United States Biochemical), were placed in the center of a lawn of P. anaerobius seeded on Brucella agar (0.7%) in 60 x 15 mm plastic Petri plates. A zone of inhibition of growth of 12 to 30 mm surrounding a disk of sodium polyanethanol sulfonate is a positive test identifying this microorganism. P. anaerobius is the only Gram positive anaerobic coccus that is inhibited to this degree by this salt (Wideman et al. 1976).

Another method used to confirm species purity was the tyrosine degradation test. P. anaerobius degrades tyrosine crystals (Sigma) suspended in Schaedler agar (BBL). A clear zone around the colony confirms the species identity. Duplicate plates of Schaedler agar containing tyrosine crystals were prepared for each culture. Tyrosine does not

dissolve in the medium as long as the pH remains near 7. When the plates were poured and the agar solidified, it appeared cloudy. One loop full from a 24 h inoculum was placed in the center of the plate. The plates were incubated at 37 C for 48 h in a GasPac anaerobic jar. P. anaerobius is the only Gram positive anaerobic coccus that degrades tyrosine and leaves a clear zone in the cloudy Schaedler agar.

Duplicate plates of the mutant and the prototype on Brucella agar (0.7%) were incubated at 37 C for 48 h. One plate was placed in the incubator aerobically and the other one was enclosed in the oxygen-free atmosphere of a BBL GasPac jar. Since P. anaerobius is a strict anaerobe (Buchanan et al. 1974; Sneath et al. 1986), growth on the plate in air would indicate either contamination or the development of aerotolerance and thus a change in cell metabolism.

Membrane viability

The ability of active membranes to remove oxygen from media and to do so for an extended period of time was monitored. Brucella broth was prepared with no regard of

oxygen exclusion. Three tubes received 0.1 ml of membranes per 10 ml of medium. The other set contained no membranes. After 5 min, to give the membranes enough time for oxygen removal, the tubes were inoculated with 0.1 ml of an actively growing culture of P. anaerobius. The plastic capped aerobic tubes were incubated at 37 C for 24 h and were observed for turbidity. Nonturbid tubes were incubated for another 24 h, and the other tubes were stored at room temperature for the remainder of the experiment. The tube contents were sampled on a weekly basis until viable cultures were no longer obtainable. The viability of the cultures with the membranes was determined weekly by removing a 0.1 ml sample from the turbid tube and incubating it in Brucella broth at 37 C for 24 h.

Inoculum for the radiation experiments

A 24 h culture grown in Brucella broth was centrifuged (International Refrigerated Centrifuge) at 5 C and 3,640 x g for 15 min. The screw capped centrifuge tubes were gassed with an oxygen scrubbed gas mixture while the broth was being transferred to the tubes. The supernatant was removed under gas and the resulting pellet was washed one time in

water containing 0.1 ml of P_2 and sodium lactate. The washed cells were ultimately suspended in 10 ml of water containing membranes and 1 M sodium lactate (Sigma) at a concentration of 0.1 ml per total volume. Sodium lactate provides H_2 to the membrane bound cytochrome system (Adler et al. 1981). Membranes included in media do not require the addition of a hydrogen source as compounds in the media provide the needed H_2.

Radiation procedure

Aliquots of the inoculum (1 ml) were dispensed into gassed modified Turner bulbs in initial experiments to determine the radiation lethality curve for P. anaerobius. Observations of nonirradiated control plates indicated that killing was taking place prior to irradiation and that it was caused by oxygen contamination in the Turner bulbs. Turner bulbs contain a very large air space compared to the small sample. The membranes were not able to contend with the oxygen diffusing into the radiation suspension fluid. Subsequent samples (1 ml) were dispensed into gassed screw capped 1 dram vials (Kimble Glass) and irradiated in a Cs-137 gamma source (J. L. Sheperd Mark 1 Model 30

S. N. 644) with an exposure rate of 0.32 Coulomb/Kg/min. The vials containing the samples were placed in the middle position of the Mark I irradiator on a revolving plate and irradiated for varying time intervals.

Radiation lethality curve

The samples were irradiated for 0, 25, 50, 90 and 130 min at 0.32 Coulomb/Kg/min. Following the irradiations the serial dilutions were plated on Brucella agar (0.7%) and incubated at 37 C for 48 h in BBL GasPac jars. After the incubation, cell counts were made using a Quebec Colony Counter (New Brunswick Scientific Co.). The actual numbers of surviving colony forming units were reported. Streptococci generally produce lower cell counts since the long strings of cocci do not dissociate and produce only one colony.

Radiation suspension fluid

Cysteine-HCl is found in most media used to culture anaerobic bacteria. It functions as a reducing agent and removes oxygen by binding it with hydrogen to form hydrogen

peroxide (Slade et al. 1981). In reported radiation work with other anaerobic bacteria (Jones et al. 1980; Jones and Woods 1981), cysteine-HCl was included in the radiation suspension fluid. The effect of the components of the radiation suspension fluid as well as the possible addition of cysteine-HCl were tested. Water, P_2 membranes and sodium lactate were investigated alone and in combination to determine their effects on the radiation lethality curve.

The inoculum was prepared in the same way as presented in the section "Inoculum for radiation experiments". After centrifugation, the pellet was washed and suspended in each testing solution. These solutions were prepared as follows: (1) Water and cysteine-HCl (water containing 50 μg/ml of cysteine-HCl), (2) Water only (distilled water was boiled and dispensed into anaerobe tubes under gas), (3) Water and sodium lactate (water containing 1 M sodium lactate at a concentration of 0.1 ml sodium lactate per 10 ml water), (4) Water and membranes (water containing membranes at a concentration of 0.1 ml membranes per 10 ml of water); and, (5) Water, membranes and sodium lactate (each 10 ml of water contained 0.1 ml of 1 M sodium lactate and 0.1 ml of membranes). All of the water was boiled and dispensed into tubes and filter sterilized through a 0.22 μm membrane

filter. All supplements tested were added to the water under an oxygen scrubbed gas mixture. The testing solutions were prepared in triplicate and irradiated with Cs-137. The samples received 0.32 Coulomb/Kg/min at the following time intervals: 0, 30, 60, 90 and 120 min.

Anaerobic medium cannot be poured into Petri plates in advance as is usual for aerobes. Media was prepared anaerobically and stored in Hungate roll tubes. The Brucella agar was remelted on the day of the experiment in the autoclave for 7 min and held at 45 C in a waterbath in a liquid state. The liquid medium was dispensed into plates just prior to use to eliminate oxygen diffusion into the agar, which would affect the radiation curve. Controls should be subjected to the same conditions as the experimental samples with the exception of the variable being tested, such as the components of the suspension fluid. The time intervals used did not allow retrieval of the irradiated sample, preparation of the plates, anaerobic dilution and plating of the samples. All tubes were kept for the 120 min maxium irradiation time, being subjected to the same temperature before dilution and plating could occur. All samples including the control were taken to the location of the Cs-137 irradiator and held at room

temperature for the duration at the radiation procedure which introduced a new variable. The effect of the 120 min holding time was tested by subjecting each of the radiation suspension fluids to room temperature for 120 min prior to plating.

Mutation technique

Once the dose-survival curve for the prototype culture of P. anaerobius was established, the amount of gamma irradiation required to kill about 96% of the colony forming units was chosen as the mutagenic dose. Samples, prepared in the same manner as for the dose-survival curve, were irradiated for 90 min. This exposure of the cells resulted in a total dose of 28.83 Coulomb/Kg, leaving 200 - 300 surviving colony forming units per ml of the irradiated suspension. Aliquots of the dilutions (0.1 ml) were spread on Brucella antibiotic gradient plates (Carlton and Brown 1981) and incubated for 48 h at 37 C in BBL GasPac jars. Multiple trials of this experimental method produced no growth on any of the antibiotic gradient plates.

Subsequently, the irradiated cells were inoculated into 10 ml of Brucella broth and incubated for 24 h. The samples

were then diluted and plated on the antibiotic gradient plates, which were incubated for 48 h at 37 C in GasPac jars. The Petri dishes contained areas of maximum antibiotic concentration to areas of almost zero. It is then possible to isolate mutants that have variable sensitivities to the same antibiotic. Colonies were picked from high antibiotic containing areas, moderate areas and near zero areas and inoculated into fresh antibioticless Brucella broth. These cultures were then tested for sensitivities. MIC's (Rotilie et al. 1975) were determined for the potential mutants to establish the amount of antibiotic resistance acquired by the isolates. The mutants thus obtained were maintained in Brucella broth containing the antibiotic to which the bacteria had become resistant.

Antibiotic gradient plates

Antibiotic gradient plates were prepared by putting 1 ml stock antibiotics in each plate and pipetting one half the medium into it, mixing it and letting it solidify into a slanted position. Then the rest of the medium was pipetted into the plate in a level position. Carlton and Brown (1981) recommended a period of equilibration before using

the antibiotic gradient plates to culture aerobes. The plates for P. anaerobius, however, were prepared and used immediately since the slower growth of the anaerobes allowed the antibiotic gradient to become established. This eliminated the possibility of oxygen diffusion into the agar.

Catalase test

Adler et al. (1981) maintained that the function of the membranes of E. coli B/r ORNL was to remove oxygen by means of an active cytochrome system. The cytochrome enzyme system binds hydrogen to oxygen with the production of water. Patel et al. (1984) reported that the biggest threat to anaerobes is the presence of peroxides in the medium. E. coli is known to posses the enzyme, catalase, which degrades H_2O_2 to H_2O and one half O_2 (Stanier et al. 1976). An experiment was done to test for the presence of the enzyme catalase and its effect on the growth of P. anaerobius in the presence of hydrogen peroxide.

Membranes were screened for the presence of catalase in the following manner. Blood is known to posses this enzyme; so a drop of fresh blood and a drop of membranes were placed

on the opposite ends of a glass slide. One drop of commercial 3% hydrogen peroxide was layed over both drops. Vigerous bubbling indicated the presence of catalase as O_2 is released.

The ability of the membranes to remove hydrogen peroxide from an anaerobic culture was tested. Anaerobe culture tubes with 10 ml of Brucella broth were prepared. Each set of tubes had a control of medium and an inoculum consisting of 0.1 ml of a 24 h culture of P. anaerobius. Both sets of tubes contained different molar concentrations of hydrogen peroxide (3%, commercial grade) at levels of 9×10^{-5}, 9×10^{-4}, 9×10^{-3} and 9×10^{-2}. One set of the peroxide tubes received membranes at a concentration of 0.1 ml per 10 ml of medium. The membrane extract was added to the medium containing the peroxide followed by a waiting period so that the bubbling action would stop before all the tubes were inoculated. The samples without membranes were prepared and inoculated under the oxygen scrubbed gas mixture while those with membranes were handled in a typically aerobic manner. Both sets of tubes were incubated at 37 C for 24 h.

Chromatography techniques

The detection of alcohols, volatile acid products and volatile methyl derivatives in the Brucella broth before and after growth of P. anaerobius were performed using the techniques as stated by Holdeman et al. (1977). Gas Liquid Chromatography (GLC) tracings presented in this dissertation are through the courtesy of Sheryl Kappus (Texas Woman's University, Denton, Texas).

CHAPTER IV

RESULTS AND DISCUSSION

Bergey's Manual of Determinative Bacteriology (Buchanan et al. 1974; Sneath et al. 1986) designates Peptostreptococcus anaerobius as a coccidial bacterium arranged in chains. Stained fixed slides viewed at 1,000X with the light microscope substantiate this observation, while visualization at 20,000X with the AVEC-DIC (Allen video-enhanced contrast/differential interference-contrast) microscope reveals the more ovoid shape as stated in Bergey's generic description (Buchanan et al. 1974; Sneath et al. 1986). The beaded appearance of the cells as seen at 20,000X seems to result from small filaments attaching one coccus to the next one (Figure 1). Brock et al. (1984) reported the opening of small holes in the macromolecular structure of cell walls by means of autolytic enzymes, while new cell material was layed down across the openings. A ridge or scar formed at the junction where the old layers of peptidoglycan met the new ones. The observations of Brock et al. (1984) did not account for the presence of connecting filaments between the cells of P. anaerobius that were clearly visible at 20,000X magnification.

Peptostreptococcus anaerobius

Figure 1--A drawing of the living cells of Peptostreptococcus anaerobius suspended in Brucella broth containing 0.1 ml of P_2 membranes. The coccobacillus was viewed at 20,000X with an AVEC-DIC microscope (Allen video-enhanced contrast/differential interference-contrast microscope).

Inclusion of Escherichia coli B/r membranes in the slide preparation for viewing with the AVEC-DIC microscope enables anaerobic bacteria to be seen in a living unstained state. Slide fixing techniques often impose the possibility of cellular distortion which is not a problem while observing materials with the visually inhanced imaging system. The membranes included in this type of preparation were used to compare the relative size of the bacteria and the particles. Since P_2 membranes were filter sterilized through a 0.22 μm filter, visualization was difficult with normal microscopic methods.

Culture purity was monitored periodically by the Gram stain and other characteristics. Willis (1977) defined P. anaerobius as being nonproteolytic but producing hydrogen sulfide gas on sulfur containing medium. A 6 - 8 hours old culture produced a distinctive unpleasant odor which seems reasonable for an organism known in German vernacular as "Stinkcoccus" (Buchanan et al. 1974; Sneath et al. 1986). A 24 h culture in a complex sulfur containing broth medium produced a sediment in the test tube with black pigmentation. Since this is a highly repeatable occurance, the appearance of the foul odor and the black sediment indicated that this was indeed P. anaerobius. This organism

stained very strongly with crystal violet as a Gram positive organism, and the destinctive beaded appearance visually confirmed that it was a pure culture.

Although a given medium may be suitable for the initiation of growth, the subsequent development of the bacterial population may cause chemical changes that severely limit further proliferation of the organism. Bacterial metabolism in a glucose containing medium, such as Brucella broth, leads to the production of organic acid wastes. Decomposition of proteins and amino acids also makes bacterial environments alkaline (Stanier et al. 1976). Bacteria may be found in a wide range of habitats with only a few species existing at pH values below 2 or higher than 10 (Brock et al. 1984). Buchanan et al. (1974) and Sneath et al. (1986) stated that the optimum pH of P. anaerobius is from 7.0 - 7.5. However, as can be seen in Table 3, the organism does quite well at a wider pH range, namely between pH 5 - 9. The pH of Brucella broth was adjusted toward the acidic range with 1 N HCl and the alkaline level with 1 N NaOH. A visual observation of turbidity was made at the end of a 24 h incubation period at 37 C. There were no observable differences in cell concentrations in tubes containing media adjusted to pH 5, 6, 7, 8 and 9. Media

Table 3--Growth of *Peptostreptococcus anaerobius* in *Brucella* broth at different pH values.

pH		
Before growth	After 24 h incubation	Amount of growth
4.0	4.0	no turbidity
5.0	5.0	heavy turbidity
6.0	6.0	heavy turbidity
7.0	6.0	heavy turbidity
8.0	6.5	heavy turbidity
9.0	7.0	heavy turbidity
10.0	9.0	light turbidity

with adjustments to pH 10 repeatably exhibited decreased turbidity and inhibition of growth occured below pH 5. Brucella broth does contain glucose as a fermentable carbohydrate that converts to organic acids, as was seen from the final pH of the broth after the 24 h incubation (Table 3).

A generic characteristic of P. anaerobius is an optimum temperature of 35 - 37 C (Buchanan et al. 1974; Sneath et al. 1986). This places this anaerobe into the mesophilic group. Since this organism was cultured from a human specimen, an optimum temperature in the body's homothermic range is appropriate. The data obtained and shown in Figure 2 indicates a somewhat more extensive optimum temperature toward the upper end of the mesophilic range. The method used may account for the difference between the observed and the recorded temperature range of Buchanan et al. (1974) and Sneath et al. (1986). Nephlo culture flasks (500 ml volume) containing 10 ml Brucella broth with 0.1 ml P_2 membranes were inoculated and incubated at different temperatures for 24 h. (Figure 2) indicates that 36 - 41 C is an optimum temperature range for the experimental organism. Because of the large air volume to fluid ratio, the use of the Nephlo flask required the inclusion of the P_2 membranes to maintain

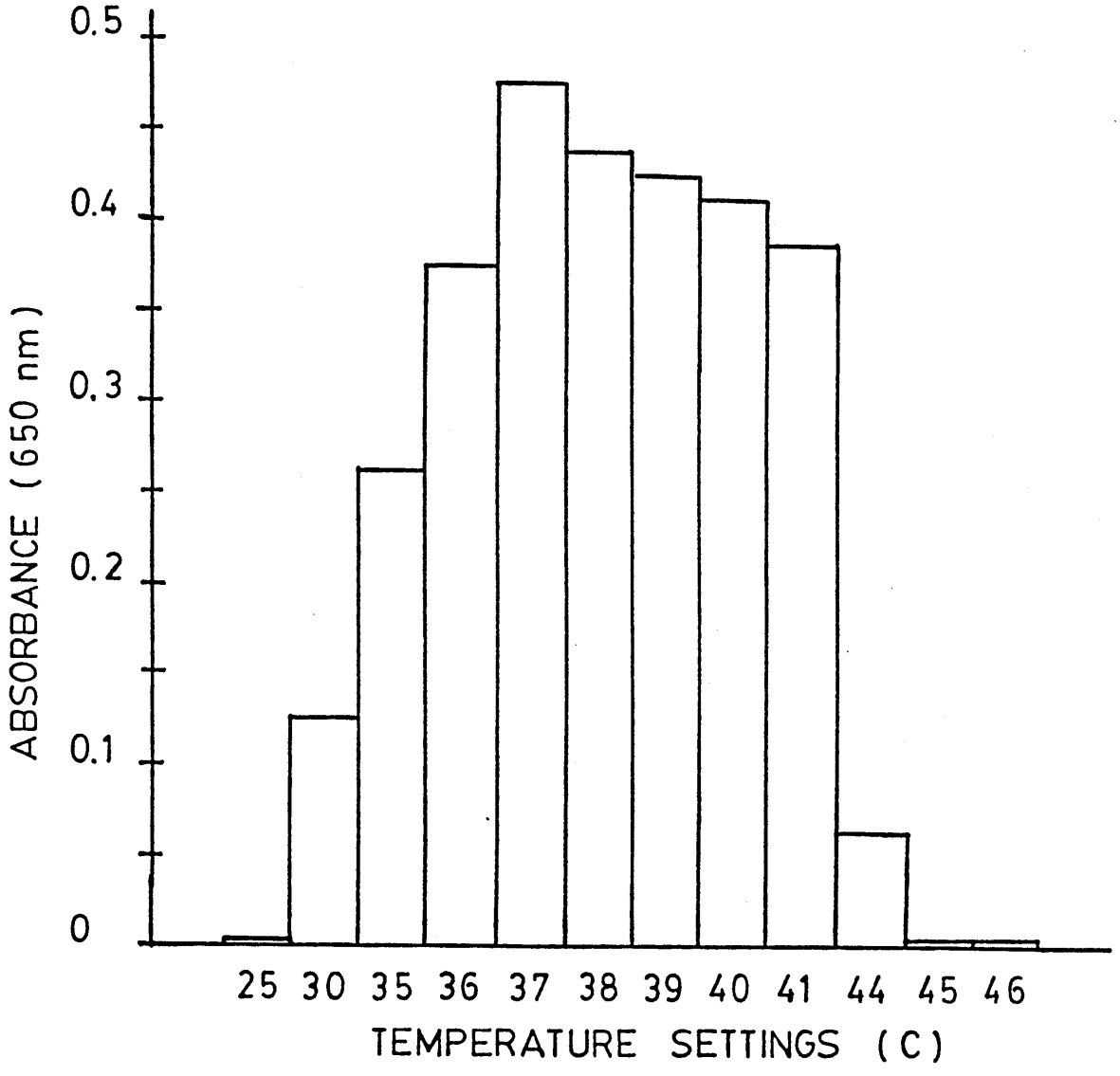

Figure 2--Measurement of growth, expressed as absorbance at 650 nm, of Peptostreptococcus anaerobius as effected by temperature. Each Nephlo culture flask contained 10 ml of Brucella broth, 0.1 ml of P_2 membranes and 0.1 ml of a 24 h inoculum.

anaerobiosis. Consequently, a control without P_2 membranes could not be included.

Following the procedure of Juni et al. (1984) the techniques were modified to construct growth curves suitable to enumerate some of the aspects of P. anaerobius nutritional requirements. Articles describing anaerobic methodology often recommend the addition of the singlet oxygen scavenger cysteine-HCl to lower the oxidation potential of media used in the culture of anaerobes (Wilkins and Thiel 1973; Kwok et al. 1975; Holdeman et al. 1977; Washington 1981; Swenson and Thornsberry 1984). Figure 3 shows the effects of various combinations of cysteine-HCl, hemin and vitamin K in Brucella broth on the growth of P. anaerobius. Membranes (P_2) at a concentration of 0.1 ml per 10 ml of Brucella broth were used as the control. A combination of the three variables had a slight stimulatory effect on the growth of the experimental organism, while single or combinations of any two of the additives resulted in less growth increase over the Brucella control.

Although cysteine-HCl is the most commonly used oxygen remover, it has been suggested that it is a metabolic toxin to the very organisms that it is supposed to be protecting (Adler and Crow 1981). According to Kari et al. (1971),

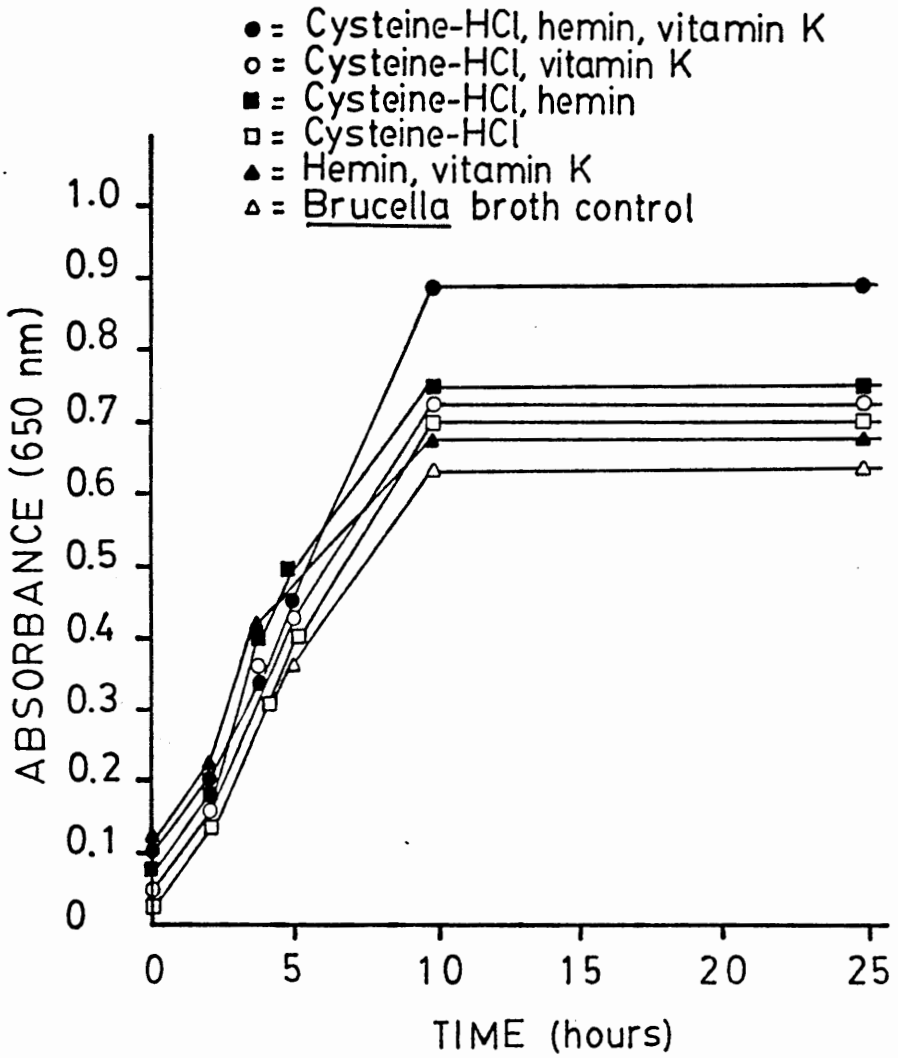

Figure 3--Effects of cysteine-HCl, hemin and vitamin K on the growth of Peptostreptococcus anaerobius. All Nephlo flasks contained 10 ml Brucella broth, 0.1 ml P_2 membranes, 0.1 ml inoculum and combinations of 50 µg/ml of cysteine-HCl, 50 µg/ml of hemin and/or 10 µg/ml of vitamin K.

cysteine-HCl seems to inhibit the growth of E. coli by two different mechanisms: (1) The biosynthesis of leucine, isoleucine, threonine and valine may be interrupted, or (2) it may interact with the function of the membrane-bound enzymes. Cysteine-HCl may be decomposed by Proteus and Escherichia species to pyruvate, ammonia and hydrogen sulfide (Sokatch 1969). Carlsson et al. (1979) stated that P. anaerobius ATCC# 27337 was rapidly killed by autooxidized cysteine containing solutions, and that this was the result of the formation of hydrogen peroxide. Other authors commented about the extreme toxicity of hydrogen peroxide on P. anaerobius and other anaerobes (Frolander and Carlsson 1977; Carlsson et al. 1978; Nyberg and Carlsson 1981; Patel et al. 1984).

The use of heme as a growth requirement has not been substantiated for a large number of organisms. Because heme is required by Bacterioides melaninogenicus and B. ruminicola (Caldwell et al. 1965), it is often included in many media used for clinical isolation. Vitamin K is also needed by only a few genera such as Mycobacterium (Gerhardt et al. 1981). This vitamin, fat soluble and destroyed by light, alkalies, and reducing agents, is generally added as menadione.

Lactate is listed by Brock et al. (1984) as an organic acid and glutamate as an amino acid. These chemicals are able to enter pathways for the anaerobic breakdown of substrates to be converted to pyruvate and finally to acetate. Juni et al. (1984) proposed that they act as possible carbon sources. Figure 4 illustrates the effect of added sodium lactate and sodium glutamate on the growth of P. anaerobius. It was unexpected to find that neither lactate nor glutamate at the concentrations used stimulated the growth of this organism. The addition of sodium glutamate resulted in an almost identical growth curve as the Brucella broth control. The depression of the growth curve produced by the addition of sodium lactate disproved its use as a carbon source for this organism. The data suggest that other possible carbon sources for P. anaerobius should be investigated.

Glucose, arabinose and xylose were listed by Stanier et al. (1976) along with many other carbohydrates, fatty acids, alcohols, amino acids and nitrogenous compounds as chemicals capable of serving as carbon sources for both aerobic and anaerobic bacteria depending on their nutritional requirements. Brucella broth contains glucose as a carbon source at a concentration of 0.1%. Figure 5

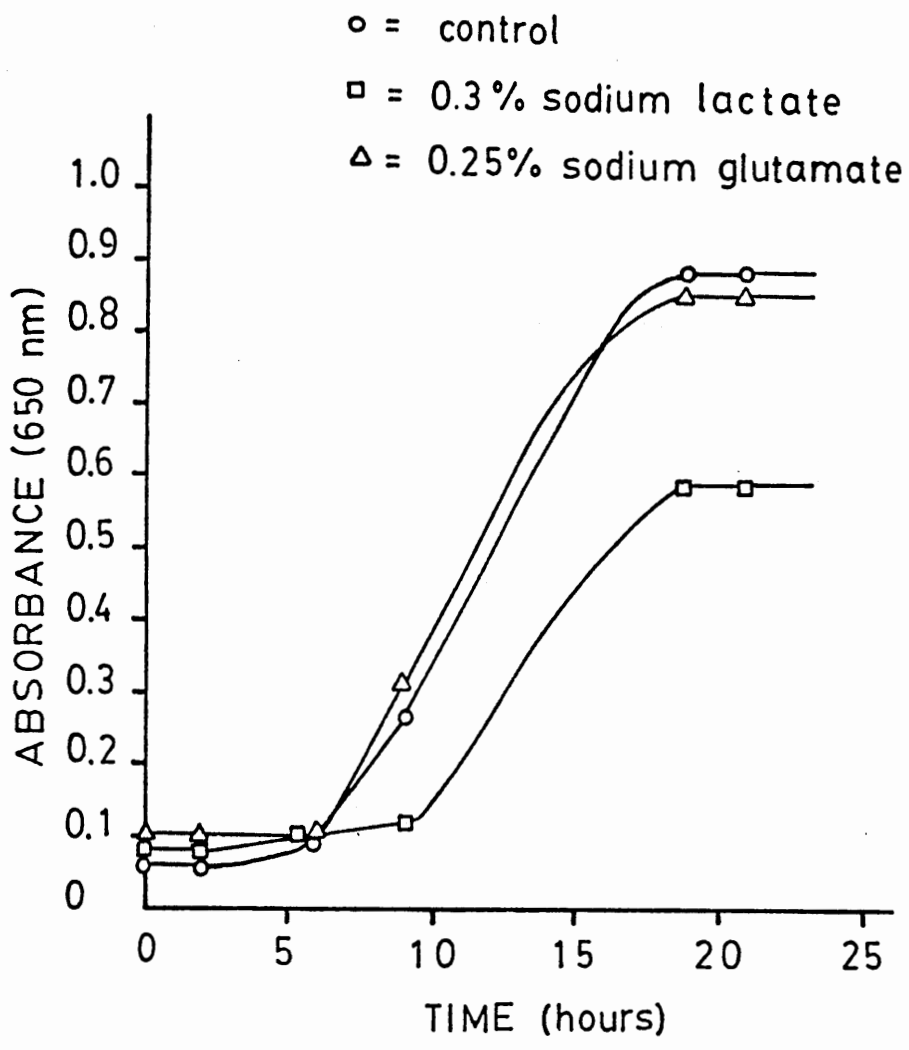

Figure 4--Effects of sodium lactate and sodium glutamate as a carbon source on the growth of Peptostreptococcus anaerobius. All Nephlo culture flasks contained 10 ml Brucella broth, 0.1 ml P_2 membranes, 0.1 ml inoculum and a final concentration of 0.3% sodium lactate or 0.25% sodium glutamate.

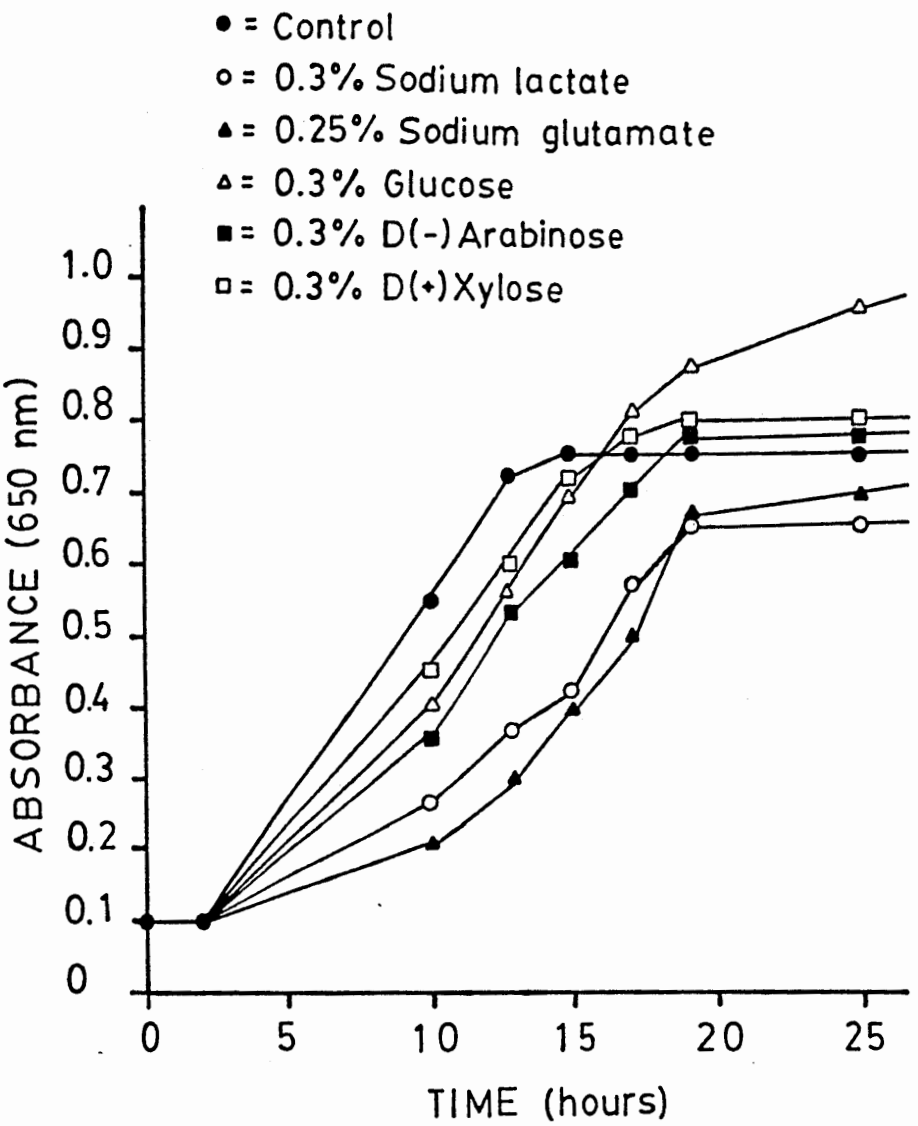

Figure 5--Effects of various carbon sources on the growth of Peptostreptococcus anaerobius. Nephlo flasks contained 10 ml Brucella broth, 0.1 ml P_2 membranes, 0.1 ml inoculum and carbon sources at the stated concentrations.

shows the effects of glucose, arabinose and xylose as well as sodium lactate and sodium glutamate on the growth of P. anaerobius. Glucose, arabinose and xylose supported growth as measured by absorbance of the organism. Glucose provided the largest stimulus. Figure 5 shows that glucose was particularly utilizable for incorporation into organic cell materials. The elevation of the glucose curve over the normal growth indicates that the stationary phase of the control was reached because the carbon source was exhausted. The addition of 0.3% glucose to the 0.1% glucose already present in Brucella broth caused the growth curve to become elevated and the stationary phase was not achieved during the 25 h testing period.

Brock et al. (1984) divided pathways involved in the breakdown of glucose by anaerobes into three major parts. First, there is phosphorylation, followed by an oxidation-reduction step, leading to the production of pyruvate and an energy gain in the form of ATP. The second oxidation reduction sequence leads to the third part of the fermentation process which results in the production of ethanol and CO_2. It was evident from the large number of gas bubbles produced during incubation that CO_2 did result from fermentation, although the formation of ethanol was not

determined.

The use of a synthetic medium for the growth of an organism is necessary to establish details of the bacterial metabolism. Juni et al. (1984) recommended a method for working out the composition of such a medium, involving the use of spent complex culture medium. Spent medium is used to provide minimal requirements for growth with other ingredients being added to supply missing nutrients. The spent medium provides a proven nutritional source in which measurable growth could occur. The organism, grown to the stationary phase, depleated the excesses of all required nutrients and the addition of any other growth factors was seen as an elevation of the curve. It is difficult to test numerous chemically defined media, since many causes may prevent growth, rather than a single missing nutrient. Testing a variety of chemically defined media is hampered by the fact that should no growth appear, the reason may be unclear. The cause may be the absence of one or more growth factors, the presence of a compound that inhibited growth, an extremely long lag period or a compound that combines with oxygen to produce a toxic effect. The amount of any nutrient needed for growth may be determined by adding concentrations of chemicals to the base spent medium and

salt solution and observing which one causes the greatest increase in the initial stationary phase. Inhibitory chemicals can be detected by noting the effect on growth that the spent broth and the additives are able to support. Elimination of the spent medium at any point in the procedure would enable one to be aware that all the nutrients required by the organism either had or had not been identified. An increased lag phase or decreased growth rate after the removal of the spent medium would serve to indicate that although all essential factors had been identified, others needed for optimum growth were still unknown.

Figure 6 demonstrates the use of 12.5, 15 and 50 percent spent Brucella broth compared to fresh Brucella broth control medium. Absorbance of the control flask culture increased from an initial reading of less than 0.2 to more than 0.9 indicating a substantial increase in growth during the 20 h incubation period. The culture in the flask containing 12.5% spent medium without glucose increased in turbidity measurement from 0.1 to slightly less than 0.2 while the 15% plus glucose (0.3%) culture did not increase in turbidity. Medium containing 12.5% and 15% spent Brucella broth do not provide enough nutrients to support

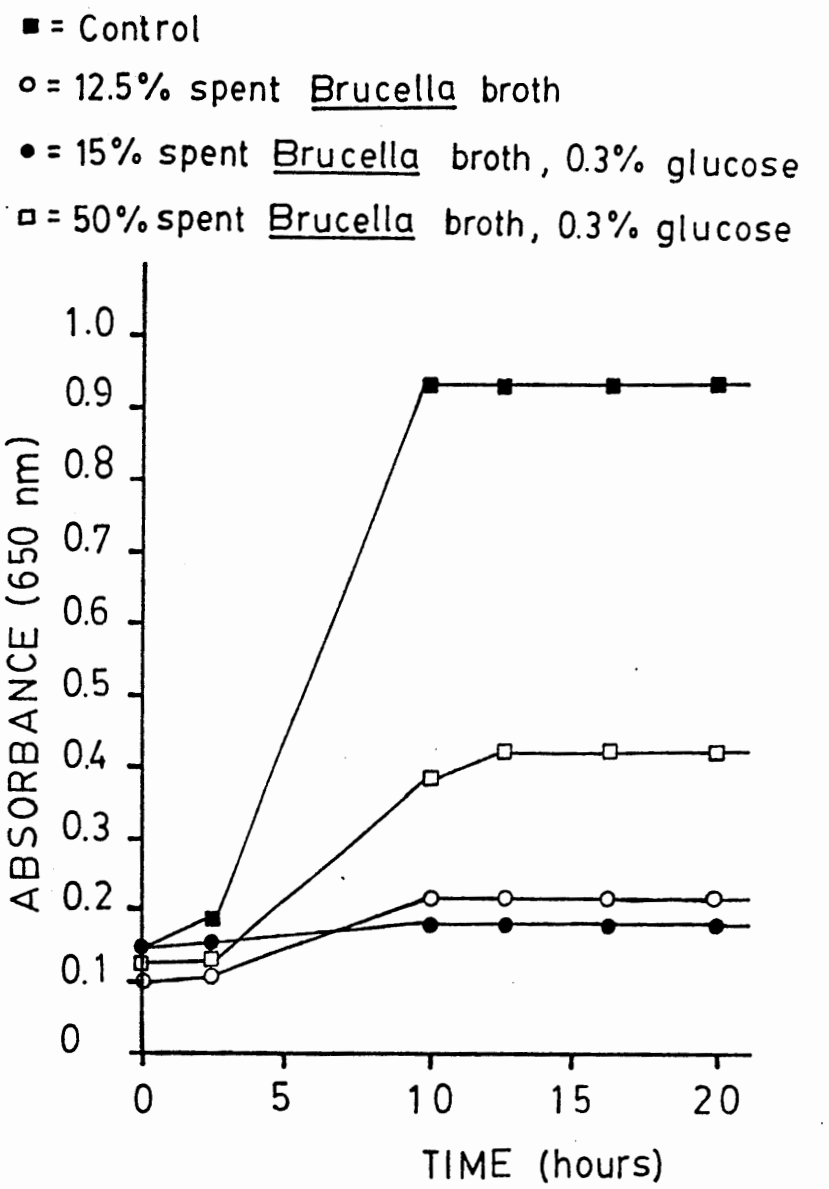

Figure 6--The growth of Peptostreptococcus anaerobius in spent Brucella broth. The Nephlo flasks contained various concentrations of spent Brucella broth diluted with half-strength salt solution, 0.1 ml P_2 membranes and 0.1 ml inoculum. The control contained 10 ml Brucella broth, 0.1 ml P_2 membranes and 0.1 ml inoculum.

adequate growth of P. anaerobius to be used in a nutrient requirement search. Medium prepared with 50% spent broth and a concentration of 0.3% glucose proved to support some growth of the culture. The absorbance readings increased from about 0.1 to a little over 0.4. A growth curve positioned in the middle range between no growth and the control growth curve would show the effect of inhibiting additives and those that enhance the growth of P. anaerobius.

Further work needs to be done to formulate a chemically defined medium suitable for the optimum growth of P. anaerobius. This research could be continued with pools of growth factors that may be tested in various combinations. Once a defined medium has been developed, auxotrophic mutants of P. anaerobius can be isolated, and studies determining biochemical pathways peculiar to this organism performed. The literature is remarkably limited with regard to information on the metabolism of Gram positive anaerobes.

Lyopholyzed cultures obtained from the American Type Culture Collection were suspended in cooked meat medium prepared fresh and according to the procedure of Holdeman et al. (1977) and incubated at 37 C for 48 h. Abundant growth was observed, as evidenced by quantities of sediment in the

tubes. Black pigment was not apparent since a sulfur-containing compound was not added to this medium.

Publications concerning the cultivation of P. anaerobius generally recommend the use of either one of the following media: Schaedler, Schaedler blood, Brucella, Brucella blood, BBL-Thioglycolate 135C, Peptone Yeast Extract Glucose, Brain Heart Infusion or CDC, all prepared in pre-reduced form (Wilkins and Thiel 1973; Wilkins and Chalgren 1976; Wren et al. 1977; Graham and Falkler 1979; Wong et al. 1980; Lambe et al. 1980). As seen in Table 4, all of these liquid media supported growth of P. anaerobius and were turbid after 24 h incubation. Plates containing solidified media with 2.0% agar, yielded different results depending if Gibco agar or other agars were used. The later were incubated for 48 h, but still contained only a few colonies while others showed no growth at all. The addition of hemin and/or vitamin K to the different complex media, did not produce an increase in colony counts. The addition of sheep blood to the basal medium encouraged the growth of a few colonies, but it was recognized that this was not a good supplement to a medium for the isolation and characterization of mutants. A new shipment of Gibco Agar-Bacteriological grade was used in one batch of medium and

Table 4--Inhibition of Peptostreptococcus anaerobius growth due to the addition of agar to complex liquid media.

Media	48 h incubation with agar other than Gibco	48 h incubation with Gibco agar	24 h incubation in broth
CDC (Tryptic Soy Agar Base)	no growth	seeded	turbid
Brucella Blood	<30 colonies	seeded	turbid
Brucella	no growth	seeded	turbid
Chopped Meat	-	-	turbid
Schaedler	<30 colonies	seeded	turbid
BBL-Thioglycolate 135C	<30 colonies	seeded	turbid
Brain Heart Infusion Blood	<30 colonies	seeded	turbid
PYG (Peptone Yeast Extract Glucose)	no growth	seeded	turbid

the results were remarkable. As shown in Table 4 when agar other than Gibco agar was used, little or no growth occured on the plates after 48 h of incubation, while plates containing Gibco agar were seeded with numerous small colonies. This cell proliferation compared well with the turbidity obtained in liquid culture.

From experiments conducted with Brucella broth and seven different brands and types of agar, it was found that Difco agar was not the only "inhibitor" of growth. This can be seen in Table 5. Oxoid Agar-Agar #3 and Gibco Agar-Bacteriological Grade were the only solidifying agents that did support heavy growth, while Fisher Laboratory Grade allowed development of only a few colonies. All other agars were unsuccessful in supporting colony formation of P. anaerobius. The use of a gellan gum called Gellrite (Kelco) or agarose (Sigma) resulted in as much growth as Gibco agar, but not more. Gibco agar was chosen as the solidifying agent with Brucella broth for further experiments.

Sealed McFarland tubes with various concentrations of barium sulfate were compared optically with a tube of inoculated broth. The cell population density of the inoculum is related to turbidity by comparison to a standard McFarland tube. Although this does not take into

Table 5--Growth responce of Peptostreptococcus anaerobius to different agars used with Brucella medium.

Agar added to medium	Growth Response
Oxoid Agar Agar #3	heavy growth
Difco Agar Nobel (low ash content)	no growth
Difco Bacto-Agar	no growth
Fisher Agar Laboratory grade	<10 colonies
Difco Agar Bacteriological technical	no growth
Sigma Agar Type IV	no growth
Gibco Agar Bacteriological grade	heavy growth

consideration dead cells in the culture, if the inoculum was obtained from organisms in an actively growing phase, the difference is insignificant. It was not expected that the cell counts obtained with the culture on Gibco agar would be higher than the cell density as related to the standard. However, the data shown in Figure 7 indicates that the "inhibitor" found in other agars was also present in Gibco agar, but in small amounts. However, this required a reduction in agar concentration from 2.0% to 0.7% so that a greater number of organisms could be recovered on the solid medium.

Agar is extracted from certain red marine algae and is washed and purified to remove algal cell debris and various impurities before it is suitable for bacteriological purposes. Since it is not biodegraded by most bacteria, it is the most widely used solidifying agent in the preparation of media. It is composed of two polysaccharides, 70% agarose and 30% agaropectin (Gerhardt et al. 1981). A search of the literature did not reveal any mention of a specific inhibitory property of agar or any agar component detrimental to growth. However, Wideman et al. (1976) used Brucella agars of several manufacters to evaluate their influence on zones of inhibition surrounding disks of sodium

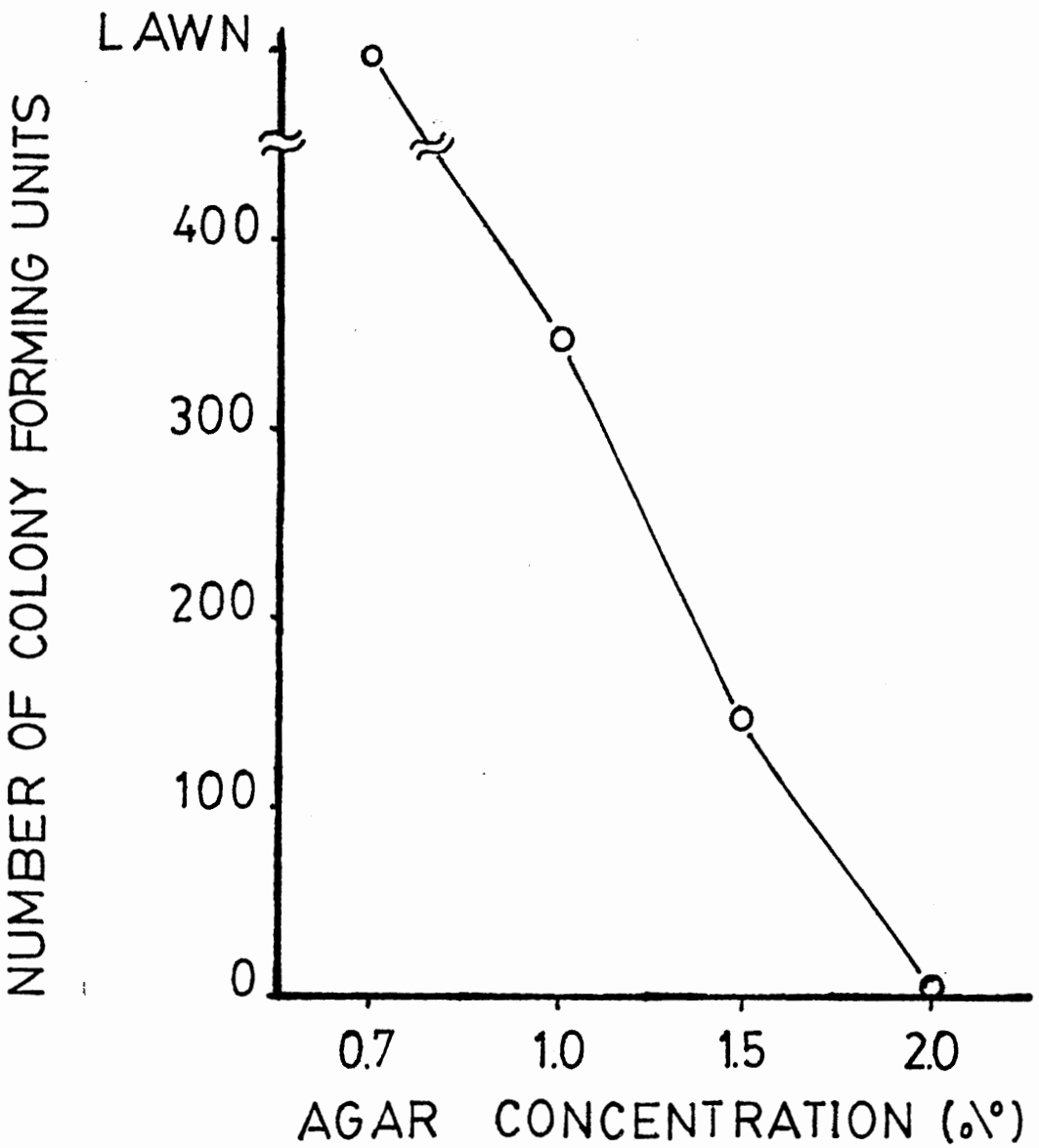

Figure 7--The inhibitory effect of agar concentration on the growth of Peptostreptococcus anaerobius. The solid medium was prepared with Brucella broth and Gibco agar.

polyanethol sulfonate. They reported that Pfizer Brucella agar yielded the largest zones of inhibition, because it did not support the growth of P. anaerobius as well as Brucella agars obtained from BBL or Difco.

In an attempt to determine possible inhibitory substances in agar the following simple experiment was performed. Difco agar, which depressed the growth of P. anaerobius was subjected to water, chloroform, ether or alcohol extractions, and the solvents were volatilized. Equivalent dilutions of the extracts were added to Gibco Brucella agar and it was found that the inhibitor was in the ether and chloroform phase.

The question arose as to whether that particular ATCC culture was unique in its ability to be inhibited by a constituent in some brands of agar. An experiment was devised to test this hypothesis. For this experiment, the original culture of P. anaerobius (VPI# 4330) was designated as A100. An derivative of A100 that had been isolated from a 2.0% Gibco agar plate was labeled, A101. Two new cultures of genetically identical P. anaerobius VPI# 4330 were ordered and labeled B100 and C100. As can be seen in Figure 8 there was total inhibition of growth of the three ATCC cultures at 0.3% Difco agar, with only a few colonies

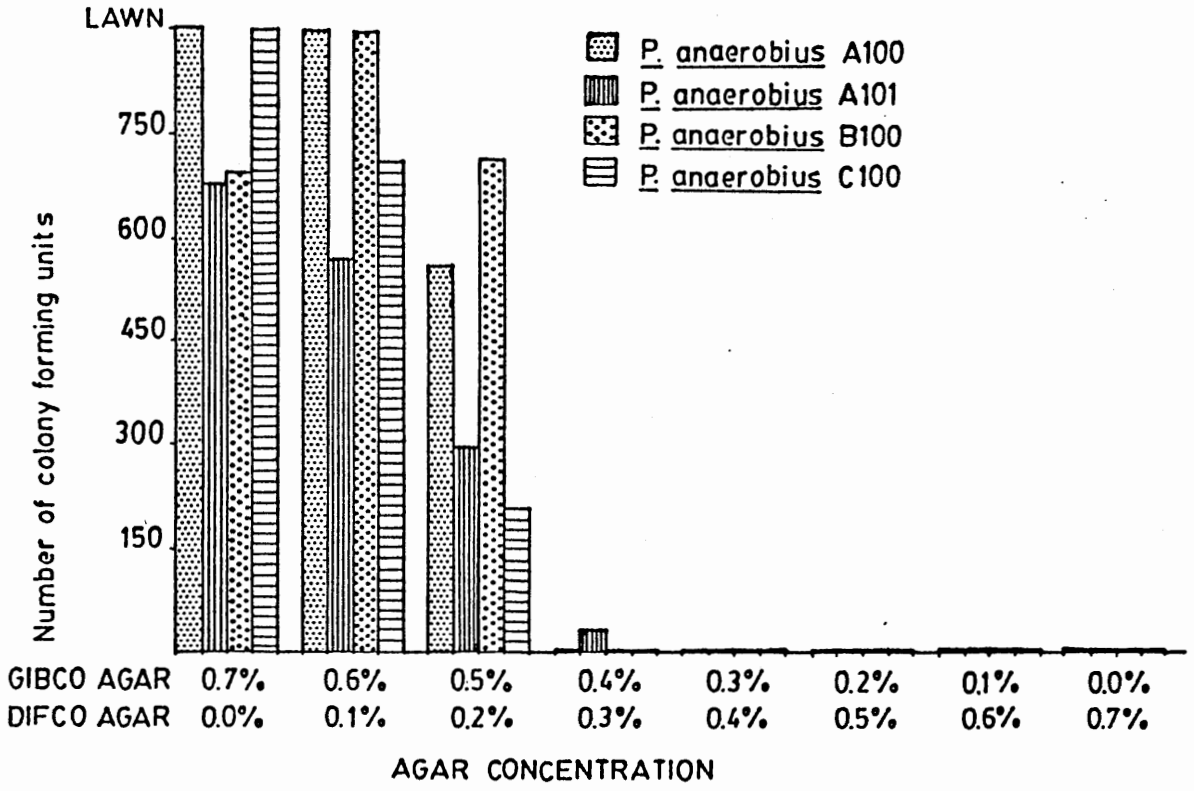

Figure 8--The inhibitory effect of Difco agar on the growth of Peptostreptococcus anaerobius. The total concentration of agar in Brucella medium was 0.7%.

present in the Al01 culture. The presence of about 50 colonies of Al01 on the plate containing 0.3% Difco agar indicated that it might be possible to select for a culture that was not inhibited by the ether/chloroform extractable substance. All of the strains purchased from the American Type Culture Collection were inhibited to the same extent by the unknown substance(s) present in Difco agar.

No further effort was made to isolate or identify the inhibitory substance(s), since Gibco agar proved satisfactory as a solidifying agent in 0.7% concentration despite the slight depressing effect it had on the growth of P. anaerobius. However, before a chemically defined medium can be formulated, the inhibitor(s) should be identified and an extraction procedure established. The purification of the agar should be stringent so that optimum growth may be observed during the formulation of a practical chemically defined medium.

When bacteria are exposed to radiation, in either the high energy or the ultraviolet range, the most prominent effect is the killing of a certain percentage of cells, the fraction being a function of the absorbed energy (Hollaender 1955). The terms bactericidal effect, lethal effect, killing or inactivation are used synonymously to indicate

the failure of the cells to form a colony visible to the naked eye when an inoculum is plated under the particular conditions of the experiment. This indicates the necessity of obtaining a solid medium that supports the growth of the bacteria being subjected to the radiation study. The decreasing number of colonies following an appropriate period of incubation was the means by which the effects of radiation could be measured.

The type of radiation used in the experiments for this dissertation was the gamma rays of Cesium-137. The portions of the electromagnetic spectrum that possess the ability to produce ionizations are the gamma rays and X-rays. The Cesium-137 source produces gamma emissions over a long time since it decays with a half-life of 30 years (Arena 1971).

Following the absorption of ionizing radiation in a biological system, the chain of events is as follows. Chemical damage develops with the production of free radicals and excited molecules, leading to biomolecular damage, which affects proteins, nucleic acids and can advance further to cell death (Coggle 1979). The formation of free radicals from both direct and indirect effects seems to be of greatest importance in experimentations.

Brock et al. (1984) reported the water content of an

average E. coli B/r cell to be about 70% of the total weight of the bacterium. Radiation may produce detrimental effects in media such as; pH changes, production of hydrogen peroxide, organic peroxide and breakdown of carbohydrates (Casarett 1968). The chemical products of irradiated water include H, OH, H_2O_2, HO_2 and O_2, as well as free radicals. These may then react with important organic molecules and thus be indirectly responsible for an observable effect such as mutation or cell death (Grosch and Hopwood 1979).

As an anaerobe, P. anaerobius is sensitive to oxygen. The oxygen effect that influences radiation damage in aerobes and facultative anaerobes is greatly magnified in this organism. The presence of oxygen either as a contaminant, or produced during exposure, would increase the number of harmful radicals or blocks restoration of damaged organic molecules (Arena 1971). Adler and Crow (1981) reported the use of a partially purified membrane fraction from E. coli B/r ORNL to remove oxygen from bacteriological media. These fragments are large and cannot penetrate bacterial cells, but remove oxygen contamination by enzymatic reactions. Membranes prepared from E. coli that cannot synthesize ubiquinone, which is part of the cytochrome system, proved the validity of Adler's theory.

These membranes were incapable of removing oxygen from the test medium (Adler and Crow 1981).

It was observed in this study that membranes would reduce oxygen load from atmospheric level to zero in 4 min. These measurements were made using a Chemical Microsensor System (Transidine General Corp.) and a miniature Clark-style electrode in a chamber containing 1 ml of medium equilibrated with air at 23 - 25 C (Adler 1983).

Adler and Crow (1981) also reported that several anaerobes reproduced better in membrane supplemented medium than that which contained the oxygen scavenger, sodium thioglycollate. Although P. anaerobius grew well in both of these media, Frolander and Carlsson (1977); Rolfe et al. (1978); Carlsson et al. (1978); Carlsson et al. (1979); Nyberg and Carlsson (1981) and Patel et al. (1984) indicated that if cysteine had been used as the oxygen scavenger, the data would have been different, since this amino acid can be destructive to the anaerobe.

P. anaerobius was suspended in water containing a concentration of cysteine-HCl as recommended by Holdeman et al. (1977) in gassed modified Turner bulbs. Washed cultures were ajusted to the turbidity of a #1 McFarland so that a standard number of cells would be subjected to the gamma

rays. Following exposure, the dilution of cells was plated on 0.7% Brucella agar by the spread plate technique as pour plates had been proven unsatisfactory because of the agar inhibition factor. No growth occured on the plates containing irradiated cells (as low as 0.16 Coulomb/Kg), although the control developed a few colonies. The experiment was repeated with the addition of a broth control for both irradiated and unirradiated cultures. The exposed cells did not proliferate but normal growth occured in the control at an extended period of time. This was a good indication of cell death rather than inhibition caused by agar.

An alternative to the use of cycteine-HCl as an oxygen scavenger is employment of P_2 membranes obtained from E. coli B/r ORNL. The modified procedure of Adler et al. (1981) is shown in Table 1. The membranes were used in these experiments as indicated in Table 6. Membranes were added to water at a concentration of 0.01 ml per ml of water. The hydrogen donor, sodium lactate, was included in the same proportion as the membranes. Adler and Crow (1981) recommended the use of sodium lactate or sodium succinate to act as suitable donors for the membrane bound cytochrome-mediated reduction of oxygen. Sodium citrate (Sigma) was

Table 6--P_2 membranes of Escherichia coli B/r ORNL applied to media used in radiation and culture techniques.

LIQUID MEDIUM
0.01 ml of membranes per ml of medium
SOLID MEDIUM (100 mm plate)
Bottom Layer a. 0.1 ml membranes b. inoculum c. 10 ml medium
ALLOW TO SOLIDIFY
Top Layer a. 10 ml medium
RADIATION SUSPENSION FLUID
for each ml of distilled water used a. 0.01 ml 1 M sodium lactate b. 0.01 ml P_2 membranes

also found to be satisfactory.

The oxygen removal activity of each batch of membranes was checked with a Yellow Springs oxygen meter, but the instrumentation setup could only provide a gross indication of membrane activity. Each batch of membranes was tested for sterility in modified nutrient broth (Adler et al. 1981). Long term activity of the membranes was determined by culturing P. anaerobius in Brucella broth and P_2 using a plastic capped test tube normally associated with an aerobic culture. On a weekly basis the anaerobe was subcultured back into prereduced Brucella broth. The usual viability was 4 - 5 weeks at room temperature, indicating the same period of activity level for the P_2 membranes. Only the first preparation of membranes failed to meet the set criteria and these had been subjected to sonnification for 120 sec prior to fractionation in the Ribi Cell Fractionator. Heat build up during sonnification was the probable cause for the loss of activity.

Since oxygen contamination was critical, the Turner bulbs were replaced with screw capped Hungate anaerobe tubes. Twenty-four cultures were washed and resuspended in water containing membranes and sodium lactate in the quanities recommended by Adler (1983) and as seen in Table

6. The inoculum was adjusted to the turbidity of a #1 McFarland. The control counts were substantially less than the bacterial numbers proposed by Gerhardt et al. (1981) as listed for the #1 McFarland. These investigators indicated that the McFarland tubes were standardized to single cell Gram negative bacteria which would, when plated on solid media, give rise to one colony per viable cell. Peptostreptococcus has a chain formation which would lower the colony per cell ratio since a chain would produce a single colony. Also, the P_2 membrane containing water produces turbidity since the fragments were at a concentration of approximately 20 to 30 mg dry weight per ml (Adler et al. 1981).

The tubes were placed on the turn table in the center position of the J. L. Sheperd MI cesium-137 irradiator and were exposed to 0.32 Coulomb/Kg/min for a total of 130 min. A total dose of 41.64 Coulomb/Kg resulted in a 99.999% killing of P. anaerobius with less than 10 colonies being formed from the surviving cells. Figure 9 shows that at 90 min exposure (28.83 Coulomb/Kg) there were 200 - 300 viable cells remaining. This amount of radiation was chosen as suitable for the production of mutants in this anaerobe.

An experiment was performed to assertain the effect of

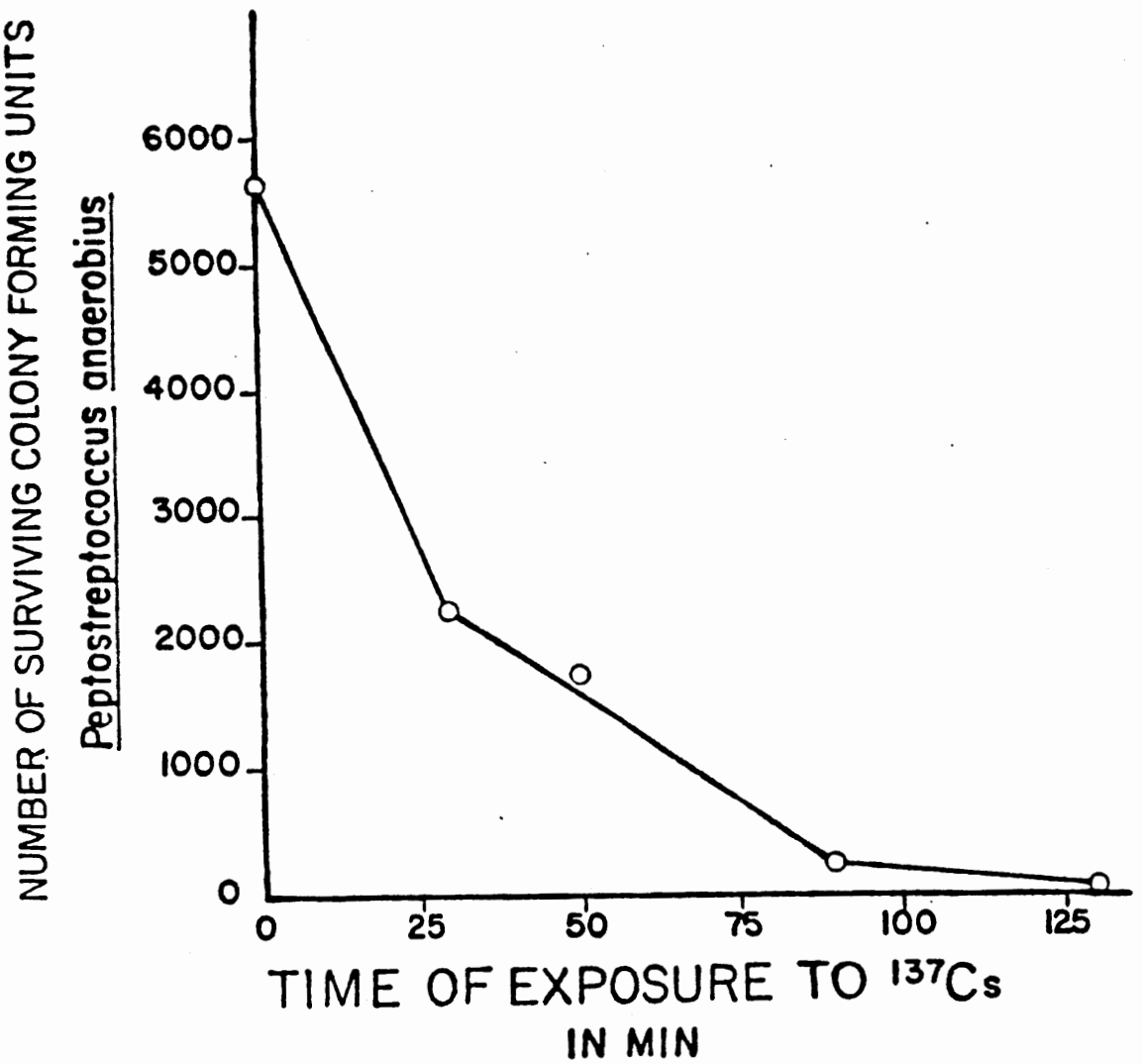

Figure 9--Effect of gamma radiation on the number of surviving colony forming units of Peptostreptococcus anaerobius. The total dose of irradiation at 125 min from the J. L. Shepard Mark I-Cs-137 source was 41.65 Coulomb/Kg.

water, cysteine-HCl, membranes and sodium lactate on the survival of P. anaerobius during radiation and during the holding period. Figure 10 indicates the results of this experiment. The normal procedure for radiation was to place all of the cultures in the radiation chamber at the same time, with the exception of the control and remove them at specific time intervals. The control was placed outside the source to be subjected to the same temperature and time period as the irradiated cultures. This was necessary for the following reasons. Plates may not be poured in advance, although the media may be made ahead of time and stored anaerobically in Hungate roll tubes. The plates, once inoculated, must be placed in BBL GasPac jars with H_2 - CO_2 generators and activated palladium catalyst. The time lag between jar closure and an oxygen free atmosphere is approximately two hours. The time interval between removal of each sample from the irradiator would have been insufficent to allow pouring the plates, solidification, dilution, plating and placement in the jars, unless all samples were held to be processed until the final sample was removed from the Cesium-137 source.

Cysteine-HCl in water had proven unsatisfactory for the radiation suspension fluid and it was unknown whether the

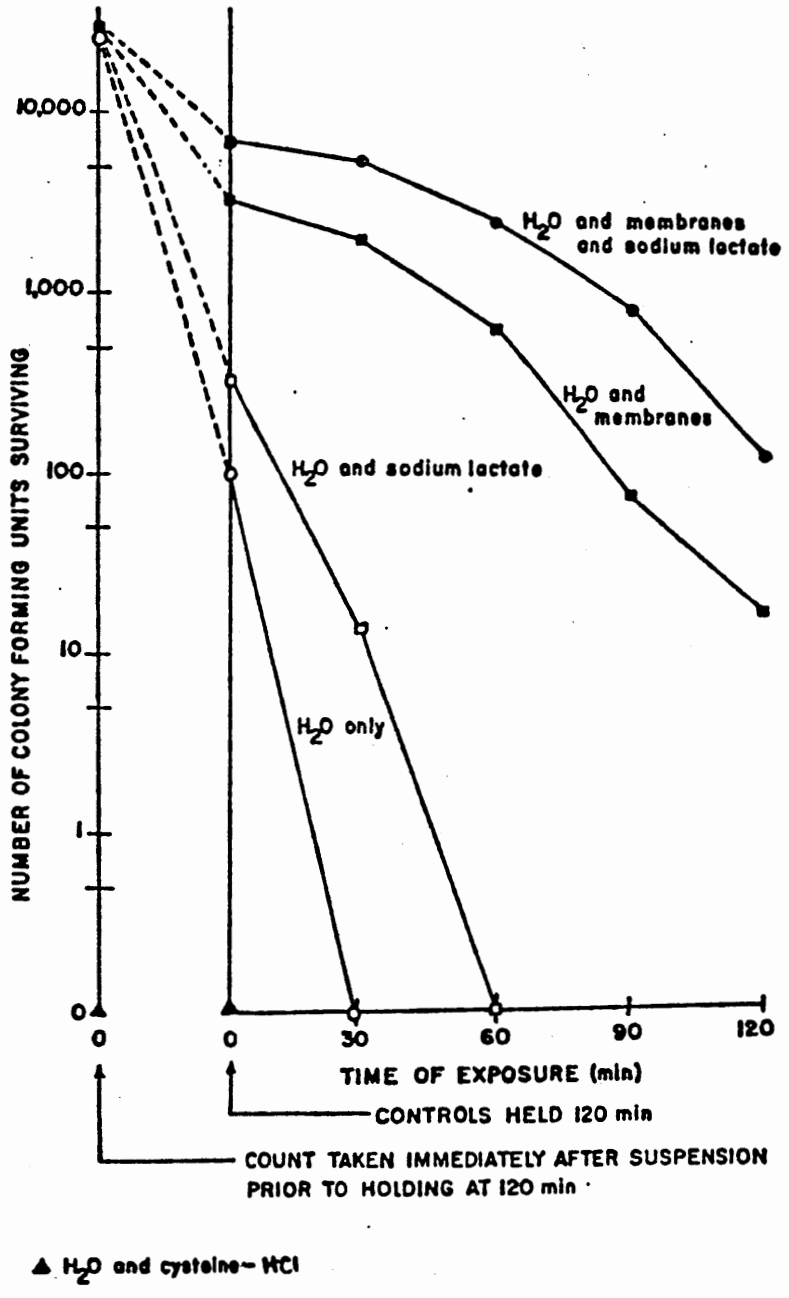

Figure 10--Effect of variation in suspension fluid on the survival of colony forming units of Peptostreptococcus anaerobius.

120 min room temperature holding period, the Turner bulb or the cysteine itself was the lethal factor. The Turner bulbs were closed by a sintered glass nonscrew slipon top. Even though the bulb had been gassed with an anaerobic atmosphere, air contamination was possibly very high. The use of Hungate screw capped tube did not change the results. The reduction of the holding period from 120 min to less than 10 min did not increase the number of viable bacteria recovered. It should be noted that there are two 0 points on the horizontal line of Figure 10. The first zero indicates the 0 time control, demonstrating no waiting period and no radiation exposure. The second zero shows the 120 min holding period control but without radiation exposure. The subsequent points reveal the number of colony forming units surviving 30, 60, 90 and 120 min of exposure to Cesium-137 at 0.32 Coulomb/Kg/min. All substances other than cysteine-HCl had the following effects on the number of colony forming units (CFU) of P. anaerobius following the 120 min holding period and the different radiation exposures. The samples with water only, with no means of removing oxygen, had approximately 100 CFUs following the 120 min nonradiated holding period. This number dropped to zero during the first 30 min of exposure. The difference

between water only and water-cysteine-HCl may be explained by the results of the research done by Carlsson et al. (1979) and Patel et al. (1984) involving effects of cysteine on the growth of P. anaerobius. In their studies anaerobic bacteria were rapidly killed in a dilution blank containing H_2O, cysteine-HCl, 0.2% gelatin, salts and reaszurin, an oxygen indicatior. The dilution blank not containing cysteine, produced the least killing while the one with the chemical showed lethality. Carlsson et al. (1979) proposed that hydrogen peroxide was formed when cysteine was exposed to oxygen and this was supported by a reported decrease in lethality when catalase and horseradish peroxidase were added to the dilution blanks containing cysteine.

As seen in Figure 10, sodium lactate seems to have a slight radiation protective effect during holding and during radiation, since 60 min exposure was required to obtain 100% killing of P. anaerobius. There was some difference between the samples containing membranes. This might be explained by the assumption that there may be enough free hydrogen associated with the membrane preparation along with hydrogen radicals produced during the radiation exposure to complete the bonding of the oxygen. The cytochrome system requires two hydrogens per atom of oxygen, so the depression of the

lethality curve of H_2O and P_2 membranes may be due to an inadequate supply of hydrogen atoms.

The possibility that E. coli B/r ORNL membranes might be affecting the amount of oxygen contamination and autooxidized products in the medium and the radiation suspension fluid by means other than the cytochrome system required testing. E. coli species are catalase positive organisms and superoxide dismutase (SOD) positive (Stanier et al. 1976). Aerobes and aerotolerant anaerobes may contain these enzymes to protect the cells from an internal buildup of superoxide (O_2^-) and hydrogen peroxide (H_2O_2). It was thought that the major difference between aerobes and anaerobes was the lack of the superoxide dismutase enzyme in the anaerobes (Sokatch 1969). However, Tally et al. (1977) and Fulghum and Worthington (1984) disproved this theory by finding measurable amounts of SOD in both clinically significant and in ruminal anaerobes. Oxygen was shown to increase the production of SOD in Bacteriodes fragilis (Tally et al. 1977) and Oberley (1982a) reported a like stimulatory effect in mammalian cells. The Sigma catalog (1986) lists superoxide dismutase of mammalian origin and from E. coli B, the organism from which E. coli B/r ORNL was derived (Adler and Haskins 1960). Superoxide dismutase

has been purified from mitochondrial membranes (Oberley 1982b). P_2 membranes may contain active SOD besides the membrane bound cytochrome system, although this has not been ascertained at this time.

However, the production of catalase by P_2 has been proven experimentally and an attempt has been made to quantitate the influence this had on the growth of P. anaerobius. It has been discussed previously in this dissertation that this anaerobe is oxygen sensitive (Buchanan et al. 1974; Reddick et al. 1980; Sneath et al. 1986) and is affected very rapidly by the presence of H_2O_2 (Morris 1976; Carlsson et al. 1978; Rolfe et al. 1978; Slade et al. 1981). Catalase in the P_2 membranes would account for the report by Adler and Crow (1981) that Peptostreptococcus grew more rapidly and better in membrane containing broth than in broth containing sodium thioglycollate. A drop of blood on one end of a microscope slide provided a control for the action of hydrogen peroxide on the P_2 membranes. One drop of 3% (commercial grade) hydrogen peroxide placed on one drop of blood and one of the membrane preparation produced massive amounts of bubbles. This is a test for the presence of catalase indicating that the enzyme breaks down hydrogen peroxide with the release of

H_2O and O_2. The membrane bound cytochrome system would then be able to bind the oxygen to hydrogen with the production of water. Table 7 indicates the effect of membranes on a dilution series of 3% hydrogen peroxide as measured by growth of P. anaerobius.

Duplicate 10-fold dilutions of 3% hydrogen peroxide were prepared in Brucella broth with a 0 control for each set of tubes. E. coli B/r ORNL membranes were added to one of the controls and a dilution series at a concentration of 0.1 ml/10 ml medium. Each tube of both sets was inoculated and incubated for 24 h. Tubes containing H_2O_2 but lacking membranes, exhibited growth in the control and in the tube with 9×10^{-5} M peroxide. The addition of membranes decreased the toxic effect of the disinfectant in all tubes except for the concentration of 9×10^{-2} M. There is the possibility that an increased incubation time of the membranes and hydrogen peroxide would have enabled P. anaerobius to proliferate in the 9×10^{-2} M concentration. There was a loss of medium from this tube after the addition of P_2 membranes since the resulting violent action caused an overflow. This line of experimentation could be extended in Nephlo sidearm flasks to eliminate loss of media and allow a controlled environment for a longer incubation period for

Table 7--Effect of molar concentrations of hydrogen peroxide in Brucella broth on growth of Peptostreptococcus anaerobius in the presence and absence of P_2 membranes, after 24 h incubation at 37 C.

	Molar Concentration of Hydrogen Peroxide				
	0	9×10^{-5}	9×10^{-4}	9×10^{-3}	9×10^{-2}
absence of membranes	+	+	-	-	-
membranes*	+**	+	+	+	-

*Membranes (P_2) were added at a concentration of 0.01 ml of membranes per ml of Brucella broth.

**The presence of growth as measured by the visibility of turbid medium.

the membranes and hydrogen peroxide before inoculation. It would be of interest to ascertain the amount of time the catalase enzyme is available after a chemical challenge. The four week maintenance of a P. anaerobius culture in a plastic capped aerobe tube suggests that the cytochrome system is not only active but retains the catalytic activity. This may also be true for the production of catalase.

Ionizing particles produced by a Cs-137 source, are known to be mutagenic agents and resulting genetic changes are a very important sublethal effect of radiation (Hollaender 1955). It is generally assumed that the kinds of viable mutations seen after radiation do not differ from spontaneously occuring natural genetic changes. Ionizing emissions just increase the rate of occurrence (Bryson and Davidson 1951). In the natural environment, mutants are often not apparent. When some selective measure is applied to a heterogeneous population, the genetic change may give an advantage to the mutant over the prototype. An example is the hospital setting. Because of the increasing importance of antibiotic resistance in anaerobic bacteria, selection for development of mutations to a variety of antibacterial agents has been accomplished, making therapy

of infectious diseases more difficult (Sutter and Finegold 1976).

Radiated samples of P. anaerobius were incubated in Brucella broth for 24 h to allow any mutated organism time to replicate and to recover from the exposure. An increased lag phase in the development of phenotypic expression following exposure has been reported by Hollaender (1955) which may account for the absence of mutants in several experiments. When the 24 h broth incubation period was omitted and the irradiated samples were immediately challanged with antibiotics and agar, mutants did not appear on the Petri plates. However, when the 24 h broth incubation period was included, antibiotic deviants developed colonies on Brucella antibiotic gradient plates.

Gerhardt et al. (1981) recommended that antibiotic gradient plates be incubated for at least 24 h prior to inoculation to allow a gradient to develop from the antibiotic layer to the antibiotic-less layer. Since the anaerobic bacteria have a long lag phase on agar and are very sensitive to autooxidized products in media, this step was omitted. The gradient developed depended on the concentration of the stock antibiotic. For example, rifampin stock had a concentration of 1,000 μg/ml and when

added to the 20 ml of Brucella agar it contained 50 μg/ml of the antibiotic. The final gradient developed in the plate was almost 0 μg/ml at one edge to 50 μg/ml on the opposite side.

The advantage of antibiotic gradient plates over those with a single antibiotic concentration was the possibility that the radiation induced mutants with varying levels of resistance to a single antibacterial agent could be identified as seen in Tables 8 and 9. This indeed occurred as shown in Table 9 which lists the two rifampin and the two carbenicillin mutants tested. Mutants other than those represented in Tables 8 and 9 were isolated and screened by determination of Minimum Inhibitory Concentrations (MIC). Mutants with a very small increase in resistance were eliminated from further characterization. Mutants were identified by their variance from the prototype in the following test.

P. anaerobius is the only Gram-positive anaerobic coccus that will degrade and metabolize tyrosine crystals (Babcock 1979). Schaedler agar was prepared anaerobically and a 0.3% concentration of L-tyrosine was added. The crystals did not go into solution and were visible as a cloudy precipitate. P. anaerobius degrades these crystals

Table 8--Tests for changes in antibiotic resistance of Peptostreptococcus anaerobius mutants using the paper disk agar diffusion method (modified Kirby-Bauer technique).

Antibiotics	Zones of inhibition (mm)*		
	P. anaerobius prototype	P. anaerobius 101 rifampin-res	P. anaerobius 102 rifampin-res
Sulfadiazine	10	10	9
Cefamandole	40	35	37
Rifampin	56	15	21
Chloramphenicol	36	38	35
Erythromycin	35	36	33
Penicillin G	37	35	38
Tetracycline	42	45	41
Carbenicillin	21	22	20
Lincomycin	21	25	21
Gentamicin	14	15	13
Clindamycin	26	29	26
Sodium polyanethol sulfonate	21	22	21

*average of three trials.

Table 9--Minimum Inhibitory Concentrations* of Peptostreptococcus anaerobius prototype and antibiotic mutants.

Organism	MICs		
	Rifampin µg/ml	Carbenicillin µg/ml	Sodium polyanethol sulfonate µg/ml
P. anaerobius prototype	0.0007	1.56	3.05
P. anaerobius 101 rifampin-res	6.25	1.56	3.05
P. anaerobius 102 rifampin-res	1.56	1.56	3.05
P. anaerobius 103 carbenicillin-res	0.0007	6.25	3.05
P. anaerobius 104 carbenicillin-res	0.0007	12.50	3.05

*MICs were not affected by aerobic culture medium.

and a clear zone is apparent around the colony. This was not a method to quantitate amounts of tyrosine used. All mutants and the prototype performed alike. No contaminants were identified during the testing period and this was confirmed by the other species identification tests used in this experiment. These data are clear and do not need to be presented here in either graph or table forms.

P. anaerobius is also the only Gram-positive anaerobic coccus to be inhibited by the presence of sodium polyanethol sulfonate. Table 8 indicates the reactions of P. anaerobius 101 and 102, the two refampin-resistant mutants, to sterile disks containing 20 µl of a 5% sodium salt of polyanetholesulfonic acid. A zone of 12 - 30 mm is an indication of positive identification. The prototype and the rif-res mutants were inhibited to the same degree by this chemical. Table 9 lists the MIC for sodium polyanethol sulfonate for the prototype, two rifampin-resistant mutants and two strains inhibited by carbenicillin. Sodium polyanethol sulfonate at a concentration of 3.05 µg/ml inhibited growth in all mutants tested and in the prototype.

Table 8 presents the results of a screening of P. anaerobius, rif-res 101 and rif-res 102 with various antibiotics by the modified Kirby-Bauer paper disk agar

diffusion test. Commercial disks containing antibiotics were placed on Brucella agar at recommended distances (Gerhardt et al. 1981). P. anaerobius showed such an extreme sensitivity to so many of the antibiotics that the zones of inhibition around the disks conjoined, preventing measurement. Further testing using disks was done in quadrilaterally divided plates to separate physically the influence of one antibiotic on another. As indicated in Table 8 the prototype and the two rifampin-resistant mutants did not exhibit a marked different response to any of the antibiotics with the exception of rifampin. Table 9 presents MIC determinations and it may be seen that rif-res 101 is more resistant to rifampin than is the rif-res 102 strain. This is confirmed by the zones of inhibition around rifampin containing disks (Table 8). A zone of 15 mm indicates a greater resistance of rif-res 101 as compared to 21 mm produced by rif-res 102 and 56 mm as exhibited by the prototype. The rifampin resistant organisms reacted equally to carbenicillin as did the prototype (Tables 8 and 9). Rifampin resistance did not confer carbenicillin resistance and visa versa.

The difference in resistance (Table 9) of rif-res 101 (MIC 6.25 μg/ml) and rif-res 102 (MIC 1.56 μg/ml) might be

explained by considering the means by which rifampin is known to block RNA synthesis. Rifamycins inhibit bacterial DNA-dependent ribonucleic acid polymerase. Since this action is highly specific to bacteria, rifampin is an important tool in the study of RNA biosynthesis and metabolism (Wehrli and Staehelin 1971). RNA polymerase is the enzyme responsible for the transcription step which allows information inherent in the large DNA molecule to be transferred to relatively small RNA molecules. Rifampin-resistant E. coli mutants have been used to map the genes of the RNA polymerase subunits responsible for resistance (Ovchinnikov et al. 1981a). These workers determined that the mutant gene contained an A.T. to T.A. transversion at position 1547 from the beginning of the rboB gene (β subunit). This transversion entails a substitution of a valine residue in the mutant RNA polymerase for an aspartic acid in the normal β subunit. A λ phage carrying this rifampin-resistant gene was obtained through the courtsey of Haywood (1986) for the purpose of comparing DNA from the λ phage with DNA from rifampin-resistant P. anaerobius to elucidate whether or not the mutation was the A.T. to T.A. transversion.

The antibiotic, carbenicillin, is a penicillin

derivative with a carboxyl side chain. The cell wall of infectious bacteria is the target for this antibiotic and this makes the penicillins very important in the treatment of mammals since mammalian cells do not posses a cell wall (Braude 1976). Tables 8 and 9 indicate that P. anaerobius prototype is sensitive to carbenicillin. The mutant carb-res. 103 is about 6 times less sensitive than the prototype, while carb-res. 104 is 12 times less sensitive. The penicillins, cephalosporins and others have recently been designated as β-lactam antibiotics which indicates their mode of action (Bryan 1984). These antibiotics act against cell wall synthesizing enzymes and mutations of the bacteria often involve the production of β-lactamases which inactivate β-lactam antibiotics (Bryan 1984). An extention of this dissertation would be the confirmation of the site of action of the carbenicillin and a comparision of β-lactamase production in the prototype and the two carbenicillin-resistant mutants.

In the past, laboratory workers encountered considerable difficulties in establishing specific identities of a variety of non-spore forming anaerobic bacteria. Virginia Polytechnic Institute developed and refined Gas Liquid Chromatography (GLC) procedures which

enabled them to speciate many anaerobes (Willis 1977). GLC is the process by which a chemical mixture is separated by solution partition between two immisible phases; a liquid stationary phase and a moving gas phase. This technique (Holdeman et al. 1977), was used to determine possible differences between the prototype and rif-res 101 and the effects of P_2 membranes as determined by GLC.

Figure 11 shows the tracing of the metabolic products of the P. anaerobius prototype (Tube A) and 101, the rifampin-resistant mutant (Tube B). P_2 membranes were added to both cultures at a concentration of 0.1 ml/10 ml. The rif-res. 101 strain produced significantly more of the following products than did the prototype: acetic acid, propionic acid, isobutyric alcohol and isocaproic acid. When the tracing of the prototype is compared with that reported by Holdeman et al. (1977) there is definitly a similarity in height of peaks of the products produced by P. anaerobius grown on peptone yeast glucose broth. The tracings of the rif-res 101 strain indicate an increase in volume of metabolites produced but not in the number of different acids and alcohols.

Media controls without the organism were tested with GLC in order to determine the effect of P_2 membranes,

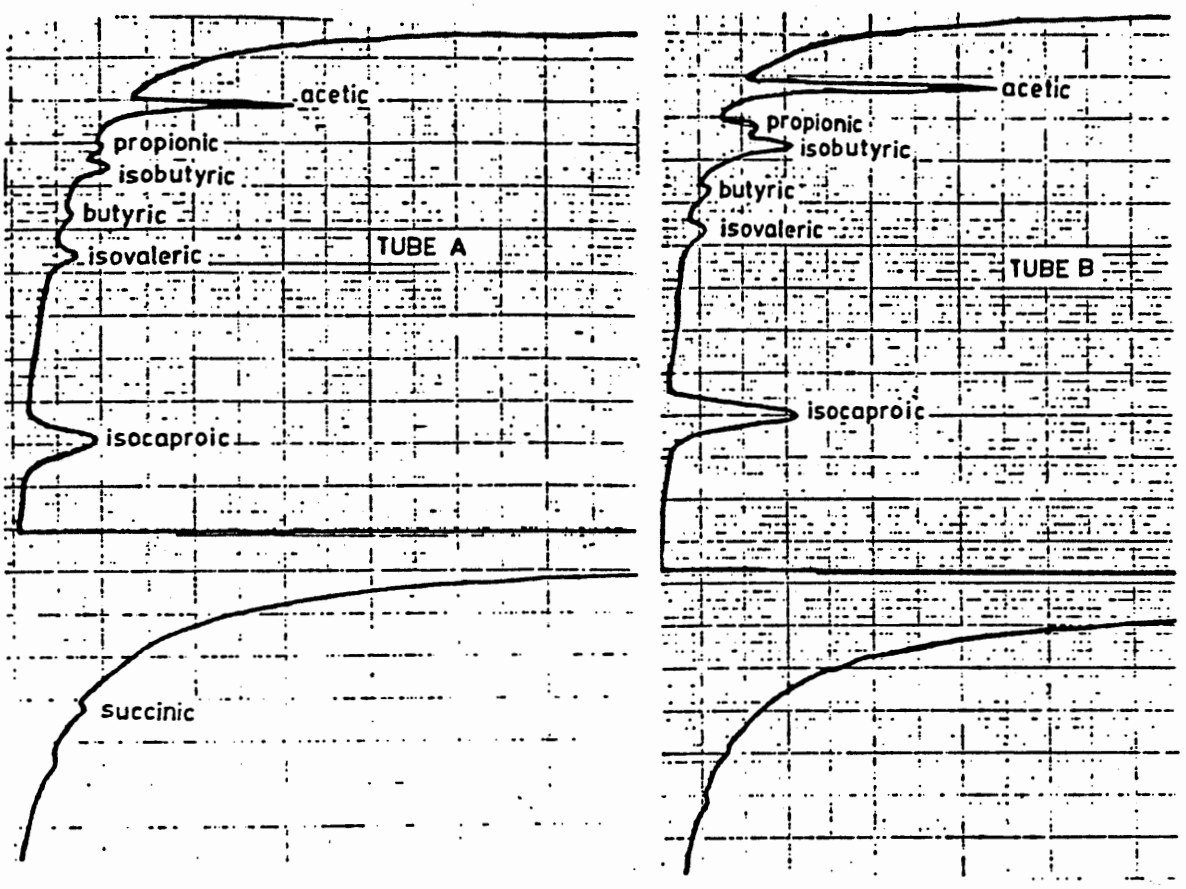

Figure 11--Gas Liquid Chromatography tracings of the products of ptototype Peptostreptococcus anaerobius and the rifampin resistant mutant, rif-res 101. Tube A contains 10 ml Brucella broth, 0.1 ml P_2 membranes and 0.1 ml of the P. anaerobius prototype. Tube B contains 10 ml Brucella broth, 0.1 ml P_2 membranes and 0.1 ml of rif-res 101. The cultures were incubated at 37 C for 24 h.

cysteine-HCl and incubation of P_2 and broth as shown in Figure 12. Acetic, lactic and succinic acids were found in all four tubes and the tracings indicate equivalent amounts despite the contents or treatments. Tubes E and F contained P_2 membranes and broth and exhibited a greater quantity of lactic acid than was produced in the broth control, or broth with cysteine-HCl. Lactic acid was not present in the inoculated tubes which may indicate that this metabolite was used by P. anaerobius.

Results presented in this dissertation disclosed difficulties encountered by using ionizating radiation as a mutagenic agent on strict anaerobes. Peptostreptococcus anaerobius provided additional problems by being extremely sensitive to cysteine and autooxidized products of media leading to the formation of hydrogen peroxide. Compounds resulting from ionizing radiation indicate that this treatment is not suitable as a mutagenic agent for organisms that have little or no internal protection mechanisms against oxygen or its products, namely anaerobes. However, the introduction of P_2 membranes from E. coli B/r ORNL eliminated many of the culturing difficulties associated with anaerobic metabolism and negates radiation induced problems. This allowed genetic changes to occur in the

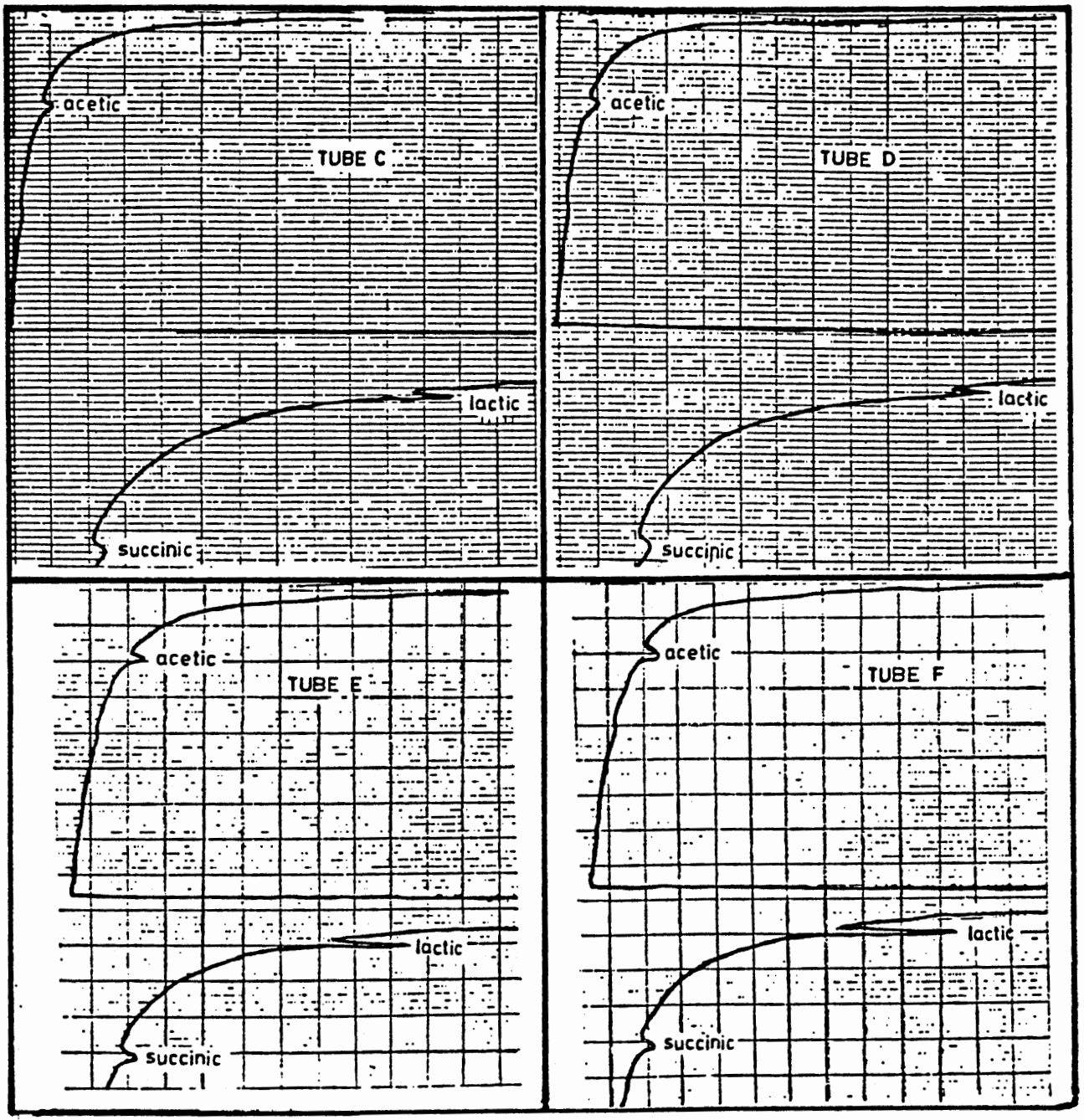

Figure 12--The effect of P_2 membranes, cysteine-HCl and incubation of GLC tracings of Brucella broth. Tube C contained 10 ml of Brucella broth only. Tube D contained 10 ml Brucella broth and 50 µg/ml cysteine-HCl. Tube E contained 10 ml Brucella broth and 0.1 ml membranes. Tube F contained 10 ml Brucella broth, 0.1 ml membranes and was incubated at 37 C for 24 h.

anaerobe, in much the same manner as they are produced in aerobes, without the formation of bactericidal agents such as peroxides and possible superoxide. Adler et al. (1981) formulated the theory that an active membrane cytochrome system could remove free oxygen from media, as was confirmed by the data reported for the experiments with P. anaerobius. The results of this dissertation indicate that the enzyme catalase is also produced by the membranes. These enzymes remove free oxygen and hydrogen peroxide from the irradiated suspension fluid.

The research results obtained indicate that bacterial membranes can be used to facilitate the production of antibiotic mutants. Further research may lead to the development of auxotrophic mutants, to be used in other studies of P. anaerobius metabolism. The effect of membranes in culture techniques used for the identification of other clinically important anaerobiccocci is highly recommended for future studies of medically significant pathogens. Bacterial P_2 membranes could be applied to recombinant DNA techniques and to the mapping of chromosomes of Gram-positive anaerobic cocci. In general, applications of membrane technology in anerobic research would add significantly to our knowledge of anaerobes. This method

makes it possible to conduct experiments with anaerobic bacteria by using basically aerobic techniques and exploring new areas where oxygen and its products previously inhibited all experimentations.

CHAPTER V

SUMMARY

1. The active stable membrane fraction (P_2) of *Escherichia coli* B/r ORNL was used to provide an oxygenless environment for the strict anaerobe *Peptostreptococcus anaerobius* VPI# 4330. This organism was grown in *Brucella* broth with 0.1 ml of the membranes in the presence of oxygen. These anaerobic cocci exhibited a sensitivity to an ether/chloroform extractable substance found in many commercial brands of agar. Gibco agar, agarose or Gellrite inhibited the growth of this anaerobic coccus only slightly. *P. anaerobius* was found to grow on the surface of *Brucella* agar when the solidifying agent was at 0.7%.
2. P_2 membranes function by removing oxygen from the environment by means of the cytochrome system. Membranes, used in this research, were capable of reducing oxygen levels about 20 ppm to zero in 4 min. It was found that there is an active catalase enzyme system in the membrane fraction. This enzyme removes hydrogen peroxide from autooxidized medium and

radiation suspension fluid. Hydrogen peroxide is formed when cysteine-HCl is included in the medium/ fluids and P. anaerobius is rapidly killed.

3. Growth parameters, when membrane culturing techniques were used, could be redefined. The inclusion of P_2 membranes in medium extended both the pH and temperature ranges reported as optimum for this organism (Breed et al. 1948; Buchanan et al. 1974; Sneath et al. 1986). Glucose was found to be a major source of carbon. Sodium glutamate and sodium lactate depressed the growth of P. anaerobius. A combination of cysteine-HCl, hemin and vitamin K stimulated proliferation. The addition of membranes in media resulted in increased growth, whereas, cysteine-HCl added to the media depressed P. anaerobius.

4. When membranes were added to radiation suspension fluids, the total dose of Cesium-137 required to kill 99.999% of the bacterial cells increased from less than 0.32 Coulomb/Kg to over 41.64 Coulomb/Kg. Cysteine-HCl and water used as the suspension fluid, killed this organism in less than 30 sec with or without radiation. When membranes and sodium lactate

were used as the oxygen scavenger in place of cysteine-HCl, 130 min of radiation were required to affect total lethality.

5. Radiation induced mutants were selected, by culturing P. anaerobius on antibiotic gradient plates containing a single antibiotic. Variants in degree of sensitivity to rifampin and carbenicillin were obtained. The rifampin resistant mutants were not resistant to carbenicillin and vice versa. The mutants exhibited an increased resistance to their respective antibiotics as compared to prototype P. anaerobius.
6. Tests used to select, differentiate and characterize the prototypic organism and the mutants were performed with and without the membranes. The Minimal Inhibitory Concentration test, the Kirby-Bauer agar disk diffusion test and Gas Liquid Chromatography evaluation indicated greater growth and increased test sensitivity when membranes were applied. The addition of membranes to the wet mount used on the Allen video-enhanced contrast/ differential interference-contrast microscope enabled the P. anaerobius culture to be viewed in a

living state. The filaments that connect the coccoidal cells become very apparent.

CHAPTER VI

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VITA

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