

STUDIES ON THE ISOLATION AND CHARACTERISATION  
OF GRAM-POSITIVE ANAEROBIC COCCI

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## Abstract

The literature on the classification, pathogenicity and laboratory procedures for the identification of Gram-positive anaerobic cocci (GPAC) belonging to the genera Peptococcus and Peptostreptococcus is reviewed.

A scheme for the identification of GPAC to species level utilising the minimum number of tests was developed. It is suggested that simplified identification procedures, based on antibiotic and liquid susceptibilities, ability to ferment glucose, but avoiding conventional speciation, could be adopted by service laboratories until a definitive classification scheme for GPAC comes into existence.

The recovery of GPAC from swabs and the role of transport media in this respect were studied. None of the transport media tested enhanced the recovery of GPAC; highest recoveries were recorded using plain cotton-wool or albumin-coated swabs held in their containers at room temperature and plated out within 2 hours of loading. Storage of GPAC on blood agar plates in air for periods up to 72 hours prior to anaerobic incubation indicated that they were not particularly oxygen sensitive: all were capable of growth after 24 hours exposure to atmospheric oxygen. The clinical and laboratory implications of these findings are discussed.

A new selective agent for anaerobic cocci, bicozamycin, was incorporated in primary culture media to aid the isolation of GPAC

from oral and vaginal specimens. Low recoveries of GPAC from the oral cavity may reflect the high standards of oral health of the subjects sampled. A greater number of GPAC was recovered from vaginal specimens, particularly from symptomatic women attending a sexually-transmitted disease clinic. The pathological significance of GPAC in the mouth and female genital tract is discussed.

Non-covalently bound cell surface components were extracted from whole cells of GPAC with EDTA and examined by polyacrylamide gel electrophoresis. Strains of Peptostreptococcus anaerobius produced distinctive and virtually identical protein patterns, but considerable heterogeneity was observed in patterns of other biotypes. Western blotting revealed two common antigens within the species Ps. anaerobius, extracted with EDTA, but these are not species-specific. Considerable cross-reactivity between various species was demonstrated by ELISA.

The antigenicity of purified wall and membrane teichoic acids extracted from Ps. anaerobius was investigated by a number of procedures and the composition of these antigens was determined by gas-liquid chromatography.

Preliminary investigations were conducted to determine the potential of each cell extract in the development of a serological classification scheme for GPAC.

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I reserve very special thanks for Mrs May Norquay, whose exceptional skills in the preparation of this manuscript made light of my task.

The results of work presented in this thesis have already been submitted for publication. The references are as follows:

1. Smith G L F, Cumming C G, Ross P W 1985 Gram-positive anaerobic cocci: aspects of their isolation and characterisation. In: Recent advances in streptococci and streptococcal diseases, Reedbooks, Surrey (in press).
2. Smith G L F, Cumming C G, Ross P W Survival of Gram-positive anaerobic cocci on swabs and their isolation from the mouth and vagina. Journal of Medical Microbiology (submitted).
3. Smith G L F, Cumming C G, Ross P W Immunochemical characterisation of the cell surface carbohydrate antigens of Peptostreptococcus anaerobius. Journal of General Microbiology (submitted).
4. Smith G L F, Cumming C G, Ross, P W Analysis of EDTA-soluble cell surface components of Gram-positive anaerobic cocci. Medical Microbiology and Immunology (submitted).



Declaration

The investigations and procedures described in this thesis were performed by the author.

## Clarification of Nomenclature used in this Thesis

The confusion surrounding the classification of Gram-positive anaerobic cocci is renowned in taxonomic circles. At the time of writing this thesis, several classification schemes exist, the results of numerous studies throughout this century. Each was intended to clarify finally the situation. It is pertinent at this stage to explain the nomenclature of organisms that appears in the text. The literature reviewed will describe 'anaerobic cocci', 'anaerobic streptococci', 'peptostreptococci' and 'peptococci'. In many cases, the organisms under study would not in fact be classified as obligate anaerobes by the rigorous standards adopted in the present study. For historical accuracy, the names applied to the organisms described in each paper will be given, rather than attempting to convert them into modern equivalents. Indeed, changes in the taxonomic status of Gram-positive anaerobic cocci have been proposed during the preparation of this thesis, which indicates not only the inadequacies of present schemes, but the continuing efforts by microbiologists to correct these by the application of recently developed techniques. The bacteria isolated and studied for this thesis will be classified within the genera Peptococcus and Peptostreptococcus, according to the Virginia Polytechnic Institute manual (Holdeman *et al.*, 1977).

## LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
CIE	Crossed immunoelectrophoresis
CMB	Cooked meat broth
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
GLC	Gas-liquid chromatography
GPAC	Gram-positive anaerobic cocci
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
NCTC	National Collection of Type Cultures
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PMNL	Polymorphonuclear leucocytes
PPY	Proteose peptone-yeast extract broth
RIE	Rocket immunoelectrophoresis
SCMB	Selective cooked meat broth
SDS	Sodium dodecyl sulphate
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TTBS	Tween-20 Tris-buffered saline
VPI	Virginia Polytechnic Institute

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CHAPTER 1

GENERAL INTRODUCTION



"Where observation is concerned, chance favours only the prepared mind."

Louis Pasteur, 1854

The role of Gram-positive anaerobic cocci (GPAC), both as commensals and potential pathogens in humans has been a subject of much speculation since the end of the last century. Despite this concern, and the major advances in laboratory techniques for the isolation and culture of obligate anaerobes which facilitate the study of such organisms, no definitive classification scheme for GPAC has yet emerged suitable for the diagnostic laboratory. This subject will be more fully discussed later in this chapter, but clearly the situation is unsatisfactory for taxonomists and clinical microbiologists alike who have no absolute criteria by which to speciate GPAC.

In this thesis, studies on the GPAC of human clinical interest, currently assigned to two genera, Peptococcus and Peptostreptococcus, are discussed. Members of these genera form part of the commensal anaerobic flora found in the oral cavity, upper respiratory tract, gut and genital tract; certain species are also found on the skin (Finegold, 1977; Evaldson et al., 1982). In common with many other indigenous anaerobes, they are frequently isolated from infections localised at sites adjacent to surfaces harbouring GPAC as commensals and also from metastatic infections.

Veillon (cited by Hare et al., 1952) first described the isolation of strictly anaerobic cocci in 1893 from cases of Ludwig's angina, perinephric abscess and Bartholinitis. Subsequently, a number of workers noted an association between 'anaerobic streptococci' and infections of the female genital tract (Schwarz and Dieckmann, 1927; Colebrook and Hare, 1933), but it quickly became apparent that these bacteria occurred in a very wide range of infections that varied both in anatomical site and severity. Many researchers have attempted to determine the commensal status of GPAC in humans as well as their occurrence and pathogenic significance in disease.

### 1.1 GPAC as Commensals

Table 1.1 indicates the various sites at which GPAC and other anaerobic bacteria may be found in health. Along with Bacteroides spp, they are prominent in the oral cavity, intestine and vagina. GPAC are often present in equivalent numbers to bacteroides and fusobacteria, e.g. GPAC account for  $10^2$ - $10^6$  bacteria per gram and Bacteroides spp and Fusobacterium spp  $10^3$ - $10^8$  bacteria per gram of ileal contents (Evaldson et al., 1982); GPAC may outnumber Bacteroides spp in the vagina or at the cervix

TABLE 1.1:

GRAM-POSITIVE ANAEROBIC COCCI AND OTHER ANAEROBES AS HUMAN COMMENSALS

	COCCI		BACILLI				
	Gram+	Gram-	Eubacterium	Clostridium	Propioni- bacterium	Bacteroides	Fuso- bacterium
Skin	1	0	+	0	2	0	0
Upper respir- atory tract	1	1	+	0	1	1	1
Mouth	2	2	1	+	+	2	2
Intestine	2	1	2	2	+	2	1
Perineum	1	0	U	0	U	1	1
Urethra	+	U	U	+	0	1	1
Vagina	1	1	+	+	1	1	+

Key: U = unknown; + = irregularly found; 0 = not found/rare; 1 = usually present;  
2 = large numbers.

adapted from Finegold, 1977

- 3 -

(Bartlett et al., 1978); different sites in the oral cavity harbour different bacterial populations (Gibbons and van Houte, 1975), but Evaldson's group (1982) stated that GPAC, Actinomyces spp and facultative Gram-positive cocci predominated at healthy gingival sites (Evaldson et al., 1982). In common with other indigenous microorganisms, GPAC seem to occupy particular ecological niches which suit their metabolic requirements. Their presence is influenced by such variable factors as oxygen tension, pH, hormonal alterations and the host response (Gibbons and van Houte, 1975; del Bene et al., 1977, cited by Evaldson et al., 1982; Bartlett and Polk, 1984).

## 1.2 GPAC in Human Infections

The following account is intended to convey the frequency with which GPAC are isolated, the nature of infections in which they are encountered and speculation on the original source of those GPAC involved.

"Virtually all anaerobic infections arise endogenously" (Finegold, 1977). Breakdown of normal skin or mucosal barriers allows normally commensal organisms access to deeper tissues. If conditions are favourable to the invading organisms, this process

commonly results in infection. Anaerobes are particularly involved, since they greatly outnumber aerobes at several surfaces (Evaldson et al., 1982). Indeed, the flora isolated from such infections often reflects the population of the adjacent surface in health. Whilst GPAC may be the sole organisms recovered from clinical material, as discussed later in this chapter, they are more commonly found with other strict or facultative anaerobes and aerobes. Bornstein et al. (1964) noted the high incidence of 'anaerobic streptococci' in abscesses and infected sebaceous cysts. Wideman et al. (1976) reported that anaerobic cocci, Bacteroides fragilis and Fusobacterium nucleatum accounted for 65% of all anaerobes isolated from their clinical specimens. The incidence of GPAC was second only to Bacteroides spp (Holland et al., 1977) and accounted for 40% of the total anaerobes recovered from clinical material over a nine-year period. Recently Citron (1984) reported a lower figure of 25% for GPAC isolated at the Wadsworth Medical Center, Los Angeles.

### 1.2.1 Infections of the central nervous system

GPAC are commonly isolated from cerebral abscesses (McFarlan, 1943; Heineman and Braude, 1963). With the exception of those arising from septicaemia, many of these lesions are regarded as metastatic foci of infection secondary to anaerobic

infections elsewhere such as the oral cavity, lungs, ears, sinuses or female genital tract. It appears that cerebral lesions often arise at the poorly vascularised, therefore poorly oxygenated junction of grey and white matter which would favour the growth of anaerobic bacteria, but such abscesses usually yield organisms of varying oxygen sensitivities.

Ingham et al (1977a), in a study of cerebral abscesses, secondary to ear infection, found GPAC to be the second most common isolates after Bacteroides spp and emphasised the importance of sound anaerobic laboratory techniques when handling such cases.

#### 1.2.2 Infections of the respiratory tract

Although less frequently encountered since the advent of antibiotics, infections such as Ludwig's angina and Vincent's angina are commonly associated with GPAC, as well as fusobacteria and spirochaetes. In addition, GPAC may be encountered in peritonsillar abscess, chronic suppurative otitis media, mastoiditis and chronic sinusitis. In the latter infection, Frederick and Braude (1974) described GPAC and Bacteroides spp as the commonest anaerobic bacteria encountered. Spread of the original infection may result in intracranial lesions, e.g. brain abscess (Busch, 1984).

Pulmonary infections arising from aspirated oral secretions or gastric contents often yield anaerobes derived originally from the oral cavity. GPAC have often been the predominant isolates from a variety of pleuropulmonary infections, including lung abscess, necrotising pneumonitis, aspiration pneumonia and empyema (Bartlett et al., 1974a,b). Bacteroides melaninogenicus and Fusobacterium nucleatum were also frequent isolates, suggesting an oral origin.

Panwalker (1982) recovered a Peptococcus spp in pure culture from a transtracheal aspirate during the course of a penicillin-resistant infection. Subsequent isolates included Bacteroides spp.

In 1979, Collee highlighted a major discrepancy between British and American experience of anaerobes in pleuropulmonary infections. He urged a careful approach by clinicians to the taking and transportation of specimens which might redress the balance, since there was no lack of technical ability to culture clinically important anaerobes in British laboratories. Collee stated that the commonest anaerobes encountered in pleuropulmonary infections were Bacteroides spp and Fusobacterium spp, but anaerobic and microaerophilic cocci were also frequently isolated; he stressed that, although all these bacteria presented some problems in their growth and isolation, they were certainly not the most difficult.

### 1.2.3 Intra-abdominal infections

The normal human colon harbours large numbers of anaerobes at a level of approximately  $10^{12}$  colony-forming units per gram of faeces. These outnumber aerobes by 1000:1 (Evaldson et al., 1982). It is therefore not surprising that anaerobes are commonly isolated from infections following bowel perforation associated with appendicitis, diverticulitis, chronic inflammatory bowel disease, intestinal surgery or carcinoma of the bowel. Gorbach et al. (1972, cited by Gorbach and Bartlett, 1974) reported that GPAC were present in 32% of intra-abdominal infections, but they were outnumbered by Bacteroides fragilis (65%) and Clostridium spp (60%). However, these organisms were later described (Gorbach and Bartlett, 1974) as a "recurring trilogy of anaerobes" and "major culprits" in such infections.

A number of recent studies have suggested a link between Peptostreptococcus productus, certain Eubacterium spp and Crohn's disease, based on a higher incidence of these bacteria in the faecal flora and serum agglutinins to them found in such patients compared with healthy controls (van de Merwe and Mol, 1980; Wensinck et al., 1981 and 1983). The diagnostic and chemotherapeutic implications of this relationship are being further investigated (Hudson et al., 1984).



Although outnumbered by coliforms and facultative streptococci, GPAC and clostridia are the commonest obligate anaerobes associated with biliary tract infection (Gorbach and Bartlett, 1974). However, the majority of liver abscesses are considered to be caused by anaerobes, an opinion put forward by Sabbaj et al. (1972), who recovered a range of such organisms, including GPAC, from 45% of these lesions.

#### 1.2.4 Infections of the skin and soft tissues

GPAC, referred to as 'anaerobic streptococci' have been associated with several characteristic syndromes:

- i. chronic burrowing ulcer (Meleney and Johnson, 1937)
- ii progressive synergistic gangrene (Brewer and Meleney, 1926)
- iii. crepitant anaerobic cellulitis (Wills and Reece, 1960)
- iv. anaerobic streptococcal myositis (MacLennan, 1943)

GPAC have been recovered from a variety of superficial ulcers associated with vascular disease (Sandusky et al., 1942; Pien et al., 1972).

Of particular relevance to medical and dental practitioners is the incidence of anaerobic infections of the fingers and hands following human bites; equivalent cases may be seen in assailants

who have struck their victims in the teeth (Barnes and Bibby, 1929). Indeed, Meleney (cited by Sandusky et al., 1942) believed that all serious infections following human bites were due to synergy between 'anaerobic non-haemolytic streptococci', fusiforms and spirochaetes.

#### 1.2.5 Infections of bones and joints

Finegold (1977) reported that his group recovered GPAC from several cases of osteomyelitis and again it was emphasised that many of these patients had foci of anaerobic infection elsewhere presumably acting as the source of organisms associated with the osteomyelitis. Finegold further suggested that, in general, anaerobic bacteria do not appear to be significantly associated with septic arthritis, but when they are, GPAC are the predominant organisms.

#### 1.2.6 Infections of the cardiovascular system

Bacteraemia following surgery is of great concern and consequently well documented. Frances and de Vries (1968) and Crawford et al. (1974) found GPAC with other anaerobes in the blood of patients following dental extraction. Urethral dilation (Pien

et al., 1972) and gynaecological procedures (Gorbach and Bartlett, 1974) have also caused GPAC bacteraemia. Gorbach and Bartlett (1974) state that the gastrointestinal and female genital tracts are the commonest portals of entry to the bloodstream for GPAC and Bacteroides spp.

A serious complication of bacteraemia in susceptible individuals is endocarditis and there are several cases in the literature ascribed to GPAC (Colebrook, 1930; Felner and Dowell, 1970; Wilson et al., 1972).

#### 1.2.7 Infections of the urinary tract

Anaerobes, including GPAC, are regarded as commensal in the urethra (Finegold, 1977) but, if they gain access to the bladder, ureters and kidneys, infection may result. Felner and Dowell (1970) demonstrated GPAC in urine and blood cultures from a case of cystitis which led to bacterial endocarditis. Shimizu and Mo (cited by Finegold, 1977) also recovered GPAC in significant numbers from urine in both acute and chronic cystitis. Abscesses associated with urinary tract structures - prostatic, periurethral, perinephric - have also yielded GPAC, as documented by Finegold (1977). However, he stressed that the significance of

many reports of anaerobes in urinary tract infections may be doubtful, since they may only have occurred as contaminants.

#### 1.2.8 Infections associated with malignancy

Although systemic anaerobic infections associated with malignancy are infrequent, localised tumour-associated infections are more common and often involve GPAC. Sandusky et al. (1942) described cases of a lung abscess secondary to bronchogenic carcinoma and an intra-abdominal abscess secondary to uterine leiomyosarcoma from which 'anaerobic streptococci' were recovered in pure culture. Aerobic and facultative organisms, e.g. Pseudomonas aeruginosa, Escherichia coli, may also be recovered from infected tumours. However, the observation that mixed infections of tumours respond as well to antimicrobials directed at the anaerobic component (e.g. clindamycin, tinidazole) as they do to agents also effective against the aerobic component, suggests that anaerobes are of greater significance (Klastersky et al., 1977; Lagast et al., 1982).

(The presence of GPAC in the oral cavity and female genital tract will be discussed in Chapter 4.)

### 1.3 The Occurrence of GPAC in Pure Culture

Occasionally GPAC are isolated in pure culture. One of the original anaerobic cocci described by Veillon in 1893 was the sole organism isolated from a case of suppurative bartholinitis. Sandusky et al. (1942) observed that 17% of 'anaerobic streptococci' recovered from clinical material were in pure culture. McDonald et al. (1937) recovered these organisms in pure culture at post-mortem from blood as well as from the lesion, 11 times out of 23 cases.

Since these early reports the literature has continued to record the occurrence of GPAC as the sole organism present in a variety of infections (Stokes, 1958; Bornstein et al., 1964; Pien et al., 1972). It has been stated that such findings are an absolute indication of pathogenicity (Bornstein et al., 1964; Brook and Walker, 1984). However, as described in the next section, several workers have focused on the association of GPAC with other organisms and there is both clinical and laboratory evidence of synergistic interactions during certain infections.

#### 1.4 GPAC in Synergistic Infections

Meleney (1931) cited Pasteur's observation, of major significance, that anaerobes could grow in the presence of oxygen if aerobic organisms were also present. Weinberg, also mentioned by Meleney, observed that certain combinations of intestinal organisms from acute appendicitis were lethal to animals in smaller doses than the individual organisms in pure culture. Some years later, Hite et al. (1949) attempted to induce necrotising abdominal wall lesions in mice by combining 'anaerobic streptococci', isolated from normal post-partum uteri or cases of vaginal trichomoniasis, with Bacteroides spp and fusiform bacteria. These organisms were mixed either with each other or with aerobic staphylococci and streptococci and injected subcutaneously. Their results, however, indicated that Bacteroides spp had greater influence in synergistic infections than 'anaerobic streptococci'.

The problem of studying such models of infection is highlighted by several reports which attest to the low virulence of 'anaerobic streptococci' in laboratory animals. According to Prevot (1925), injection of live anaerobic cocci in pure culture into guinea pigs and mice resulted in small abscesses. Only when bacteria were combined with Vibrion septique toxin did widespread crepitant lesions arise. All but three of the 57 strains of

strictly 'anaerobic streptococci' from the uteri of cases of puerperal sepsis tested by Harris and Brown (1929) were non-pathogenic in pure culture for animals and similar results were reported by McDonald et al. (1937). Possible explanations for this were that organisms had become avirulent after passage in artificial media and were not "in symbiosis" with other organisms when injected. However, McDonald and his colleagues clearly believed that 'anaerobic streptococci' were incapable of inducing clinical infections alone.

Colebrook and Hare (1933) found that only one out of 13 strains of 'anaerobic streptococci' injected into a variety of laboratory animals resulted in a fatal septicaemia. MacDonald et al. (1956) noted that, although anaerobic cocci persisted throughout serial transmissions in animals, they were not essential for the production of fusospirochaetal-like infections in guinea pigs. In subsequent papers, concerned particularly with periodontal disease as a model for other mucous membrane-associated infections (MacDonald et al., 1960, 1962 and 1963) MacDonald reasoned that such mixed infections were "bacteriologically non-specific, but probably specific in the biochemical sense", i.e. different combinations of organisms could produce substances required for the development of a typical infection. Bacteroides melaninogenicus seemed particularly important since its omission from mixtures of anaerobes, including anaerobic cocci, fusobacteria, spirochaetes and diphtheroids, led to failure of the



inoculum to produce infection. This discovery did not rule out some role for anaerobic cocci in these lesions: MacDonald's group demonstrated that some strains produced a vitamin K analogue, shown to be a growth factor for B. melaninogenicus; some strains produced hyaluronidase and, although this enzyme did not play a significant part in guinea pig infections, the authors speculated that it might be of some importance in periodontal disease.

Mergenhagen and his colleagues (1958) demonstrated that intracutaneous injection of 'anaerobic streptococci', either alone or mixed with a strain of Staphylococcus aureus, regularly produced infection. <sup>in rabbits</sup> In pure culture, injections of  $10^8$  cells of 'anaerobic streptococci' resulted in only small abscesses, although one oral strain that produced hyaluronidase induced a larger, less circumscribed lesion. This was presumably due to depolymerisation of the connective tissue ground substance by the enzyme. As culture supernates of the anaerobes had no effect on tissues following intracutaneous injection, toxin was presumably absent. The authors suggested, however, that this might happen under certain conditions in vivo. Fewer 'anaerobic streptococci' were required to induce infection when injected in a reduced medium or mixed with Staph. aureus; this latter combination resulted in the type of lesion described by Brewer and Meleney (1926), i.e. a central necrotic area, from which Staph. aureus could be recovered, with a spreading, erythematous margin containing only 'anaerobic streptococci'. Thus, although Brewer and Meleney's original



description involved a microaerophilic streptococcus, the same effects could be demonstrated with obligately 'anaerobic streptococci'. From further experiments by Mergenhagen's group it emerged that even a cell-free filtrate of the Staph. aureus culture injected with the anaerobes produced this spreading necrotic lesion, but injected alone had no visible effect on the skin. Thus it seemed that, under certain conditions, 'anaerobic streptococci' alone could invade tissue and cause local necrosis.

Aspects of this early work on synergy have recently been elaborated. In the first of two papers published by Brook et al. (1984) synergistic potential between various aerobic and anaerobic species was assessed in terms of abscess formation and mortality following subcutaneous injection of organisms into mice. Synergy between most GPAC and Bacteroides spp, Fusobacterium spp, Pseudomonas aeruginosa and Staph. aureus was demonstrated; the involvement of the latter organism with 'anaerobic streptococci' in synergistic gangrene has already been described. No interaction occurred between GPAC and Clostridium spp. Inoculation of two anaerobic species together induced abscess formation with minimal mortality, whereas the reverse was true of anaerobes combined with aerobes. The LD50, defined as the number of organisms killing 50% of mice within seven days of inoculation, was higher for most GPAC than for Bacteroides spp, confirming the observations of Hite et al. (1949). The second of Brook's papers (Brook and Walker, 1984) concentrated on the pathogenicity of facultative and anaerobic

Gram-positive cocci, using the same subcutaneous abscess model described earlier. In this instance, however, mice were treated with various antimicrobial agents directed against the infecting components in an attempt to assess the relative importance of each organism in a particular lesion. In only six out of 70 combined inocula were the facultative or anaerobic cocci more important than the coinjected species; combined with Proteus mirabilis, Escherichia coli or Staph. aureus, GPAC were less important. The authors correctly point out that their results are based on combinations of only two organisms, whereas in reality a greater number of species is usually recovered from any infection. However, these workers stated that all GPAC tested were resistant to metronidazole, which contradicts current therapeutic and identification principles (Watt and Jack, 1977; Eykyn, 1983). The first conclusion to draw from this statement is that the strains examined were not in fact obligate anaerobes.

### 1.5 Polymorphonuclear Leucocytes and Anaerobic Bacteria

Several studies have investigated the interactions of anaerobic bacteria with polymorphonuclear leucocytes (PMNL), cells involved in the body's first line of defence against organisms

breaching skin or mucosal barriers. The ability of PMNL under aerobic and anaerobic conditions to kill a variety of facultative and strictly anaerobic bacteria, including Peptostreptococcus anaerobius and Peptococcus magnus, was examined by Mandell (1974). One of the bactericidal mechanisms in these cells, involving hydrogen peroxide, requires oxygen. Both strains of GPAC were phagocytosed and killed equally efficiently by both aerobic and anaerobic PMNL. This suggested that oxygen independent mechanisms were involved, certainly under anaerobic conditions. This was borne out by the observation that individuals with chronic granulomatous disease, whose PMNL oxidative killing mechanisms are defective, experienced no greater incidence of anaerobic infections than normal individuals. Similar conclusions were drawn by Vel et al. (1984) who further suggested that PMNL could vary their bactericidal mechanisms to suit different conditions in vitro and possibly in vivo.

The inhibition of phagocytosis of aerobic organisms by the anaerobic component of a mixed infection has been offered as an explanation for the success of metronidazole to which the anaerobes succumbed in such situations (Lancet, 1980). However, Namavar et al. (1980) took issue with this, citing evidence that intact phagocytes had no greater effect than disrupted phagocytes on the viable counts of mixed B. melaninogenicus - P. mirabilis cultures. Ingham et al. (1977b) reported that both Gram-positive and

Gram-negative anaerobic cocci inhibited phagocytosis less effectively than B. melaninogenicus and B. fragilis.

#### 1.6 Pathological Features associated with Anaerobic Coccal Infections

As previously mentioned, most anaerobes isolated from human infections are resident on skin or mucosal surfaces adjacent to or distant from foci of infection. Circumstances predisposing to endogenous anaerobic infection may be divided into two groups: firstly, those following damage to a normally intact surface - accidental trauma, surgery, malignant neoplasm - which allows the bacteria access to deeper tissues. Secondly, certain systemic conditions and diagnostic or therapeutic procedures carry an increased risk of anaerobic infection. These are shock, vascular disease, diabetes mellitus, corticosteroid therapy, radiotherapy and cytotoxic agents. In many of these situations, tissues may be poorly vascularised or already infected with aerobic and facultative bacteria. The concomitant lowering of the tissue oxidation-reduction potential (Eh) favours the multiplication of anaerobic organisms, hence their designation as "opportunistic pathogens". Tally et al. (1975) have suggested that pathogenic

anaerobes are less oxygen sensitive than non-pathogenic anaerobes since extremely oxygen sensitive (EOS) strains, known to be present in the normal flora (Attebery et al., 1974, cited by Tally et al., 1975), have not yet been recovered from clinical material. Such a situation seems possible, for it would favour the initial establishment of more oxygen tolerant strains in newly invaded tissues.

All the pathological changes in tissues infected with anaerobic organisms involve necrosis to some extent:

- i. gangrenous spreading lesions of the skin: microaerophilic or anaerobic cocci are the predominant isolates;
- ii. necrosis of mucous membranes and adjacent tissues: 'anaerobic streptococci' or Bacteroides spp, commonly both, are frequently involved;
- iii. abscess formation: the centre of the lesion is necrotic;
- iv. thromboembolic disease: suppuration and necrosis involve blood vessels.

The synergistic gangrene of Brewer and Meleney (1926) presented as a very painful, enlarging ulcer, often associated with sutures; the chronic burrowing ulcer described later by Meleney and Johnson (1937) caused little pain, but gradually enlarged to involve subcutaneous tissue, including muscle and bone. Wills and Reece (1960) noted that crepitant anaerobic cellulitis was often associated with diabetes mellitus, possibly due to the high sugar content of the tissues. It was later stressed (MacLennan, 1962;

Anderson et al., 1972) that this acute anaerobic infection be carefully distinguished from clostridial gas gangrene to avoid the drastic surgical intervention required in the latter condition. Diabetic patients are also susceptible to gas-gangrene due to Escherichia coli (Wills and Reece, 1960), which must be identified to allow the correct antimicrobial therapy.

Colebrook and Hare (1933) described "characteristic" findings at post-mortem of women who had succumbed to puerperal sepsis associated with 'anaerobic streptococci'; thrombophlebitis of the pelvic veins, with coagulated blood and pus often extending up to the inferior vena cava. Suppuration was present in the surrounding tissues and embolic foci of infection could be found in the lungs and spleen. Such evidence proved to the authors' satisfaction that, in certain circumstances, 'anaerobic streptococci' were capable of invading and proliferating in human tissues. McDonald et al. (1937) seemed uncertain as to the role of anaerobic cocci in infections. Their paper begins by stating that anaerobic cocci are "rare as pathogens", but of great aetiological significance in "certain inflammatory conditions". In general they found these bacteria in chronic granulomatous lesions, e.g. subdiaphragmatic, pelvic and lung abscesses, which suggested they were of low virulence. However, two rapidly fatal cases (phlegmon of the neck with extensive necrosis and pelvic cellulitis with peritonitis, in which anaerobic cocci were found in



pure culture) led them to conclude that these organisms were significant "whether or not they were the sole pathogens present".

Sandusky et al. (1942) were less equivocal: 17% of 'anaerobic streptococci' isolated by them occurred in pure culture, which in their opinion was strong evidence of pathogenicity. In contrast to other clinical descriptions, the recovery of these organisms was persistently higher than Gram-negative anaerobic bacilli. Abscesses at a variety of sites and wound infections were regular sources of GPAC. Recently Brook et al. (1984) described the histology of experimentally induced abscesses involving GPAC. Each consisted of a central area of necrosis containing bacteria and PMNL, surrounded by a well-formed fibrous capsule.

No specific virulence factors have been identified in GPAC, but Brook and Walker (1985) demonstrated that the possession of a capsule by these bacteria influenced abscess formation in mice: subcutaneous injection of inocula in which more than 50% of Gram-positive facultative or anaerobic cocci were encapsulated resulted in abscess formation, although no mortality was observed. Coinjection of unencapsulated GPAC with other encapsulated bacteria, e.g. Bacteroides spp, or capsular material, produced similar results. The authors suggested that capsular material prevented phagocytosis of encapsulated strains of GPAC or facultative streptococci and allowed their multiplication;

possession of a capsule by GPAC may therefore be a virulence factor.

To summarise, it appears that GPAC are capable of inducing tissue damage under certain circumstances, as witnessed by their isolation in pure culture from various infections. Such instances are, however, numerically uncommon (Pien et al., 1972) and more often GPAC are found with other anaerobes, particularly Bacteroides spp; the precise contribution of GPAC to the overall process is therefore a matter of conjecture. The problems of reproducing clinical situations in vitro exactly are a hindrance that prolongs the controversy surrounding the pathogenic potential of GPAC in humans.



## 1.7 The Classification of Anaerobic Cocci

"An adequate system for the classification of the anaerobic non-haemolytic streptococci is lacking."

(Sandusky et al., 1942)

".... the current classification of anaerobic cocci is unsatisfactory ...."

(Huss et al., 1984).

These quotations leave no room for misinterpretation. In 40 years, no solution to the classification problem has emerged, yet there are good reasons for this. A fundamental obstacle encountered in the taxonomy of anaerobes generally is their frequent occurrence in mixed cultures and the difficulties associated with separating different species in pure culture. It is quite likely that descriptions of many 'new' species are actually based on mixtures of organisms behaving slightly differently to their individual components. GPAC in particular vary in their microscopic morphology, e.g. chain length and Gram reaction. Their colonies on solid media are not distinctive and many species are inert or variable in their biochemical characteristics, therefore it is difficult to determine if isolates are pure or a mixture of species.

Attempts to classify clinically important microorganisms may be justified on the following grounds.

- (1) Epidemiological studies of infectious diseases depend on precise identification of the species involved.
- (2) There is a need to monitor antimicrobial resistance patterns amongst pathogens as they develop.
- (3) The determination of distinctive serological characteristics not only aids diagnosis of infections, but provides the basis for immunisation against pathogenic species.

It is only in very recent years that the most significant accomplishments in anaerobic bacteriology have occurred chiefly as a result of improvements in culture techniques. Also, clinicians have been made aware of the involvement of these bacteria in many infections and can therefore instigate the appropriate treatment more promptly.

Veillon (cited by Hare et al., 1952) was the first to describe strictly 'anaerobic streptococci' in 1893. He isolated these in pure culture from cases of suppurative bartholinitis, perinephric abscess and also from a fatal case of Ludwig's angina in which the 'anaerobic streptococci' were part of a mixed flora.

Both the pus from these lesions and cultures of the isolates were foul-smelling and accordingly he named this organism Micrococcus foetidus. On microscopic examination, the cells appeared in short chains and a further feature was the production of gas in artificial media. Veillon was the first to report the association of these organisms with female genital tract infections.

In 1895, Kroenig and Menge (cited by Colebrook, 1930) independently described Streptococcus anaerobius, which had been isolated from the vaginas of healthy pregnant women. Later, in 1899, they isolated similar strictly 'anaerobic streptococci', from cases of parametrial suppuration and peritonitis; these produced gas in culture media. Another species of obligately 'anaerobic streptococci' which failed to produce gas was named Streptococcus anaerobius micros by Lewkowicz (cited by Hare et al., 1952) in 1901. He isolated this organism from the mouths of suckling infants. In 1905, six strains of strictly 'anaerobic streptococci' which produced gas and odour in culture media were isolated from the vaginas of pregnant women by Natvig (cited by Hare et al., 1952). Ignorant of, or ignoring Lewkowicz' report, he concluded that all such organisms produced gas and odour and grouped them under the name Streptococcus anaerobius. The significance of 'anaerobic streptococci' in puerperal sepsis was established by Schottmuller (cited by Colebrook and Hare, 1933) who in 1910 described the isolation of Streptococcus putridus from such cases; this organism differed from the strains of Kroenig and

Menge in that the former only produced gas when cultured in the presence of blood

Hitherto, no detailed studies or attempts at classification of 'anaerobic streptococci' had been undertaken. Undoubtedly, the first major study in this field was conducted by Prevot, working at the Pasteur Institute in Paris. In 1925 he published his first scheme based on 25 strains of 'anaerobic streptococci' isolated from pulmonary gangrene, appendiceal abscesses and puerperal sepsis. These he divided into three groups on their morphological and biochemical characteristics:

Group A: large streptococci producing odour and gas in artificial media. It consisted of Streptococcus (Micrococcus) foetidus (Veillon, 1893), Streptococcus anaerobius (Kroenig, 1895) and Streptococcus putridus (Schottmuller, 1910).

Group B: small streptococci producing neither gas nor odour, composed of Streptococcus anaerobius micros (Lewkowicz, 1901) and Streptococcus intermedius, a medium-sized diplococcus.

Group C: producing neither gas nor odour and comprising only one member, Streptococcus evolutus (Graf and Witneben, 1907). Prevot described this organism as an 'anaerobe by predilection', that is it required anaerobic conditions for primary isolation, but subsequently became microaerophilic.

Prevot's work with anaerobes continued and in 1948 he produced his 'Manuel de Classification et de Determination des Bacteries Anaerobies' (cited by Thomas and Hare, 1954). Using microscopy primarily, followed by examination of colonial morphology and biochemical tests, particularly fermentations, he allocated anaerobic cocci to the following genera: Neisseria, Veillonella (both Gram-negative), Micrococcus, Staphylococcus, Diplococcus, Streptococcus, Gaffkya and Sarcina. This scheme was used in Bergey's 7th Manual of Determinative Bacteriology (1957) with the addition of three species named by Foubert and Douglas (1948); Gaffkya was omitted. The genus Peptococcus included those GPAC described as staphylococci, micrococci and diplococci and the genus Peptostreptococcus included all those previously known as streptococci.

In 1929 Taylor (cited by McDonald et al., 1937) had studied 11 strains of anaerobic cocci isolated from a variety of infected sites. The citation suggests that Taylor found such variation in carbohydrate fermentation, he concluded that a classification based on this method would be of little use. Such foresight went unheeded, however, and many investigators continued to pursue an identification scheme involving fermentation tests.

Writing in 1933, Colebrook and Hare, who were convinced of an association between 'anaerobic streptococci' and puerperal sepsis and were critical of Prevot's scheme for its reliance on morphology, stated: "We have not as yet any satisfactory criteria

for their [anaerobic streptococci] differentiation and classification." They attempted to correct this with a study of 60 strains of these bacteria isolated from the blood and uteri of puerperal fever cases. Using a range of biochemical tests, including gas production in artificial media and fermentation tests, as well as microscopic and colonial morphology, isolates were allocated to four groups: A and B, which were commonly found and probably equivalent to M. foetidus (Veillon, 1893) and S. anaerobius micros (Lewkowicz, 1901) respectively, and C and D (unnamed) which were rare. However, they experienced some difficulty with carbohydrate fermentation tests and admitted that not all of their isolates were classifiable by these methods.

This was the start of a long association of Hare with this subject and resulted in the eponymous classification scheme for anaerobic cocci as used in the National Collection of Type Cultures catalogue. Hare and his colleagues (Hare et al., 1952; Thomas and Hare, 1954) continued to seek a system for the identification of anaerobic cocci that depended more on biochemical tests than on morphological criteria. Eventually there emerged a scheme of 11 groups of anaerobic cocci, I - IX, including two subdivisions, VIa and b and VIIa and b (Table 1.2).

Having investigated the effects of various fatty acids in culture media on the characteristics of anaerobic cocci used as the basis of Prevot's classification scheme, Wildy and Hare (1953)

TABLE 1.2:

HARE'S CLASSIFICATION OF ANAEROBIC COCCI IDENTIFIED

Hare's group	Possible synonyms
I	S. putridus
II	-
III	S. activus
IV	-
V	V. alcalescens
VIa	-
VIb	-
VIIa	-
VIIb	M. abscedens ovis
VIII	St aerogenes
IX	? St anaerobius (Jungano, 1907)

Key: S = Streptococcus  
M = Micrococcus  
St = Staphylococcus  
V = Veillonella

(from Hare, 1967)



observed considerable changes in the growth, morphology and biochemical activity of certain strains. They concluded that such parameters would be completely unreliable for identification purposes if media were not standardised, echoes of Taylor's earlier experience. It seemed that fatty acids were present as impurities in agar and peptone and varied from one batch to another, leading to inconsistent results. However, certain fatty acids, added in known and standardised amounts, enhanced growth, gas formation and fermentation reactions of groups III, IV and VIII. Since the cells of groups III and IV were also smaller and better formed in the presence of fatty acids, the authors concluded that, for some strains at least, these compounds provided an essential growth factor. It also seemed that a sulphur compound was required for gas production by group I strains. Thus their standard test medium contained peptone, yeast extract, sodium oleate and sodium thioglycollate, the latter substance providing sulphur and also reducing the Eh of the medium.

Reviewing the taxonomic status of his groups, Hare (1967) asserted that groups II, IV, VIa and b, VIIa and IX did not correlate definitely with any known species, although group IX could possibly be Staphylococcus anaerobius (Jungano, 1907). However, group I was probably the same as Streptococcus putridus (Schottmuller, 1910), group III Staphylococcus activus (Prevot and Taffanel, 1945), group V Veillonella alcalescens (Lewkowicz, 1901, named Micrococcus lactilyticus by Foubert and Douglas in 1948,



because of its Gram-positive appearance in young cultures), group VIIb Micrococcus abscedens ovis (Morell, 1911, important in sheep disease) and group VIII Staphylococcus aerogenes (Schottmuller, 1912).

Other studies on the same subject were published throughout this period. Stone (1940) had also attempted to improve matters by studying both the biochemical and serological characteristics of 26 strains of 'anaerobic streptococci' isolated from the uteri of parturient and post-abortal females. Despite using previously untried tests, such as growth in bile and hydrolysis of sodium hippurate, as well as established fermentation tests, he was unable to formulate any distinct scheme from his results. His serological findings will be discussed in Chapter 5.

Also in 1940, Dack reviewed the confused situation surrounding the importance and classification of non-sporing anaerobic bacteria in clinical specimens. In his opinion, descriptions of most species up to that time were so unsatisfactory and the isolation and identification procedures so varied that many different species had been named when a number of them probably represented the same organism. Of non-sporing anaerobes in general he said: "... they have been assigned names and characteristics on the basis of inadequate study". He listed only four species of anaerobic cocci out of the many already in the literature because he felt that only these had been adequately described: Streptococcus putridus (Schottmuller, 1910),

Staphylococcus parvulus (a Gram-negative organism later transferred to Veillonella), Streptococcus anaerobius (Kroenig and Menge, 1899) and Streptococcus foetidus (Veillon, 1893). Dack laid down the challenge that "careful study of this group of bacteria using uniform methods" was required.

After some time this challenge was taken up by Foubert and Douglas who in 1948 published their findings on the taxonomy of anaerobic micrococci within the genera Staphylococcus and Micrococcus. These appeared in Prevot's scheme of eight genera published in the same year, as described earlier. Their study was again based on the morphological and biochemical characteristics of 52 strains and resulted in descriptions of three new species, M. prevotii, M. saccharolyticus and M. variabilis, as well as confirmation of previously described species. They noted that some of their organisms regularly formed tetrads and could thus be classified as Gaffkya anaerobia, as described by Choukevitch in 1911. However, Foubert and Douglas held the opinion that this organism was in fact an anaerobic sarcina, an assertion to be challenged by Ezaki et al. (1983).

In another study, Mergenhagen and Scherp (1957) observed considerable variation in the requirements of anaerobic streptococci for amino acids, purines, pyrimidines and vitamins which they determined by omitting each nutrient from a defined medium. However, since they studied only eight strains, few conclusions could be drawn from their results.

An alternative approach to classification, based on nitrogen metabolism rather than carbon metabolism in the form of carbohydrates and organic acids, was made by Whiteley. In 1957, he reported that anaerobic cocci could be divided into three groups: the first fermented only glycine, the second only purines and the third both purines and amino acids. This was later criticised by Hare (1967) as being of little use for classification, since most pathogenic strains fell into the third group.

The 1970s witnessed a certain 'weeding out' of names: Rogosa (1971) proposed the new family Peptococcaceae, in the order Eubacteriales, to include three genera of obligately anaerobic Gram-positive cocci (Table 1.3).

Organisms in the genus Peptococcus are described by Rogosa as spherical, occurring singly, in pairs, tetrads or irregular masses. Short chains could occasionally be formed, but long chains were not characteristic. Frankly saccharolytic strains were excluded and the type species of the genus was Pc. niger (Hall) Kluver and van Niel.

Rogosa described Peptostreptococcus spp as being spherical to ovoid cells, occasionally lengthened and pointed, in pairs and chains of varying length - a source of confusion with Peptococcus if relying on morphological distinctions. The type species was Ps. anaerobius (Kroenig) Kluver and van Niel. Rogosa went on to describe individual species with their historical synonyms

TABLE 1.3:

CHARACTERISTICS OF THE GENERA PEPTOCOCCUS AND PEPTOSTREPTOCOCCUS

Genus	Chain formation	G + C, mol%	Carbohydrates fermented	Peptones or amino acids as main nitrogen and energy source
Peptococcus	not characteristic	35.7-36.7	- +	+
Peptostreptococcus	characteristic	33.5	+ -	+

Key: + positive

+ usually positive

- usually negative or limited

adapted from Rogosa, 1971

(Table 1.4) in Bergey's 8th Manual of Determinative Bacteriology. Of the genus Peptostreptococcus he concluded that Ps. foetidus and Ps. putridus were synonymous with Ps. anaerobius and that Ps. intermedius was probably a strain variant of Ps. evolutus, but because lactic acid was a major end product, it should be classified in the genus Streptococcus. This is now its accepted classification. He suggested that Pc. prevotii and Pc. asaccharolyticus were identical, bar the latter's production of indole and that Pc. prevotii and its basionym M. prevotii were nomina confusa and should be rejected. He also considered Pc. glycinophilus, Pc. variabilis and Pc. anaerobius to be synonyms of Pc. anaerobius, but West and Holdeman (1973) argued convincingly that Pc. anaerobius was a nomen confusem based as it was on descriptions of frankly different species and its similarity to Ps. anaerobius. This name was formally rejected by a Judicial Commission in 1982.

Thus, a definitive classification of anaerobic cocci by morphological and biochemical criteria persistently eluded the most erudite and stalwart investigators over many decades. Yet, not content to sweep the entire group under the taxonomic carpet in the face of such demoralising historical evidence, other workers have stoically sought alternative approaches to this apparently insoluble problem.

TABLE 1.4:

SYNONYMS OF PEPTOCOCCUS AND PEPTOSTREPTOCOCCUS SPP (Rogosa, 1974)

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<u>Peptococcus</u>	
Pc. niger	M. niger
Pc. asaccharolyticus	St. asaccharolyticus, M. saccharolyticus
Pc. aerogenes	St. aerogenes, M. aerogenes
Pc. activus	St. activus
Pc. constellatus	Dip. constellatus
Pc. anaerobius	Dip. magnus anaerobius, St. anaerobius, Dip. magnus, Dip. glycinophilus, M. variabilis, M. anaerobius, Ps. magnus Pc. glycinophilus, Pc. variabilis

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<u>Peptostreptococcus</u>	
Ps. anaerobius	M. foetidus, S. anaerob [sic], S. anaerobius, S. putridus, S. foetidus, Ps. putridus, Stinkcoccus
Ps. productus	S. productus
Ps. lanceolatus	Coccus lanceolatus anaerobius, S. lanceolatus
Ps. micros	S. anaerobius micros, S. micros
Ps. parvulus	S. parvulus non liquefaciens, S. parvulus

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## 1.8 Alternative Techniques for Classification

### 1.8.1 DNA-DNA homology and base ratios

Probably the ideal basis for the classification of micro-organisms is by their nucleic acid content, since this is a 'blueprint' for every feature of a particular cell and is unlike many other criteria used in taxonomy which are merely a reflection of the cell's response to a particular environment. The bacterial DNA or RNA can be studied by a number of techniques, each of which indicates relationships at different levels of classification. A commonly used method determines the guanine-plus-cytosine content of DNA. However, since this value indicates the quantity of these bases and not their sequence, organisms with similar G + C content may be phenotypically different. A more accurate method of assessing the relatedness of strains and species is DNA-DNA homology, i.e. to what extent the nucleotide sequence in sections of DNA from one strain matches that of another, usually expressed as a binding percentage. The meaning of results, however, depends on the technique used, but in general homologies of 10-60% indicate the same genus; >60% indicates members of the same species. A further improvement measures ribosomal RNA (rRNA)-DNA homology, since rRNA cistrons have evolved less rapidly than DNA cistrons.



All of these techniques have been applied to GPAC: Romond et al. (1966) divided 'anaerobic streptococci' into six groups on the basis of G + C content which was characteristic for various species. To support her proposal that Ps. parvulus should be transferred to the genus Streptococcus, Cato (1983) determined the G + C content of the type strain of this species to be 46 mol%, similar to Streptococcus sobrinus and Streptococcus ferus. Later that year, Cato and her colleagues (Cato et al., 1983) at the Virginia Polytechnic Institute proposed that Pc. glycinophilus should be considered a later synonym of Ps. micros on the basis of similar G + C content (28 mol% and 27 mol% respectively), 84% DNA-DNA homology and identical protein patterns in polyacrylamide gel electrophoresis experiments.

It certainly appears that during 1983-4, the nucleic acids of GPAC held considerable fascination for certain groups. Ezaki et al. (1983) shook the inherently weak foundations of the genus Peptococcus by their revelations of the G + C content and DNA homologies of certain species. He determined that the G + C ratios of Pc. asaccharolyticus, Pc. indolicus, Pc. prevotii and Pc. magnus ranged from 29-30 mol% and as such were closer to Ps. anaerobius (33 mol%), the type species of that genus, than to Pc. niger (51 mol%), the type species of the genus they were presently classified in. DNA-DNA homology between Ps. anaerobius and the four peptococci was in the range 23-36%, indicating a relationship at the genus level. No homology was demonstrated



between the four peptococci and Pc. niger. In the newly described species Ps. asaccharolyticus, DNA homology studies revealed three groups, one of which had different biochemical characteristics to the other two. In the species Ps. prevotii, G + C analyses plus DNA homologies of the type strain and clinical isolates indicated a heterogeneity within this species which contrasted with Ps. magnus strains. This paper also revived interest in the species Gaffkya anaerobia (Choukevitch) Prevot, which had disappeared from classification schemes when its generic name was rejected in 1971. Since its growth was not enhanced by carbohydrate in media, it could not be classified in the genus Sarcina which initially seemed appropriate because the organism regularly formed tetrads. Ezaki and his group (1983) proposed the name 'Peptostreptococcus tetradius', on the basis of its G + C content being similar to the type species of that genus.

If their proposal is accepted, only Pc. niger will remain in the genus Peptococcus, since the transfer of Pc. saccharolyticus to the genus Staphylococcus was proposed by Kilpper-Balz and Schleifer in 1981 (cited by Ezaki et al., 1983) on the basis of biochemical, cell wall and nucleic acid studies.

Huss et al. (1984) also studied various aspects of the nucleic acids of GPAC of known murein types and could divide their strains into seven groups on this basis. Whilst agreeing with the proposed transfer of some species currently in the genus Peptococcus to Peptostreptococcus, they felt that, based on DNA-DNA

hybridisation studies, Ps. micros, Ps. anaerobius and Ps. parvulus should not be included in the same genus. They could not support Cato's proposal (1983) that Ps. parvulus be transferred to Streptococcus. Other results indicated a close relationship between Ps. anaerobius, Eubacterium tenue and Clostridium litusburense, that Hare's groups I (NCTC 9801) and III (NCTC 9814) belonged in the same species and that both of these groups plus group VIII (NCTC 9810) were closely related to Pc. prevotii. Hare's group VIII (NCTC 9820) resembled Pc. asaccharolyticus, group IV Pc. magnus and group IX was related to Ps. micros.

#### 1.8.2 Cellular fatty acids

Analysis of the chemical composition of microorganisms, e.g. gas chromatography of cellular fatty acids for classification, was advocated by Abel et al. (1963) since, under controlled growth conditions, any differences between species are the result of evolutionary adaptations. Wells and Field (1976) applied this technique to peptococci and peptostreptococci and examined 82 strains representing 12 species identified according to the VPI Manual (Holdeman et al., 1977). On the basis of whole cell long-chain fatty acid profiles, they assigned their strains to four groups:

I = Ps. anaerobius

II = Ps. intermedius, Ps. parvulus, Ps. morbillorum,  
Ps. constellatus (all now placed in the genus Streptococcus) and  
Ps. micros.

III = Pc. variabilis, Pc. magnus (considered synonymous by  
Holdeman and Moore in the 2nd Edition of the VPI Anaerobe  
Laboratory Manual, 1973), Pc. asaccharolyticus, Pc. prevotii,  
Ps. productus.

IV = Pc. saccharolyticus (transferred to the genus Staphylococcus  
by Kilpper-Balz and Schleifer, 1981).

The authors concluded that, while of some help in taxonomic studies of GPAC, analysis of long-chain fatty acids could not provide reliable identification to species level. Following this study, Lambert and Armfield (1979) analysed the cellular fatty acid profiles with GLC of strains grown in three different culture media and similarly examined derivatised spent culture media for characteristic end products containing 1-7 carbon atoms. The fatty acids of Pc. magnus were similar to Pc. asaccharolyticus and Pc. prevotii, but these species could be distinguished by their volatile fatty acid end products. Ps. anaerobius was unique in both cellular and metabolic fatty acids. An apparent advantage to

emerge from their study was the overall similarity between profiles from different culture media, therefore different laboratories using different media could compare results. Whilst the technique could distinguish facultative and obligately anaerobic strains and Peptococcus spp from Peptostreptococcus spp, the authors experienced difficulty separating Pc. magnus, Pc. variabilis, Pc. prevotii and Pc. asaccharolyticus. Since this problem may also be associated with biochemical identification procedures, the analysis of cellular fatty acids offers little in the way of progress.

In their study of the nucleic acids of certain peptococci, Ezaki et al. (1983) included evidence that the cellular fatty acid profiles of the four species examined resembled that of the type species Ps. anaerobius, supporting their proposed transfer of these peptococci to the genus Peptostreptococcus. In contrast to Lambert and Armfield, this group noted variation in profiles depending on culture media used. This important feature will be mentioned further in Chapter 2.

### 1.8.3 Cell wall studies

The biochemical and serological characteristics of bacterial cell walls have been cited as taxonomic aids (Cummins and Harris, 1956). The structure of the Gram-positive bacterial cell wall and its serological implications will be discussed in Chapters 5 and 6.

An early study by Bahn et al. (1966) described the chemical composition of the cell walls of Peptostreptococcus spp. However they studied only five strains, one of which was Gram-negative (Ps. elsdenii, now in the genus Megasphaera) and two were Ps. intermedius (now in the genus Streptococcus). The two remaining strains of Ps. putridus, synonymous with Ps. anaerobius according to Rogosa (1974), were similar in their amino acid and sugar contents.

More recently, Weiss (1981) studied the peptidoglycan structure of anaerobic cocci belonging to a number of genera and certain Hare groups. He distinguished 20 peptidoglycan types based on variations in peptide subunits and interpeptide bridges. He endorsed the transfer of Pc. saccharolyticus to the genus Staphylococcus and suggested it was equivalent to Hare's group VIIb; he also agreed with Cato's proposed transfer of Ps. parvulus to the genus Streptococcus (Cato, 1983) but did not support the proposed synonymy of Ps. micros with Pc. glycinophilus (Cato et al., 1983). Weiss further concluded that Hare group IV comprised

Pc. anaerobius, Pc. variabilis and Pc. magnus, and that group VIa included S. intermedius, S. constellatus and S. morbillorum.

Huss et al. (1984) determined murein types of the GPAC included in their nucleic acid study and found considerable variation between closely related strains, particularly Pc. prevotii. Because of this these authors urged caution if attempting to classify anaerobic cocci on this basis.

#### 1.8.4 Enzyme production and substrate hydrolysis

Little work has been reported on the use of pre-formed substrate hydrolysis tests in the classification of anaerobic cocci; this technique has proved useful in the characterisation of certain capnophilic streptococci (Humble et al., 1977). The production of extracellular enzymes was examined by Marshall and Kaufman (1981), but they found such variation within species of GPAC that results were of little use for identification purposes and did not correlate with virulence in vivo. Similar variation was observed by Ezaki et al. (1983) using the API-ZYM system.

Recently, Taylor (1984, Ph.D. thesis, University of London) concluded that such an approach was far superior to carbohydrate fermentation tests in the characterisation of certain GPAC, but modification of available methods was required before it could be regarded as practical for diagnostic laboratories.

The relationships between the two most commonly used classification schemes for GPAC (VPI, CDC), their approved names (Skerman et al., 1980) and proposed reclassifications are outlined in Table 1.5.

The use of microculture and serological techniques, antibiotics, GLC analysis of metabolic end products and polyacrylamide gel electrophoresis in the classification of GPAC will be discussed in later chapters.

To summarise, it appears that despite the devotion of much time and energy to a morphological and biochemical classification scheme for GPAC, nothing in existence at present is universally accepted. The application of more advanced analytical techniques, such as nucleic acid hybridisation and fatty acid analysis offered great promise, but different groups who have advanced evidence of new relationships between various species often contradict each other. In addition, such techniques are expensive, time-consuming and require considerable expertise; they are hardly practical for diagnostic laboratories. The situation remains confused.



TABLE 1.5:

COMPARISON OF APPROVED NAMES WITH CURRENT CLASSIFICATION AND PROPOSED RECLASSIFICATIONS

Approved name <sup>1</sup>	VPI <sup>2</sup>	CDC <sup>3</sup>	Proposed reclassification
Pc. asaccharolyticus	Pc. asaccharolyticus	Ps. CDC group 1	Ps. asaccharolyticus <sup>4</sup>
Pc. glycinophilus	-	-	Ps. micros <sup>5</sup>
Pc. indolicus	Pc. indolicus	-	Ps. indolicus <sup>4</sup>
Pc. magnus	Pc. magnus	-	Ps. magnus <sup>4</sup>
Pc. niger	Pc. niger	-	-
Pc. prevotii	Pc. prevotii	Ps. CDC group 2	Ps. prevotii <sup>4</sup>
Pc. saccharolyticus	Pc. saccharolyticus	Pc. CDC group 2	St. saccharolyticus <sup>6</sup>
Ps. anaerobius	Ps. anaerobius	Ps. CDC group 3	-
Ps. micros	Ps. micros	-	-
Ps. parvulus	Ps. parvulus	-	S. parvulus <sup>7</sup>
Ps. productus	Ps. productus	-	-

- Key: 1. Skerman et al., 1980  
 2. Holdeman et al., 1977  
 3. Dowell and Hawkins, 1974

4. Ezaki et al., 1983  
 5. Cato et al., 1983  
 6. Kilpper-Balz and Schleifer, 1981  
 7. Cato, 1983



### 1.9 Aims of the Present Study

The aims of work conducted for this thesis were as follows:-

- 1 - to develop a procedure for the identification of GPAC based on a minimum number of biochemical and antibiotic sensitivity tests;
- 2 - to develop a technique for the isolation of GPAC, using a new selective agent, from oral and vaginal samples;
- 3 - to attempt to develop a serological classification scheme for GPAC using a variety of cell extracts in both new and well-established immunological techniques.

CHAPTER 2

LABORATORY CULTURE AND BIOCHEMICAL IDENTIFICATION  
OF GRAM-POSITIVE ANAEROBIC COCCI

## Introduction

The previous chapter described the development of various classification schemes for GPAC based on morphological and biochemical criteria. Problems associated with morphological differentiation include the effect of media components on cell size and form (Wildy and Hare, 1953), variable chain length (Mergenhagen and Scherp, 1957) and nondescript colonial appearance. A further difficulty encountered with microscopic observation of GPAC, in common with other Gram-positive bacteria, is the variability of their reaction to Gram's stain.

The following account is intended to highlight particular achievements in the development of suitable conditions for the culture and identification of anaerobic bacteria; their application to the study of GPAC will be stressed where relevant. Conclusions drawn by specialists in the field regarding the efficacy of individual methods will also be described.

## 2.1 Methods for the Culture and Identification of Anaerobic Bacteria

### 2.1.1 Culture media

Satisfactory growth of anaerobic bacteria, as with any microorganisms, can only be achieved if a medium fulfilling their metabolic requirements is provided. This is in addition to the provision of an anaerobic gaseous environment which will be discussed in the following section. Various selective and non-selective media have been described for the recovery and identification of anaerobes from clinical specimens (Holdeman et al., 1977; Wren, 1980) and a number of fundamental principles have been defined over years of study as follows:

- i. It is easier to isolate anaerobes in liquid media than on solid media (Watt and Collee, 1974).
  
- ii. The use of media that have been stored under aerobic conditions should be avoided, unless they are reduced prior to inoculation (Watt and Collee, 1974). Oxygen dissolves rapidly in culture media exposed to air and has a number of deleterious effects: the oxidation-reduction potential (Eh) of the medium is



raised, often to a level incompatible with the growth of anaerobes; certain constituents of the medium are oxidised with the production of amounts of hydrogen peroxide that may prove toxic to bacteria, e.g. cysteine, added to media as a reducing agent (Carlsson et al., 1979); oxygen sensitive enzymes involved in cellular metabolism are inhibited.

iii. Any medium used for the isolation or identification of pure cultures of anaerobic organisms must contain the requisite nutrients to support consistent growth, e.g. agar containing 5-10% human or equine blood and cooked meat broth. The meat particles in the latter medium contain glutathione which serves as a reducing agent (Rutter, 1970).

iv. The isolation of clinically important anaerobes from specimens requires the use of selective media in parallel with non-selective media, otherwise facultative and aerobic species also present in samples will overgrow the anaerobes (Wren, 1980). To ensure the recovery of all anaerobes present, at least two selective media should be used. Finegold et al. (1971) found that nalidixic acid was not superior to neomycin incorporated in blood agar for GPAC, but Wren (1980) proposed that nalidixic acid - Tween agar was suitable for recovering these bacteria.

Recently, Watt and Brown (1983a) suggested the use of bicozamycin (bicyclomycin, CGP 3543E; FR1881), as a selective

agent for anaerobic cocci. Bicozamycin was originally developed as an oral anti-diarrhoeal agent and is bactericidal for aerobic Gram-negative intestinal pathogens, e.g. Shigella spp, Campylobacter jejuni (Vanhoof et al., 1982, cited by Watt and Brown, 1983b). Watt and Brown (1983a) reported that all strains of anaerobic cocci tested by them (including Veillonella spp) grew on blood agar containing  $500 \text{ mg l}^{-1}$  bicozamycin. This concentration inhibited all but three of 58 strains of Gram-negative anaerobic rods and these three strains grew only with difficulty. Addition of  $70 \text{ mg l}^{-1}$  neomycin to bicozamycin blood agar did inhibit some strains of anaerobic cocci, suggesting synergy between the two antimicrobial agents. However, no such inhibition occurred if the concentration of neomycin was reduced to  $30\text{-}40 \text{ mg l}^{-1}$ . Watt and Brown (1983b) tested bicozamycin with further strains of obligate anaerobes, including anaerobic cocci, clostridia and bacteroides. Its activity was unaffected by storage at  $4^{\circ}\text{C}$  for one week or by changes in medium pH. The compound is bacteriostatic for Gram-negative anaerobes below  $400 \text{ mg l}^{-1}$  and much higher concentrations are required to exert a bactericidal effect. The selective inhibition of most Gram-negative anaerobes, particularly Bacteroides spp, can thus be exploited in clinical studies where anaerobic cocci are of prime interest.

v. Media may be supplemented with reducing agents to maintain a low Eh, e.g. cobalt for Clostridium tetani (Dedic and Koch, 1956), thioglycollate, cysteine and dithiothreitol (Moore, 1968); palladium chloride (Aranki et al., 1969); iron filings (Collee et al., 1971).

Culture media for the isolation and identification of anaerobic bacteria fall into two categories: conventional media, used immediately after preparation or reduced prior to use following storage under aerobic conditions, and pre-reduced anaerobically sterilised (PRAS) media. In each case, the aim is to attain an Eh in the medium as close as possible to that of the tissues from which the organisms have been isolated, thus enhancing their chances of survival and isolation. According to Holdeman et al. (1977), "isolation of anaerobes requires more exacting conditions than subsequent culture of the pure isolates" and they cite an Eh of  $\leq -250$  mV for tissues harbouring "actively metabolizing" anaerobes.

Conventional media may have their oxygen content reduced prior to inoculation by either storing under anaerobic conditions for at least 24h, which is suitable for both solid and liquid media, or holding at 100°C for at least 10 min, which is suitable for liquid media. This is at present the most practical form of media for service laboratories or laboratories with no specific interest in anaerobic bacteria. PRAS media, in contrast, are

prepared and sterilised under oxygen-free conditions. Furthermore, exposure to atmospheric oxygen during inoculation is avoided by either injecting culture material into the stoppered tubes through a rubber diaphragm or opening the tube and inoculating the medium under a flow of oxygen-free gas (Beaucage and Onderdonk, 1982). PRAS media have an Eh of  $\leq -150$  mV and incorporate an Eh indicator such as resazurin, which remains colourless up to an Eh of  $-42$  mV at pH 7.0 (Smith, 1975). This system is the basis of the Hungate roll-tube technique (Hungate, 1950) in which stoppered tubes of media form individual anaerobic chambers. This technique will be discussed further in the following section but its advantages include a saving of work space, continuous anaerobiosis, rapid growth of cultures and the facility to examine them without exposure to oxygen. In addition, PRAS media have a good shelf life. For several reasons, however, many laboratories find this system impractical. The preparation of PRAS media requires specialised facilities and, although commercial preparations are available, they are expensive. Many argue that it is more difficult than a conventional system to use, but proponents of the roll-tube technique state that the necessary skills are easily acquired. Colonies are difficult to separate and media incorporating an opaque substance, such as blood, make visual examination of colonial morphology difficult. Moreover, antibiotic sensitivities cannot be determined with discs.



Using either conventional or PRAS media, the speciation of most anaerobic bacteria depends to a great extent on their ability to ferment a range of substrates. These may be added to solid media by flooding the agar with a solution of substrate (Phillips, 1976), or by using them in disc form (Occhionero et al., 1982), and incorporating them in liquid media (Rutter, 1970; Sutter et al., 1975; Holdeman et al., 1977). Carbohydrates are usually added to give a final concentration of  $\frac{1}{2}$ -1% in the medium. Acid production, indicating a positive reaction for a particular substrate relative to a control without substrate, may be detected by pH indicator or pH electrode. Spent culture media may be analysed by gas-liquid chromatography (GLC) for end-products of metabolism (see section 2.1.4).

The procedures described thus far require an oxygen-free environment for the successful growth of organisms. A method designed to obviate this necessity was described recently by Hussain et al. (1984) who successfully identified 93% of anaerobes tested in coculture with Acinetobacter lwoffii, incubated under aerobic conditions. This non-fermentative aerobic Gram-negative bacillus metabolised oxygen in the culture medium, thus allowing the anaerobic bacteria to grow and express their biochemical characteristics. However, some discrepancies between this method and conventional test procedures were encountered; in addition, this coculture method could not be used in the form described for

the isolation of anaerobes from clinical specimens, nor the determination of antibiotic susceptibilities.

A number of microculture systems containing a range of biochemical tests are now available for the characterisation of anaerobes. Their main attraction lies in the saving of time and space. The use of vast numbers of media-containing tubes is avoided and many more specimens can be processed at one time. However, the media supplied with microculture kits must support the growth of a wide range of anaerobic bacteria and a considerable drawback that has been noted (Citron, 1984) is the frequent failure of GPAC to grow in the media provided; this may be due to the small amounts of growth media incorporated in these systems.

Wilkins and Walker (1975) compared conventional media with agar containing fermentable substrates and phenol red (a pH indicator) dispensed into the wells of microtitre plates for the identification of anaerobic bacteria. They observed a 97% correlation between the two methods and 99% reproducibility for the microculture method. Furthermore, the majority of results could be read within 24h. However, possible sources of confusion lay in the reduction of phenol red to its colourless form by non-acid producing anaerobes and the fall in pH caused by some anaerobes in media without additional carbohydrate.

Hansen and Stewart (1976) compared two commercially available kits, API and Minitex, with the conventional procedures

of Dowell and Hawkins (1974). The Minitex system compared most favourably with conventional tests, achieving a 99% correlation, whereas the API system showed an 88% correlation. However, "the most difficult genera to speciate were the Peptostreptococcus and Peptococcus"; indeed 17 out of 23 GPAC could not even be placed in a genus. Greater success was experienced by Lombard et al. (1982) and Buesching et al. (1983) using the Anaerobe-Tek system, in which test results are converted to a six-figure number which is then compared with a pre-existing data base for identification. This system correctly speciated all but one of the GPAC tested, but problems were encountered with other genera including Bacteroides, Clostridium and Fusobacteria. The authors suggested this was due to an inadequate data base.

The problem of poor growth of GPAC in microsystems was discussed by Citron (1984). Her paper described a promising new system employing chromogenic substrates for the rapid detection of preformed enzymes in bacteria. Suspensions of organisms from a plate prepared on a panel of tests are incubated aerobically and reactions read after 4h; no growth of bacteria is required. GPAC displayed considerable activity within the short incubation period.

### 2.1.2 Provision of anaerobic growth conditions

The anaerobic jar is the most widely used piece of laboratory equipment designed to provide an oxygen-free atmosphere for the growth of anaerobic bacteria. Following closure of the jar and removal of most of its content of air, internal catalysts promote the combination of added gaseous hydrogen with residual oxygen to form water. The first anaerobic jar described by McIntosh and Fildes in 1916 incorporated platinum catalysts that required heating for activation. Subsequently the Brewer jar was developed, in which the catalyst was electrically heated (Brewer, 1938-39, cited by Willis, 1977). Stokes (1958) reported isolating anaerobes from clinical samples using modified McIntosh and Fildes' anaerobic jars (Baird and Tatlock Ltd.) bearing palladium-coated alumina catalysts (Deoxo) active at room temperature; jars also had a side arm holding an Eh indicator (methylene blue). It is important to remember that such catalysts may be 'poisoned' by moisture or gases from bacterial cultures, e.g.  $H_2S$ , therefore fresh catalysts should be reactivated at frequent intervals by heating either in a bunsen flame or to  $160^{\circ}C$  for 2h (Watt et al., 1973).

Carbon dioxide gas is now added to enhance bacterial growth Collee et al., 1972) and the mixture of gaseous  $CO_2$  and  $H_2$ , at concentrations of 10% and 90% respectively, are available mixed in a single cylinder. Catalytic activity is indicated by the

development of a secondary vacuum of at least -20 mm Hg; a further test of catalyst efficiency is the inclusion of a highly oxygen sensitive anaerobe such as Clostridium tetani and a strict aerobe such as Pseudomonas aeruginosa (Willis, 1977): the former organism should grow and the latter should not if conditions in the jar are adequately anaerobic, provided the medium does not contain nitrate.

A disadvantage of the evacuation-replacement method is the need for a vacuum pump, gas cylinder and manometer. This can be avoided using disposable CO<sub>2</sub> and H<sub>2</sub> generators, e.g. the GasPak system (Becton, Dickinson UK Ltd.), developed in 1966 by Brewer and Allegeier (1966). Aluminium foil sachets contain two tablets, one of sodium bicarbonate and citric acid which generates CO<sub>2</sub> and another composed of sodium borohydride and cobalt chloride which generates H<sub>2</sub> after the addition of water to the sachet. Polycarbonate jars with room temperature palladium catalysts are available for this system, but standard jars can also be used. A disadvantage of the polycarbonate jar is its lack of venting which makes it impossible to assess catalytic activity manometrically.

Martin (1971) suggested "an inexpensive, but practical and simple" method for creating a suitable atmosphere for clinically significant anaerobes. It involved the storage of media for up to 72h prior to use under a constant stream of CO<sub>2</sub> in an air-tight cabinet. Following inoculation of plates in air, they were placed in an open anaerobic jar also being flushed with oxygen-free gas.

When a jar was filled with plates, a GasPak sachet was added and the jar closed for incubation.

Over several years, Hungate developed a roll-tube technique for the isolation of highly sensitive rumen and sewage bacteria utilising PRAS media (Hungate, 1950; also 1966, 1969, cited by Willis, 1977). As already mentioned, this technique has been adopted by the Virginia Polytechnic Institute group for the isolation and identification of commensal and pathogenic anaerobes (Holdeman et al., 1977).

Anaerobic cabinets and glove boxes have been designed to provide an air-tight, oxygen-free environment for the handling of anaerobic bacteria (Socransky et al., 1959; Aranki et al., 1969). Such equipment combines the advantages of avoiding exposure of cultures to air as in the roll-tube technique, with the use of conventional media and inoculation techniques. A large volume of oxygen-free gas is required to fill these devices, therefore the mixture is usually 10% CO<sub>2</sub>, 10% H<sub>2</sub> and 80% N<sub>2</sub> to avoid the risk of explosion associated with larger concentrations of hydrogen. The mixture of gases circulates over palladium catalysts. Many cabinets incorporate an incubator; alternatively, inoculated media may be removed for incubation in anaerobic jars; the main advantage of the former system is that growth can be easily assessed at any stage without exposure to the air.

There have been a number of comparative studies on the relative efficiency of a standard anaerobic jar procedure against



disposable gas generators, cabinets and glove boxes and PRAS media in roll-tubes. Dowell (1972) obtained similar quantitative recoveries using both Brewer jars and the GasPak system with stock cultures of anaerobes, with the exception of Clostridium haemolyticum and Clostridium oedematiens type B; with these organisms the glove box improved recovery. He included PRAS media and a roll-tube method in his study of clinical isolates, but found no single technique offered any advantage. Similarly, Rosenblatt et al. (1973) found the anaerobic jar as efficient as PRAS media in roll-tubes or an anaerobic chamber, but stressed that this applied to clinical samples placed immediately into anaerobic transport containers, rather than to samples of normal flora containing extremely oxygen sensitive anaerobes. These findings contradicted those of McMinn and Crawford (1970), whose evidence indicated that PRAS fluid media recovered twice as many anaerobes from clinical samples as conventional thioglycollate broths or blood agar plates incubated with GasPak sachets. However, these authors gave no indication that the conventional media used were pre-reduced or fresh; if media had been stored aerobically, this might account for the superior performance of the PRAS media.

Collee et al. (1972) noted variation in the rate of gas production from different GasPak sachets, but stated that the recovery rate of demanding anaerobes using this system was only slightly lower than a standard evacuation-replacement technique. These authors suggested that the GasPak system would be practical

and reliable for busy service laboratories or laboratories with limited experience in handling anaerobes.

Watt et al. (1974) supported Rosenblatt et al. (1973) and took issue with the prevalent opinion that absolutely oxygen-free conditions, provided by a roll-tube technique or anaerobic cabinets, were necessary for the isolation of all anaerobic bacteria. Indeed, they questioned the assertion that air could be completely excluded at all stages using the Hungate method and suggested that an anaerobic cabinet was more reliable in this respect. Watt and his colleagues found no difference in the recovery of Clostridium oedematiens type D, Clostridium tetani type V and Clostridium welchii using either plates stored and inoculated in a cabinet or plates stored in a cabinet, but inoculated at the bench. They concluded that specialised equipment, such as cabinets and roll-tubes, could not surpass a sound anaerobic technique involving regularly serviced jars, fresh catalysts and fresh or anaerobically stored media, in the isolation of clinically significant anaerobes. Recently Berry et al. (1982) found essentially no difference in the recovery of anaerobes from clinical samples using a prototype anaerobic incubator or a standard anaerobic jar procedure.

Although Watt et al. (1973) recommended the use of BTL jars (Baird and Tatlock Ltd.) bearing two or three active room temperature catalysts combined with a standardised evacuation-replacement procedure, this group detected considerable



'jar variation'. They observed significant differences in colony counts of demanding anaerobes, e.g. Clostridium oedematiens types A, B and D, incubated in different jars of the same type. Possible contributory factors, such as mercury contamination (from the manometer) or H<sub>2</sub>S gas from cultures, were eliminated but the age and number of catalyst sachets did appear to be important. Three fresh catalyst sachets in a jar significantly increased colony counts compared to one catalyst, but five did not give better recoveries than three. However, no significant jar variation was noted for other anaerobes tested (including one GPAC) or faecal anaerobes.

In the same year, Watt (1973) had investigated the effect of CO<sub>2</sub> concentrations on the recovery of clostridia and bacteroides inoculated onto solid media. Growth was enhanced and colony size increased up to a concentration of 10% CO<sub>2</sub>, but above this level no improvement was observed. It is interesting to compare this evidence with the studies of Ferguson et al. (1975, 1976) on the production of CO<sub>2</sub> and H<sub>2</sub> by disposable generators. Ferguson and his colleagues found that GasPak tablets used in 2500 ml polycarbonate jars had the potential to create an atmosphere containing 6% CO<sub>2</sub> by volume; this was greater than the 4% stated by the manufacturers, but well below the 10% recommended by Watt. In practice, however, only 0.5-2.7% CO<sub>2</sub> by volume was produced after overnight incubation at 37°C. Although demanding anaerobes such as Clostridium oedematiens type D could be grown using this

system, the CO<sub>2</sub> production was too unreliable for antibiotic sensitivity testing, the results of which depend greatly on the CO<sub>2</sub> content of the environment. In contrast, Ferguson's group found the Gaskit system reliably produced 10% and 12% CO<sub>2</sub> by volume in BTL (3000 ml) and BBL (2500 ml) jars respectively.

In summary, the evidence suggests that the use of fresh or anaerobically stored, suitably enriched solid or liquid media combined with adequately maintained catalysts and anaerobic jars will suffice for the isolation of clinically significant anaerobes from human infections. There is no advantage to be gained by the use of sophisticated, expensive and technically demanding procedures. Anaerobic cabinets, however, are convenient for the storage of large quantities of media and permit examination of cultures at any stage of incubation without exposure to air.

### 2.1.3 Determination of Gram reaction

Certain Gram-positive organisms decolourise more readily than others and, furthermore, the Gram reaction may vary with the age of the culture (Conn et al., 1957, cited by Halebian et al., 1981). Bacteria with a typical Gram-positive cell wall structure may appear Gram-negative within 24-48h of incubation in artificial media (Holdeman et al., 1977). Although a number of modifications to Gram's original method have been suggested, for example that of Kopeloff and Beerman (1922), which is used by Holdeman et al. (1977), these have had limited success with Gram-positive

anaerobes. A number of supplements to Gram staining have been described. To distinguish Gram-positive from Gram-negative bacteria, Halebian et al. (1981) compared the relative efficacies of vancomycin discs (to which Gram-positive organisms are sensitive), colistin discs (to which Gram-negative organisms are sensitive) and a 3% solution of potassium hydroxide. The latter substance disrupts Gram-negative cell walls and releases DNA, resulting in a viscous, 'stringing' solution, but it has no effect on Gram-positive cells. The authors considered it a reliable and rapid method for differentiating the two types of cell, particularly since disc tests require at least 24h for growth of the test organism; vancomycin discs were a suitable alternative, however. Wiegel (1981) proposed the abandonment of the terms Gram-positive, negative and variable, to be replaced by 'Gram type' indicating the cell wall structure for taxonomic purposes, and 'Gram reaction' positive, negative or zero to indicate staining reaction. Wiegel and Quandt (1981) described the polymixin-B-dependent formation of blebs on the surface of Gram-negative cells visualised with an electron microscope. While of interest to taxonomists, the technique could not be described as practical for service laboratories.

#### 2.1.4 Gas-liquid chromatography

Cherry and Moss (1969) described gas chromatography as a rapid and sensitive technique for the analysis of certain microbial cell components or their products that would aid taxonomists and clinicians. The application of gas-liquid chromatography (GLC) to the analysis of cellular fatty acids of GPAC was described in Chapter 1.

Phillips et al. (1976), Ladas et al. (1979) and Watt et al. (1982a,b) have all emphasised the value of GLC in the analysis of pus, which provides a rapid presumptive diagnosis of anaerobic infection. Certain short chain volatile fatty acids are characteristic of anaerobes that are subsequently isolated from cultures of the pus - proprionic, butyric, isobutyric, valeric and isovaleric acids. GLC profiles cannot specifically identify a particular organism. Watt's group, however, did find a correlation between the presence of isocaproic acid and Ps. anaerobius in the specimen; samples containing no acid or only acetic acid virtually always yielded facultative species on culture.

Nord and Berg (1981) applied GLC specifically to the cellular and metabolic fatty acids of GPAC isolated from clinical specimens. They concluded, however, that the technique could not

definitely speciate these organisms without additional biochemical information.

A number of authors have reported the effects of growth medium composition on GLC profiles of metabolic fatty acids. Deacon et al. (1978) concluded that glucose in culture media influenced the amounts of acids produced by saccharolytic Bacteroides spp, but not the range. Lombard (1979) spoke of the "profound influence" of carbohydrates, peptones and growth supplements present in culture media and later Turton et al. (1983a,b) also noted the effects of glucose on a strain of Ps. anaerobius (ATCC 27337): this organism produced increased amounts of acetic acid and ethanol as glucose in the medium increased from 0 - 1.5%. They also found that differences in GLC profiles of fatty acids from a single species grown in different medium to be as great as different species grown in the same medium. Turton's group recommended the addition of 1% glucose to media for fermentation tests or the use of media known to have a low glucose content.

#### 2.1.5 High-performance (high-pressure) liquid chromatography (HPLC)

HPLC has been used to analyse the volatile and non-volatile fatty acids produced by certain anaerobes including Ps. anaerobius (Guerrant et al., 1982). This technique has the advantages of a one-step sample preparation along with detectors that are more sensitive to non-volatile fatty acids. Guerrant and his colleagues concluded: "The method has potential for routine use within the diagnostic microbiological laboratory."

#### 2.1.6 Current identification schemes for GPAC

There can be no dispute over the major contributions to anaerobic bacteriology made by the Virginia Polytechnic Institute (VPI) group. The first edition of their laboratory manual appeared in 1972; the fourth and current edition, edited by Holdeman et al., appeared in 1977. The identification scheme is based on Gram reaction and morphology, biochemical tests and GLC analysis of metabolic fatty acids and alcohols. The fourth edition does not list characteristics of several recently isolated (in 1977) intestinal anaerobes, including Coprococcus spp (Moore and Holdeman, 1974). In addition, Ps. parvulus had not been

transferred to the genus *Streptococcus* (Cato, 1983) and *Pc. saccharolyticus* was not yet classified in *Staphylococcus* (Kilpper-Balz and Schleifer, 1981, cited by Ezaki et al., 1983). The classification of GPAC, as it appears in this manual, forms the basis of the corresponding section in the Approved List of Bacterial Names (Skerman et al., 1980).

The isolation and identification methods for anaerobes in the VPI manual involve the use of PRAS media, but the editors state that "any method which provides a low Eh and protects organisms and media from exposure to oxygen will probably be effective". They recommend the addition of 0.02% Tween-80 to media used for Gram-positive organisms since it enhances the growth of many of these bacteria and has inhibitory effects on almost none. Short chain metabolic fatty acids and alcohols produced by organisms grown in chopped meat-glucose or peptone yeast extract-glucose broths are identified by GLC.

A criticism that may be levelled at the GPAC identification scheme is that many species are defined on the basis of results from very few strains, e.g. *Ps. productus* 2, *Pc. indolicus* 4, *Pc. niger* 8. However, this may be a reflection of the frequency with which they are encountered in clinical samples compared with other species isolated in greater numbers, e.g. *Ps. anaerobius* and *Pc. magnus*. A further problem with this scheme, as with its predecessors, is the dearth of positive reactions displayed by GPAC. The scheme offers a list of 52 tests for the identification



of anaerobic cocci in which most species of GPAC are inert, weak or variable. The exceptions are Ps. productus and Ps. parvulus which ferment a wide range of carbohydrates, but are not common clinical isolates. The asaccharolytic GPAC, some of which are frequently associated with infection, do not produce distinctive profiles with GLC, therefore their identification to species level is difficult; errors or variations in identification between operators are inevitable. Two species showing characteristic GLC profiles are also the easiest to identify by conventional means: Ps. anaerobius and Pc. niger produce isocaproic and caproic acids; the former is sensitive to sodium polyanethol sulphonate (see section 2.1.7) and the latter produces pigmented colonies after prolonged incubation.

Thus, although the scheme of Holdeman et al. is probably the most commonly used of those presently available for the classification of GPAC, it has not solved the problems encountered by previous workers in this field. Other current classification schemes for GPAC, based on morphological and biochemical criteria, are also less than adequate: Dowell and Hawkins (1974) at the Center for Disease Control in Atlanta divide clinically significant anaerobic cocci into five groups (see Table 1.5). Sutter et al. (1975) include antibiotic sensitivities (vancomycin for Gram-positive cocci and colistin for Gram-negative cocci) in the Wadsworth Anaerobic Bacteriology Manual.

Recently, Watt et al. (1984) described a simplified identification scheme for anaerobic cocci that they consider ideal



for diagnostic laboratories. This scheme recognises four groups (A-D) based on glucose fermentation and susceptibility to liquid and vancomycin. The authors admit that it is of "doubtful taxonomic validity", but advocate it as a base from which further tests can be conducted.

#### 2.1.7 Antibiotic sensitivities

A number of antibiotics, used in disc form with cultures on solid media, have been proposed as aids to the identification of GPAC. As mentioned earlier, sensitivity to vancomycin (5 µg) indicates a Gram-positive organism (Sutter et al., 1975). The in vitro use of metronidazole (5 µg) to indicate the nature of an isolate (only obligate anaerobes are sensitive) has paralleled its clinical success in the treatment of anaerobic infections. Watt and Jack (1977) proposed criteria for the definition of anaerobic cocci which included sensitivity to metronidazole. Of 91 strains tested, all but one had MIC values of  $\leq 2.5 \mu\text{g ml}^{-1}$ ; the remaining strain had an MIC value of  $5 \mu\text{g ml}^{-1}$ . The wide range of MIC of metronidazole ( $\leq 0.2 \rightarrow 100 \mu\text{g ml}^{-1}$ ) cited by Chow et al. (1975) was questioned by Ingham et al. (1975), who felt that such results were more likely to reflect "taxonomic uncertainty, rather than genuine differences in sensitivity to metronidazole within different species".

The use of novobiocin (5 µg) discs was proposed by Wren et al. (1977) as a means of distinguishing the genera Peptococcus and Peptostreptococcus: organisms belonging to the latter genus were sensitive whereas peptococci were resistant. The authors admit, however, that very few peptostreptococci other than Ps. anaerobius were tested; this presumptive distinction of the genera is not infallible (Watt et al., 1984).

Watt et al. (1979) detected a wide range of susceptibilities within reference strains and clinical isolates of anaerobic cocci to such antimicrobials as penicillin, erythromycin, lincomycin and clindamycin. This group also reported that 26 strains of biochemically heterogeneous anaerobic cocci had displayed 25 antibiotic susceptibility profiles. Such an approach, therefore, did not aid speciation.

Watt and Brown (1975) and Phillips (1982) highlighted a number of factors that should be borne in mind when testing the antibiotic sensitivities of anaerobic bacteria. A fall in medium pH, due to CO<sub>2</sub> in the atmosphere and fermentable carbohydrate in the medium, will decrease the activity of certain antibiotics, including erythromycin and novobiocin. Watt and Brown (1975) urged that the control of media constituents should be considered before the exclusion of CO<sub>2</sub> from the gaseous mixture since the former had a more pronounced effect on media pH and because of the latter's growth enhancing effect. Phillips (1982) found that partial anaerobiosis resulted in an increased MIC value for

metronidazole and decreased zones of inhibition around metronidazole discs. He recommended using the same or a closely related species as a control organism in antibiotic susceptibility tests.

Although not an antimicrobial agent, another substance of value in the presumptive identification of Ps. anaerobius is sodium polyanethol sulphonate, commonly known by its trade name, liquoid (Roche Products Ltd., P.O. Box 8, Welwyn Garden City, Herts, AL7 3AY). Liquoid is a heat-stable anticoagulant often added to blood culture bottles to reduce the bactericidal action of blood and thus increase the recovery of organisms. However, Hoare (1939, cited by Kocka et al., 1974) observed inhibition of the growth of certain organisms in blood cultures containing liquoid, notably anaerobic cocci. Kocka et al. (1974) compared liquoid with sodium amylosulphate, another anticoagulant, and reported that only Ps. anaerobius was inhibited by liquoid, but sodium amylosulphate had no effect. Theoretically therefore, sensitivity to liquoid demonstrated by GPAC was a presumptive identification of Ps. anaerobius. In the same year, however, Graves et al. (1974) found that liquoid delayed the growth of a few strains of Pc. prevotii and Pc. magnus in liquid media; on solid media growth was reduced around discs containing this substance, but nonetheless visible. Ps. anaerobius showed 12-14 mm zones of complete inhibition.

Wideman et al. (1976) were aware that Ps. anaerobius accounted for one-fifth to one-third of anaerobic cocci recovered from clinical specimens by them and were anxious to evaluate the reliability of the liquoid disc test as a presumptive identification for this organism. Their results did indeed confirm this, but they observed variations in zone size depending on inoculum dilutions and culture media used. Although a few fresh clinical isolates not subsequently identified as Ps. anaerobius showed zones of inhibition around liquoid discs, these were smaller than zones displayed by equivalent strains of Ps. anaerobius, therefore incorrect identification was unlikely. Wilkins and West (1976) determined that proteose peptone, gelatin and casein present in commercial media might inhibit the effect of liquoid on cultures of Ps. anaerobius. They recommended that media should be tested prior to use; Columbia blood agar was a suitable medium in this situation.

## 2.2 Aims of This Study

The intention of the following study was to determine a minimum number of conventional tests that would speciate GPAC. These tests comprise Gram reaction and cell morphology, fermentation of certain carbohydrates, antibiotic and liquid susceptibilities and analysis of metabolic fatty acids by GLC. The bacteria used include fresh, unidentified clinical isolates, previously identified GPAC and NCTC strains, identified as Hare groups. The scheme developed is utilised later in studies of GPAC isolated from the oral cavity and vagina (Chapter 4).

### 2.3 Materials and Methods

**Bacteria:** the organisms tested belonged to three separate groups:

- i. fresh clinical isolates on blood agar plates identified only as 'anaerobic cocci', from the Royal Infirmary of Edinburgh;
- ii. GPAC previously identified to species level at the Western General Hospital, Edinburgh, and the Department of Bacteriology, University of Edinburgh; these were received as lyophilised cultures or cooked meat broth cultures in screw-capped bottles;
- iii. lyophilised cultures of NCTC strains, listed as 'anaerobic cocci' and classified according to Hare (1967).

A total of 56 strains was examined, comprising 21 fresh clinical isolates, 18 previously identified strains and 17 NCTC strains.

**Culture of bacteria:** cooked meat broth cultures were firstly subcultured onto 5% human blood agar (BA) plates and incubated anaerobically for 48h at 37°C to ascertain viability and purity; subsequently ten colonies were picked from the agar and transferred to 10 ml modified Robertson's cooked meat medium (CMB; Watt, 1972) pre-reduced by boiling for 10-15 min. Cultures received on blood agar plates were assessed for purity by a Gram film. From pure

cultures, ten colonies were picked and transferred to CMB as described. Lyophilised cultures were reconstituted with pre-reduced CMB, then transferred to 10 ml of the same medium. After incubation these CMB cultures served as stock cultures for further tests.

**Anaerobic procedure:** all CMB and BA plates were placed in anaerobic jars (Baird and Tatlock Ltd.) carrying three fresh Deoxo catalysts. The evacuation-replacement method of Collee et al. (1972) was used, but CO<sub>2</sub> and H<sub>2</sub> (10% and 90% respectively) were drawn premixed from a single cylinder. Jars were incubated at 37°C for 48h unless otherwise stated.

For the identification of GPAC, stock cultures were used in the following tests:

**Gram films:** 5 ml of pre-reduced thioglycollate medium (Appendix 1) containing 1% glucose was inoculated with 0.02 ml CMB culture and incubated anaerobically for 48h at 37°C. The Gram reaction and morphology of organisms in this medium were recorded.

**Fermentation of sugars:** the following sugars were added to 5 ml pre-reduced proteose peptone-yeast extract medium (PPY; Holbrook et al., 1977): glucose, sucrose, maltose and lactose, at a final concentration in PPY of 1%. Each PPY then received one drop of

CMB culture. A further PPY without additional carbohydrate (PPYC) was also inoculated to serve as a control. Cultures were incubated anaerobically for 24h at 37°C, then assessed visually for turbidity; if growth was satisfactory at this stage, culture pH was determined using a pH electrode. If growth was not adequate, cultures were reincubated for a further 24h before pH was determined. A positive fermentation was recorded if the pH of a sugar-containing PPY culture was  $\geq 0.5$  units below that of the PPYC.

Sensitivity to liquid and antibiotics: swabs of CMB cultures were plated onto BA using Stokes' method (1960). The following discs were added: liquid (100 µg), metronidazole (5 µg), novobiocin (5 µg), vancomycin (5 µg). A strain of Ps. anaerobius known to be sensitive to each substance was inoculated onto the plates as a control. Plates were incubated anaerobically at 37°C for 24-48h, after which the inhibition zones of test strains were compared with those of the control organisms.

Growth in the presence of bile: growth on agar containing 0.05% bile distinguished Pc. prevotii and Pc. indolicus from the remaining species of GPAC which will not grow on such a medium (Dr Brian Watt, personal communication).

Indole production: 2 ml of a CMB culture were mixed with 0.2 ml xylene and 0.2 ml Erlich's reagent. Indole production was



indicated by the appearance of a deep pink band at the top of the mixture. An overnight culture of E. coli in CMB was included as a positive control.

**Pigment and coagulase production:** a loopful of CMB culture was inoculated onto a BA plate which was then incubated anaerobically for 7 days at 37°C. The appearance of black-pigmented colonies provisionally identified the organism as Pc. niger. A positive slide coagulase test, including positive and negative control organisms, distinguished Pc. asaccharolyticus (coagulase-negative) from Pc. indolicus (coagulase-positive).

**Analysis of metabolic fatty acids:** to identify volatile and non-volatile short-chain fatty acids produced by individual strains, control and glucose-containing PPY cultures described earlier (PPYC and PPYG) were treated as follows: 5 drops of 50% H<sub>2</sub>SO<sub>4</sub> were added to PPYC and PPYG and well mixed. The PPY cultures were then centrifuged at 1000g for 15 min and the supernate collected for analysis of volatile fatty acids. Methyl derivatives of non-volatile fatty acids were prepared according to Holdeman et al. (1977). Samples (1 µl) were then analysed by GLC using a column of SP1220 on Chromosorb WAW at a temperature of 147°C. Gas flow rates were as follows: oxygen-free nitrogen (the

carrier gas) 35 ml min<sup>-1</sup>; hydrogen 35 ml min<sup>-1</sup>; air 525 ml min<sup>-1</sup>. A Pye Unicam 104 chromatograph with a flame-ionisation detector (200°C) was used. Standard solutions containing volatile and non-volatile fatty acids (see Appendix 1) were injected into the column for comparison prior to samples. The amount of a particular acid in a sample was calculated from the size of the corresponding peak traced on a recorder by the following equation:

Concn of acid =

$$\frac{\text{Peak height of test-Peak height PPYC}}{\text{Peak height of standard}} \times \text{Concn of Standard}$$

Amounts of acid produced, represented by lower case or capital letters, e.g. acetic a, A, are designated as follows:

- 0 - 0.2  $\mu\text{mol ml}^{-1}$  = acid not produced
- 0.21- 1  $\mu\text{mol ml}^{-1}$  = trace amount, (a)
- >1 - 10  $\mu\text{mol ml}^{-1}$  = significant amount, a
- >10  $\mu\text{mol ml}^{-1}$  = major product, A

Confirmation of anaerobic status: In addition to the above tests, CMB were subcultured onto BA plates and incubated both aerobically and in air plus 10% CO<sub>2</sub> for 7 days at 37°C; failure to grow under these conditions would establish their status as obligate anaerobes, rather than microaerophilic or capnophilic bacteria (Watt and Jack, 1977). The scheme is summarised in Table 2.1.

TABLE 2.1:

IDENTIFICATION PROFILE OF GRAM-POSITIVE ANAEROBIC COCCI

Species of GPAC	Fermentation			Sensitivity				Growth on bile	Indole	Coagu- lase	GLC profile adapted from (Holdeman et al, 1977)*
	Glu	Suc	Mal Lac	Mz	Liq	Nov	Van				
Ps. anaerobius	+	-	+	-	S	S	S	variable	-	-	A (iv ib p ic b l s)
Ps. productus	+	+	+	+	S	R	S	-	-	-	A S (1 p)
Ps. parvulus	+	-	+	+	S	R	S	-	-	-	L a (s)
Ps. micros	-	-	-	-	S	R	S	-	-	-	A (1 s)
Pc. indolicus	-	-	-	-	S	R	R	+	+	+	A B p
Pc. asaccharolyticus	-	-	-	-	S	R	R	-	+	-	A b (s l)
Pc. magnus	-	-	-	-	S	R	R	-	-	-	A (1 s)
Pc. prevotii	-	-	-	-	S	R	R	+	-	-	A (b L s p)
Pc. niger	-	-	-	-	S	R	R	-	-	-	G B iv ib (a l s)
Pc. saccharolyticus	+	-	-	-	S	R	R	-	-	-	A

Key: \* The flame-ionisation detector used in this study does not detect formic acid.

S = sensitive; R = resistant.

## 2.4 Results

A total of 56 strains was examined. The identification profiles of ten selected strains of GPAC are shown in Table 2.2. All GPAC in this study were both metronidazole- and vancomycin-sensitive. Inhibition of growth around discs ranged from 24-45 mm for metronidazole, 12-30 mm for vancomycin, 15-19 mm for novobiocin and 12-17 mm for liquid. Those strains subsequently classified as peptostreptococci formed long chains in thioglycollate-glucose broth, whereas peptococci appeared as single cells, short chains and clusters. The number of each species of GPAC identified is shown in Table 2.3. The predominant species recovered from clinical specimens, both fresh and previously identified, were Ps. anaerobius and Pc. magnus. No strains of Ps. productus, Ps. parvulus, Pc. indolicus or Pc. niger were identified.

Five of the NCTC strains designated 'anaerobic cocci' were resistant to metronidazole and grew in air plus 10% CO<sub>2</sub> within 7 days at 37°C. These were NCTC 9806 (Hare group VIa), 9807 (VIb), 9809 (VIIb), 9817 (VIa) and 9819 (VIIb). Two NCTC strains (9805 and 9816, both Hare group V) and two anaerobic cocci isolated from a dental abscess appeared Gram-negative and were also vancomycin-resistant; GLC analysis established their identity as Veillonella spp. The identities of the remaining NCTC strains are

TABLE 2.2: IDENTIFICATION PROFILE OF SELECTED STRAINS OF GRAM-POSITIVE COCCI

Strain+	FERMENTATION										Growth on bile	Indole	GLC profile	Identification
	Glu	Suc	Mal	Lac	Mz	Liq	Nov	Van	S	S				
NCTC 9801	-	-	-	-	S	R	R	R	S	S	-	+	b 1	Pc. asaccharolyticus
NCTC 9808	+	-	-	-	S	R	R	R	S	S	-	-	b s	Pc. saccharolyticus
WGH 151	-	-	-	-	S	R	R	R	S	S	-	-	a 1	Pc. magnus
WGH 152	-	-	-	-	S	R	R	R	S	S	-	-	a	Pc. magnus
WGH 163	-	-	-	-	S	R	R	R	S	S	+	-	b 1	Pc. prevotii
EDH 2	-	-	-	-	S	R	R	R	S	S	-	-	a 1	Pc. magnus
GS 3	-	-	-	-	S	R	R	R	S	S	-	-	a (s)	Pc. magnus
GS 4	+	-	+	-	S	S	S	S	S	S	-	-	A (i b s b) i v i c	Ps. anaerobius
GS 15	-	-	-	-	S	R	R	R	S	S	-	+	a	Pc. asaccharolyticus
56393	+	-	+	-	S	S	S	S	S	S	-	-	A (i b b i v s) i c 1	Ps. anaerobius

+ None produced pigment or coagulase.

TABLE 2.3:

TOTAL NUMBERS OF GRAM-POSITIVE ANAEROBIC COCCI IDENTIFIED

Species	No. identified
Ps. anaerobius	12
Pc. magnus	18
Pc. prevotii	6
Pc. asaccharolyticus	10
Pc. saccharolyticus	1
Total	47

shown in Table 2.4. These proved to be obligately anaerobic Gram-positive cocci by their sensitivity to metronidazole and vancomycin and their failure to grow in air plus 10% CO<sub>2</sub>. Here groups I and III were identified as Pc. asaccharolyticus, IV and IX as Pc. magnus and VIII as Pc. prevotii.

In the majority of cases, identification of strains in this study agreed with the species names previously assigned to them. However, the behaviour of strains WGH 151, WGH 152 and WGH 163 (Table 2.2) did not conform with their previous identities of Ps. anaerobius, Pc. prevotii and Ps. micros respectively: WGH 151 was resistant to liquoid and novobiocin and the GLC profile was not characteristic of Ps. anaerobius; WGH 152 did not grow on bile-agar, nor did it produce butyric acid; WGH 163 was novobiocin-resistant, grew on bile-agar and produced butyric acid. In several other cases, previous identities were only confirmed after GLC analysis, i.e. fermentation and antibiotic sensitivity tests were insufficient for identification to species level. However, certain GLC profiles were uncharacteristic in their absence of acetic acid, e.g. NCTC 9801, NCTC 9808 (Table 2.2). Indeed, the latter strain proved to be an atypical strain of Pc. saccharolyticus; this species usually produces large amounts of acetic acid only. Acetic acid was a major end product of all strains of Ps. anaerobius which produced a range of volatile fatty acids including isocaproic.



TABLE 2.4:

IDENTIFICATION OF NCTC STRAINS OF OBLIGATELY ANAEROBIC COCCI

NCTC No.	Hare group	Identification
9801	I	Pc. asaccharolyticus
9803	III	Pc. asaccharolyticus
9804	IV	Pc. magnus
9805	V	Veillonella spp
9808	VIIa	Pc. saccharolyticus
9810	VIII	Pc. prevotii
9811	IX	Pc. magnus
9814	III	Pc. asaccharolyticus
9815	IV	Pc. magnus
9816	V	Veillonella spp
9820	VIII	Pc. prevotii
9821	IX	Pc. magnus

## 2.5 Discussion

A total of 47 strains of GPAC was examined. All were identified to species level. Ps. anaerobius proved the easiest to identify because of its sensitivity to liquoid and a characteristic GLC profile. For many of the asaccharolytic strains it was only after GLC analysis was compared with previous biochemical and antibiotic sensitivity tests that speciation was possible. However, considering the unreliability of some of the tests, described in the introduction to this chapter, some strains may have been misidentified. The use of previously characterised strains was designed to test the reliability of the procedures and in most cases identities were confirmed.

The colonies formed by GPAC on blood agar incubated anaerobically are not distinctive; they range in size from a pinpoint to 2 mm in diameter and are grey or translucent, occasionally with a white centre. Although chain formation by members of the genus Peptostreptococcus is considered variable, therefore an unreliable characteristic by which to distinguish this genus from Peptococcus (Mergenhagen and Scherp, 1957), a good correlation between morphology and genus was observed in the present study. The formation of long chains by peptostreptococci in thioglycollate-glucose broth may have been enhanced by the presence of 0.075% agar in the medium. Novobiocin has been

recommended as an aid to distinguishing these two genera (Wren et al., 1977), but its reliability has been questioned (Watt et al., 1984). Results with novobiocin in this study agreed with previous identifications, therefore it was decided to omit growth in thioglycollate-glucose broth from the final scheme.

All but one of the saccharolytic GPAC involved in this study were later identified as Ps. anaerobius. All members of this species were sensitive to liquoid. These strains grew well enough in fermentation media for pH to be assessed after 24h incubation. Asaccharolytic strains were given 48h incubation prior to pH measurement, although growth was not greatly increased by this longer incubation period. A pilot study showed that the addition of rabbit serum to PPY at a final concentration of 2% in the medium did not significantly enhance the growth of GPAC. Gas production in PPY cultures was noted, but was of no use in the speciation of isolates.

It was concluded from this study that the number of fermentation tests could be reduced to the use of glucose only, along with a control for comparison. The saccharolytic GPAC examined in this study all fermented glucose and could be further distinguished by liquoid sensitivity, in the case of Ps. anaerobius, novobiocin resistance in Pc. saccharolyticus and GLC profile in general. Additional fermentation tests may be carried out if results are dubious.

The identification of NCTC strains was confirmed by Mr Robert Brown (Department of Bacteriology, University of Edinburgh) and further compared with results reported by Taylor (1984). There was disagreement in two cases: Taylor identified NCTC 9811 and 9821 (both Hare group IX) as Ps. micros. In the present study, these strains were identified as Pc. magnus, based on the appearance of large Gram positive cocci and novobiocin resistance. However, since both these characteristics vary and the GLC profiles of the two species are similar, misidentification is possible. Although their names imply that Pc. magnus and Ps. micros should be easily distinguished by comparison of cell sizes in Gram films, Hare (1967) indicated that Ps. micros (referred to as Streptococcus micros) assumes a larger size on subculture.

Metronidazole distinguished obligately anaerobic Gram-positive cocci from microaerophilic or capnophilic NCTC strains. This observation confirmed the report of Watt and Jack (1977). Three of the five metronidazole-resistant strains were identified by R. Brown as Streptococcus intermedius (two strains of Hare group VIa) and Streptococcus morbillorum (VIb). This suggests that these strains should not be included in the section for anaerobic cocci defined by Holdeman et al. (1977). It also serves to highlight the need for a well-defined reference collection of strictly anaerobic cocci.

It was decided to omit the coagulase test from the final scheme. Pc. indolicus, the only coagulase-positive species, is rarely encountered in clinical specimens, but has been recovered from individuals working with sheep (Bourgault and Rosenblatt, 1979). Any indole-positive peptococci are almost certainly Pc. asaccharolyticus. In addition, Pc. indolicus produces propionic acid, which Pc. asaccharolyticus does not.

The need for GLC analysis to identify strains is a drawback because of the additional time and effort involved. Indeed, not all laboratories possess such equipment. In the present study, a small number of strains did not produce typical profiles. The absence of acetic acid from analyses of some asaccharolytic strains is difficult to account for, but it did not interfere with identification. The growth medium (PPYG) contained 1% glucose, as recommended by Turton et al. (1983a). The ether extraction method for volatile fatty acids outlined by Holdeman et al. (1977) is designed to reduce column contamination by cellular proteins, but it was not followed in this study for a number of reasons: it is more complex than the acidification procedure used; the SP 1220 column used can withstand considerable contamination before its performance is impaired; ether tends to evaporate from samples, thus concentrating the acids present and invalidating quantitative analyses.

In conclusion, the method developed for the identification of GPAC involves the minimum number of biochemical tests, but

relies heavily on susceptibility to antibiotic and liquid discs, plus GLC analysis of acid end products. The reliability of the scheme was demonstrated by including strains of known identity. It proved simple to identify Gram-negative cocci and microaerophilic or capnophilic Gram-positive cocci and eliminate them from the study. A major obstacle in the study of GPAC however is that, even under ideal growth conditions, many of these bacteria isolated from clinical specimens are biochemically inert in such cases. Speciation is usually based on a series of negative results., This is not a satisfactory basis for the identification of bacteria. Furthermore, these tests are time-consuming and service laboratories are often reluctant to carry them out, particularly with the uncertainties surrounding the status of many species. Workers who do attempt speciation of GPAC may obtain dubious or contradictory results for certain tests. If growth is satisfactory, such results often indicate a mixture of species hitherto unsuspected because of microscopic and colonial similarities. From such a mixture it is extremely difficult to purify the component strains. The scheme outlined in this study suffers from the same shortcomings as any present or previous using similar criteria, which confirms the opinion of most workers in the field that an alternative system for the classification of GPAC must be found. Until this is forthcoming, however, currently available schemes will still be used, despite their inadequacies.

CHAPTER 3

SURVIVAL OF GRAM-POSITIVE ANAEROBIC COCCI  
ON SWABS AND IN AIR

## Introduction

A prerequisite to the culture and study of any clinically significant organism is a reliable means of transferring it from the patient to the laboratory. In the case of anaerobes, consideration must be given to their likely oxygen sensitivity. This chapter describes the development of various transport methods and their application to anaerobic bacteria.

### 3.1 Swabs and Transport Media

The use of plain cotton-wool swabs for the recovery of bacteria was first recommended towards the end of the 19th century (Councilman, 1893; Preston, 1896; Hewlett and Nolan, 1896; all cited by Cooper, 1957). It was much later, however, that attention was paid to the survival of bacteria on these. Downie (1940) found that meningococci on cotton swabs remained viable for up to 24h if the swabs were in contact with a small amount of blood agar in tubes. Later, Pollock (1948) discovered that cotton-wool fibres contained long-chain unsaturated fatty acids that inhibited



the growth of some bacteria, but enhanced the growth of others; charcoal absorbed fatty acids and neutralised these effects. Rubbo and Benjamin (1951) observed that different types of cotton-wool varied in their effects on Gram-positive bacteria. Furthermore, moisture accelerated the death of these organisms on cotton swabs, but was essential for the survival of Gram-negative enteric pathogens. However, the survival of Streptococcus pyogenes, for example, on non-absorbent cotton swabs coated with ox serum was "constant and prolonged", regardless of the type of cotton-wool used and was further enhanced by storage of swabs at 4°C. The protection afforded by serum was not due to neutralisation by lipids or toxic water-soluble swab components. Rubbo and Benjamin stated that "organisms leaving their host in serous exudates will live longer than those discharged in secretions of low protein content". These authors cited van Reimsdijk (1924) who, like Downie (1940), observed that diphtheria bacilli survived up to 24h on swabs in contact with agar. Some years later, Bartlett and Hughes (1969) criticised the continuing use of plain cotton swabs and recommended in their place cotton swabs coated with bovine serum albumin and sterilised by steam. Although these swabs permitted overgrowth of certain bacteria, they were non-absorbent and had no antiviral properties. Of the total number of Streptococcus pyogenes cells that could be recovered from swabs used to inoculate blood agar plates immediately after loading, Bartlett and Hughes could recover 92% from similar swabs

stored for 4h, and 61% from swabs stored for 24h at room temperature.

The method of sterilisation of swabs appears to affect the survival of organisms. White (1965), Mair and McSwiggan (1965) and Dadd et al. (1970) concluded that gamma radiation altered cotton swabs in some way that reduced the recovery of Streptococcus pyogenes. This was disputed by Anderson (1965) who asserted that it was the low pH of cotton-wool prepared for swabs, rather than irradiation that was responsible for low recoveries of this organism. Serum-coated swabs were effectively neutral therefore less damaging to bacteria, whereas irradiated swabs had a pH of 4.0.

The early findings of Van Reimsdijk and Downie led to the development of transport media. Ideally, a transport medium should maintain the viability of all organisms in contact with it until they can be transferred to suitable growth media; it should not allow multiplication of any species which would invalidate quantitative assessments and obscure the presence of particular pathogens. Thus, a transport medium should contain no nutrients and be poised at an Eh compatible with the survival of any anaerobes encountered. The addition of agar reduces convection currents of air that might oxidise bacteria or medium components. Stuart et al. (1954) were faced with the rapid death of gonococci on swabs in transit due to drying in air, but they overcame this problem by placing swabs in a soft, non-nutrient agar medium.

This contained sodium thioglycollate, which minimised the problem of oxidation, and charcoal, which absorbed toxic fatty acids in agar. However, since it also absorbed methylene blue included as an Eh indicator, the charcoal was incorporated in swabs previously boiled in phosphate buffer. This medium allowed the recovery of organisms from 90% of samples for up to 24h and the authors surmised that it might be of "general applicability to bacteriological diagnosis" where a delay in plating out organisms was anticipated. In 1957, Cooper confirmed the value of Stuart's transport medium: various pathogens tested survived on swabs for several weeks. However, in contrast to the earlier findings of Rubbo and Benjamin, he stated that for Streptococcus pyogenes and Corynebacterium diphtheriae the "rapid loss of moisture is undoubtedly deleterious to these organisms".

Amies (1967) described modifications to Stuart's transport medium prompted by observations that coliforms and other Gram-negative bacteria multiplied in this medium if plating out was delayed. It seemed that these bacteria produced a glycerophosphatase which released glycerol, a nutrient, from glycerophosphate in the medium. Amies therefore omitted glycerophosphate but reinstated charcoal in the new version. Barry et al. (1972) found that Amies' medium without charcoal (for ease of preparation) greatly increased the recovery of a range of pathogenic bacteria (including an unnamed strain of Peptostreptococcus) held on plain cotton or calcium alginate swabs

compared with similar swabs left in dry containers. In common with most studies, this group observed a considerable decrease in viability of bacteria with storage in transport medium. In the authors' opinion, anaerobes survived better in cotton-wool fibres than on the surface of calcium alginate swabs; the strain of Peptostreptococcus tested was, however, "very sensitive to brief exposures to air".

Ellner et al. (1973) compared commercial Amies' medium (without charcoal) to PRAS transport media containing cysteine, thioglycollate and dithiothreitol. The latter, however, offered no significant advantage, even with Clostridium novyi type B, a fastidious anaerobe. This group chose Amies' medium as opposed to gassed-out oxygen-free tubes because of the former's availability and familiarity to clinicians. Yrios et al. (1975) decided that a further comparative study was required to assess the value of CO<sub>2</sub>-filled tubes, air-filled tubes and a transport medium for the storage of aerobic and anaerobic bacteria in transit to laboratories. The medium used in this study was a further modification of Stuart's that lacked glycerophosphate (Grover et al., 1965) and the test organisms comprised stock cultures of varying oxygen-sensitivity. A considerable loss of viability over 24h was recorded for anaerobic strains stored on cotton swabs in dry CO<sub>2</sub>-filled tubes and air-filled tubes, but Ps. anaerobius could still be recovered after 48h storage in the transport medium.

Cary and Blair (1964) developed a transport medium similar to Stuart's from which Salmonella and Shigella species could be recovered after 49 days and Pasteurella pestis after 75 days of storage. This medium has been recommended for the transport of anaerobic bacteria on swabs (Watt and Collee, 1974; Finegold, 1977) and successfully used in studies of the female genital tract microflora (Bartlett et al., 1978; Bartlett and Polk, 1984).

Ross (1977) and Ross and Lough (1978) found that recovery rates of various upper respiratory tract bacteria decreased as the time that organisms were held on cotton or albumin-coated swabs increased, but holding swabs at 4°C significantly prolonged viability. This confirmed the work of Rubbo and Benjamin (1951). Cumming et al. (1981) obtained similar results with a range of beta-haemolytic streptococci. They stated that transport media did not enhance, but may endanger the survival of these bacteria in transit to the laboratory. In their study on the recoveries of a number of anaerobic bacteria from simulated exudates taken up by albumin-coated swabs, Collee et al. (1974) also noted decreased viability with time. Initially, however, they could only recover 3-5% of original inocula from swabs plated out onto blood agar immediately after loading. They subsequently produced evidence showing that these low recoveries were due mainly, but not entirely, to the retention of bacteria on swabs. Both Collee et al. and Ross (1977) demonstrated greatly increased recoveries of bacteria if swabs were agitated or squeezed with forceps in

nutrient broth. Thus, low recoveries were in the main not due to death of bacteria caused either by toxic materials in the swabs or exposure to air, although the anaerobes tested (Clostridium welchii and Bacteroides spp) <sup>had been previously</sup> considered to be sensitive to oxygen. These authors cautioned, however, that such in vitro experiments could not simulate the in vivo environment, regarding physiological, immunological and interbacterial influences, from which organisms of clinical interest were removed.

Moller (1966) described four transport media, VMGI-IV, which provided good recoveries of facultative streptococci and non-sporing anaerobes from endodontic specimens. Gastrin et al. (1968) compared VMGIV with Stuart's original medium and their own modified version of Stuart's medium, SBL; this contained thioglycollic acid, cysteine hydrochloride and methylene blue. They studied the survival of a range of pathogenic bacteria on plain cotton swabs previously boiled in buffer (pH 7.4), treated with charcoal and sterilised by gamma radiation. They did not, however, include any non-sporing anaerobes in their study. Conventional Stuart's and SBL media, particularly if held at room temperature, prolonged the survival (72h) of strains tested, which included gonococci and meningococci. VMGIV medium also performed well in this respect, but a major drawback observed was the multiplication of most test bacteria. Syed and Loesche (1972) reported similar findings using VMGII medium to store human dental plaque bacteria. They compared VMGII and SBL medium, as described



by Gastrin's group, with a reduced transport fluid (RTF) containing dithiothreitol. Over 14 days of storage, VMGII gave the best recoveries of bacteria from healthy mouths, but RTF was the best for bacteria from diseased periodontal sites; this was explained by the low Eh of RTF creating a suitable environment for anaerobes which predominate in such areas. Bacteria taken from patients with active caries multiplied in VMGII. The authors urged caution with the use of VMGII, particularly in quantitative studies. They recommended RTF held at 4°C for oral samples because it was non-nutrient and had a low Eh. In contrast, Holbrook et al. (1978) recommended VMGII for the recovery of Bacteroides melaninogenicus from the oral cavity. However, this was not a quantitative study and specimens were processed rapidly, therefore the effect of VMGII on bacteria held in it for prolonged periods could not be assessed.

A number of synthetic alternatives to cotton-wool have been tested for their suitability as swabs, e.g. dacron, rayon, fortrel. Hollinger and Lindberg (1958) recommended dacron swabs stored in silica gel for the recovery of pharyngeal streptococci. Ellner and Ellner (1966) compared the survival times of Streptococcus pyogenes, C. diphtheriae, H. influenzae and N. gonorrhoeae held on a variety of synthetic swabs and plain cotton swabs. The latter swabs were tested with and without Stuart's transport medium. From their results, it appeared that plain cotton swabs used with Stuart's medium allowed the survival of all organisms tested for up

to 120h, with the exception of gonococci which remained viable for up to 21h. Synthetic swabs offered no advantages. Dadd et al. (1970) observed good recoveries of group A streptococci from swabs composed of synthetic fibres, but calcium alginate swabs were unsuitable. None of the papers reviewed presented data on the performance of synthetic swab materials with GPAC. Indeed, there is a relative scarcity of literature describing the survival of anaerobes on swabs compared to the considerable work on facultative and aerobic bacteria that has been reported. Although the use of swabs to collect specimens likely to contain anaerobes is criticised (Jousimies-Somer and Finegold, 1984), many service laboratories continue to receive such samples. Investigations into the survival of anaerobic bacteria on swabs would therefore seem prudent.

### 3.2 The Transport of Anaerobes

It is generally thought that specimens likely to contain anaerobes require specialised handling techniques to avoid loss of significant organisms. Holdeman et al. (1977) recommend the following procedures: specimens to be cultured anaerobically should be processed as quickly as possible without refrigeration



and without enrichment or transport media. The efficiency of a particular regime is indicated by the recovery of all morphotypes observed in Gram films of the original specimens. Aspirated fluid or pus may be transferred to CO<sub>2</sub>-filled tubes for transport, but a syringe itself is just as effective if air has been expelled prior to aspiration. A rubber stopper should be placed over the needle after use and the specimen processed within 30 min. Samples of tissue may also be placed in CO<sub>2</sub>-filled tubes. If swabs are used, the VPI group recommend a two-tube system where both tubes contain CO<sub>2</sub>, a salt solution and agar and are closed with butyl rubber stoppers. The first tube holds the uninoculated swab which is quickly transferred after sampling to the second tube for transport to the laboratory. Thus, swabs are held in an oxygen-free environment before use and exposure of bacteria to air is kept to a minimum thereafter.

Aspirated material or excised tissue are regarded as ideal specimens from which to recover anaerobic bacteria (Holdeman et al., 1977), since the organisms are still in contact with their immediate in vivo environment. These surroundings provide Eh, pH and interactions with other organisms that are conducive to the survival and replication of the bacteria sought. The use of swabs in such situations is thought to incur a greater loss of anaerobes, partly due to removal from protective surroundings and also from exposure to atmospheric oxygen. The situation for service laboratories was neatly summed up by Martin (1971):

Whatever method one considers for recovery of anaerobic bacteria, the major factors limiting the efficacy are the proper collection and transporting of specimens to the laboratory. If neither of these is adequate, there is little chance of isolating these bacteria.

### 3.3 Oxygen Sensitivity of Anaerobes

A number of studies of anaerobic cocci have included organisms that require strictly oxygen-free conditions for primary isolation, but which subsequently become aerotolerant on repeated culture. It is essential, therefore, to define exactly what is meant by the term 'obligate anaerobe'. Morris (1976) applied this description to organisms whose metabolism did not depend on molecular oxygen and which were in fact incapable of growth in air. Watt and Jack (1977) formulated the following definition for anaerobic cocci: "cocci that grow well under satisfactory conditions of anaerobiosis and do not grow on suitable solid media in 10% CO<sub>2</sub> in air even after incubation for 7 days at 37°C".

Several theories have been proposed to account for the oxygen intolerance of certain organisms (discussed below), as well as their surprising, but regular occurrence on surfaces exposed to air, e.g. skin, oropharyngeal mucosae. It has already been stressed that consideration must be given to the Eh of a culture

medium, which must be sufficiently low for growth to occur. However, different anaerobes vary in their oxygen sensitivity and Eh requirements. In his classic study, Loesche (1969) defined three groups of anaerobes as follows:

i. Strict anaerobes: these would not grow on the surface of reduced agar in the presence of  $>0.5\%$   $O_2$ . They survived only 60-80 min exposure to air.

ii. Moderate anaerobes: these grew in 2-8%  $O_2$  and could still be recovered after 6h in air.

iii. Microaerophiles: these could multiply in atmospheres containing 5-10%  $O_2$ .

The only anaerobic coccus included in Loesche's study, Peptostreptococcus elsdenii (a Gram-negative organism, now in the genus Megasphaera) was classified as a moderate anaerobe. Strict anaerobes included Treponema spp and Clostridium haemolyticus, whereas Vibrio spp were classified as microaerophiles. Loesche admitted, however, that since he had tested only stock strains which had been subcultured on several occasions, these may have been less oxygen sensitive than fresh clinical isolates. This point was taken up by Tally et al. (1975), who tested 57 anaerobes recently isolated from clinical samples. They defined oxygen

tolerance as the time that isolates survived exposure to air prior to anaerobic incubation and oxygen sensitivity as the greatest concentration of oxygen in which organisms would grow. The strains examined included Bacteroides spp, Fusobacterium spp, Clostridium spp and anaerobic cocci. All survived 8h exposure to air on the surface of blood agar plates. Three of the six Peptostreptococcus spp and six of the eight Peptococcus spp survived 72h. The oxygen sensitivity of GPAC varied, however, with different isolates categorised as strict, moderate and even aerotolerant. (The latter term corresponds with 'microaerophilic' as defined by Loesche.) Inoculum size did not significantly influence results. These studies have important implications for the handling of clinical specimens containing anaerobes. It must be borne in mind that the oxygen tolerance of anaerobes in pus, aspirate or on swabs, possibly in contact with transport media, may differ considerably from that displayed by the same organisms on blood agar. Tally's group failed to isolate any extremely oxygen sensitive organisms from clinical material, despite using an anaerobic chamber. This led them to suggest that pathogenic anaerobes were more oxygen tolerant, therefore could be safely handled in air following primary isolation, if not before. However, more care would be required in studies of normal flora containing extremely oxygen sensitive forms.

The Eh of a medium tends to decrease as metabolising organisms release reducing substances into their surroundings.

The Eh value rises as medium pH falls and this has been shown to affect bacterial growth, e.g. at pH 6.4, the growth limiting Eh for Clostridium welchii is +160 mV, but at pH 6.0, it is +106 mV (Hanke and Bailey, 1945, cited by Hentges and Maier, 1972). The Eh can be lowered by the addition to media of reducing agents such as cysteine or thioglycollate.

The growth limiting factors of different anaerobic species vary in nature. Some organisms are limited by the Eh of a medium regardless of environmental oxygen concentrations. Others will grow in media poised at a high Eh as long as oxygen is excluded from the environment. Hentges and Maier (1972) record that Bacteroides spp tested by them grow at an Eh of +370 mV in the absence of oxygen.

The toxic effects of oxygen may be direct or indirect. The former result from oxidation of intracellular substances vital to metabolism, from the increase in medium Eh and the 'diversion' of the cells' intrinsic reducing capabilities away from normal biochemical pathways. The indirect effects of oxygen are mediated by substances arising from its interaction with medium constituents or the bacteria themselves. The most significant of these substances are hydrogen peroxide ( $H_2O_2$ ), superoxide anion ( $O_2^-$ ), singlet oxygen and hydroxyl radicals.

Hydrogen peroxide, formed by the combination of  $O_2^-$  radicals with hydrogen ions, is removed by its conversion to water plus molecular oxygen in a reaction catalysed by the enzyme catalase.

Originally, lack of catalase, leading to toxic accumulations of  $H_2O_2$ , was considered to be responsible for the oxygen sensitivity displayed by anaerobes. Subsequently, however, it was shown that catalase added to culture media did not aid growth. Some anaerobes do produce catalase and indeed some oxygen dependent bacteria do not. The catalase theory has been superseded by the superoxide dismutase theory: this group of metalloprotein enzymes catalyse the conversion of superoxide radicals to  $H_2O_2$  and molecular oxygen. The danger of highly reactive  $O_2^-$  anions, which result from the monovalent reduction of  $O_2$ , lies in three reactions:

- i. spontaneous dismutation of  $O_2^-$  to form singlet oxygen, which is equally reactive and therefore toxic;
- ii. the reaction of  $O_2^-$  with  $H_2O_2$  to form hydroxyl radicals;
- iii. hydroxyl radicals formed above combine with more  $O_2^-$  to yield more singlet oxygen.

All aerobic and facultative organisms that metabolise oxygen possess superoxide dismutase to guard against this chain of events. Obligate anaerobes either lack or have ineffective quantities of this enzyme, hence their sensitivity to oxygen. McCord et al. (1971) found no superoxide dismutase and very little catalase in

the anaerobes they examined. They suggested, however, that such bacteria could survive in an oxygen-containing atmosphere if they produced only small quantities of  $O_2^-$ . Two studies by Gregory and Fridovich (1973a,b) confirmed that the  $O_2^-$  radical was toxic to bacterial cells and superoxide dismutase protected against its effects. Hyperbaric oxygen induced the formation of superoxide dismutase in E. coli and Strept. faecalis, and catalase in B. subtilis. Despite this, however, the latter organism was unable to tolerate high concentrations of oxygen, suggesting that catalase was not important in this respect.

The apparent paradox of anaerobes as commensals at sites exposed to air is resolved when the ecology of those areas is considered. These bacteria form part of a complex flora comprising organisms with a wide spectrum of oxygen requirements. Aerobic and facultative species utilise oxygen that would otherwise prove toxic to strict anaerobes. A similar situation is envisaged in mixed infections and this is particularly well-illustrated in periodontal disease, discussed in Chapter 4.



### 3.4 Aims

Two areas of work were undertaken in this study with the following aims:

- i. to determine a suitable swab for the recovery of GPAC and whether or not a transport medium enhanced the survival of GPAC on swabs;
  
- ii. to assess the aerotolerance of stock cultures and fresh clinical isolates of GPAC.



### 3.5 Materials and Methods

#### 3.5.1 Swab trial

Swabs and transport media: two types of swab were selected for this study: plain buffered absorbent cotton-wool and albumin-coated non-absorbent cotton-wool (Exogen Ltd, Clydebank Industrial Estate, Beardmore Street, Clydebank). The transport media used were Amies' (1967), Stuart's (Stuart et al., 1954) and VMGII (Moller, 1966).

Bacteria: the strains of GPAC used included six clinical isolates from the Royal Infirmary and Western General Hospital, Edinburgh, and four NCTC strains previously identified to species level in the Department of Bacteriology, University of Edinburgh. They comprised three strains each of Peptostreptococcus anaerobius and Peptococcus magnus, two strains of Peptococcus prevotii and one strain of Peptococcus saccharolyticus and Peptococcus asaccharolyticus. Modified Robertson's cooked meat medium (Watt, 1972) and 5% human-blood agar plates were used for culture. All incubations were performed at 37°C for 48h (unless otherwise stated) using anaerobic jars (Baird and Tatlock Ltd; Don Whitley Scientific) according to the method of Collee et al., 1972.

Preparation of swabs: strains were incubated for 24h in 10 ml of pre-reduced cooked meat medium and 0.02 ml was mixed in air with 0.08 ml sterile saline (0.85% w/v NaCl). This 0.1 ml volume was loaded onto each swab. Half blood agar plates were streaked with swabs either immediately or after having been held in their containers or in transport medium for up to 6h, at both room temperature and at 4°C. At intervals of 1h, 2h and 6h, a set of swabs held under the various conditions was plated out and plates were incubated anaerobically for 48h at 37°C. Colony-forming units were counted and recovery rates calculated on the basis of the number of organisms originally loaded onto the swabs. This was determined from dilutions of the parent culture by a spread plate viable count procedure.

Statistical methods: these comprised analysis of variance and t tests.

### 3.5.2 Sensitivity to atmospheric oxygen

Bacteria: GPAC selected for study comprised eight stock strains and five fresh clinical isolates, the latter supplied by the Bacteriology Laboratory, Royal Infirmary of Edinburgh. These clinical isolates of GPAC were present in mixed cultures, that

included other facultative and anaerobic organisms, on gentamicin-blood agar plates. GPAC were subcultured onto blood agar and incubated anaerobically and aerobically a minimum number of times (average was three) to obtain pure cultures and ascertain metronidazole sensitivity.

**Culture:** pre-reduced cooked meat broths (CMB) were inoculated with 2-3 drops of thawed skimmed milk preparations or 10 colonies picked from a pure culture on blood agar. Skimmed milk preparations were also subcultured onto blood agar to assess purity. Inoculated CMB and blood agar plates were incubated anaerobically for 48h at 37°C.

**Sensitivity to air:** after incubation, Gram films and subcultures of CMB to blood agar for anaerobic and aerobic incubation were made to confirm purity. Ten-fold dilutions of a portion of CMB culture were made in pre-reduced nutrient broth and one drop from a Pasteur pipette (0.03 ml) of a  $10^{-4}$  and  $10^{-5}$  dilution was spread over the surface of a pre-reduced or freshly prepared 5% human-blood agar plate, using a sterile glass spreader. This procedure was duplicated for each strain at each dilution. Plates were transferred to anaerobic jars for immediate incubation at 37°C or left in air on the bench for 6h, 24h, 48h and 72h before incubation. The numbers of colony-forming units present on each plate after 48h anaerobic incubation were calculated. The effects

of delayed incubation were assessed by comparison with recoveries from plates incubated immediately. Clinical isolates were subsequently identified according to the scheme outlined in Chapter 2.

### 3.6 Results

#### 3.6.1 Swab trial

The mean recovery of 10 strains of GPAC from swabs plated onto blood agar immediately after loading was 9.5% of the initial inoculum from the cotton-wool and 13.2% from the albumin-coated swabs (Table 3.1). Approximately 90% of bacteria, therefore, were not recovered from swabs used in this way.

The recoveries of GPAC from swabs stored in and without transport media for periods up to 6h at either room temperature (17-22°C) or 4°C are summarised in Tables 3.1 and 3.2. There was no difference in recovery of organisms from either type of swab held without transport medium at room temperature for 2h. The highest mean recovery rates at 6h were obtained from albumin-coated swabs stored at 4°C without transport medium or in Amies'. Of the three transport media tested, only Amies' compared favourably with 'dry' swabs. In general, if GPAC were held on swabs for longer than 2h, a significantly decreased recovery resulted compared with recoveries from swabs plated immediately, or within 2h of loading ( $p < 0.05$ ).

There was no significant difference in the recovery of bacteria from swabs stored either at room temperature or 4°C

TABLE 3.1:

MEAN PERCENTAGE RECOVERY OF TEN STRAINS OF GRAM-POSITIVE  
ANAEROBIC COCCI HELD ON SWABS AT ROOM TEMPERATURE

Swab	Time on swab before plating	Type of System*				
		I	II	III	IV	
Cotton-wool	0	9.5				
	1		8.7	3.4	2.9	3.3
	2		6.3	6.0	1.3	3.3
	6		1.2	2.9	0.6	2.0
		Mean (1-6h)	5.4	4.1	1.6	2.9
Albumin	0	13.2				
	1		9.3	8.8	3.1	4.5
	2		5.5	8.2	2.7	4.8
	6		0.5	6.6	0.5	2.8
		Mean (1-6h)	5.1	7.8	2.1	4.0

Key:

\* I: swab replaced in container, no transport medium

II: swab held in Amies' medium

III: swab held in Stuart's medium

IV: swab held in VMGII medium

TABLE 3.2:

MEAN PERCENTAGE RECOVERY OF TEN STRAINS OF GRAM-POSITIVE  
ANAEROBIC COCCI HELD ON SWABS AT 4°C

Swab	Time on swab before plating	Type of System*			
		I	II	III	IV
Cotton-wool	1	5.8	6.6	1.4	3.2
	2	7.4	6.8	2.4	3.6
	6	3.8	4.1	1.5	2.3
	Mean	5.7	5.8	1.8	3.0
Albumin	1	10.2	8.7	4.4	5.1
	2	9.8	9.6	3.3	6.1
	6	5.2	5.9	2.9	2.8
	Mean	8.4	8.1	3.6	4.7

\*See Table 3.1.

( $p > 0.1$ ) if cotton-wool swabs held without transport medium were plated out 2h after loading (Tables 3.1 and 3.2). Under the same conditions, however, albumin-coated swabs gave significantly higher recoveries if held at 4°C for the same period ( $p < 0.01$ ). There was no difference in recovery from either type of swab held 'dry' at room temperature for 2h.

Amies' medium offered no significant advantage over storing cotton-wool swabs 'dry' at room temperature for 2h. However, this medium did provide significantly higher recoveries with albumin-coated swabs ( $p < 0.05$ ) under the same conditions. Of particular interest were the poor recoveries of GPAC from either type of swab held in Stuart's transport medium at both temperatures throughout the study. At the 6h limit, less than 2% of the original inocula could be recovered from cotton-wool swabs, and less than 3% from albumin-coated swabs stored in this medium. Albumin swabs held in VMGII gave slightly higher recovery rates. Over the 6h period, no single transport medium offered any particular advantage, with the exception of albumin-coated swabs held in Amies' medium at either temperature. This combination, or albumin-coated swabs held without transport medium at 4°C, gave significantly higher recoveries of GPAC than any others.

Tables 3.3 and 3.4 show recoveries of individual species of GPAC used in this study. The mean immediate recoveries calculated from individual species were 8.7% for cotton-wool and 12.2% for albumin, which differ slightly from the mean immediate recoveries



TABLE 3.3: MEAN PERCENTAGE RECOVERIES OF INDIVIDUAL SPECIES OF GRAM-POSITIVE ANAEROBIC COCCI FROM DIFFERENT SYSTEMS AT ROOM TEMPERATURE

Species	Time held on swab before plating (h)		Type of System*											
	Immediate Plating		I		II		III		IV		A		A	
	C	A	C	A	C	A	C	A	C	A	C	A	C	A
<u>Ps. anaerobius</u> (3 strains)	0	12.5	16.7	6.3	10.7	3.3	11.4	2.9	3.3	2.4	2.2	2.4	2.4	2.2
	1			4.5	0.9	5.3	8.9	1.4	1.2	2.7	2.1	2.7	2.7	2.1
	2			0.3	0	2.5	8.0	0.2	0	2.3	1.4	2.3	2.3	1.4
	6													
<u>Pc. magnus</u> (3 strains)	0	9.9	15.0	3.5	8.6	3.2	9.1	2.8	1.9	3.1	4.8	3.1	2.5	6.6
	1			9.1	10.4	6.5	8.5	0.8	3.1	2.1	6.6	2.1	2.1	1.8
	2			0.9	0.7	2.8	4.6	0.3	0	2.1	1.8	2.1	2.1	1.8
	6													
<u>Pc. prevotii</u> (2 strains)	0	6.9	7.2	3.8	4.1	3.6	7.2	4.5	5.7	7.0	6.7	7.0	6.8	8.0
	1			3.2	1.2	6.5	7.2	2.7	2.8	6.8	8.0	2.8	6.8	8.0
	2			0.4	0	1.7	6.4	0.8	1.1	1.7	6.1	1.1	1.7	6.1
	6													
<u>Pc. asaccharolyticus</u> (2 strains)	0	2.6	3.3	2.3	10.1	1.7	5.3	0.7	0.6	2.6	2.0	0.6	2.6	2.0
	1			0.9	0.2	2.5	6.7	0	0.1	2.9	3.4	0.1	2.9	3.4
	2			0	0	0.7	3.4	0	0	2.2	1.5	0	2.2	1.5
	6													
<u>Pc. saccharolyticus</u> (1 strain)	0	11.8	18.8	16.6	16.5	5.2	6.5	2.9	3.5	2.1	8.6	3.5	2.1	8.6
	1			13.6	18.5	9.4	9.2	0.8	8.3	2.6	2.7	8.3	2.6	2.7
	2			7.2	2.4	9.2	11.4	2.7	2.8	0.9	4.4	2.8	0.9	4.4
	6													

\* See Table 3.1. Key: C = cotton-wool swabs; A = albumin-coated swabs

TABLE 3.4: MEAN PERCENTAGE RECOVERIES OF INDIVIDUAL SPECIES OF GRAM-POSITIVE ANAEROBIC COCCI FROM DIFFERENT SYSTEMS AT 4°C

Species	Time held on swab before plating (h)	Type of System*															
		I		II		III		IV		C		A					
Pc. anaerobius	1	4.1	9.8	8.4	11.7	1.4	4.8	1.5	2.7	4.1	9.8	8.4	11.7	1.4	4.8	1.5	2.7
	2	5.8	10.8	6.2	10.9	2.3	4.5	2.1	2.3	5.8	10.8	6.2	10.9	2.3	4.5	2.1	2.3
	6	4.1	2.0	2.8	6.0	0.8	2.3	2.0	1.6	4.1	2.0	2.8	6.0	0.8	2.3	2.0	1.6
Pc. magnus	1	7.0	8.7	7.2	6.9	1.6	4.0	2.8	4.9	7.0	8.7	7.2	6.9	1.6	4.0	2.8	4.9
	2	5.4	10.3	8.4	9.2	0.9	2.4	4.5	6.6	5.4	10.3	8.4	9.2	0.9	2.4	4.5	6.6
	6	2.7	10.6	3.9	8.2	0.6	2.1	2.8	3.9	2.7	10.6	3.9	8.2	0.6	2.1	2.8	3.9
Pc. prevotii	1	5.4	7.5	4.0	6.6	2.0	6.5	6.3	7.6	5.4	7.5	4.0	6.6	2.0	6.5	6.3	7.6
	2	4.6	4.2	6.1	9.2	4.9	3.9	5.6	14.4	4.6	4.2	6.1	9.2	4.9	3.9	5.6	14.4
	6	2.5	0.2	3.2	1.8	3.8	3.8	2.7	2.7	2.5	0.2	3.2	1.8	3.8	3.8	2.7	2.7
Pc. asaccharolyticus	1	4.0	8.4	3.0	7.1	0.9	2.6	4.7	4.8	4.0	8.4	3.0	7.1	0.9	2.6	4.7	4.8
	2	2.8	11.2	3.7	8.1	1.3	1.1	2.3	3.6	2.8	11.2	3.7	8.1	1.3	1.1	2.3	3.6
	6	1.8	0	2.5	4.3	0.4	0.3	1.8	1.8	1.8	0	2.5	4.3	0.4	0.3	1.8	1.8
Pc. saccharolyticus	1	9.8	23.4	8.0	10.8	1.6	2.1	1.9	8.6	9.8	23.4	8.0	10.8	1.6	2.1	1.9	8.6
	2	17.8	14.7	6.7	8.6	2.9	3.4	2.5	2.5	17.8	14.7	6.7	8.6	2.9	3.4	2.5	2.5
	6	11.4	14.1	11.9	8.9	2.4	8.4	1.6	4.9	11.4	14.1	11.9	8.9	2.4	8.4	1.6	4.9

\* See Table 3.1

calculated from individual strains of 9.5% and 13.2% respectively (Table 3.1). It should also be noted that some strains did not survive 6h storage under certain conditions, e.g. Pc. asaccharolyticus on cotton-wool or albumin-coated swabs without transport medium or in Stuart's medium at room temperature (Table 3.4). There was, however, some variation in recovery rates within single species.

There was no indication of multiplication of GPAC on swabs held at room temperature. The increase in number of organisms recorded for cotton-wool swabs held in Amies' medium over 2h (Table 3.1) was not significant ( $p > 0.05$ ).

### 3.6.2 Sensitivity to atmospheric oxygen

The recoveries of stock and fresh clinical isolates of GPAC exposed to air for various periods are summarised in Tables 3.5 and 3.6. With the exceptions of Pc. magnus strains 1, 2 and 3 (Table 3.5), all figures are derived from  $10^{-4}$  dilutions of the original cooked meat broth culture.

After 6h exposure to atmospheric oxygen on the surface of pre-reduced or fresh blood agar plates prior to anaerobic incubation, the mean recoveries of stock strains and fresh clinical isolates were 88.4% and 84.4% of the original inocula respectively. This is in contrast to figures of less than 10% of original inocula

TABLE 3.5:

EFFECT OF EXPOSURE TO AIR ON STOCK STRAINS OF GRAM-POSITIVE ANAEROBIC COCCI ON BLOOD AGAR

Strain	Inoculum*	Percentage of viable count recovered after exposure to air				
		6h	24h	48h	72h	
Pc. magnus 1	101	135.6	103.9	110.9	67.3	
Pc. magnus 2	237	90.3	72.2	65.8	46.0	
Pc. magnus 3	234	91.0	107.3	85.5	76.1	
Pc. asaccharolyticus 1	358	69.4	76.9	29.7	11.0	
Pc. asaccharolyticus 2	167	89.8	53.3	36.5	3.0	
Ps. anaerobius 1	46	95.7	102.2	63.0	0	
Ps. anaerobius 2	50	90.0	72.2	12.0	32.0	
Pc. prevotii 1	183	45.4	36.1	12.0	4.9	
	Mean	88.4	78.0	51.9	30.0	

\* Colony-forming units from 0.03 ml of  $10^{-4}$  or  $10^{-5}$  dilution of CMB culture.

TABLE 3.6:

EFFECT OF EXPOSURE TO AIR ON CLINICAL ISOLATES OF GRAM-POSITIVE ANAEROBIC COCCI ON BLOOD AGAR

Strain	Inoculum*	Percentage of viable count recovered after exposure to air				
		6h	24h	48h	72h	
GPAC 1 (Ps. anaerobius)	148	116.9	127.7	62.2	30.4	
GPAC 2 (Pc. magnus)	196	73.5	95.9	65.8	66.3	
GPAC 3 (Ps. anaerobius)	116	91.4	94.0	27.6	23.2	
GPAC 4 (Pc. magnus)	> 500	< 17.0	100	< 14.4	< 12.4	
GPAC 5 (Ps. anaerobius)	65	123.1	140.0	66.2	21.5	
	Mean	84.4	111.5	47.2	30.8	

\* Colony-forming units from 0.03 ml of  $10^{-4}$  dilution of CMB culture.

recovered from cotton-wool and albumin swabs stored for 6h under the various conditions described earlier. All stock strains and fresh clinical isolates remained viable over 48h exposure to air and mean recoveries after this period were 51.9% and 47.2% of the original inocula respectively. Recoveries of certain strains, e.g. Pc. magnus 1, GPAC 1, GPAC 5, appeared to increase over 24 or 48h.

All but one of the 13 strains of GPAC tested survived 72h in air. Two strains, Pc. prevotii 1 and Pc. asaccharolyticus 2, exhibited much lower recoveries than the remaining strains after this period. The mean percentage recovery of stock strains exposed to air for 72h was 30.0% of the original inocula. The equivalent figure for fresh clinical isolates was 30.8%.

The identities of the clinical isolates were subsequently determined and appear in Table 3.6.

### 3.7 Discussion

The diagnosis of any infection and the study of the pathogenic organisms involved require reliable means for the removal of samples from the patient and their transportation to the laboratory. Such procedures should maintain the viability of the most sensitive organisms, yet prevent overgrowth by others present in the sample that might hinder identification of the true pathogen. Samples received on swabs are not ideal for the recovery of anaerobes, but many service laboratories still receive such specimens. There is little information regarding the survival of GPAC on swabs, therefore this study was intended to assess the possibility of recovering GPAC from swabs held in a variety of transport media and also without any transport medium in an air-filled tube. It was further hoped to gain an impression of the likely oxygen sensitivity of GPAC recently isolated from clinical material and thence some idea of the precautions that would be required in a clinical study.

Figures for the mean recoveries of GPAC from cotton-wool and albumin-coated swabs (9.5% and 13.2% respectively) used to seed blood agar plates immediately after loading are considerably higher than those reported by Collee et al. (1974). They recovered 3-5% of the original inocula of the anaerobes tested with albumin-coated

swabs treated similarly. Both Collee's group and Ross (1977) proved that organisms remained viable on swabs, but resisted plating out. This observation led the former authors to state "the traditional procedure of plating a swab on a series of primary plates seems to have little chance of prompt success unless large numbers of organisms present in an exudate are plated promptly." Collee et al. attempted to reproduce in vivo conditions as far as possible by using drops of cooked meat broth cultures mixed with sterile defibrinated horse blood as inocula for swabs, thus creating a 'simulated exudate'. They admitted, however, that even this model did not include the many other factors at work in vivo. In the present study, no such attempt was made and bacteria were suspended in aerobically-stored saline for loading onto swabs. The absence of inflammatory exudate, host immune factors, chemotherapeutic agents, and particularly other bacteria in this artificial inoculum, all contribute to the results observed; the use of an organic suspending medium such as blood could not entirely compensate for this lack. The recovery of GPAC from mixtures of bacteria possessing various oxygen sensitivities will be influenced by any persistent metabolic activity of their companions on the swab, e.g. utilisation of oxygen by aerobes and facultative anaerobes, production of  $O_2^-$  radicals and hydrogen peroxide. This is feasible when one considers the presence of substrates and an adequate environment that will exist for some period in the sample. None of these influences can be adequately



reproduced in vitro. This study does highlight, however, the relative performances of cotton-wool and albumin-coated swabs with or without transport media in the recovery of GPAC.

Cumming et al. (1981) described recoveries of beta-haemolytic streptococci ranging from 4.3-13.5% from cotton-wool and albumin-coated swabs plated out immediately after loading. The use of saliva instead of saline as a suspending medium did not greatly enhance results. This group recommended plain cotton-wool swabs without transport medium, but held at 4°C to prevent multiplication over short periods of storage and ensure recovery of bacteria if longer periods of delay before laboratory processing were envisaged. In the present study, the majority of strains survived 6h delay on swabs without transport medium and did not multiply if held at room temperature in any medium. A lower temperature (4°C) did enhance recoveries from albumin-coated swabs held for 6h before plating out.

Attempts to maintain the viability of anaerobic bacteria using various transport media have been described in the introduction to this chapter. Barry et al. (1972) reported improved survival of a strain of Peptostreptococcus on cotton swabs placed into reduced Amies' medium that did not contain charcoal. In the present study, charcoal was included in Amies' medium and results were similar to those of Barry's group, particularly if the inoculated medium was held at 4°C. On the basis of results in the present study and contrary to the findings of Gastrin et al. (1968)

and Holbrook et al. (1978), VMGII cannot be recommended for the storage of GPAC, although no multiplication of organisms was observed. Stuart's transport medium gave the poorest performance of the four systems over 6h storage. In fact, none of the transport media investigated significantly improved recovery rates, although Amies' medium was suitable for the storage of GPAC on albumin-coated swabs. It is concluded, therefore, that plain cotton-wool or albumin-coated swabs were most efficient in the recovery of GPAC if held in their containers without transport media for periods up to 2h at room temperature.

Collee et al., (1974) expressed surprise that clinical specimens likely to contain obligate anaerobes were not treated in the same way as those containing other delicate bacteria such as gonococci. By this they meant immediately inoculating culture media with a sample as it was taken from the patient. In the case of anaerobes, transferring samples directly to pre-reduced solid or liquid media would minimise both exposure to air and the lag period of viable organisms. There is, however, the practical problem of clinics having a store of pre-reduced uninoculated media and anaerobic jars in which to place media after inoculation. The theoretical problem of exposure of samples to air may not be as great as many fear. Tally et al. (1975) expressed the belief that pathogenic anaerobes were less oxygen sensitive than non-pathogens, a feature which would aid their colonisation of tissues normally having a high oxidation-reduction potential. Results obtained in

the present study lend support to this theory. Fresh clinical isolates of GPAC were included in the study, as well as stock strains, because a criticism levelled by Tally's group (1975) at Loesche's (1969) original work on oxygen sensitivity was the use of stock strains only. These may have acquired greater oxygen tolerance with repeated subculture and storage. In the current study, fresh clinical isolates survived equally as well as stock strains and all remained viable over at least 24h exposure to atmospheric oxygen. However, to call these bacteria 'fresh' clinical isolates is not entirely accurate: GPAC were received from the diagnostic laboratory on primary plates which also carried several other organisms, therefore it required several subcultures onto fresh blood agar to separate and purify the GPAC. 'Fresh' strains of GPAC had been subcultured at least four times, including finally into cooked meat broth, prior to oxygen sensitivity testing. It may, therefore, be impossible to ascertain the exact oxygen sensitivity of a freshly-isolated anaerobe in pure culture. Indeed, in the present study, an initial selection of less sensitive strains may have occurred, since the diagnostic laboratory does not use pre-reduced media. Thus, any anaerobic organisms growing within 48h under these conditions may be intrinsically more robust and it is impossible to determine the loss of delicate organisms that may have taken place. Tally's group (1975), however, failed to recover extremely oxygen sensitive

bacteria from clinical samples using an anaerobic chamber, which suggests that such organisms are not present in the first place.

The contrast in recoveries of GPAC from swabs stored in or without transport media compared with recoveries from blood agar plates stored in air for 6h is evidence of the 'protection of the plate' theory proposed by Collee et al. (1974). This group observed less than a 50% reduction, and occasionally no loss of viability, in recoveries of Bacteroides spp exposed to air for 24h on blood agar plates, prior to anaerobic incubation. The low recoveries of GPAC from swabs after 6h storage may be ascribed to such factors as retention of organisms by swab material combined with gradual inactivation of organisms on the swab. Evidence of the latter are the lower recoveries from swabs stored up to 6h compared with recoveries from swabs plated out immediately.

The apparent multiplication of certain strains in air at room temperature is highly unlikely since parallel blood agar purity plates incubated anaerobically and aerobically indicated that none of the strains tested grew in air at 37°C within 48h. Alternative explanations are clumping of the bacterial cells in the diluting nutrient broth despite repeated aspiration into a narrow-bore pipette and counting errors.

From the present results it may be concluded that, following the primary isolation of GPAC, subsequent procedures for their identification may be undertaken at the bench without risk to the organisms' viability. GPAC need not be transferred immediately to

an anaerobic environment to ensure their survival following subculture to another growth medium. However, this evidence should not be used as an excuse for careless practice, since there may be inter- and intra-species variation in oxygen tolerance. In addition, organisms may be irreparably damaged by antibacterial agents, administered to patients and present in specimens, or exposure to oxygen during transit to the laboratory (Collee et al., 1974). Ultimately, therefore, it is impossible to know what organisms, originally present in high numbers at an infected site, are lost in transit or found in lower proportions in the laboratory.

CHAPTER 4

ISOLATION OF GRAM-POSITIVE ANAEROBIC COCCI FROM  
THE ORAL CAVITY AND VAGINA

## Introduction

Since their first description by Veillon (1893), anaerobic cocci have been found in association with infections of the female genital tract. Subsequent studies have concluded that many of the bacteria isolated from such infections are derived from the normal commensal flora of that region. Similarly, the oral cavity harbours a complex anaerobic commensal flora, many members of which have been implicated in orofacial, pulmonary and cerebral infections. The following sections review reports of the incidence of GPAC as commensals and suspected pathogens in both the oral cavity and the female genital tract.

### 4.1 GPAC in the Oral Cavity

#### 4.1.1 GPAC as commensals

The oral cavity is densely populated with a complex mixture of bacteria in which anaerobic species predominate. The composition of this microflora, however, is not uniform throughout

the mouth (Socransky and Manganiello, 1971); different areas provide different ecological niches, therefore in each area resident organisms differ in type and in number. In the gingival crevice, for example, loosely attached debris (not dental plaque) has been found to contain  $10^{11}$  organisms per gram (wet weight) and most of these are obligately anaerobic (Socransky et al., 1963). Gibbons et al. (1963) reported that GPAC (not identified to species level) accounted for 10% of isolates recovered from pooled gingival crevicular debris obtained from five 'normal' individuals. The predominant organisms isolated from these subjects were Gram-positive anaerobic rods and facultative streptococci; Gram-negative anaerobic cocci accounted for 10.5% of the crevicular flora.

Little attention has been paid to GPAC at other intraoral sites, but Socransky and Manganiello (1971) found that GPAC represented 12.6% of the total cultivable flora in dental plaque (of unstated maturity), 4.2% of the tongue microflora, 13% of salivary bacteria and 7.4% of gingival crevicular isolates. However, the authors stressed that these data were from pooled samples, therefore individual variations were obscured. Williams et al. (1976) failed to find GPAC in only one out of six samples from healthy gingival sites in six individuals; the number of GPAC recovered from the remaining five sites ranged from 3 to 11 isolates, but these were not identified to genus level. In a



further attempt to determine the microflora in healthy gingival crevices, Slots (1977b) examined samples from seven periodontologists. Out of 350 isolates, 85 were obligate anaerobes and only three were GPAC; Gram-positive facultative cocci and rods predominated.

Thus, GPAC have been found in low numbers at healthy gingival sites, but in common with other oral bacteria, there is evidence of quantitative variation between individuals. The problems associated with sampling intraoral sites and difficulties in interpretation of results posed by different techniques are discussed in the following section.

#### 4.1.2 GPAC in orofacial infections

The normal flora surrounding the teeth, particularly those organisms normally present in the densely populated gingival crevice, are the organisms usually isolated in soft-tissue infections of dental origin.  
(Busch, 1984)

A number of investigations into the microbial composition of such infections have been described in recent years. Sabistan et al. (1976) found that obligate anaerobes accounted for 66% of the total species cultured from dental abscess aspirates. In general, these lesions contained a complex mixture of bacteria. Ps. micros was

found in 22% of cases and Ps. anaerobius in 16%. The numerically predominant organisms in all the abscesses, however, were facultative Gram-positive cocci. The authors speculated that the recovery of several anaerobic species from individual abscesses indicated the inability of a single species to induce infection alone.

Bartlett and O'Keefe (1979) studied 21 cases of perimandibular closed-space infections and found an average of six bacterial species per specimen. Anaerobic species outnumbered aerobes 2:1 and the predominant isolates of the former were GPAC, Bacteroides melaninogenicus, Fusobacterium nucleatum and Eubacterium lentum. Similar findings in dentoalveolar infections were reported by von Konow et al. (1981). This group recommended direct GLC analysis of pus to distinguish an anaerobic component.

One of the commonest afflictions of mankind is periodontal disease. The majority of people suffer to some extent from one or both of its chronic forms, gingivitis and periodontitis, during the course of their adult lives. There is little doubt that bacteria derived from the commensal oral flora are involved in chronic periodontal disease (Kelstrup and Theilade, 1974; van Palenstein Helderman, 1981; Newman, 1984), but the host's inflammatory and immune responses contribute significantly to the tissue damage observed (Genco et al., 1974; Lehner et al., 1976).

Chronic gingivitis appears to be bacteriologically non-specific, resulting instead from the host's response to

increases in plaque mass and numbers of various bacteria, e.g. Actinomyces spp, Bacteroides spp, Fusobacterium nucleatum, Streptococcus milleri and Ps. micros (Marsh and Martin, 1984) in contact with the gingival margin. In some individuals, untreated chronic gingivitis may progress to destructive periodontitis, in which inflammation extends into the periodontal ligament, gingival connective tissue and bone with concomitant loss of collagen and ground substance. The pathogenic mechanisms involved are divided into two groups: firstly the direct damage due to bacterial enzymes, e.g. collagenase, hyaluronidase, and toxic waste products such as acids and ammonia. Secondly, as already mentioned, there is the host's response to tissue damage and bacterial antigens, involving phagocytic leucocytes, humoral antibody, cell-mediated lymphokines and complement. Many believe that bacteria themselves do not enter the gingival tissue, but there is recent evidence to the contrary (Saglie et al., 1982).

It has been suggested that periodontal "disease" represents a group of diseases with different microbial aetiologies (Socransky, 1977). A number of bacteria are strongly implicated by virtue of their frequent occurrence in relatively high numbers at diseased sites, e.g. Actinomyces spp, Bacteroides spp, Actinobacillus actinomycetemcomitans and spirochaetes (Listgarten and Hellden, 1978; Tanner et al., 1979). Thus, the predominant organisms isolated from periodontal pockets are anaerobes and microaerophiles that may also be present in low numbers in the

healthy gingival crevice. Such organisms can flourish under conditions of low oxidation-reduction potential that prevail in periodontal pockets; Onisi et al. (cited by Busch, 1984) have recorded an Eh of -300 mV for gingival crevices. In theory, GPAC could also thrive in such an environment and contribute directly or indirectly to tissue damage. The pathogenic potential of GPAC and their synergism with other bacteria, including Bacteroides spp have been discussed in Chapter 1. However, the literature is not conclusive about the role of GPAC in periodontal disease. Gram-negative anaerobic cocci, belonging to the genus Veillonella, have been found in similar numbers to GPAC at healthy and diseased sites (Gibbons et al., 1963; van Palenstein Helderma, 1975).

Dwyer and Socransky (1968) used periodontal curettes to sample surfaces of periodontally-involved teeth and found that, on average, GPAC accounted for 15% of the total cultivable flora. They suggested that these bacteria "warrant further investigation". Four years later, Sabistan and Grigsby (1972) isolated peptostreptococci (identified as Ps. anaerobius) in significant numbers, along with anaerobic Lactobillus spp and Actinomyces spp from 31 teeth extracted from individuals with advanced periodontal disease. They encountered Gram-negative anaerobic rods and fusobacteria in low numbers.

Williams et al. (1976) compared the bacteria found subgingivally in five periodontally healthy and four diseased individuals, all over 30 years of age. They found that

Gram-negative anaerobic rods constituted 30% of the predominant cultivable flora in both control and disease groups. There were no consistent bacteriological differences between groups and GPAC represented 6% of the flora at healthy and diseased sites. Slots (1977a,b) raised the question of whether subgingival Gram-negative organisms initiated the disease or multiplied only after supragingival, predominantly Gram-positive bacteria created a suitable environment.

In a study of the bacteria associated with early periodontitis, Darwish et al. (1978) used a retraction suture to expose as much root surface as possible for sampling. Peptostreptococci predominated in samples from one subject and were found in significant numbers in the remaining three. All these isolates were identified as Ps. intermedius or Pc. constellatus, which have since been transferred to the genus Streptococcus. In contrast to Dwyer and Socransky (1968), Tanner et al. (1979) recovered Gram-positive cocci, including peptostreptococci, "in low numbers and with no apparent consistent pattern from the diseased apical pocket sites." They used an oxygen-free gas-flushed syringe to obtain samples from the most apical extent of periodontal pockets. Gram-negative anaerobic rods were recovered most frequently and in the greatest concentrations. However, Moore et al. (1982), studying severe periodontitis in young adults, found Ps. micros in greater numbers in the subgingival flora compared to the supragingival flora at diseased sites; indeed, 48%

of subgingival samples were positive for Ps. micros. This species could, therefore, be involved in tissue destruction. The reverse was true for Ps. anaerobius, however: this species was more prevalent in supragingival samples. The authors commented that periodontal pockets were a "significant reservoir" of bacteria frequently recovered from other serious infections, e.g. aspiration pneumonia, lung and brain abscesses and sinusitis. Both Tanner et al. (1979) and Moore et al. (1982) found significant quantitative differences between the subgingival microflora of healthy individuals and those with periodontal disease, i.e. a predominance of Gram-negative rods in the latter group, although their opinions differed over the significance of GPAC.

Povolotskii et al. (1982) used passive haemagglutination and complement fixation tests to detect antibodies to anaerobic streptococci in periodontitis sufferers. Of 48 individuals sampled, 65% had serum antibodies that reacted with HCl extracts of unspecified strains compared with 18% of 'healthy' controls. Since Povolotskii's group made no attempt to culture periodontal pocket bacteria from their patients and did not assess the periodontal status of their control subjects, it is difficult to draw conclusions from their study.

The differences in recoveries of various bacteria recorded in the studies quoted thus far may be attributable to several factors. Among these are variations in sampling techniques, methods of transportation of samples and their subsequent handling



in the laboratory. The teeth sampled and the population studied have considerable influence on results.

MacDonald et al. (1960) speculated on the significance of hyaluronidase production by anaerobic streptococci. This enzyme destroys connective tissue ground substance and thus causes direct damage, but it can lead to further damage by enhancing the penetration of bacterial enzymes, metabolic waste and endotoxin (from Gram-negative organisms) into the gingivae. Tam and Chan (1983, 1985) recovered extracellular hyaluronidase-producing peptostreptococci in significantly higher numbers from periodontal pockets than healthy gingival crevices. (These bacteria produced acetic acid only in fermentation reactions, which suggests they may be Ps. micros.) Purification of this enzyme revealed that it is significantly different in some respects from other bacterial hyaluronidases. Not only can it rapidly degrade hyaluronic acid, but it can also attack chondroitin sulphates A and C. Furthermore, its optimum pH is higher (around neutral) than other bacterial hyaluronidases, which normally function most efficiently in acidic conditions. The authors do not indicate whether or not they believe that an optimum environment for this enzyme exists at the advancing edge of periodontal inflammation. Kelstrup and Theilade (1974) thought it unlikely that enzymes synthesised by bacteria in the gingival crevice or a periodontal pocket could diffuse into the connective tissue in high enough concentrations to cause significant damage. If, however, enzyme-producing bacteria

are themselves present and metabolising in the tissues (Saglie et al., 1982), their effects will be more severe.

There are major difficulties involved in the study of periodontopathic bacteria. Access to the base of a pocket is a practical problem and carries the risk of contamination with more superficial bacteria not involved in the disease process. If a quantitative study is desired, it is almost impossible to determine the surface area of a tooth that has been sampled, far less reproduce the manoeuvre accurately. If bacteria are successfully cultured, a further problem is the indeterminate taxonomic status of some organisms encountered in the oral cavity; this is amply demonstrated by GPAC, as discussed in Chapter 1. All these obstacles combine to render comparative studies extremely difficult.

Periodontal disease is episodic in nature, i.e. the destruction is not continuous. Absolute indications of active tissue destruction have not been agreed upon, although bleeding from a pocket after gentle probing (WHO, 1978) or increased loss of attachment (Haffajee et al., 1983) are currently accepted criteria. Thus, investigators face the problem of not knowing whether GPAC isolated from a pocket form part of a periodontopathic flora or not. Furthermore, different sites in the oral cavity, even around the same tooth, vary in their bacterial population at any given time (Socransky and Manganiello, 1971) and a single site may yield



a different flora if sampled on separate occasions (Darwish et al., 1978; van Palenstein Helderma, 1981).

In summary, several studies have revealed that significant quantitative differences exist between the composition of plaque taken from healthy gingivae compared with diseased sites. Similarly, there are marked differences in the microflora associated with chronic gingivitis and chronic periodontitis. However, investigations into these conditions are fraught with problems due to numerous variables that can affect final results and other workers have failed to determine significant differences in the microflora associated with health or disease. The precise contribution of GPAC to periodontal pathology remains to be defined, but it is reasonable to hypothesise that they may be significant in some individuals.

## 4.2 GPAC in the Female Genital Tract

### 4.2.1 GPAC as commensals

In bacteriological terms, great similarities exist between the vagina and the oral cavity (Bartlett et al., 1978). In general, anaerobes outnumber aerobes 10:1, although this ratio may be higher at certain sites in the oral cavity. Bacterial concentrations in the mouth and vagina approximate  $10^9$  cells per ml. (Again, this figure varies in different areas of the mouth.). The resident flora in the mouth and vagina are influenced by fluctuations in hormone levels (Kornman and Loesche, 1980; Bartlett and Polk, 1984). Bacteria resident in these two sites in health, or exogenously acquired organisms, may gain access to deeper tissues and cause local or disseminated infections (Bartlett and Polk, 1984; Busch, 1984).

One of the earliest studies in this field was conducted by Doderlein (1894, cited by Bartlett et al., 1978), in which he found aerobic lactobacilli to predominate in the vaginal microflora of healthy premenopausal women. With the benefits of improved laboratory techniques, more recent studies have indicated the importance of anaerobes as both commensals and pathogens in the female genital tract. Gorbach et al. (1973) recovered obligate

anaerobes from the cervix of 21 out of 30 healthy women sampled with swabs. Bacteroides spp were isolated from 57% of women and Peptostreptococcus spp from 33%. No anaerobes were isolated in the absence of aerobes. Two years later, Ohm and Galask (1975) published their findings on the cervical flora of a wide age range of women about to undergo hysterectomy. Swabs were also used to sample the endocervix, via a speculum. In contrast to the findings of Gorbach's group, GPAC, not Bacteroides spp, were the most frequently isolated anaerobes. The commonest species were Pc. asaccharolyticus and Ps. anaerobius.

A study conducted by Tashjian et al. (1976) revealed GPAC to be present in vaginal samples taken from four groups of women - pregnant, premenopausal (one group taking oral contraceptives, another not) and postmenopausal. However, no significant difference in the isolation rates of GPAC existed between these groups. In a quantitative study of the vaginal bacteria in healthy adult females of reproductive age, Bartlett et al. (1977) noted that peptococci were the most frequently encountered anaerobes, confirming the findings of Ohm and Galask (1975). Peptostreptococci were encountered less frequently and in lower concentrations. Bartlett et al. (1978) described the vaginal and cervical microflora and concluded that qualitative and quantitative differences found between bacterial populations at each site in an individual indicated two distinct ecological niches. These authors drew a comparison with the situation known to exist in the

oral cavity due to differences in local environments and cell types. Cultures from one site in the female genital tract, therefore, were not representative of the whole microflora. Overall, Bartlett's group found that peptococci (not identified to species level) predominated at both cervical and vaginal sites. Peptostreptococci were less frequently isolated, but differences between individual subjects were not discussed. Approximately half of the isolates (species are not described therefore figures presumably include aerobes and anaerobes) were isolated from only one site, i.e. the cervix or vagina, but not both; the cervical and vaginal microflora were qualitatively identical in only two of the 14 subjects. No particular organisms were associated with only the cervix or vagina, hence the similarity in populations when data from all subjects were combined. More details on any differences in proportions of species found at each site would have been of interest, since the authors cite evidence (Hunter and Long, 1958) of more acidic conditions in the vagina than at the cervix.

Against established opinion, Hammann (1982) disputed the idea that anaerobes were commensal in the female genital tract. Culturing cervical and vaginal swabs for aerobic and anaerobic bacteria and yeasts, Hammann found that only 34% yielded clinically significant anaerobes. The majority of these were Bacteroides spp; only three strains of GPAC were recovered. However, in this study, swabs were held in a transport medium for up to 6h, which could account for the low recovery of anaerobes.

Bartlett and Polk (1984) recently gave a quantitative description of the vaginal microflora of 78 premenopausal women. Obligate anaerobes were recovered from 86% of specimens. Peptococci were isolated from 80% of women (predominantly Pc. prevotii and Pc. asaccharolyticus) and peptostreptococci from 28% (predominantly Ps. anaerobius). Bacteroides spp also occurred frequently. It is worthy of note that a comparison between swabs and calibrated loops used to collect specimens revealed no statistically significant difference in the concentration of bacteria recovered. Sequential studies showed that concentrations of aerobic bacteria increased during menstruation, but mean concentrations of anaerobes remained relatively constant throughout the menstrual cycle. No significant differences were recorded in concentrations of aerobes or anaerobes at either site associated with age or method of contraception. Considerable variation existed between the vagina and cervix in individuals. This confirmed earlier work (Bartlett et al., 1978) that these two sites harbour significantly different bacterial populations.

GPAC have also been demonstrated in the urethra. Marrie et al. (1980) encountered these bacteria frequently in urethral samples from premenarchal, reproductive and postmenopausal women but less often in women with urinary tract infections. Rosenstein et al. (1982), however, found no difference in the anaerobes recovered from the periurethral area of healthy women compared with those giving a history of recurrent urinary tract infection.

#### 4.2.2 GPAC in female genital tract infections

Following Veillon's description of anaerobic cocci isolated from a case of Bartholinitis, Schottmuller (1910, cited by Hite *et al.*, 1949) and Schwarz and Dieckmann (1927) established a role for these bacteria in puerperal sepsis. Later Colebrook and Hare (1933) isolated strictly anaerobic streptococci from blood cultures of 40 cases of puerperal sepsis. These bacteria were second in number only to beta-haemolytic streptococci as causative agents in this condition. There appeared to be an association with difficult labour involving premature rupture of the membranes and trauma to local tissues. It was further noted that symptoms manifested three days post-partum, coinciding with a fall in lochia pH, absence of lochia anti-tryptic activity and ischaemia of the uterus during involution. All these factors would favour the growth of anaerobes. There is now little doubt about the source of organisms in such cases: "It is generally conceded that bacteria involved in puerperal infection, either amnionitis or endometritis, are originally from the vaginal flora." (Finegold, 1977).

In addition to puerperal sepsis, GPAC have been implicated in other infections of the female genital tract including postabortal sepsis (Thadepalli *et al.*, 1973), pyometra (Carter

et al., 1951), post-operative gynaecological infections (Hall et al., 1967) and tubo-ovarian abscesses associated with pelvic inflammatory disease. Recently Sweet (1981) reported that anaerobes, predominantly GPAC, were present in the fallopian tubes of 30% of females with pelvic inflammatory disease.

Anaerobic bacteria present in the vagina have received considerable attention as contributors to vaginitis associated with Gardnerella vaginalis. Spiegel et al. (1980) and Blackwell and colleagues (1982, 1983) found a significant association between GPAC, Bacteroides spp, G. vaginalis and non-specific vaginitis. Both groups noted that metronidazole, to which G. vaginalis is resistant, effected a cure in the majority of cases, which was a strong indication of the importance of the anaerobic component. This prompted Blackwell et al. (1983) to suggest the term "anaerobic vaginosis" for this condition. Taylor et al. (1982) and Tabaqchali et al. (1983) recovered GPAC in particularly high numbers from women also harbouring Trichomonas vaginalis. Indeed, GPAC have been isolated from the vagina of women with various sexually-transmitted diseases and also their contacts (Tabaqchali et al., 1983; Wilks et al., 1984). Numbers of GPAC were comparable to those of anaerobic Gram-negative bacilli and the commonest species were Pc. magnus, Pc. asaccharolyticus, Ps. anaerobius and Ps. micros. However, it is significant that in the study of Wilks et al., GPAC were found in all control patients (those without sexually-transmitted disease) in similar numbers to



those found in symptomatic patients. Wilks' group suggest that, since there is still no overall agreement on the significance of anaerobes in the vagina, future studies should involve sequential sampling to determine changes in the flora associated with specific conditions.

The vaginal microflora during pregnancy was investigated by de Louvois et al. (1975), who found the isolation rate of GPAC increased in the third trimester. GPAC from the female genital tract have been implicated in both intrauterine and neonatal infections. This is similar to the involvement of group B beta-haemolytic streptococci in neonatal septicaemia and meningitis (Franciosi et al., 1973). Intrauterine infection of an infant with GPAC is usually due to vaginal bacteria ascending into the amniotic sac following premature rupture of the membranes, or prolonged labour with trauma to the maternal tissues. During the neonatal period, a fatal pneumonia may complicate infections acquired in utero and GPAC have been isolated post-mortem from such cases.



#### 4.3 Aims of This Study

The purpose of the present research was to develop procedures for the isolation of GPAC from the oral cavity and vagina. Bicozamycin was incorporated in primary culture media as a selective agent. Isolates were identified according to the scheme determined in Chapter 2. The feasibility of applying these procedures to large-scale epidemiological surveys is discussed.

#### 4.4 Materials and Methods

##### Subjects

Fifteen dental students were randomly selected as sources of oral GPAC; none was undergoing any form of periodontal treatment, had any intercurrent systemic illness, e.g. diabetes mellitus, or signs of upper respiratory tract infection. Vaginal strains were obtained from 20 asymptomatic women attending an antenatal clinic in the first trimester of pregnancy and 20 symptomatic women attending a sexually-transmitted disease clinic. Any individuals who had been prescribed antibiotics within a month prior to the study were excluded.

##### Sampling

Oral cavity: sterile dental floss in 3 cm lengths was passed through three interproximal sites: between the upper left canine and first premolar, the lower central incisors and the lower right first molar and second premolar teeth. Each sample from the first five students was transferred into selective media consisting of 10 ml pre-reduced cooked meat medium containing  $20 \mu\text{g ml}^{-1}$

neomycin,  $16 \mu\text{g ml}^{-1}$  nalidixic acid and  $500 \mu\text{g ml}^{-1}$  bicozamycin (Watt and Brown, 1983a). Floss from the next five students was transferred into 7 ml volumes of phosphate-buffered saline (PBS), pH 7.4, containing  $4 \text{ mg ml}^{-1}$  L-cysteine hydrochloride and  $3 \text{ mg ml}^{-1}$  dithiothreitol (Williams et al, 1976). Samples from the final five students were transferred into 7 ml proteose peptone-yeast extract medium (PPY). Gingival crevices were probed to measure depth and assess gingival health (WHO, 1978); subsequent bleeding indicates inflammation associated with active gingivitis or periodontitis. An albumin-coated swab was used to sample each student's throat and two saliva samples were obtained using Pasteur pipettes. The swabs were returned to their containers and saliva samples transferred to either PBS or PPY. All specimens were transported to the laboratory within one hour.

In the laboratory, selective cooked meat broths (SCMB) containing samples were transferred to an anaerobic cabinet (Forma Scientific System Model 1024, Marietta, Ohio) for 48h incubation at  $37^{\circ}\text{C}$ . Swabs were placed in the cabinet, transferred to SCMB and also incubated for 48h at  $37^{\circ}\text{C}$ . Samples in PBS and PPY were sonicated for 30 sec in an ultrasonic bath before transfer to the cabinet; 0.5 ml of each was inoculated into separate SCMB and incubated for 48h at  $37^{\circ}\text{C}$  in the cabinet. Following incubation, SCMB were serially diluted in nutrient broth and 0.2 ml of  $10^{-4}$  and  $10^{-5}$  dilutions spread over the surface of 5% human blood agar plates with sterile glass spreaders. These plates were removed

from the cabinet after 72h incubation at 37°C, giving a total of five days without exposure of organisms to atmospheric oxygen. Non-haemolytic grey, white or translucent colonies, ranging in size from a pin-point to 2 mm in diameter were subcultured onto blood agar plates for 48h parallel anaerobic and aerobic incubation at 37°C. Approximately 30 colonies could be placed on a single plate marked out in a grid pattern. Gram films were made of any colonies present on the anaerobic plates which failed to grow on the aerobic plates. Organisms thought to be GPAC were subcultured onto half blood agar plates with 5 µg vancomycin and 5 µg metronidazole discs. After 48h incubation, metronidazole- and vancomycin-sensitive isolates were checked for purity and ten colonies selected to inoculate 10 ml of cooked meat broth. Mixtures of metronidazole- and vancomycin-sensitive and resistant organisms were separated by repeated subcultures of individual colonies onto blood agar with these discs until pure. Cooked meat broth cultures were incubated for 48h; these served as stock cultures for identification tests, as described in Chapter 2.

An additional isolation procedure was investigated with duplicate samples in PBS from the first three dental students. After sonication these samples were inoculated onto non-selective and selective blood agar plates, the latter containing 500 µg ml<sup>-1</sup> bicozamycin (BBA). Both plates also received 5 µg metronidazole discs and BBA plates received 25 µg gentamicin discs. After 48h

anaerobic incubation, metronidazole-sensitive, gentamicin-resistant colonies were selected and processed as already described.

Vagina: samples were taken from the posterior fornix of 40 female subjects using plain cotton-wool swabs. These swabs were left in their containers and transferred to the anaerobic cabinet within 2h. They were placed in SCMB and processed in an identical manner to the oral samples.

Throughout the study, all media were reduced prior to inoculation, either by storage under anaerobic conditions for 24-48h or, in the case of liquids, by holding at 100°C for 10-15 min. The anaerobic status of isolates was confirmed by failure to grow in air plus 10% CO<sub>2</sub> at 37°C within seven days (Watt and Jack, 1977).

#### 4.5 Results

The numbers and species of GPAC recovered from the oral cavity and vagina are shown in Tables 4.1, 4.2 and 4.3. Of the 45 interproximal sites sampled with dental floss, 11 (24%) yielded a total of 15 isolates of GPAC (Table 4.1). All but one of these were identified as Pc. magnus. GPAC were isolated from five of the 15 throat swabs and all were identified as Pc. magnus.\* However, GPAC were not found in any of the 30 saliva samples processed. Other organisms recovered from oral specimens, but not identified to genus level, included facultative and aerobic Gram-positive cocci and pleomorphic rods, as well as Gram-negative anaerobic cocci. All the dental students examined had a high standard of oral hygiene; there were no visible deposits of dental plaque or gingival inflammation. All gingival crevices at the sample sites were < 3.5 mm in depth and none bled after gentle probing.

Samples inoculated on to BBA plates did not yield higher numbers of GPAC than the SCMB method. Incubation of these primary plates for a total of seven days did not produce additional colonies of GPAC.

A greater proportion of vaginal samples contained GPAC than samples from the oral cavity. GPAC were isolated from 11 (55%) of

\*Eight of the 15 dental students harboured GPAC interproximally; throat swabs from five of these eight individuals also yielded GPAC.

TABLE 4.1:

ISOLATION OF GRAM-POSITIVE ANAEROBIC COCCI FROM THE ORAL CAVITY

Sample	No. of samples	No. +ve for GPAC (%)	No. of isolates
Interproximal	45	11 (24)	15
Saliva	30	0 (0)	0
Throat swabs	15	5 (33)	7
Total	90	16 (18)	22

TABLE 4.2:

ISOLATION OF GRAM-POSITIVE ANAEROBIC COCCI FROM THE VAGINA

	No. of samples	No. +ve for GPAC (%)	No. of isolates
STD Clinic	20	16 (80)	89
Antenatal Clinic	20	11 (55)	56
Total	40	27 (68)	145



TABLE 4.3:

SPECIES OF GRAM-POSITIVE ANAEROBIC COCCI ISOLATED FROM THE  
ORAL CAVITY AND VAGINA

Species	Inter- proximal	Throat	Antenatal (%)	STD (%)
Ps. anaerobius	-	-	12 (21)	28 (32)
Pc. magnus	14	7	15 (27)	13 (15)
Pc. asaccharolyticus	-	-	2 ( 4)	15 (17)
Pc. prevotii	-	-	16 (29)	25 (28)
Pc. saccharolyticus	-	-	1 ( 1)	2 ( 2)
Ps. micros	1	-	1 ( 1)	3 ( 3)
Pc. spp.	-	-	7 (13)	3 ( 3)
Unidentified	-	-	2 ( 4)	-

the antenatal and 16 (80%) of the STD samples respectively (Table 4.2). Species of GPAC most frequently encountered were Ps. anaerobius (32% of STD isolated, 21% of antenatal), Pc. prevotii (28% and 29%) and Pc. magnus (15% and 27%). Diagnoses in the STD group were as follows: four had gonorrhoea, three had gonorrhoea and were also carrying Chlamydia trachomatis, one harboured Chlamydia trachomatis and two patients had genital warts. Cultures from the remaining ten patients were negative for any sexually-transmitted pathogens. Four samples failed to yield any GPAC: one from a case of gonorrhoea, both cases of genital warts and one negative for any sexually-transmitted disease. There was no apparent pattern to the numbers and species of GPAC recovered from any of these patients. Other organisms recovered, but not identified further, included facultative Gram-positive cocci, anaerobic Gram-positive coccobacilli and rods, together with Gram-negative anaerobic cocci and filaments.

Laboratory tests failed to speciate 12 isolates of GPAC, two of which could not be assigned to a genus, but sensitivity to metronidazole and vancomycin indicated that they were GPAC. Isolation and identification required 12 days following receipt of samples.

#### 4.6 Discussion

Anaerobic bacteria are an important component of the commensal microflora in the oral cavity and vagina, but on occasion they are involved in both local and disseminated opportunistic infections. In the present study, a considerable difference was found in the isolation rates of GPAC from the mouth compared with rates from the vagina. The low numbers recovered from the former site may reflect the excellent oral health of the subjects investigated. Slots (1977b) identified only three strains of GPAC ("presumably Peptostreptococcus species") out of 350 bacteria recovered from seven periodontologists using a roll-tube technique. Slots found conventional anaerobic jars only half as efficient as roll tubes for the recovery of plaque bacteria. Williams et al. (1976) dismissed the roll-tube technique as impractical in studies of the predominant cultivable flora present in the oral cavity because of the difficulties involved in picking separate colonies out of the numerous types that appear. This group used an anaerobic chamber as described and successfully employed by Aranki et al. (1969) in a study of human gingival bacteria. In the present study, the use of pre-reduced media and an anaerobic cabinet should have minimised the loss of GPAC, which in any case are not extremely oxygen sensitive organisms as demonstrated in

Chapter 3. The practice of examining primary cultures in air after 24h anaerobic incubation reduces the number of anaerobes recovered (Dowell, 1972). In this study, primary cultures had 48h and subcultures 72h uninterrupted anaerobic incubation. All intermediate procedures were conducted within the anaerobic cabinet.

Failure to isolate GPAC from saliva was initially surprising following the findings of Gordon and Jong (1968, cited by Socransky and Manganiello, 1977) that GPAC accounted for 13% of the bacteria cultured from adult saliva. The present results may indicate a low incidence of GPAC in the healthy oral cavity. Gram-negative anaerobic cocci, however, were found in a small number of samples (interproximal and saliva). A comparative study of samples from periodontal pockets processed in the manner developed in this study may provide valuable information regarding the presence of GPAC in such areas and their contribution to the disease process.

Although PPY is a rich source of nutrients for many bacteria, samples were held in it for less than one hour and it may be that this protein-containing medium affords some protection for delicate organisms, particularly if pre-reduced.

Robrish et al. (1976) determined that Gram-negative rods were very sensitive to sonication and suggested that it served as an enrichment method for Gram-positive cocci and rods present in dental plaque. This is clearly undesirable in quantitative studies of the total cultivable flora, but useful where

Gram-positive bacteria are sought. Selection of GPAC was further enhanced in this study by the combination of agents in cooked meat broths. This had previously been tested with stock strains of GPAC and did not interfere with their growth. Neomycin is normally used at a concentration of 70-75  $\mu\text{g ml}^{-1}$  in selective media, but in the present study was used at a lower concentration following evidence of synergy with bicozamycin which may inhibit some anaerobic cocci (Watt and Brown, 1983a). The use of bicozamycin in blood agar plates was no more beneficial for isolating GPAC than the alternatives described. However, it did show, by the presence of black pigmented colonies on the non-selective agar plates, the inhibitory effect of bicozamycin on Bacteroides spp.

A greater number and variety of GPAC were encountered in vaginal samples compared with oral samples. Results in the present study are in agreement with those of Ohm and Galask (1975) and Bartlett and Polk (1984). The former authors identified the most frequently encountered GPAC as Pc. asaccharolyticus, Pc. magnus, Pc. prevotii, Ps. micros and Ps. anaerobius. Bartlett and Polk recovered obligate anaerobes from 86% of healthy premenopausal women sampled with swabs. GPAC, particularly Pc. prevotii, Pc. asaccharolyticus and Ps. anaerobius predominated.

In the current study, the number of women sampled in each group is too small to determine significant associations between

the presence of certain species of GPAC and pregnancy or sexually-transmitted diseases. However, it is of interest that a much higher proportion of symptomatic women attending an STD clinic carried GPAC in the vagina. Half of these symptomatic women were culture-negative for 'classical' sexually-transmitted pathogens. These findings bear comparison with the evidence produced by Spiegel et al. (1980) and Taylor et al. (1982), which strongly suggests a pathogenic role for anaerobes in non-specific vaginitis.

Failure to isolate GPAC from almost half the pregnant women sampled may support the statement of Bartlett and Polk (1984) "that the genital flora is unstable and that a single sampling .... does not always detect the carrier state."

A total of 167 GPAC were isolated from 130 oral and vaginal specimens processed; 12 of these could not be speciated. This was attributable to poor growth, either on subculture with novobiocin and liquid discs or in fermentation tests; repetition did not improve results. All GPAC conformed to the criteria of Watt and Jack (1977) that they would not grow in air, nor in 10% CO<sub>2</sub> and air after seven days' incubation at 37°C. It is impossible to state whether or not several isolates of the same species recovered from a single specimen represent the same or different isolates. This is a reflection of the failure of GPAC to display distinctive colonial morphologies; several similar colonies picked from primary culture plates often proved to belong to different species of GPAC.

At present, most diagnostic laboratories do not identify GPAC to species level for several reasons: it is a time-consuming and sometimes inconclusive exercise; individual laboratories may obtain different results for the same isolate and the names applied may prove to be taxonomically invalid; because of their susceptibility to a range of antimicrobials, they do not present a therapeutic problem. The scheme developed in this study in its entirety does not solve the service laboratory's problem but certain tests, such as liquoid, vancomycin and novobiocin sensitivity could easily be incorporated in present procedures. The complete scheme could be employed in epidemiological surveys of the carriage of GPAC in the mouth and female genital tract as well as other sites, such as the skin or gut. This study highlights the need for practical, rapid yet reliable tests for the identification of GPAC, based on a universally accepted classification scheme.

CHAPTER 5

SEROLOGICAL ASPECTS OF THE CELL SURFACE PROTEINS  
OF GRAM-POSITIVE ANAEROBIC COCCI



## Introduction

The detection of a specific immune response to invading pathogens by a host has both diagnostic and taxonomic applications. The demonstration of high concentrations of antibody or antigen in clinical samples indicates recent infection of that individual by a specific organism. Serological identification can be extended to determine the epidemiological significance of particular species. Hare (1967) stated: "There can be no question that the most satisfactory method for the classification of bacteria is by serology." Although recent developments in nucleic acid technology allow examination and comparison of the most fundamental characteristics of a bacterial cell, these sophisticated techniques cannot be practised by service laboratories. Serology remains a widely-adopted approach to the identification and classification of many microorganisms. It is of particular value where conventional morphological and biochemical procedures have consistently failed to deliver a satisfactory identification scheme, e.g. beta-haemolytic streptococci. Lancefield (1933) stated that strains of these bacteria "are occasionally encountered which are difficult or impossible to classify" by biochemical and cultural techniques. From her original work, a classification scheme for beta-haemolytic streptococci developed, based on cell wall carbohydrate antigens.

A further approach to the classification of bacteria which has gained favour recently is the use of polyacrylamide gel electrophoresis (PAGE) to examine cellular protein profiles. This, combined with serology in the technique known as Western blotting, allows visualisation of cellular antigens; these methods are described later in this chapter.

A variety of cell preparations may be used as antigens in serological studies. Whole cells or cell surface antigens are considered appropriate, since these are exposed to the host's immunocompetent cells during infection and become the targets of a specific response. An awareness of the cell envelope structure of the organism under study is fundamental to an understanding of antigen-antibody interactions. The components of the Gram-positive cell wall and membrane are outlined in detail in Chapter 6, but a brief description of the cell surface is pertinent at this point. External to the cytoplasmic membrane is the cell wall, a rigid structure which maintains cell shape, protects the cell against osmotic lysis and exerts some control over the movement of solutes in and out of the cytoplasm. In Gram-positive cells, this wall is composed of peptidoglycan, teichoic or teichuronic acids and polysaccharides; the thickness and composition of cell walls can vary according to growth conditions. Associated with, but non-covalently bound to the external surface of the cell wall may be a highly organised protein coat, which can

be removed with agents such as urea or guanidine hydrochloride. Gram-positive organisms may also possess a capsule, usually composed of polysaccharides, but which may consist of polyamino acids. Each of these structures may contain antigenic determinants that are 'seen' by the host's immunocompetent cells; some of these surface features may contribute to the organism's defence against the host response; e.g. Streptococcus pyogenes possesses the M-protein and Streptococcus pneumoniae a capsule which inhibit phagocytosis.

The persistent failure of conventional morphological and biochemical procedures to classify GPAC has prompted several investigations into the immunological features of these bacteria and these are reviewed in the following section.

## 5.1 Serological Studies of GPAC

Colebrook and Hare (1933) included agglutination tests in their study of 'anaerobic streptococci' associated with puerperal sepsis. They raised antisera in rabbits to eight strains of 'anaerobic streptococci' and tested these antisera against homologous and heterologous strains, as well as 12 other strains of 'anaerobic streptococci'. Although the antisera only agglutinated homologous strains and confirmed their identities achieved by conventional biochemical tests, the authors drew a comparison with the problems of classifying the Streptococcus pyogenes group [sic] by serology. With GPAC, however, this problem was "difficult to solve on account of the less vigorous growth obtained in artificial media." The 'anaerobic streptococci' commonly isolated by Colebrook and Hare, divided into groups A and B by biochemical and morphological tests, appeared to be serologically distinct, i.e. each strain reacted only with its group antiserum. These results were confirmed by McDonald et al. (1937), who found that nine strains of 'anaerobic streptococci' reacted only with homologous antisera raised in rabbits to killed whole cells. Three strains that displayed serological cross-reactivity proved to belong to the same biotype.

Weiss and Mercado (1938) extracted proteins from two strains of 'anaerobic streptococci' (one of which subsequently became

microaerophilic). Precipitin tests (Lancefield, 1933), using antisera raised in rabbits by intravenous injection of these protein extracts, indicated that the two strains were serologically distinct. Cross-reactions were observed with antisera to haemolytic streptococci, but these were easily removed by absorption with cells of the cross-reacting strains. Weiss and Mercado (1938) noted that the protein extract from the obligately anaerobic strain also contained carbohydrate; they speculated on whether the latter might function as a hapten and convey group specificity as in the haemolytic streptococci.

Stone (1940) investigated the classification of 'anaerobic streptococci' from a biochemical and immunological standpoint, in an effort to differentiate virulent and non-virulent strains. He raised antisera in rabbits to 24 heat-killed strains injected intravenously. Cell extracts for use as antigens in tube precipitin tests were prepared according to Lancefield's method (1933). Of the 24 strains tested, 20 gave good homologous antibody titres, detected in precipitin tests. Numerous cross-reactions between different biotypes were observed, however, and Stone was unable to group his test strains by the biochemical or serological methods employed. His explanation was that such "crudely prepared" extracts, containing a mixture of antigens, may not represent the entire cell surface. Thus the amount of antigen present in an extract may be significantly less than that present on whole cells used to raise antisera. Cross-reactions were also

observed between several 'anaerobic streptococci' and antisera to beta-haemolytic streptococci of Lancefield's groups A, B and C; cross-reactions with the latter group were particularly strong. This suggested to Stone the possession of at least one common antigen between these species. He concluded, however, that future serological studies of GPAC should be based on antisera raised against more precisely defined antigens.

Bahn et al. (1966) raised antisera in rabbits to cell wall extracts from two strains of Ps. putridus (synonymous with Ps. anaerobius), two strains of Ps. intermedius (now in the genus Streptococcus) and one strain of Ps. elsdenii (now in the genus Megasphaera). Agglutination tests detected no cross-reactions between any of the species. The two strains of Ps. putridus showed reciprocal cross-reactivity, but did not cross-react with any grouping antisera to haemolytic streptococci.

Porschen and Spaulding (1974) used intravenous and subcutaneous injection of live GPAC, representing five commonly isolated species, to raise antisera in rabbits, but no significant difference in titres was obtained by these methods. Fluorescent antibody conjugates were prepared from these antisera and tested against 49 homologous and heterologous strains of GPAC which included Pc. magnus, Pc. asaccharolyticus, Pc. prevotii, Ps. anaerobius and Ps. intermedius. This investigation produced no evidence of a common genus antigen for Peptococcus or Peptostreptococcus, but cross-reactions were encountered between

Ps. anaerobius and Pc. magnus. All ten strains of the latter species gave strong reactions with the two homologous antisera prepared, suggesting the possession of a major species specific surface antigen detectable by fluorescence in the clinical laboratory. Fluorescent antibody conjugates of antisera prepared against Pc. prevotii, Ps. anaerobius and Ps. intermedius were highly strain-specific, although one of the Ps. anaerobius conjugates did react with two other members of the same species suggesting common surface antigens. No cross-reactions occurred between GPAC antibody conjugates and group A, B or D haemolytic streptococci. The authors concluded that much more work was required before "useful polyvalent antisera" could be developed and utilised in the specific identification of GPAC.

In contrast to Porschen and Spaulding, Markowitz and Lerner (1977) found three clinical strains of Pc. magnus to be serologically distinct. They used bacteria disrupted by sonication as antigens both for the intravenous immunisation of rabbits and the detection of antibodies in serum by counter-immunoelectrophoresis. Such cell preparations will contain cytoplasmic as well as surface antigens which makes interpretation of results more difficult. These authors could not demonstrate cross-reactivity between the strains of Pc. magnus tested or with antiserum raised in a similar manner to Ps. anaerobius; the reverse reaction, i.e. between Ps. anaerobius antigen and



Pc. magnus antisera also gave negative results, suggesting that no antigens were shared between these strains.

Graham and Falkler (1978) injected rabbits subcutaneously with soluble antigens derived from sonicated GPAC and used the same type of extracts in immunodiffusion, immunoelectrophoresis, indirect fluorescent antibody and tanned-cell passive haemagglutination tests. Of the strains examined - two strains of Ps. anaerobius, one each of Ps. micros, Ps. parvulus, Ps. productus and Strept. morbillorum, all sera reacted strongly with their homologous antigen preparation; each strain of Ps. anaerobius demonstrated reciprocal cross-reactivity with antiserum to the other. The only inter-species cross-reactivity observed was exhibited by Ps. productus antiserum and Ps. parvulus antigen, but the reverse reaction was negative.

These results had suggested to Graham and Falkler the existence of shared antigens within the species Ps. anaerobius. The following year (Graham and Falkler, 1979) they continued their exploration of the serological characteristics of this species by testing antisera prepared to the two strains of Ps. anaerobius described previously with antigens extracted from these and six other clinical isolates of Ps. anaerobius. In this case, however, antigens were prepared by autoclaving cell suspensions and using supernatants obtained following centrifugation in capillary precipitin tests. All eight strains of Ps. anaerobius reacted with the two homologous antisera, but none reacted with antisera to



Lancefield's groups A-G streptococci. Similarly, antigens prepared from other species of GPAC and streptococci did not react with the Ps. anaerobius antisera in precipitin tests.

Wong et al. (1980) also concentrated their studies on Ps. anaerobius, using antisera raised to whole cells of reference strains and sonicated cell extracts or culture medium supernatants as antigens. In Ouchterlony tests, they demonstrated precipitin lines of identity between all Peptostreptococcus spp and uninoculated culture medium, but these could be eliminated by a single absorption of antisera with dehydrated culture medium. Subsequent tests revealed all the antisera to be species-specific. These vaccine strains, together with 16 clinical isolates representing each species of Peptostreptococcus were tested in Staphylococcus protein A coagglutination (SPAC) reactions, in which the protein A had been sensitised with each of the antisera. These tests again indicated only species-specific reactions and gave no evidence for the existence of common genus antigens. All strains biotyped according to VPI criteria (Holdeman et al., 1977) reacted strongly and exclusively with antisera raised to the corresponding reference strains. Furthermore, no cross-reactions occurred between peptostreptococci and strains of Pc. asaccharolyticus, Pc. magnus or Streptococcus intermedius tested similarly. SPAC proved to be a more rapid and sensitive test than the Ouchterlony method. The authors advised that, although useful as antigens in serological tests, sonic extracts

were not ideal for the production of antisera; the presence of cytoplasmic antigens released by disrupted cells results in an unnecessarily complex range of antibodies. Whole cells expressing surface antigens are more practical in this respect.

In summary, attempts to classify GPAC using traditional serological techniques have met with as little success as existing biochemical and morphological schemes. Conflicting evidence has emerged regarding common antigens within and between species and even genera. This may be accounted for by different methods of antigen and antiserum preparation, or techniques used to demonstrate their interaction. It may also indicate that the antigenic determinants possessed by GPAC are complex, therefore careful selection of antigens, both as immunogens and detectors of specific antibodies, is a prerequisite to success in this approach.

## 5.2 Polyacrylamide Gel Electrophoresis

In recent years, a technique that has received much attention as an aid to taxonomic studies of bacteria is polyacrylamide gel electrophoresis (PAGE). The procedure involves the separation of cell extracts, usually proteins, according to molecular size by the sieve-like effect of the porous acrylamide gel and their visualisation by staining with, e.g. Coomassie blue, silver, amido black. Such proteins may be made soluble with sodium dodecyl sulphate (SDS); this anionic detergent is used to resuspend samples before their application to gels and is also included in electrophoresis buffers and gels themselves. SDS dissociates proteins into monomeric polypeptides and coats them with a uniform negative charge, thus promoting their migration towards the anode during electrophoresis. The rate of migration of these molecules, and therefore their final position in the stained gel, is determined by their molecular weight and the pore size of the gel. An additional function of SDS is to prevent reaggregation of polypeptides into their native configurations.

Proponents of PAGE for taxonomic studies stress the fact that such an examination of these genetically-determined cellular components reveals relationships between organisms at generic and species levels, but avoids the complex, expensive and

time-consuming procedures necessary for DNA homology studies. Many authors have reported that PAGE often supports taxonomic data obtained from nucleic acid studies. Several of these are reviewed below.

Polyacrylamide gels consist of acrylamide and methylene bis-acrylamide, polymerised by the addition of ammonium persulphate and tetramethylethylenediamine. Gels may be of two types:

- i. gradient, either discontinuous or linear, where the concentration of acrylamide usually increases, therefore the pore size decreases, away from the point of sample application. Low molecular weight molecules migrate further from the origin than heavier molecules.
- ii. homogeneous, which is more commonly used because of its greater reproducibility, and ease of preparation.

An early study by Razim and Rottem (1967) demonstrated the value of PAGE in the classification of Mycoplasma spp. Whole cell proteins of various species were extracted with a phenol-acetic acid-water mixture and applied to gels containing acetic acid and urea; these substances prevented reaggregation and enhanced the reproducibility of patterns. The authors reported that individual species gave almost identical and reproducible patterns which thereafter aided in the identification of fresh isolates. Morris (1973) conducted a similar study of Brucella spp. using the methods of Razim and Rottem. All but one of the strains tested gave

similar patterns, indicating a close relationship and confirming nucleic acid studies. Morris included in his study two strains of Yersinia spp, one of which showed serological cross-reactivity with Brucella spp. These two strains also had identical patterns, which differed from Brucella patterns, despite the serological similarity between this genus and one of the Yersinia strains. Morris emphasised the practicality of using whole cell proteins as opposed to purified specific cellular components such as enzymes since the former required smaller volumes of cells and less preparation than the latter.

Biavati et al. (1982) observed good correlations between PAGE patterns of soluble cellular proteins, biochemical tests and DNA homology data for 1094 strains in the genus Bifidobacterium; identical or almost identical PAGE patterns corresponded to  $\geq 80\%$  DNA homology between strains. Biavati's group proposed four new species within the genus on the basis of their results. A similar correlation between PAGE profiles and DNA homology was found by Cato et al. (1982) for the genus Clostridium. As well as  $\geq 80\%$  DNA homology between strains exhibiting very similar patterns, Cato's group determined that minor differences represented 70% homology. This group obtained proteins by shaking cells with glass beads; after centrifugation to remove the beads and cell debris, the supernatants were applied to polyacrylamide gels. In common with other genera, different species of clostridia produced individual profiles and the technique proved useful for the

presumptive identification of unknown strains within 24h of obtaining a pure culture. Problems were encountered with certain strains, however: despite applying a standard amount of protein, their tracks stained so darkly that individual bands were indistinguishable. This problem was solved by photographing these tracks with an increased lens aperture.

Several authors have studied relationships within the genus Bacteroides using PAGE. Strom et al. (1976) examined the profiles of proteins extracted by sonication of five species of bacteroides and two species of fusobacteria run in discontinuous gradient gels. No general pattern was apparent between these genera, but Swindlehurst et al. (1977) noted that groupings of strains in the genus Bacteroides suggested by PAGE matched those indicated by biochemical and serological tests, as well as G + C ratios. They observed "minor strain differences" in patterns within certain subspecies which were qualitative and quantitative, but overall similarities in protein profiles allowed the recognition and classification of strains.

Poxton and Brown (1979) used ethylenediaminetetra-acetic acid (EDTA), followed by sonication, to remove the outer membrane complexes of species in the B. fragilis group. When subjected to homogeneous SDS-PAGE, individual species exhibited very similar protein profiles, but these varied between different species.

The gingival crevice flora of humans was investigated by Moore et al. (1980) who used PAGE to identify unknown organisms by



comparison of their protein profiles with those of reference strains; proteins were released from cells by vortexing. Moore's group did not use SDS, since they had observed that cells broken in the presence of SDS did not yield greater amounts of protein, nor did they produce more bands in PAGE; SDS added to gels and electrode buffers, however, increased the number of bands and made visual assessment difficult. Gingival crevice isolates were first grouped according to cell morphology and Gram reaction prior to PAGE and subsequently identified according to VPI criteria (Holdeman et al., 1977). Patterns in gels were compared visually and band mobilities assessed by reference to a Streptococcus faecalis protein. Comparison of up to 30 protein bands proved adequate to distinguish between serotypes within a species. The majority of isolates with similar patterns proved to belong to the same species and comparison with reference strain profiles often provided a presumptive identification before biochemical testing. Occasional isolates, however, proved to belong to different species, or even genera, than those suggested by their protein profile.

The technique of PAGE has also been applied to GPAC in further attempts to resolve their taxonomic status. Cato et al. (1983) used the procedures described by Moore et al. (1980) to examine the soluble cellular proteins of ten strains of Pc. magnus, nine strains of Ps. micros and one strain of Pc. glycinophilus; all were VPI or ATCC reference strains. The patterns produced by

Pc. magnus were more heterogeneous than those of Ps. micros, but similar enough to indicate a close relationship between strains of the former species. The patterns produced by Ps. micros were quite different to those of Pc. magnus; in fact, the authors found that 13 strains previously thought to be Pc. magnus produced PAGE patterns typical of Ps. micros. This serves to highlight the recognised difficulties in distinguishing these species biochemically and the potential value of PAGE in such cases. Pc. glycinophilus produced a profile identical to the type species of Ps. micros and the respective DNAs of the two species demonstrated 84% homology. This prompted the authors to suggest that Pc. glycinophilus was a later synonym of Ps. micros. PAGE also indicated that Pc. variabilis was not closely related to Pc. magnus, as suggested by West and Holdeman (1973) on the basis of phenotypic testing. The exact status of Pc. variabilis awaits confirmation by DNA homology studies. Cato and her colleagues conclude that the precise identification of isolates, by methods including PAGE, could reveal the initial source of infection. This presupposes, however, the existence of GPAC with distinctive characteristics in different areas of the body.

In contrast to many of the authors mentioned above, Fox and McClain (1974) found PAGE to be of little help in determining taxonomic relationships between Neisseria spp, Branhamella catarrhalis and Micrococcus cryophilus. In some cases, relationships suggested by PAGE contradicted evidence from nucleic



acid studies. The authors attributed this to the difficulty in determining precise relationships from complex patterns examined by eye. Most of the studies mentioned thus far involved visual comparison of different tracks within a gel itself or <sup>in</sup> photographic reproductions. This approach is criticised on the grounds that it is essentially subjective and therefore variation between individual examiners is inevitable. Although the human eye can pick out identical patterns and can compensate for minor variations between very similar patterns, precise assessment of similarities between slightly different profiles is difficult.

Two alternative methods exist for the assessment of protein profiles in polyacrylamide gels.

i. measurement of the relative front (Rf): this involves direct measurement of the distance moved by protein bands relative to a selected reference point. This technique was used by Biavati et al. (1982) in their study of bifidobacteria. This technique also has a subjective element in the selection of bands to be measured. Since most cell extracts reveal many protein bands following electrophoresis, they cannot all be distinguished and accurately measured.

ii. computer-assisted numerical analysis: this involves stained gels being scanned by a densitometer, which produces a profile of peaks and troughs corresponding to band densities. Individual

patterns are then 'normalised' relative to a standard protein, usually thyroglobulin, included in each track; such patterns can then be compared with others to obtain the 'best fit', which may involve the realignment of certain traces. Correlation coefficients derived from pairs of patterns are combined and subjected to cluster analysis from which dendrograms of related strains are constructed. Such an approach demands rigorously standardised gel preparation and running conditions if reproducibility is to be obtained, since slight differences in patterns induced by experimental procedures alter the correlation coefficients and can affect final groups.

Kersters and De Ley (1975) utilised this technique in their study of Agrobacterium and Zymomonas species, declaring visual comparisons of protein patterns in polyacrylamide gels to be unsatisfactory, since they were "subjective, confusing and not suitable for large numbers of extracts". They outlined in considerable detail culture methods, extract preparation and gel running conditions to enhance reproducibility. Numerically-analysed results allowed the formation of groups that correlated well with DNA homology studies. In the authors' opinion, the advantages of numerical analysis included the rapid grouping of organisms it allowed, the identification of unknown strains by comparison with fully-characterised organisms and the storage of large numbers of patterns in a data bank for this purpose.

Despite their meticulous experimental procedure, they found slight variations in protein mobilities of the same samples run in different gels. They further admitted that numerical analysis was not always as sensitive in the definition of slight differences between patterns as conventional visual or photographic comparisons. This occasionally led to organisms being incorrectly grouped.

Other bacteria whose classifications have been examined by PAGE of their proteins linked to computer-assisted numerical analysis include Corynebacterium (Jackman, 1982), Pseudomonas (Owen and Jackman, 1982) and Acinetobacter (Alexander *et al.*, 1984). The latter authors suggested that, as well as its value in taxonomic studies, SDS-PAGE had considerable potential for 'fingerprinting' organisms in epidemiological studies; in this case, numerical analysis could be replaced by less time-consuming visual comparisons.

Taylor (1984) applied proteins released from reference strains and clinical isolates of GPAC by lysozyme to polyacrylamide gels and compared the resultant profiles with numerical analysis. In common with Ezaki and Suzuki (1982), Taylor found that many strains of GPAC were resistant to lysis by this enzyme. The majority of strains tested, however, could be placed into one of eight groups: all strains of Ps. anaerobius produced very similar patterns which included a broad, darkly-staining high molecular weight band; Ps. micros strains also produced identical patterns,

but these differed from Ps. anaerobius. The patterns produced by Pc. asaccharolyticus were more varied, but formed four groups, two of which displayed a large band of high molecular weight similar to Ps. anaerobius; strains of Pc. magnus and Pc. prevotii could each be divided into three groups according to their protein profiles. The remaining isolates were described as Pc. asaccharolyticus-like, Pc. magnus-like or Pc. variabilis-like; the latter species did not produce the same pattern as Pc. magnus, confirming the opinion of Cato et al. (1983) that they are not closely related. Taylor concluded that PAGE of whole cell proteins was the best method available for taxonomic studies of GPAC.

The need for a standardised PAGE procedure has been stressed by many of the authors whose studies have been reviewed. Factors affecting pattern reproducibility include bacterial growth conditions, centrifugation time and speed, gel running conditions (voltage, temperature) and oxygen in the acrylamide solution which inhibits polymerisation; Moore et al. (1980) regarded the latter as the most significant, therefore gel solutions should be de-gassed under vacuum, or by flushing with an inert gas, prior to the addition of polymerising agents. Reproducibility does not seem to be affected by variations in culture media or the age of the culture when cells are harvested but identical growth conditions are recommended (Razim and Rottem, 1967; Morris, 1973).

### 5.3 Immunoblot Transfer from Polyacrylamide Gels

An extension of PAGE of major significance was reported by Towbin et al. (1979), who successfully transferred separated proteins from polyacrylamide gels to nitrocellulose membranes by further electrophoresis; synonymous terms are Western blotting and electroblot transfer. Staining of nitrocellulose treated in this way for protein revealed exact replicas of the original gel patterns. Towbin's group then demonstrated the serological application of this technique: antigens contained in extracts that had adsorbed to the membrane could be detected by incubation with a specific antiserum, followed by a second antibody (directed against the first) conjugated to an enzyme or radioactive isotope. Thus, the number and molecular weight of antigens in the original extracts could be assessed by chromogenic enzyme substrates or autoradiography. However, it is critical that adsorption of protein antigens to nitrocellulose does not alter their configuration and should leave antibody-binding portions exposed. In addition, any sites on the membrane that have not adsorbed protein from the gel should be blocked with another protein, such as bovine serum albumin or gelatin, to prevent the non-specific binding of immunoglobulin. An important advantage <sup>that</sup> this technique has over immunoelectrophoresis in agarose gels is that it avoids

the need for antigen-antibody complexes to precipitate; this technique cannot be utilised with non-precipitating antibodies such as monoclonals. Furthermore, it can detect nano- or even picograms of antigen (Towbin et al., 1979), therefore is more sensitive than alternative serological techniques.

Immunoblot transfer can, therefore, be used to ascertain the components in a mixture of microbial proteins, to which a human or animal has been exposed, that elicit a humoral immune response. Class-specific immunoglobulin conjugates can be utilised in the procedure to determine the nature of this response more accurately.

In summary, the transfer of cell extracts to nitrocellulose, following their separation by SDS-PAGE, allows the detection of minute amounts of antigen. This technique has considerable potential in both taxonomic and epidemiological studies of pathogenic microorganisms.

#### 5.4 Aims of the Present Study

The purposes of investigations conducted in the present section were as follows:

- i. to extract non-covalently bound cell surface compounds from various species of GPAC and compare the profiles of their component proteins produced by SDS-PAGE;
- ii. to raise antisera in rabbits against commonly isolated species of GPAC;
- iii. to use these antisera and cell surface extracts in enzyme-linked immunosorbent assays (ELISA) and immunoblot transfer following PAGE to determine the existence of common genus or species antigens;
- iv. to assess the relationships between 'biotypes' and 'serotypes' and the relevance of these results to the classification of GPAC.



## 5.5. Materials and Methods

**Bacteria:** Seventy six strains of GPAC were selected for study. These comprised five reference strains and 71 clinical isolates stored as lyophilised preparations or in skimmed milk at -20°C.

**Culture:** organisms were reconstituted with pre-reduced modified Robertson's cooked meat broth (Watt, 1972) and incubated anaerobically at 37°C for 48h. After Gram films were made to assess purity, CMB cultures were then used to inoculate a culture medium selected from a pilot study: five strains of GPAC were cultured in 200 ml of pre-reduced BM broth per strain (see Appendix 1), 200 ml BM broth plus horse serum (final concentration 2%) and 200 ml PPY medium (Holbrook et al., 1977). A further method involved resuspending the entire surface growth of GPAC on blood agar plates in 5 ml nutrient broth and using this suspension in a flood-plate technique: 5 ml of nutrient broth were poured onto the first blood agar plate, then from this plate to a second, third etc. until the entire volume was absorbed. These plates, together with BM and PPY broths were incubated anaerobically for 48h at 37°C. Purity was assessed following incubation with both Gram films and blood agar plates inoculated with cultures for 48h parallel aerobic and anaerobic incubation.



**Harvest of bacteria:** after 48h incubation, cells cultured in broths were harvested by centrifugation (20 000g, 10 min) and the supernatant discarded. Cells were washed twice in 40 ml phosphate-buffered saline (PBS: 50 mM phosphate buffer, pH 7.5, with 0.15M NaCl) and harvested by centrifugation (20 000g, 10 min). Blood agar cultures were harvested by flooding each plate with 5 ml PBS and removing colonies by gently scraping the agar surface with a sterile glass spreader. Cells suspended in PBS were then pipetted off and harvested by centrifugation (4000g, 15 min) and washed twice with PBS.

**Extraction of cell surface components:** washed cells were resuspended in 2 ml PBS containing 10 mM EDTA (pH 7.4) and transferred to a 45°C water-bath for 30 min. This suspension was then centrifuged (10 000g, 3 min) and the extract containing supernatant was transferred to acid-washed Bijou bottles.

**Protein assay:** the protein content of individual EDTA extracts was estimated by the Lowry method (Lowry et al., 1951) using 2 mg ml<sup>-1</sup> bovine serum albumin as the standard.

**Preparation of antisera:** four clinical isolates of GPAC were selected for the production of antisera in rabbits, based on the method of Poxton (1979). The strains comprised Ps. anaerobius (GPAC 56393) from a case of otorrhoea; Pc. magnus

(GPAC 149) and Pc. prevotii (GPAC 150), both from wound infections; Pc. asaccharolyticus (STD 34) from the vagina of a symptomatic female. All strains had been stored in skimmed milk at  $-20^{\circ}\text{C}$ . Bacteria were reconstituted in pre-reduced CMB and incubated anaerobically for 48h in  $37^{\circ}\text{C}$ . After purity checks, 1 ml of each CMB was used to inoculate separate 20 ml volumes of pre-reduced PPY medium, which were incubated anaerobically for 24h at  $37^{\circ}\text{C}$ . Cells were harvested by centrifugation (4000g, 15 min) and washed three times in PBS. After the final wash, cell pellets were resuspended in 5 ml PBS and, after counting under phase contrast microscopy, adjusted to a concentration of  $10^9$  organisms per ml. Two New Zealand white rabbits per strain were inoculated intravenously with  $10^9$  live cells according to the following schedule: three daily injections of  $10^9$  cells each week for four weeks. Antibody titres were then determined by an ELISA technique (see below) and the majority of animals were exsanguinated in the fifth week; those inoculated with Pc. prevotii, however, received two further booster injections of  $10^{10}$  and  $10^{11}$  cells in week five and six respectively before exsanguination.

Enzyme-linked immunosorbent assay: the technique used was that of Engvall and Perlmann (1972) adapted for microtitre plates and described by Voller et al. (1976). EDTA extracts, adjusted to  $30\ \mu\text{g ml}^{-1}$  protein with antigen buffer (50 mM carbonate buffer, pH 9.6, with 0.02% sodium azide) were added to wells in 50  $\mu\text{l}$

amounts and incubated at 37°C for 4h, then at 4°C overnight. After thorough washing of wells with 0.15N NaCl containing 0.05% Tween 20, plates were shaken dry prior to the addition of 50 µl of antisera (diluted in PBS containing 0.05% Tween 20 and 0.02% sodium azide); plates were incubated for 4h at room temperature and washed as described. Anti-rabbit IgG conjugated to alkaline phosphatase (Miles Laboratories (UK) Ltd., Stoke Poges, Slough, Bucks.) was diluted 1:600 (determined by checker-board titrations) in antiserum buffer, added to wells in 50 µl amounts and incubated overnight at room temperature. The following day, 50 µl of a 1 mg ml<sup>-1</sup> solution of p-nitrophenylphosphate (Sigma) were added to each well and results were read after 60 min in a Titretrek Multiskan (Organon Teknika, St. Neots); end-points of titration were taken as the first dilutions of antisera with an A<sub>405</sub> (absorbance value at 405 nm) of 1.0. Sera taken from rabbits prior to immunisation gave no reaction in ELISA against homologous and heterologous strains, therefore animals did not have pre-existing antibodies to these bacteria.

SDS-PAGE: a modified version of the method of Laemmli (1970) was used, as described by Poxton and Brown (1979). Details of buffers are included in Appendix 2. Vertical slab gels (170 x 140 x 1 mm) consisted of a 10 mm 4% acrylamide stacking gel above a 10% acrylamide separating gel. EDTA extracts containing 100 µg protein, suspended in 100 µl single strength sample buffer

containing SDS and boiled for 3 min, were added to wells in the stacking gel and run under the following conditions: 60V were applied to the gel to move samples through the stacking gel (1-2h), followed by 150V until the sample buffer front (indicated by bromophenol blue) was approximately 2 cm from the bottom of the separating gel (2-3h). Gels were then stained with Coomassie blue (Poxton and Sutherland, 1976).

**Immunoblot transfer:** the technique described by Cousland and Poxton (1983) was adopted. EDTA extracts adjusted to 10 µg protein in 50 µl of sample buffer (boiled for 3 min) were run in duplicate SDS-polyacrylamide gels as described, but electrophoresis was halted when the gel front had travelled 7.5 cm into the separating gel. One gel was stained with silver (Oakley et al., 1980) to demonstrate protein profiles; the other gel, containing the same extracts, was used in an immunoblot transfer, according to the method of Towbin et al. (1979) using a BioRad ImmunBlot Assay kit as follows: the gel was placed in contact with a sheet of nitrocellulose membrane (initially pore size 0.45 µm, but replaced by 0.2 µm early in the study) pre-soaked in electrophoresis buffer (Tris, glycine and methanol, pH 8.3) and electrophoresed at 12V (40 mA) for 18h. The membrane was then washed in Tris-buffered saline (TBS: 20 mM Tris, 500 mM NaCl, pH 7.5) and transferred to a blocking solution of 3% gelatin-TBS for 45 min. After blocking, the membrane was placed in the first antibody solution for 2½h:

antisera to Pc. magnus, Pc. asaccharolyticus and Pc. prevotii were diluted 1:50 in 1% gelatin-TBS (antibody buffer), whereas Ps. anaerobius antiserum was used at a dilution of 1:250 (see results of antibody titre assays). Excess unbound antisera were removed with two 10 min washes in 0.025% Tween 20-TBS (TTBS) and the nitrocellulose was then transferred to a second antibody solution containing goat anti-rabbit IgG-horseradish peroxidase conjugate, diluted 1:3000 in antibody buffer. After 60 min, the membrane was washed as before, then placed in a horseradish peroxidase colour development solution consisting of 4-chloro-1-naphthol (the substrate), ice-cold methanol, hydrogen peroxide and TBS; the appearance of purple bands indicated that the first antibody solution had reacted with antigens transferred from the polyacrylamide gel. The reaction was stopped by immersing the nitrocellulose in distilled water, usually after 15 min, but with faint bands it was allowed to continue up to a maximum of 30 min. Clean glassware was used for each stage of the procedure to avoid extraneous protein appearing on the developed membrane; for the same reason nitrocellulose membranes were handled with forceps. Plastic containers are not suitable, since proteins may stick to their surfaces and contaminate the nitrocellulose. Gentle agitation of containers throughout ensured uniform exposure of the membrane to each reagent.

## 5.6 Results

### 5.6.1 Selection of culture medium and cell protein yields

Five strains, representing four species, of GPAC were used in a pilot study to compare protein yields from different culture and results are shown in Table 5.1. The lowest yields were obtained from cells grown in PPY medium, particularly asaccharolytic strains; these always exhibited less growth than Ps. anaerobius after 48h incubation. The flood-plate technique resulted in the highest protein yields, but PAGE profiles of EDTA extracts from organisms grown in this manner revealed additional bands when compared to those grown in broths. This suggested that blood proteins had been incorporated in the extracts, despite thorough washing of cells before incubation with EDTA; this may also account for the higher protein yields. The addition of serum to BM broth did not markedly enhance protein yields and problems were encountered on occasion with contaminated serum. From this study, it was decided to culture further strains in BM broth without additional serum. Attempts were made to increase protein yields by prolonging the EDTA incubation period up to 90 min, followed by sonication, but these had no significant effect on results. A period of 60 min incubation with EDTA was chosen for

TABLE 5.1:

PROTEIN YIELDS FROM GRAM-POSITIVE ANAEROBIC COCCI GROWN IN  
DIFFERENT CULTURE MEDIA ( $\mu\text{g ml}^{-1}$ )

Strain	PPY	Blood Agar	BM	BM + Serum
<i>Ps. anaerobius</i> 1	232	698	571	229
<i>Ps. anaerobius</i> 2	229	210	221	434
<i>Pc. asaccharolyticus</i>	124	533	175	298
<i>Pc. magnus</i>	61	522	383	290
<i>Pc. prevotii</i>	91	476	156	163



future extractions, since there was no microscopic evidence of cell lysis occurring within this period.

In general, protein yields from Ps. anaerobius were higher than other species, which may reflect their more prolific growth under these conditions. Considerable variation was observed within this species, however: different strains yielded 200-1000  $\mu\text{g ml}^{-1}$  protein, even if grown in the same batch of medium under identical conditions. Protein yields from other species were in the range of 100-600  $\mu\text{g ml}^{-1}$ .

#### 5.6.2

##### Antibody titres

Antibody levels to whole cells of four species of GPAC were determined by ELISA, using EDTA extracts from each strain as antigens. Sera from each pair of rabbits were initially tested separately, and then pooled from which the following end-points were recorded: Ps. anaerobius 1:8000; Pc. magnus 1:1600; Pc. prevotii 1:200; Pc. asaccharolyticus 1:1600. Despite two additional booster injections with increased numbers of live cells, Pc. prevotii proved to be poorly immunogenic relative to the other strains.



5.6.3 PAGE

Figure 5.1 shows the protein profiles of five strains each of Ps. anaerobius and Pc. magnus stained with Coomassie blue. Samples added to each track contained 100 µg protein. Striking similarities are apparent between strains of Ps. anaerobius (tracks 2-6) and the patterns are dominated by a single darkly-staining band corresponding in mobility to ovotransferrin (M.W. 76 000-78 000; track 1) in the molecular weight marker (BDH); other regions in the patterns show slight qualitative and quantitative variations. In contrast, the profiles of Pc. magnus (tracks 7-11) show more variation, particularly in track 10, which contained the extract from NCTC 9815.

Strains of Pc. prevotii also demonstrated heterogenous protein profiles (Figure 5.2), but patterns in tracks 4 and 5 and 8 and 9 are very similar. Despite receiving the same amount of protein, bands in track 7 stained very faintly. All strains shown in this figure were vaginal isolates from symptomatic women; tracks 2 and 3 show different patterns, yet the strains were obtained from a single patient; tracks 6, 7 and 8 represent isolates from three different patients and show different patterns; tracks 4 and 5 represent isolates from the same patient, as do tracks 9, 10 and 11.

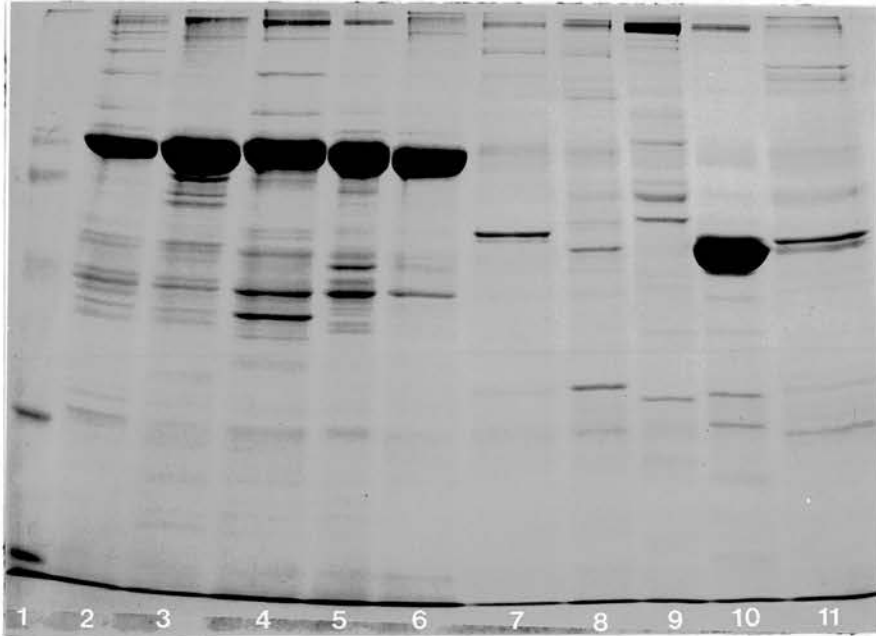


FIGURE 5.1:

PAGE of EDTA extracts from five strains of *Pc. anaerobius* (tracks 2-6) and five strains of *Pc. magnus* (tracks 7-11). Track 1 contains molecular weight marker. Coomassie blue stain; 100  $\mu$ g per track.

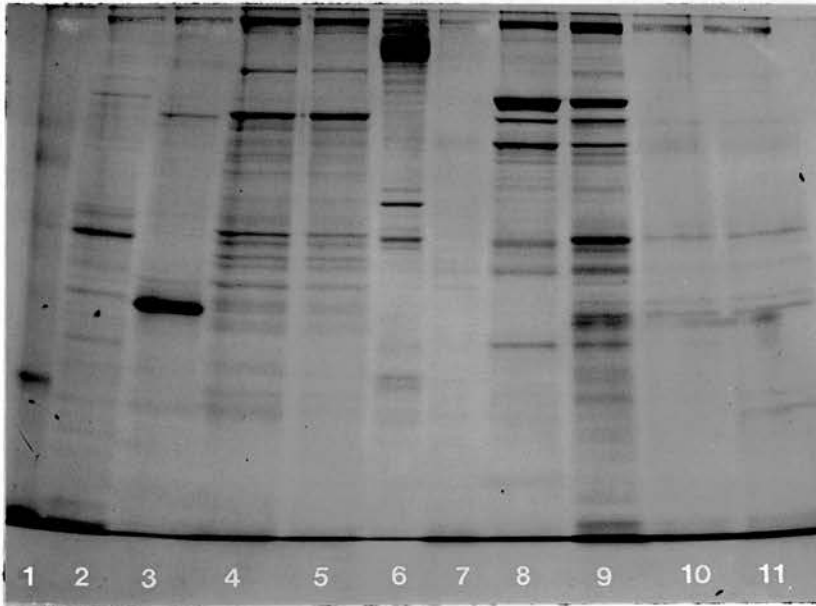


FIGURE 5.2:

PAGE of EDTA extracts from ten strains of Pc. prevotii (tracks 2-11). Track 1 contains molecular weight marker. Coomassie blue stain; 100  $\mu$ g protein per track.

Figure 5.3 indicates qualitative similarities but considerable quantitative differences in the patterns of four Pc. asaccharolyticus extracts. All possess two identical high molecular weight bands, but the extract in track 4 stained poorly despite the application of a standard amount of protein. Tracks 2 and 3 represent NCTC 9801 and NCTC 9803. Five further clinical isolates of Ps. anaerobius from different sources (tracks 6-10) show considerable similarity and a common high molecular weight band. This band caused some distortion of others immediately in front of it and may have masked significant polypeptides. In an effort to reduce the size of this band and clarify patterns, extracts from a further six vaginal strains of Ps. anaerobius from two symptomatic females were run in a gel, but only 50 µg protein were applied to each track (Figure 5.4). Much clearer patterns were obtained and, although reduced in size, the high molecular weight band was still visible in each extract.

In general, patterns varied within and between the species Pc. magnus, Pc. prevotii and Pc. asaccharolyticus, but certain strains within each species produced almost identical profiles suggesting possible groups. All the strains of Pc. magnus isolated from the oral cavity produced similar patterns. Some of the numerous protein bands demonstrated in EDTA extracts appeared to be shared by different species. Only Ps. anaerobius, however, produced distinctive patterns which were visually almost identical in many regions. This similarity prompted the following

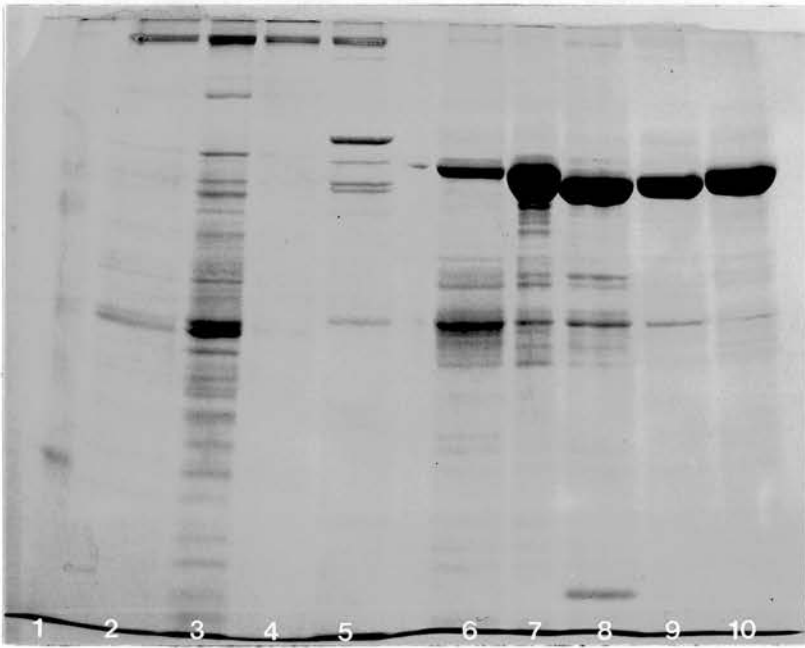


FIGURE 5.3:

PAGE of EDTA extracts from four strains of *Pc. asaccharolyticus* (tracks 2-5) and five strains of *Ps. anaerobius* (tracks 6-10). Track 1 contains molecular weight marker. Coomassie blue stain; 100  $\mu$ g protein per track.

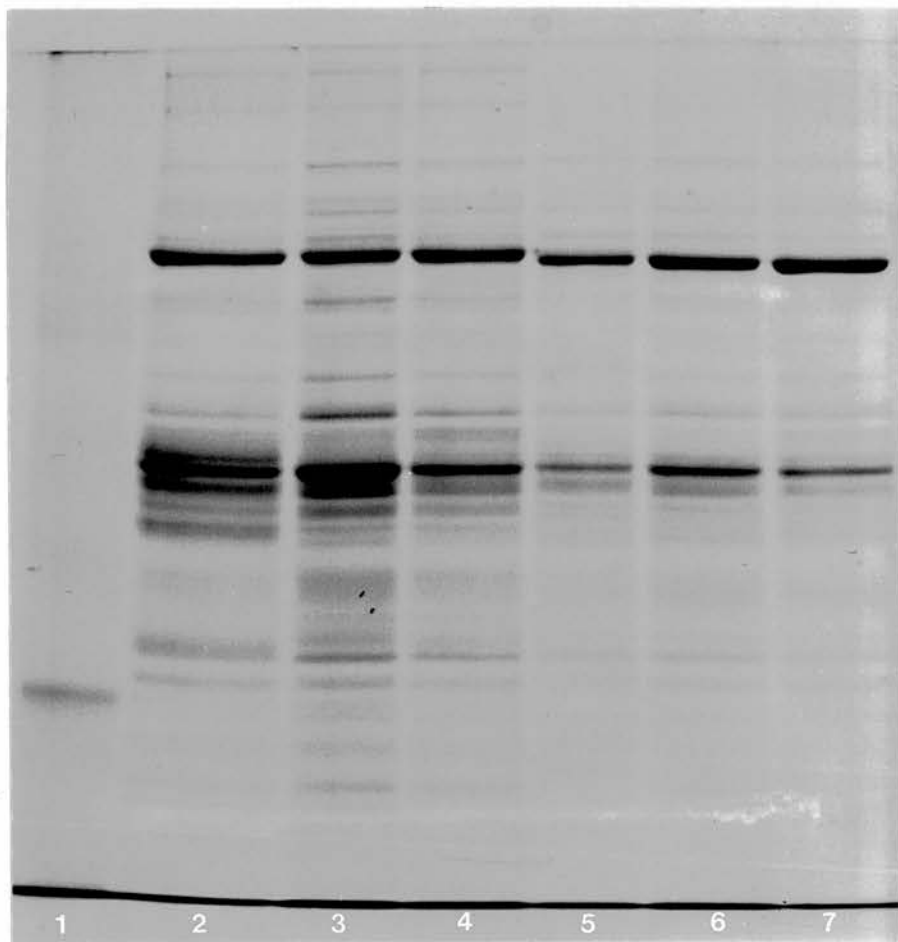


FIGURE 5.4:

PAGE of EDTA extracts from six strains of *Ps. anaerobius* (tracks 2-7). Track 1 contains molecular weight marker. Coomassie blue stain; 50  $\mu$ g protein per track.

investigations into the antigenic content of EDTA extracts using Western blotting.

#### 5.6.4

##### Immunoblot transfer from polyacrylamide gels

Figure 5.5 shows EDTA extracts from six strains of Ps. anaerobius, including the vaccine strain (GPAC 56393, track 5) run in a polyacrylamide gel (Figure 5.5a) and transferred electrophoretically to nitrocellulose pore size 0.2  $\mu\text{m}$  (Figure 5.5b). Each sample contained 10  $\mu\text{g}$  protein and the gel was stained with silver; despite the addition of standard amounts of protein, tracks 1 and 2 stained so darkly that certain regions were obscured. This figure also illustrates the greater sensitivity of the silver staining technique compared with Coomassie blue. The high molecular weight band was, however, still visible and, as demonstrated on the corresponding nitrocellulose membrane incubated with homologous antiserum, proved to be an antigen common to all six strains. A second low molecular weight antigen, of equivalent mobility to myoglobin, MW 17200, was also demonstrated. The extracts in tracks 2 and 6 displayed further antigens not shared by the other strains. Both of these common antigens were demonstrated in a further seven

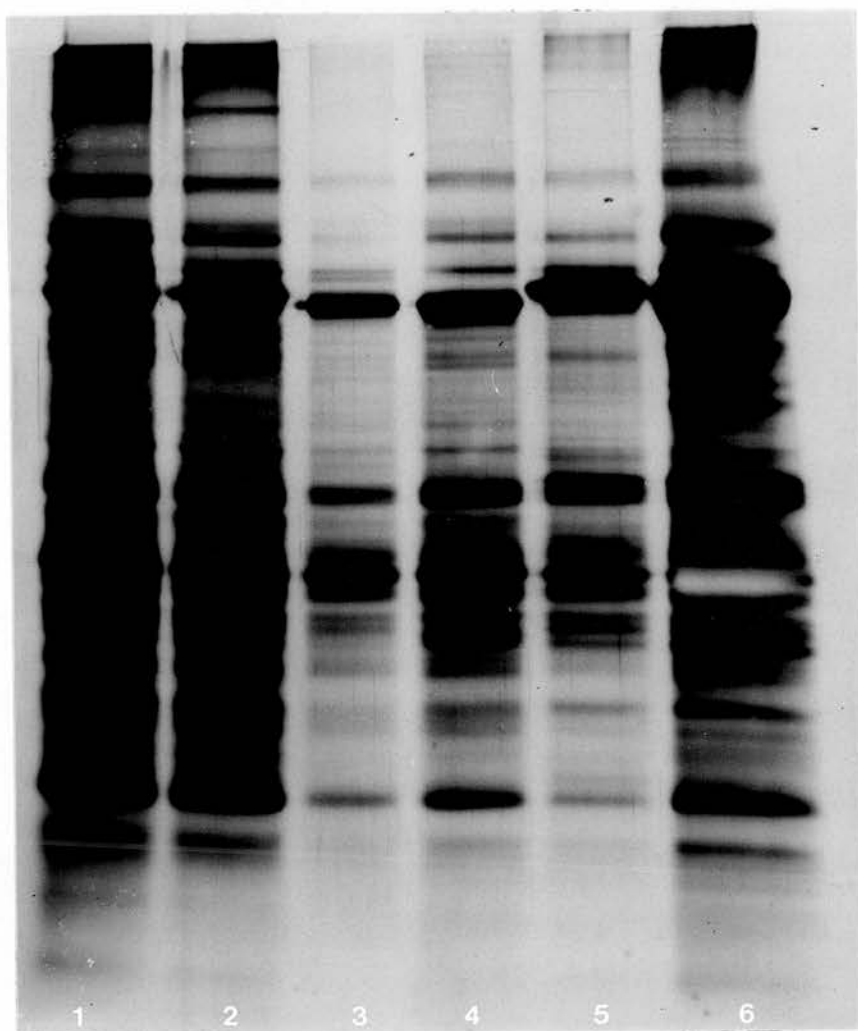


FIGURE 5.5a:

PAGE of EDTA extracts from six strains of Ps. anaerobius stained with silver; 10  $\mu$ g protein per track.



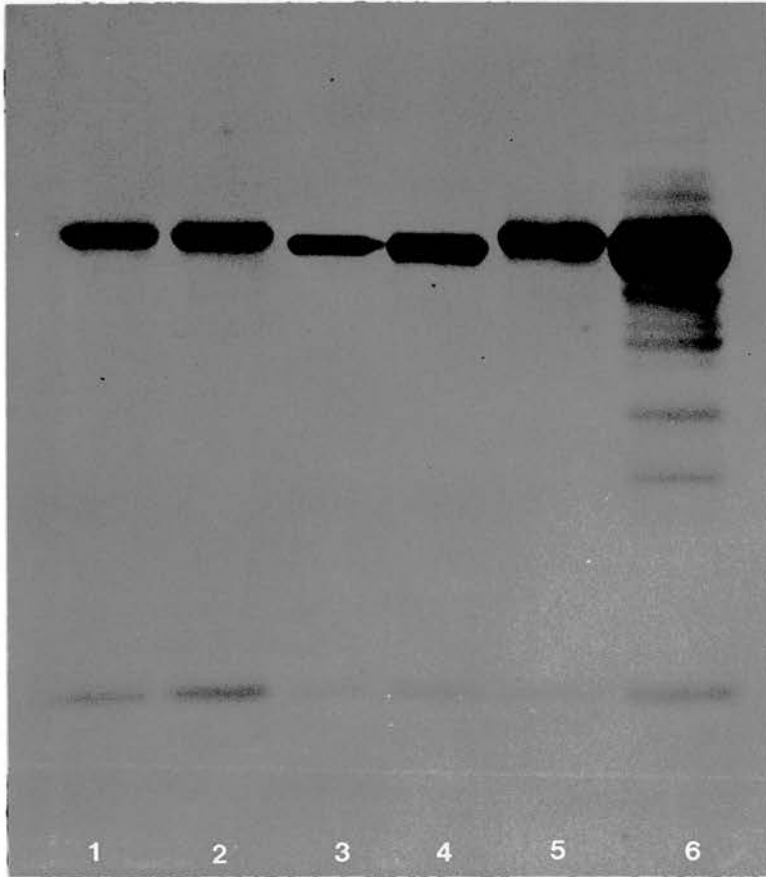


FIGURE 5.5b:

Immunoblot transfer from gel in Figure 5.5a incubated with antiserum to Ps. anaerobius GPAC 56393 (track 5).

strains of Ps. anaerobius from different sources, including ATCC 27337 by immunoblot transfer following PAGE.

The variation in protein profiles of EDTA extracts from strains of Pc. prevotii is further demonstrated in Figure 5.6; as described above, 10 µg of protein were applied to each track and the gel was stained with silver (Figure 5.6a). Incubation of the corresponding nitrocellulose membrane with antiserum raised against Pc. prevotii (GPAC 150, track 1), revealed a single high molecular weight antigen common to six of the seven strains, with additional unshared antigens in two strains (Figure 5.6b, tracks 4 and 5). The extract in track 7, however, from an isolate biochemically identified as Pc. prevotii and displaying several protein bands in common with the other six extracts, did not possess antigens detectable by antiserum to this species. This suggested misidentification of this strain or the vaccine strain by conventional means.

Strains of Pc. magnus (Figure 5.7) and Pc. asaccharolyticus (Figure 5.8) were investigated in a similar manner. Six of the Pc. magnus extracts tested revealed considerable similarities in gels stained with silver (Figure 5.7a). One extract (track 7) stained too darkly to discern a pattern; the vaccine strain (GPAC 149, track 8) produced a distinctly different pattern to other isolates. Examination of the corresponding nitrocellulose after incubation with homologous antiserum revealed a high molecular weight antigen common to four strains (Figure 5.7b,

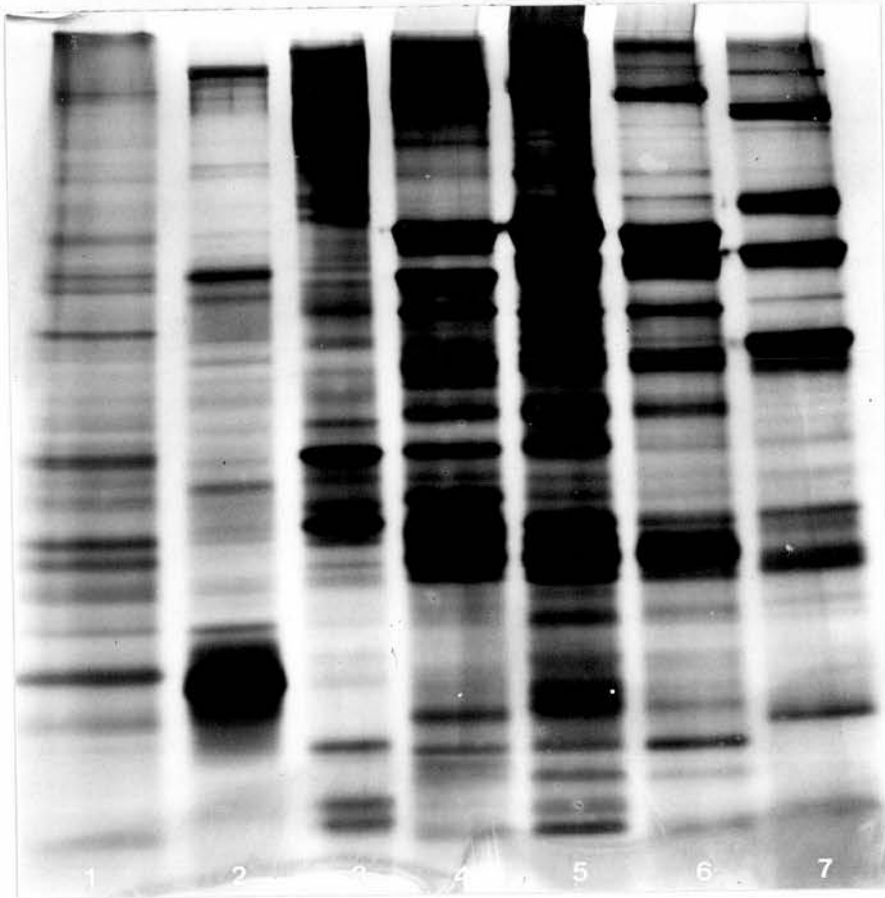


FIGURE 5.6a:

PAGE of EDTA extracts from seven strains of Pc. prevotii stained with silver; 10  $\mu$ g protein per track.

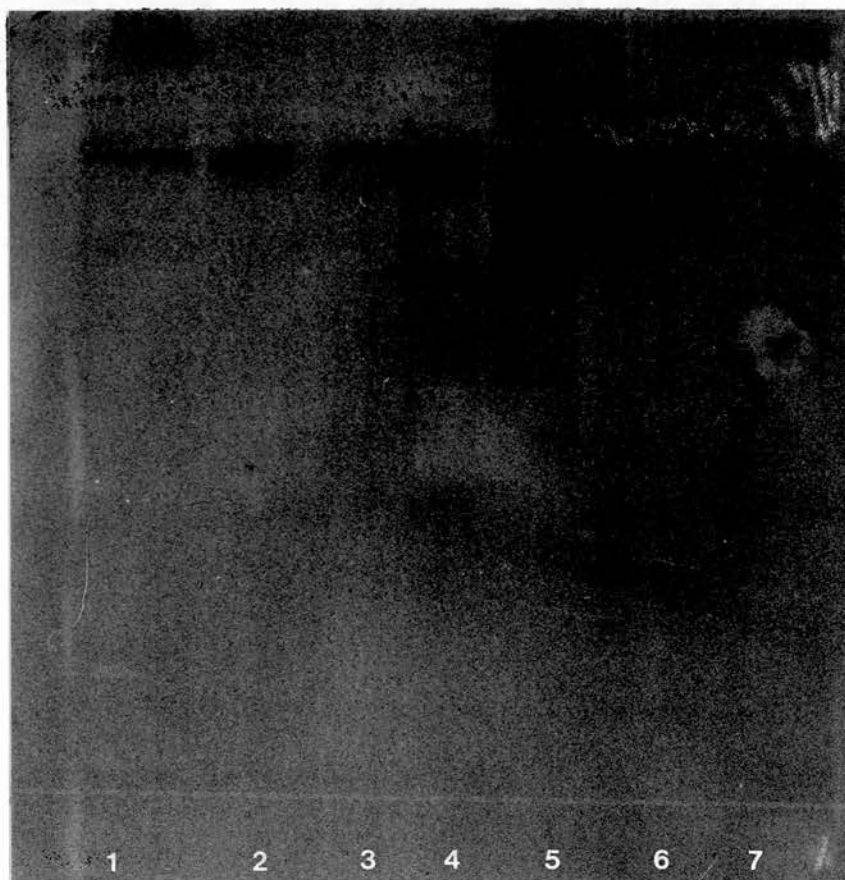


FIGURE 5.6b:

Immunoblot transfer from gel in Figure 5.6a incubated with antiserum to Pc. prevotii GPAC 150 (track 1).

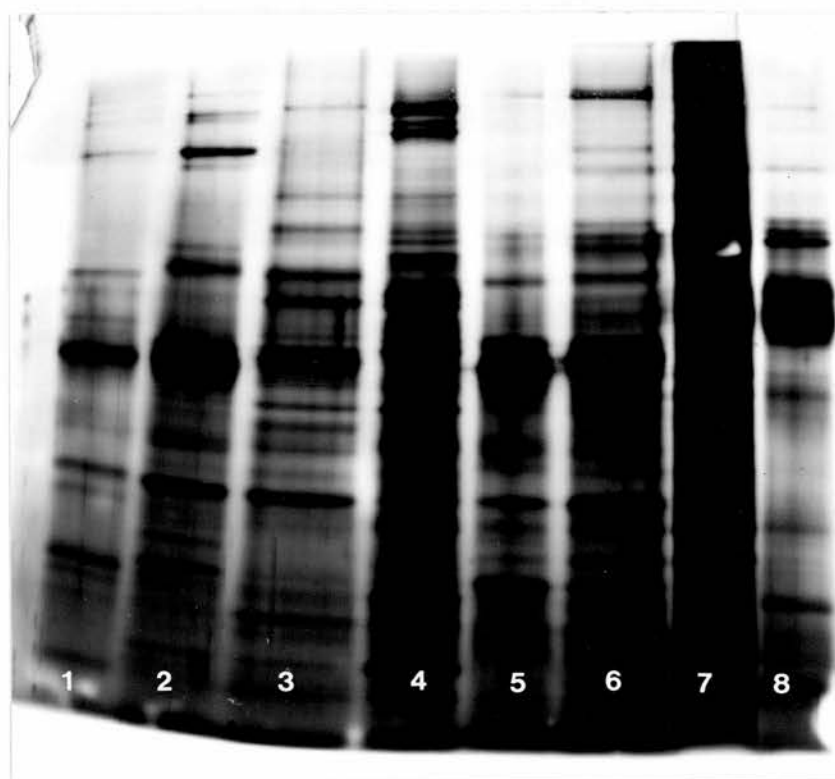


FIGURE 5.7a:

PAGE of EDTA extracts from eight strains of Pc. magnus stained with silver; 10  $\mu$ g protein per track.

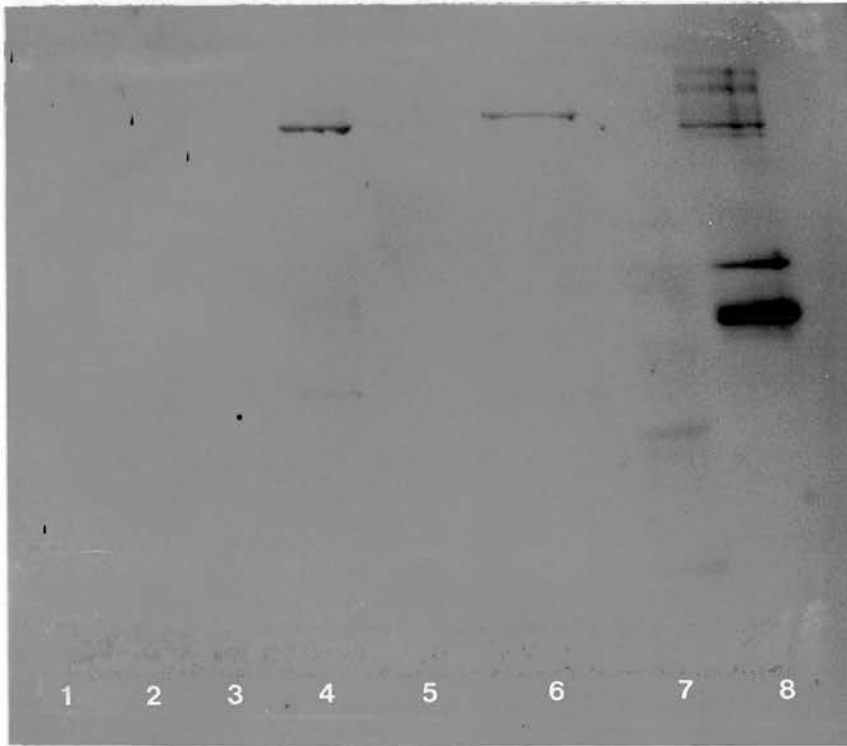


FIGURE 5.7b:

Immunoblot transfer from gel in Figure 5.7a incubated with antiserum to Pc. magnus GPAC 149 (track 8).

tracks 4, 5, 6 and 8) and additional unshared antigens (tracks 4, 6, 7 and 8). Extracts from three strains, however (tracks 1, 2 and 3) did not react with antiserum to Pc. magnus, despite having virtually identical profiles to three other strains which did react. A similar situation occurred with Pc. asaccharolyticus (Figures 5.8a and b). Track 1 contained the vaccine strain (STD 34); tracks 1, 4, 5, 6 and 7 (Figure 5.8b) show antigens of slightly different mobilities, therefore it is impossible to state from this evidence that they represent the same antigen shared between these strains; track 2 also possesses a faintly staining antigen in this region. No antigens were detected in the extract run in track 3.

Failure of extracts from several strains to react with antiserum to their biotype, suggesting incorrect identification previously, prompted further investigations, using both ELISA and immunoblot transfer from polyacrylamide gels, to establish the correct serological identity of these strains and determine the existence of cross-reactivity between species of GPAC.

#### 5.6.5 ELISA and immunoblot transfer

EDTA extracts from 50 strains of GPAC were tested in an ELISA procedure against each of the four antisera. The test organisms comprised 15 strains each of Ps. anaerobius and



FIGURE 5.8a:

PAGE of EDTA extracts from seven strains of Pc. asaccharolyticus stained with silver; 10  $\mu$ g protein per track.



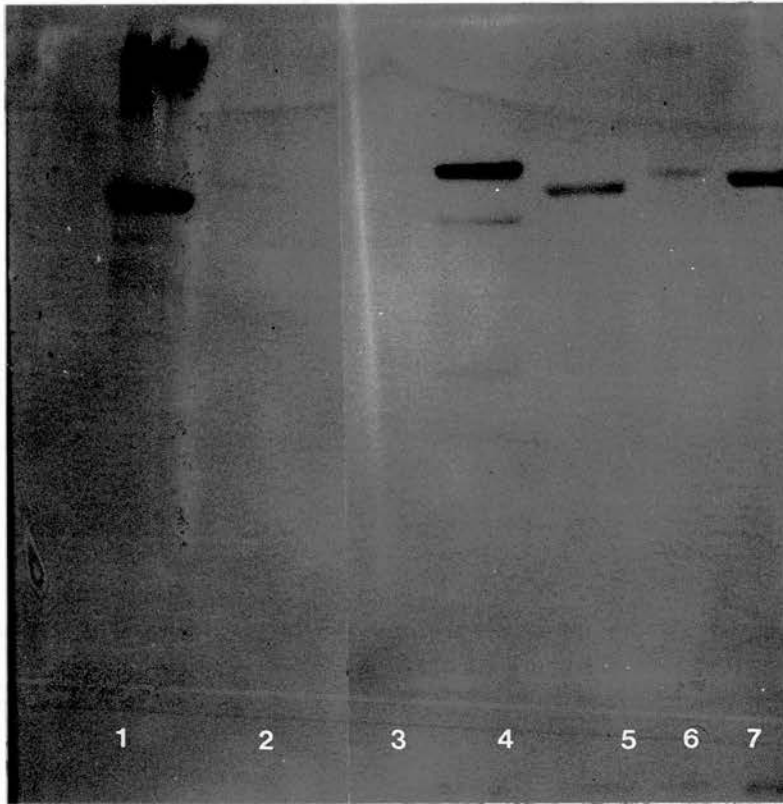


FIGURE 5.8b:

Immunoblot transfer from gel in Figure 5.8a incubated with antiserum to Pc. asaccharolyticus STD34 (track 1).

Pc. magnus and ten strains each of Pc. prevotii and Pc. asaccharolyticus. A single dilution of each antiserum was used: Ps. anaerobius 1:1000; Pc. magnus and Pc. asaccharolyticus 1:500; Pc. prevotii 1:100. Reagents were used as described in section 5.5. An  $A_{405}$  value of  $\geq 1.0$  indicated a positive value, but a value of 0.8 was included as a 'borderline' reaction.

Results from this investigation are summarised in Table 5.2. All 15 strains of Ps. anaerobius reacted strongly with the homologous antiserum. However, six strains also reacted with Pc. magnus antiserum and three of these also reacted with Pc. prevotii antiserum, but the  $A_{405}$  values were much lower than those for the homologous reaction; one of the cross-reactions with Pc. prevotii antiserum was borderline.

Only six strains of Pc. magnus were identified as such by reaction with homologous antiserum and one of these was borderline. Two strains of Pc. magnus also reacted with Ps. anaerobius antiserum. One strain of Pc. magnus displayed borderline reactions with the homologous antiserum and Pc. prevotii antiserum; two strains reacted with Pc. asaccharolyticus antiserum and, although one reaction was borderline, this extract did not react with homologous antiserum. Apart from this example, no strain biochemically identified as Pc. magnus was serologically identified as anything else.

The identification of seven strains of Pc. asaccharolyticus by ELISA correlated with previous conventional test results, but

TABLE 5.2:

IDENTIFICATION OF GRAM-POSITIVE ANAEROBIC COCCI BY ELISA

Species (number)	Correct identifications (borderline)	No significant reaction with any antiserum	Number of cross-reactive strains
Ps. anaerobius (15)	15 (0)	0	6
Pc. magnus (15)	6 (1)	9	5
Pc. prevotii (10)	3 (2)	7	2
Pc. asaccharolyticus (10)	7 (2)	3	2

two of the ELISA reactions were borderline. One strain displayed cross-reactions with both Ps. anaerobius (borderline) and Pc. magnus antisera; the latter reaction gave a higher  $A_{405}$  value than the homologous reaction. A further strain of Pc. asaccharolyticus reacted with antiserum to Pc. prevotii.

Only three of the extracts from ten strains identified as Pc. prevotii by conventional tests reacted with homologous antiserum and two of these were of doubtful significance. Only the vaccine strain itself reacted strongly with its antiserum. One of the 'borderline' strains of Pc. prevotii cross-reacted with Pc. asaccharolyticus antiserum; one strain of 'Pc. prevotii' that did not react with the homologous antiserum, reacted strongly with Pc. magnus antiserum and, to a lesser extent, with Ps. anaerobius antiserum. Thus, two strains biotyped as Pc. prevotii were serologically identified as Pc. asaccharolyticus and Pc. magnus respectively but the latter may also have had antigens in common with Ps. anaerobius.

Some of these were compared with results from PAGE followed by immunoblot transfer to nitrocellulose. In Figure 5.6, the extract in track 7 did not reveal any antigens when incubated with Pc. prevotii, although the original organism had been identified as such. ELISA and a subsequent immunoblot identified this strain as Pc. asaccharolyticus. However, the extract in track 4 from an isolate biotyped as Pc. prevotii, which reacted in this immunoblot with homologous antiserum gave only a borderline reaction in ELISA

with homologous antiserum, as well as with Pc. magnus antiserum, suggesting shared antigens between these species. Track 6 contained a 'Pc. prevotii' extract that in ELISA displayed a borderline reaction with homologous antiserum, but a strong reaction with Pc. asaccharolyticus antiserum. This suggests that immunoblot transfer may detect antigen-antibody reactions that ELISA classifies as being of borderline significance.

The two strains biotyped as Pc. magnus whose extracts appear in tracks 1 and 2, Figure 5.7, which did not react with homologous antiserum, were not identified by ELISA; the extract in track 3 gave only borderline reactions in ELISA with both Pc. magnus and Pc. prevotii antisera. The extract in track 5 cross-reacted strongly with antiserum to Pc. asaccharolyticus in ELISA, but repetition of this reaction in a subsequent immunoblot failed to identify any cross-reacting antigens.

In Figure 5.8, the extract in track 3 did not react with antiserum to Pc. asaccharolyticus, but ELISA suggested that its original biochemical identification was correct. The extract in track 5 reacted with homologous antiserum in ELISA, but gave a greater  $A_{405}$  value in reactions with Pc. magnus antiserum and a borderline reaction with Ps. anaerobius antiserum, suggesting antigens shared between the three species.

Details of cross-reactions detected by ELISA and immunoblot transfer procedures are summarised in Table 5.3.

TABLE 5.3:

CROSS-REACTING STRAINS OF GRAM-POSITIVE ANAEROBIC COCCI DETECTED BY ELISA AND IMMUNOBLOT TRANSFER

EDTA extract	ELISA		
	SIGNIFICANT	BORDERLINE	BLOT
<b>A.</b>			
GPAC 455	Ps. anaerobius	Pc. magnus	
GPAC 455	Ps. anaerobius Pc. prevotii	Pc. magnus	Pc. magnus Pc. prevotii
STD 20	Ps. anaerobius	Pc. magnus	
STD 29	Ps. anaerobius	Pc. magnus	
STD 26	Ps. anaerobius Pc. prevotii	Pc. magnus	
STD 30	Ps. anaerobius Pc. magnus	Pc. prevotii	
<b>B.</b>			
STD 62	Pc. asaccharolyticus Pc. magnus <sup>z</sup>	Ps. anaerobius	Pc. magnus (Ps. anaerobius not tested)
STD 3	Pc. asaccharolyticus Pc. prevotii		Pc. prevotii
<b>C.</b>			
STD 6 AN 13	Pc. magnus	Ps. anaerobius Pc. magnus Pc. prevotii	N.D.
GPAC 149	Pc. magnus Ps. anaerobius		
STD 16	Pc. magnus Pc. asaccharolyticus		
STD 53*		Pc. asaccharolyticus	
<b>D.</b>			
AN 8	Pc. asaccharolyticus	Pc. prevotii	Pc. asaccharolyticus
STD 11*	Pc. magnus	Ps. anaerobius	Pc. prevotii (Pc. magnus and Ps. anaerobius not tested)

Key:

A = Ps. anaerobius; B = Pc. asaccharolyticus; C = Pc. magnus; D = Pc. prevotii

N.D. = cross reaction not detected.

\* = strain did not react with homologous antiserum.

z = reaction with this antiserum stronger than with homologous antiserum.

## 5.7 Discussion

The incubation of Gram-positive cells with 10 mM EDTA results in the destabilisation of their walls and membranes by the chelation of divalent cations such as  $Mg^{2+}$  and  $Ca^{2+}$ . EDTA thus removes non-covalently bound polymers including proteins and membrane-associated lipocarbohydrates into solution. It is a simple technique to perform and, since no autolysis of GPAC was observed over the incubation periods tested, it avoids the problems associated with extracts containing cytoplasmic antigens. It is thus an appropriate procedure for the extraction of cell surface antigens. The protein content of such extracts from GPAC was low compared with other Gram-positive anaerobes treated similarly: EDTA extracts from Clostridium spp yield an average of 1.5-2.0 mg ml<sup>-1</sup> protein, although different species vary considerably (Mr Robert Brown, personal communication). Highest yields in the present study were obtained from strains of Ps. anaerobius which invariably demonstrated better growth in liquid media. EDTA extraction from cultures at the same optical density may produce less varied results. Prolonged incubation, followed in some cases by sonication, did not improve protein yields.

The method of immunising rabbits produced antibody titres that compared favourably with previous reports. Stone (1940)

obtained maximum end-points of 1:12 for homologous reactions in precipitation tests; Markowitz and Lerner (1977) recorded homologous titres of only 1:16-1:64, using counterimmuno-electrophoresis, in antisera raised against sonicated cell suspensions of Pc. magnus injected intravenously. Wong et al. (1980) injected rabbits intramuscularly and subcutaneously with live cells of Ps. anaerobius in Freund's complete adjuvant and obtained titres of 1:2560 and 1:10 240. In the present study, a titre of 1:8000 was determined by ELISA for antiserum to Ps. anaerobius which confirms the observation made by Wong's group that this species is strongly immunogenic. Titres for the three asaccharolytic strains were lower, despite injection of the same number of organisms; a titre of 1:200 was recorded for Pc. prevotii, which is lower than the range of 1:640-1:2560 quoted for this species by Porschen and Spaulding (1974). The intravenous injection of live GPAC appeared to have no detrimental effect on the rabbits, thus confirming their low virulence for laboratory animals.

Attempts were made to standardise sample preparation and PAGE procedures: all cells were harvested after 48h incubation and centrifuged at the same speed for the same period; EDTA incubation times and the boiling of samples with SDS-containing sample buffer prior to their application to a gel were also strictly controlled; solutions of stacking and separating gels were de-gassed for 10 min before polymerisation. Since gels were run at room temperature,



this variable may have had some effect on profile reproducibility. This did not have any visible effects on overall patterns in repeated gels, but differences may have been detectable by more sophisticated techniques, such as densitometry and numerical analysis. Such facilities were not available. The application of standardised amounts of protein to individual tracks did not always result in uniform staining. This problem was encountered by Fox and McLain (1974) and Cato et al. (1982). One explanation for this, suggested by Davies and Gottlieb (1973), was the presence of phenolic compounds in cell extracts interfering with the protein assay and rendering it unreliable.

The technique of SDS-PAGE has been used with some success in taxonomic studies of microorganisms which present problems when examined by conventional morphological, biochemical and serological techniques. Cato et al. (1983) and Taylor (1984) examined whole cell proteins from GPAC in PAGE, the former by visual comparison of a limited number of species and the latter by computer-assisted numerical analysis with a larger number of strains and species. The present study involved visual comparison of the surface protein profiles of a variety of GPAC and examined the antigenicity of EDTA extracts by ELISA and immunoblot transfer from polyacrylamide gels. In general, these extracts produced heterogeneous profiles in gels stained with Coomassie blue that were not characteristic of a biotype. A notable exception to this was Ps. anaerobius, which consistently produced virtually identical protein patterns

dominated by one high molecular weight band. This finding confirms evidence from GLC analysis of end-products and liquid sensitivity that Ps. anaerobius is easily distinguished from other species of GPAC. Immunoblots of 13 strains revealed that this band, and another of lower molecular weight, were common species antigens. Graham and Falkler (1978, 1979) and Wong et al. (1980) also produced evidence of common antigens within this species, and the latter group suggested that they were not possessed by other peptostreptococci. The present study involves different antigen and antiserum preparations, therefore results are not directly comparable, but they do suggest that the antigens contained in EDTA extracts of Ps. anaerobius are not unique to this species; they were also detected by antisera to Pc. magnus and Pc. prevotii. In addition, antiserum to Ps. anaerobius recognised antigens in extracts from Pc. magnus and Pc. asaccharolyticus. Further investigations are required to clarify the nature of antigens possessed by several strains within a biotype, detected by homologous antiserum, but displaying slightly different electrophoretic mobilities in polyacrylamide gels. These antigens may possess identical determinant sites and thus bind identical antibodies, but the carrier portions of the molecules may vary in composition; alternatively these may represent different determinant molecules exposed on cell surfaces which are detected by the polyvalent antiserum.

Immunoblotting allows the visualisation of EDTA-extracted antigens separated by electrophoresis and immobilised by hydrophobic bonding to nitrocellulose; it is thus similar in principle to crossed-immunoelectrophoresis, described in Chapter 6. It must be remembered, however, that these antigens are not necessarily protein, since the extract contains a mixture of cell surface components and polysaccharides can also adsorb to nitrocellulose. A further point of some importance is that not all polypeptides bind to nitrocellulose. Lin and Kasamatsu (1983) studied the movement of polypeptides out of polyacrylamide gels and their interaction with nitrocellulose membranes. They determined that the rate of elution of polypeptides from gels depended on their molecular weight, gel pore size and the current applied during electrophoresis, i.e. larger molecules required longer to be transferred to nitrocellulose; alternatively the gel pore size or current could be increased, but the latter resulted in a greater loss of low molecular weight polypeptides. Molecules of < 20 000 Daltons passed through 0.45  $\mu\text{m}$  pores and escaped detection by specific antibody, but those > 30 000 did not; 0.1  $\mu\text{m}$  pores retained virtually all polypeptides and the authors detected no differences in the binding of various polypeptides to membranes with different pore sizes. In the present study, most immunoblots involved membranes with 0.2  $\mu\text{m}$  pores which, according to Lin and Kasamatsu, cause the loss of 12% of 14 300 Dalton polypeptides. This may account for certain cross-reactions in the present study

being detected only by ELISA and not immunoblotting. Alternative explanations are that during boiling with SDS or binding to nitrocellulose the antigens were denatured, or the latter process involved masking of the antibody sites.

Although PAGE highlighted considerable variations in protein profiles of EDTA extracts from peptococci, certain strains within each biotype produced virtually identical patterns. Extracts from NCTC strains 9801 and 9803, which belong to two different Hare groups (I and III respectively), produced identical patterns, thus confirming their classification in the same species, Pc. asaccharolyticus, described in Chapter 2. These results suggested that groups of GPAC could be formed on the basis of PAGE. Of greater value, however, would be a system based on the antigens present in such extracts. ELISA demonstrated numerous cross-reactions between biotypes, indicating the presence of shared antigens. For certain strains, this technique suggested identities contrary to those deduced from biochemical tests; others could not be identified with the antisera used. Immunoblotting confirmed several of the 'reidentifications' suggested by ELISA and revealed the number of antigens involved. Certain strains biotyped as Pc. magnus failed to react with homologous antiserum, despite exhibiting almost identical patterns in polyacrylamide gels to strains which did react; this may be due to the presence of polypeptides of similar mobility, but different composition.

Shrinkage of polyacrylamide gels during the initial fixing stages of the silver staining procedure made comparisons of band positions in the gels with antigens on the nitrocellulose difficult, particularly with narrow or faintly-stained bands. This problem was not encountered with Ps. anaerobius. Silver staining has two drawbacks: its sensitivity results in the appearance of more polypeptide bands than are seen with Coomassie blue, which makes visual interpretation difficult. In addition, development continues approximately 10 min after the reaction has been 'stopped' by transferring the gel to distilled water. Thus, the operator has to estimate an appropriate moment at which to terminate the reaction to avoid dark bands being completely obscured, but allow lighter bands to appear. An advantage of the technique's sensitivity in the case of GPAC, however, is the small amount of protein required in a sample to produce adequate patterns in gels.

A finding that may be of some significance is the production of different protein profiles by vaginal isolates of the same species from a single patient. In addition, all the isolates of a single species from different individuals with healthy gingivae produced identical patterns. The use of EDTA extracts and immunoblot transfer in epidemiological studies of healthy and symptomatic individuals, suffering from a range of infections in which GPAC are implicated, <sup>could possibly</sup> detect serological differences between pathogenic and non-pathogenic strains. Future serological

investigations should involve antisera raised against whole cells or EDTA extracts from a larger number of strains to encompass a greater variety of cell surface antigens. These vaccine strains should comprise well-characterised reference strains (VPI and ATCC, but not present NCTC strains) and clinical isolates. Such antisera could then be used in ELISA procedures to classify large numbers of clinical isolates without recourse to biochemical tests, other than those confirming their identity as GPAC. Any cross-reactions could be more closely examined with immunoblots, bearing in mind that all the antigens present in an extract may not adsorb to nitrocellulose and thus escape detection. Conversely, not all antigens adsorb to polystyrene microtitre plates (Gray, 1979), therefore immunoblots may detect antigens that ELISA procedures do not. The possession of antigens cross-reacting with other genera, e.g. beta-haemolytic streptococci, should also be investigated. The identification, extraction and purification of cross-reacting and specific antigens could lead to the development of more appropriate antisera and thus to a serological classification of GPAC.

CHAPTER 6

FURTHER IMMUNOCHEMICAL STUDIES ON THE CELL SURFACE  
ANTIGENS OF PS. ANAEROBIUS

## Introduction

Morphological differences between bacterial cells observed by light or electron microscopy have been clarified by biochemical and immunological analyses of cell envelope components. In turn, these investigations have contributed to the understanding of host-parasite interactions such as the adhesion of bacteria to host tissues and the stimulation or suppression of the host's immune response. Furthermore, the classification of certain bacteria has been based on such differences revealed between species. The following account describes the structure of the Gram-positive bacterial cell wall and membrane, illustrated in Figure 6.1, and the role of specific components as antigens and aids to identification.



## 6.1 The Gram-positive Cell Wall

The major component of Gram-positive bacterial cell walls is peptidoglycan (synonyms are murein, mucopeptide and glycopeptide) which comprises 50-60% of the wall substance and determines not only the cell's shape, but also its resistance to osmotic lysis. As the name implies, this polymer has both polysaccharide and peptide moieties, consisting of chains of  $\beta$ 1-4-linked N-acetyl-D-glucosamine and N-acetyl-muramic acid residues which are cross-linked by short peptide chains. The muramic acid is usually N-acetylated, but some bacteria, including mycobacteria and Nocardia spp possess glycollyl groups (Rogers et al., 1980). The peptide linkages between peptidoglycan chains are composed of a limited number of amino acids; there may be three to six in a single type of peptidoglycan, although the range of amino acids found in different species is much larger. These are attached to the carboxyl groups of muramic acid residues, where the first amino acid is usually L-alanine. Cross-links exist not only between individual glycan chains, creating two-dimensional chains, but also between these sheets, thus contributing to the characteristic strength of the cell wall. Analysis of peptidoglycan components may not be of benefit to taxonomists, since despite quantitative and qualitative similarities between organisms, the amino acid sequence or the peptide cross-linking may vary.

Covalently bound to the peptidoglycan backbone in Gram-positive bacteria are secondary wall polymers, known as the teichoic acids or their analogues. These compounds were initially defined as polymers of glycerol or ribitol phosphate, but now the term 'teichoic acid' may be applied to polymers containing phosphodiester groups, polyols, possibly sugar residues or glucose, rhamnose, and often D-alanine ester residues (Baddiley, 1970, cited by Ward, 1981). This definition includes both wall teichoic acids and membrane lipoteichoic acids (the latter are discussed in section 6.2). A single bacterial species may have more than one type of teichoic acid in its wall and these molecules are covalently linked via phosphodiester bonds to the 6-hydroxy groups of muramic acid residues in the peptidoglycan. An intermediate linkage unit, consisting of N-acetylglucosamine and glycerol phosphate, commonly forms the attachment between the secondary wall polymer and peptidoglycan. Such linkages may be broken by treatment with acid or alkali which releases teichoic acids from the peptidoglycan backbone. Organisms grown under conditions of phosphate limitation form teichuronic acids, containing uronic acid residues; these molecules are directly linked to peptidoglycan. The secondary wall polymers carry a strong negative charge and it has been suggested that, by binding cations, such as  $Mg^{2+}$ , they create suitable ionic conditions at the cytoplasmic membrane (Archibald et al., 1961).

Electron microscopy of sections of Gram-positive cells stained with osmium tetroxide indicate two or three layers within the cell wall, an inner layer of low electron density, appearing lighter, surrounded by two dark electron-dense layers. Whilst some authors have concluded that the teichoic acids are confined to the outer layers (Weibull, 1973), it is widely believed that they are distributed throughout the cell wall (Garland et al., 1975; Anderson et al., 1978). Ward (1981) stated that "it is not possible to reach firm conclusions about the relationship between the banded appearance of gram-positive [sic] walls and the distribution of polymers within them", but Millward and Reaveley (1974) suggested that in Staphylococcus aureus and Bacillus licheniformis these layers were due to the distribution of peptidoglycan rather than the secondary wall polymers. The ability of teichoic acids to interact with specific antibodies suggests a surface location, since it is thought that immunoglobulin molecules cannot penetrate the cell wall (Ward, 1981).

Lancefield (1933) based her classification of beta-haemolytic streptococci on the different serological activities of polysaccharide antigens, including secondary wall polymers, extracted from whole cells with hot HCl. She discovered that antigens with group specificity were present in the cell wall, whereas those conveying type specificity within a group could be recovered from capsular material. In group B streptococci, for example, rhamnose is an important component of the group-specific

cell wall antigen and glucose, galactose, glucosamine and sialic acid are among the components of the type-specific capsular antigens (Baker et al., 1976; Kane and Karakawa, 1977; Cumming et al., 1983). However, treatment of cells with hot HCl results in the recovery of incomplete, low molecular weight antigens; cold trichloroacetic acid (TCA) extracts more 'complete' antigens (Lancefield and Freimer, 1966), but prolonged treatment with this substance may hydrolyse some phosphodiester bonds in the teichoic acids. To avoid acid hydrolysis of the secondary wall polymers, Archibald et al. (1969) used dilute NaOH to extract these molecules from the cell walls of Lactobacillus plantarum and Staphylococcus spp. This treatment, they observed, removed teichoic acids in their polymeric form, but did not significantly affect the peptidoglycan backbone.

There is little in the literature regarding the cell wall composition of GPAC. Bahn et al. (1966) studied five strains of 'Peptostreptococcus', but only two of these, Ps. putridus (synonymous with Ps. anaerobius), are now considered to belong to this genus. The cell walls of these two strains contained mainly glucose, although one also had a small amount of mannose. Other constituents common to both strains included alanine, lysine, glucosamine, galactosamine, aspartic acid and glutamic acid, but neither contained teichoic acids. Weiss (1981) analysed the peptidoglycan structure of over 50 strains of anaerobic cocci in an attempt to solve the classification problem. The test organisms

exhibited 20 different peptidoglycan types, based on the peptide subunit and interpeptide bridge composition, including eight which had not been previously described. Weiss suggested that such differences might aid taxonomists.

## 6.2 The Cytoplasmic Membrane

Singer and Nicholson (1972) proposed a 'fluid mosaic' model to describe the cytoplasmic membranes of prokaryotic and eukaryotic cells. Such a membrane is composed of proteins and lipids (60-70% and 30-40% of the dry weight respectively) arranged in a structure possessing the minimum free energy. This membrane is attached to the inner surface of the cell wall at several points. As illustrated in Figure 6.1, phospholipid molecules form a bilayer with their hydrophilic polar heads facing outwards (to the cytoplasm and cell wall respectively) and their hydrophobic non-polar tails directed inwards. Associated with the lipid bilayer are two classes of protein: peripheral proteins held by ionic bonds, but easily removed with, for example, EDTA, and integral proteins held by hydrophobic bonds, requiring more powerful agents, e.g. deoxycholate, for their removal. Both the

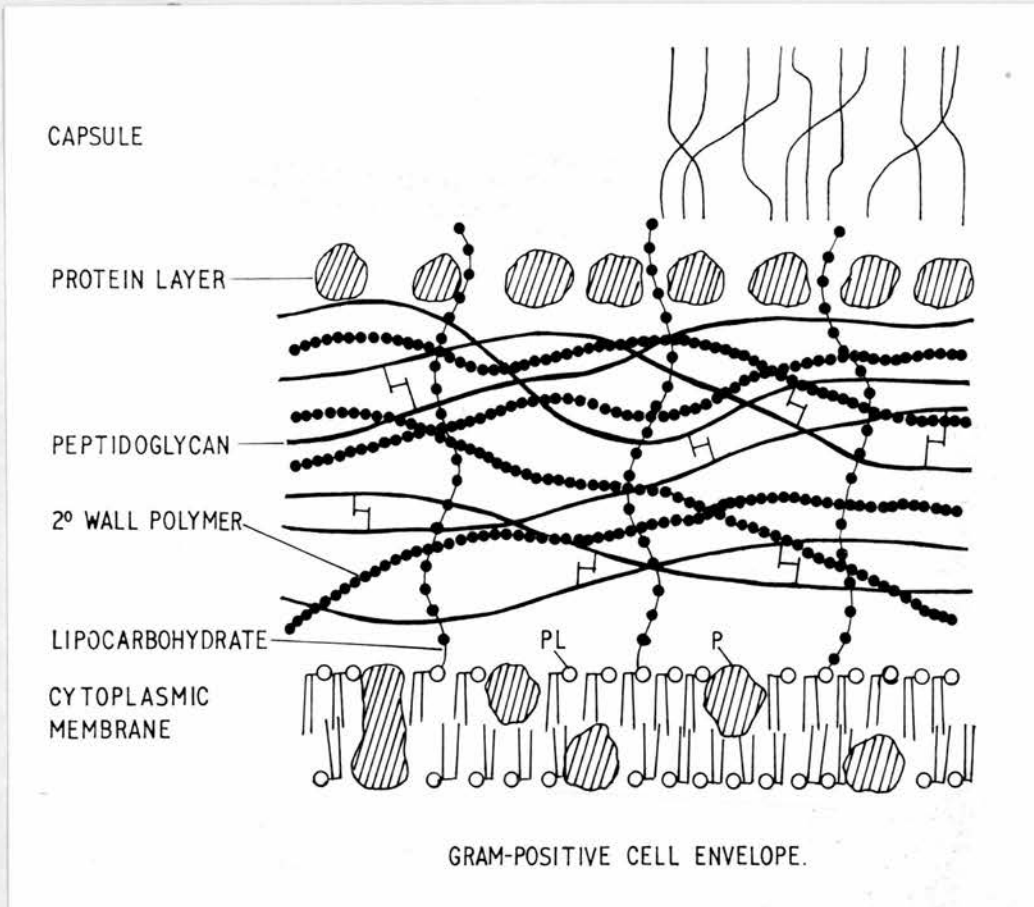


FIGURE 6.1: Gram-positive cell envelope

P = protein

PL = phospholipid molecule

H = cross-linking peptide chain

(courtesy of Dr I.R. Poxton)



phospholipid and protein moieties are capable of lateral movement within the membrane.

In general, bacteria possess a higher proportion of protein relative to phospholipid than eukaryotic cells, but unlike mammalian or eukaryotic cells, their membranes do not contain sterols and few contain polyunsaturated fatty acids. Important constituents of Gram-positive cell membranes are the lipoteichoic acids (LTA) which consist of a poly-glycerol phosphate backbone linked to a hydrophobic glycolipid residue which attaches the LTA onto or within the cytoplasmic membrane. These molecules are not covalently bound to the membrane. The remainder of the LTA molecule, which is hydrophilic, may traverse the entire cell wall and appear on the surface (Van Driel et al., 1973). Certain bacteria, including oral streptococci and lactobacilli (Markham et al., 1975) also release LTA into the surrounding culture medium; this LTA may interact with peptidoglycan and thus reattach to the outer surface of the cell. Two functions have been suggested for LTA: inhibition of autolytic enzymes and regulation of  $Mg^{2+}$  supplied to membrane enzymes (Rogers, 1983).

Cell walls can be obtained from whole cells by mechanical disruption of cells via ultrasound, pressure reduction or shaking with glass beads; cells may also be disrupted enzymatically with, for example, lysozyme, lysostaphin, achromopeptidase or by intrinsic autolytic enzymes. Cell membranes and cytoplasmic contents can be removed from these preparations by washing with a

detergent, such as SDS. An alternative means for obtaining cell membranes is to lyse bacteria lacking a cell wall; these are known as protoplasts, in the case of Gram-positive cells, and sphaeroplasts, in the case of Gram-negative cells. This procedure avoids contamination of membrane preparations with wall material.

Coley et al. (1975), discussing the problems associated with extracting LTA from Gram-positive cells, stated: "The structural similarity and functional relationship between the wall and membrane teichoic acids could account for the frequent contamination of one type with the other in isolated preparations." Extraction with TCA causes deacylation of the lipid residues, but alternative milder techniques utilising hot or cold phenol produce an extract contaminated with protein, nucleic acids and cell wall components. These authors stressed that procedures for the extraction of teichoic and lipoteichoic acids should be performed with isolated preparations of walls and membranes rather than whole cells; the latter inevitably leads to contaminated extracts.

The peptidoglycan and teichoic acids (wall and membrane) of Gram-positive bacteria have immunomodulatory effects, reviewed by Friedman et al. (1984). Extracts from staphylococci have received particular attention, but other Gram-positive bacteria possess polymers with similar activity. Teichoic acids may induce hypersensitivity in humans when injected intradermally (Martin et al., 1967); teichoic and lipoteichoic acids may stimulate an immune response if administered to laboratory animals in small



amounts (Ekstedt, 1974), but large amounts may be immunosuppressive. Ekstedt (1974) commented that the surface antigens of staphylococci possess "a very significant capacity to be changed by mutation, and can also be markedly altered by regulatory mechanisms determined by the conditions of growth." He speculated that surface antigens, including teichoic acids, might vary in their composition or configuration according to different growth conditions associated with e.g. an abscess cavity, pneumonia, bacteraemia. The time required for the host to mount a suitable immune response to these different antigens might account for repeated infections with the same organism.

Peptidoglycan from various bacteria may also stimulate or suppress the humoral and cell-mediated immune responses by its effects on lymphocytes, macrophages and PMNL. From experiments both in vitro with mouse spleen cells and in vivo with immunised mice, Friedman's group found that the immunomodulating effects of streptococcal LTA and LPS from Escherichia coli and Serratia marcesens were mediated by macrophages stimulating the release of B-cell helper factors, thus promoting antibody production. Stewart-Tull (1980) proposed that, since T-suppressor cells are formed in response to substances with repeating antigenic determinants, this might explain tolerance to commensal bacteria whose peptidoglycan possesses repeating disaccharide units.

Wicken et al. (1982) observed that the amounts of cell wall teichoic acids formed by Streptococcus mutans and Lactobacillus

plantarum remained constant under various conditions of growth; in the latter organism, however, changes in growth medium pH affected the extent of secondary glycosyl substitution of teichoic acids, which in turn affected their serological activity. Streptococcus mutans LTA was unaffected in terms of composition or serological activity, but different growth conditions did affect the amount of LTA formed. The apparent stability of the cell wall in certain bacteria, regardless of growth conditions, led the authors to conclude that phenotypic changes in oral microorganisms induced by environmental pressures may occur in surface-associated components, such as lipoteichoic acid or protein, rather than in covalently bound cell wall polymers.

The antigenicity of cell wall and membrane extracts can be detected using various established serological techniques including capillary precipitin tests, simple immunodiffusion in agarose gels (Ouchterlony, 1948), immunoelectrophoresis in agarose gels and ELISA. A further technique that has recently developed from immunoblot transfer procedures is dot-immunobinding.

### 6.3 Direct Application of Antigens to Nitrocellulose

When it was realised that proteins separated in polyacrylamide gels could adsorb non-covalently to nitrocellulose membranes, a number of workers investigated the detection of various antigens in samples applied directly to nitrocellulose (Hawkes et al., 1982; Jahn et al., 1984). Hawkes' group applied the term 'dot-immunobinding' to this procedure which is now commonly known as 'dot-blotting'. Nitrocellulose membranes treated in this way are then incubated with blocking, first and second antibody solutions in a similar manner to immunoblot transfers from polyacrylamide gels. In principle, the technique is identical to ELISA, but it has certain advantages over the latter: much less antigen is required, since a few  $\mu$ l suffice to form the 'dot'; it is more sensitive than ELISA, being capable of detecting picograms of antigen or antibody; the chromogenic substrate-conjugate interaction develops against the white background of nitrocellulose, therefore it is easier to detect positive reactions by eye, relative to controls. In common with ELISA, many samples can be rapidly assayed for their antigen or antibody content. However, neither technique allows the visualisation and enumeration of different antigens in a sample

offered by CIE and Western blotting. Brooks et al. (1985) recently described the value of dot-blotting in the detection of Toxoplasma gondii antigens in human serum and cerebrospinal fluid. Since antibodies to this pathogen are difficult to detect by conventional serological methods due to its predilection for neonates and immunocompromised individuals who cannot mount an adequate immune response, the ability to detect antigen in body fluids is a considerable breakthrough. The diagnostic potential of this technique is being thoroughly explored in a wide range of infectious and non-infectious diseases.

#### 6.4 Aims of the Present Study

The following sections describe the extraction, purification and analyses of secondary cell wall polymers and membrane lipoteichoic acids from Ps. anaerobius, as well as procedures used to determine their immunological activity. From this work, it was hoped to gain an impression of the suitability of such antigens as the basis for a serological identification scheme. Further investigations were conducted to elucidate the nature of the two common antigens belonging to Ps. anaerobius, previously extracted with EDTA and visualised by immunoblot transfer from polyacrylamide gels.

## 6.5 Materials and Methods

**Bacteria:** a single strain of Ps. anaerobius, GPAC 56393, was examined in this study. This strain had been used to raise antisera in rabbits (Chapter 5) and was stored in 10% skimmed milk at  $-20^{\circ}\text{C}$ . A stock culture of GPAC 56393 was prepared by adding a few drops of thawed skimmed milk preparation to 10 ml pre-reduced CMB and incubated anaerobically for 48h at  $37^{\circ}\text{C}$ .

**Culture:** the CMB stock culture was used to inoculate 10 litres of pre-reduced PPY medium which was then incubated in an anaerobic cabinet at  $37^{\circ}\text{C}$  for 18h.

**Preparation of wall antigen:** bacteria were harvested by centrifugation at 1000g for 30 min at  $4^{\circ}\text{C}$  and washed twice in phosphate buffered saline (PBS). The cell pellet was resuspended in 40 ml distilled water, at  $4^{\circ}\text{C}$ , and bacteria were disrupted by twice passing through a French pressure cell (Aminco, American Instrument Co. Inc., Silver Springs, Md, USA) at 7000 lbf in<sup>-2</sup> (48 MPa). The resulting suspension was centrifuged at 52 000g for 15 min at  $4^{\circ}\text{C}$  and the membrane-containing supernate was removed and freeze-dried for subsequent extraction of antigen. The pellet of cell walls was resuspended in distilled water and heated at  $80^{\circ}\text{C}$  for

3 min to destroy autolytic enzymes. To remove non-covalently bound protein and membrane fragments, an equal volume of 5% (v/v) sodium dodecyl sulphate (SDS) was added and this solution stirred at room temperature for 4h. SDS was removed by six successive washes in distilled water, centrifuging between each wash at 50 000g. Finally, the cell wall pellet was freeze-dried. The carbohydrate secondary wall polymers were extracted according to the method of Archibald et al. (1969): a portion of the freeze-dried cell wall pellet was suspended in 0.5M NaOH and stirred at room temperature for 2h. This was centrifuged at 50 000g for 15 min and the supernate containing the secondary wall polymer was passed through an ion-exchange column (Dowex 50, BDH) to remove Na<sup>+</sup> ions. The eluent from the column was freeze-dried. Further purification of the carbohydrate was achieved by applying the freeze-dried extract, resuspended in distilled water, to a 30 x 1.5 cm DEAE cellulose ion-exchange column (DE52, Whatman). The extract was eluted with a downward flow of 0-1M pyridine acetate buffer, pH 5.3, from a gradient mixer. Fifty fractions of 2 ml were collected and assayed for their carbohydrate content.

Preparation of membrane antigen: a portion of the freeze-dried supernate obtained after cell breakage was treated according to the method of Coley et al. (1975): material was defatted with three successive washes in chloroform and methanol (2:1, v/v). The residue was left to dry in air then resuspended

in 80% (w/v) phenol and stirred at 4°C for 40 min. After centrifugation at 16 000g for 30 min, the upper aqueous layer was dialysed overnight against distilled water, then reduced in volume by rotary evaporation. This solution was incubated under toluene at 37°C for 72h with RNAase (salt + protease-free) and DNAase (crude) (Sigma: 10 µg ml<sup>-1</sup> of each) in sodium acetate-acetic acid buffer, pH 5.0, with 10<sup>-3</sup>M MgCl. After further rotary evaporation, the phenol extraction and dialysis procedures were repeated and the solution of lipoteichoic acid (LTA) finally freeze-dried. This crude phenol extract was then purified on a 40 x 1.6 cm Sepharose 6B column (Pharmacia) with 0.2M ammonium acetate buffer, pH 6.9, with 0.01% sodium azide. Fifty fractions of 3 ml were collected and assayed for carbohydrate, nucleic acid and phosphorus.

Analytical techniques: carbohydrates, as glucose equivalents, were assayed by the phenol-sulphuric acid method (Dubois et al., 1956), phosphorus by the method of Chen et al. (1956) and sialic acid by the thiobarbituric acid assay of Aminoff (1961). Protein content was determined by the Lowry method (Lowry et al., 1951).

Immuno-electrophoresis: rocket (Weeke, 1973a), fused rocket (Svendson, 1975) and crossed immuno-electrophoresis (Weeke, 1973b) were performed using Shandon Southern apparatus (Camberley, Surrey,



UK). The agarose gel and buffers used for these procedures are described in Appendix 2. Antigens were added in 6  $\mu$ l volumes to wells cut in the agarose; concentrations of antigen solutions ranged from 0.1-5.0 mg ml<sup>-1</sup> protein or carbohydrate. Antiserum to Ps. anaerobius was diluted 1:48 with agarose; after electrophoresis gels were stained with Coomassie blue.

Preparation of alditol acetates: cell wall and membrane carbohydrates were hydrolysed in 2M HCl at 100°C for 2h and then converted to alditol acetates for analysis by gas-liquid chromatography (GLC) as described by Poxton and Cartmill (1982). For detection of pentose and hexose derivatives, 1  $\mu$ l samples were injected into a column of 3% SP2330 on Supelcoport; amino sugar derivatives were analysed on a column of 3% OV225 on Gas-Chrom Q. For both columns, temperatures were programmed to run between 190 and 240°C, increasing by 2°C per minute (Pye Unicam model 104 gas chromatograph).

Preparation of methyl esters: methyl esters of fatty acids in the IgG-purified membrane lipoteichoic acid (see below) were prepared by heating the LTA with 0.5M HCl in methanol at 65°C for 2h. After partitioning into ether, GLC analysis was performed using a column of 3% SP2330 on Supelcoport at 150°C.



Preparation of an immunoabsorbent column: immunoglobulin G was prepared from 3 ml of pooled antisera raised against GPAC 56393 by the addition of 2 ml saturated ammonium sulphate. This mixture was stirred at room temperature for 15 min. After centrifugation at 600g, the supernate was removed and the precipitated IgG dissolved in 2 ml PBS for dialysis against distilled water for 18h.

The column was prepared according to methods outlined in 'Affinity Chromatography, Principles and Methods' (Pharmacia): precipitated IgG was coupled to 10.5 ml CNBr-activated Sepharose 4B (Pharmacia) and this material packed into a 10 x 1<sup>cm</sup> column. The storage buffer consisted of 0.1M phosphate, pH 7.0, with 0.5% (v/v) Tween 80 and 0.01% (w/v) merthiolate.

Paper chromatography: to detect teichoic acids (ribitol and glycerol phosphate polymers), acid hydrolysates of wall and membrane antigens were chromatographed on Whatman no. 1 paper with a 6:3:1 propan-1-ol/ammonia/water-solvent and stained with periodate-Schiff's reagents (Baddiley et al., 1956).

Dot-immunobinding procedure: the procedure of Hawkes et al. (1982) was followed, using the BioRad ImmunBlot Assay kit. This system differs from the original method of Hawkes' group in the use of gelatin, as opposed to bovine serum albumin in the blocking solution. Antigens were suspended in distilled water to a concentration of 1 mg ml<sup>-1</sup> carbohydrate (wall) or protein

(membrane) and added directly to nitrocellulose membrane (0.2  $\mu\text{m}$  pores) in four 1  $\mu\text{l}$  amounts.

ELISA procedure: as described in Chapter 5.

Treatment of EDTA-extracted antigens: an EDTA extract from GPAC 56393 was adjusted to 200  $\mu\text{g ml}^{-1}$  protein and subjected to the following treatments, as described by Poxton and Byrne (1981).

- i. heat treatment, in the form of 121°C for 15 min.
- ii. formalin, as 20% w/v formaldehyde for 16h at 20°C, followed by dialysis for 18h at 4°C against PBS. This treatment denatures proteins.
- iii. 0.1M periodate ( $\text{NaIO}_4$ ) for 16h at 20°C in the dark; excess periodate was removed with ethylene glycol and this was followed by dialysis for 18h at 4°C against PBS. This treatment denatures carbohydrates.

Controls were included for all treatments. Control and treated extracts (75  $\mu\text{l}$ ) were mixed with equal volumes of SDS-containing sample buffer, boiled for 3 min and run in duplicate polyacrylamide gels (10  $\mu\text{g}$  protein per track). One gel was stained with silver for proteins; the other was used in an immunoblot transfer to nitrocellulose. Incubation of this nitrocellulose with antiserum to Ps. anaerobius revealed denaturation of antigens associated with each treatment relative to untreated controls and gave an indication of their chemical nature.

## 6.6 Results

Cell wall and carbohydrate preparation: the yield of cell walls from 10 litres of an 18h culture of Ps. anaerobius was 285 mg. Total carbohydrate extracted with NaOH was 28 mg.

Cell wall antigen: the carbohydrate content of the 50 fractions from the DEAE cellulose column is shown in Figure 6.2. A single major peak eluted between 0.15M and 0.22M pyridine acetate buffer. The fractions corresponding to this peak were pooled and concentrated by freeze-drying, as were the remaining column fractions. The antigenic nature of each was assessed by RIE, ELISA and a dot-immunobinding procedure. The latter technique indicated that peak material was antigenic, but no precipitin arcs were demonstrated in RIE; similarly, ELISA proved negative. GLC analysis of alditol acetates prepared from the peak material indicated that it comprised glucose and an unknown sugar in approximately equal proportions, with traces of galactosamine and glucosamine. The unknown had a retention time between those of the fucose and ribose standards, but coinjection ruled out these sugars and mannose as possibilities. In addition, the antigen contained  $123 \mu\text{g ml}^{-1}$  total phosphorus. No sialic acid or teichoic acids were detected in this antigen.

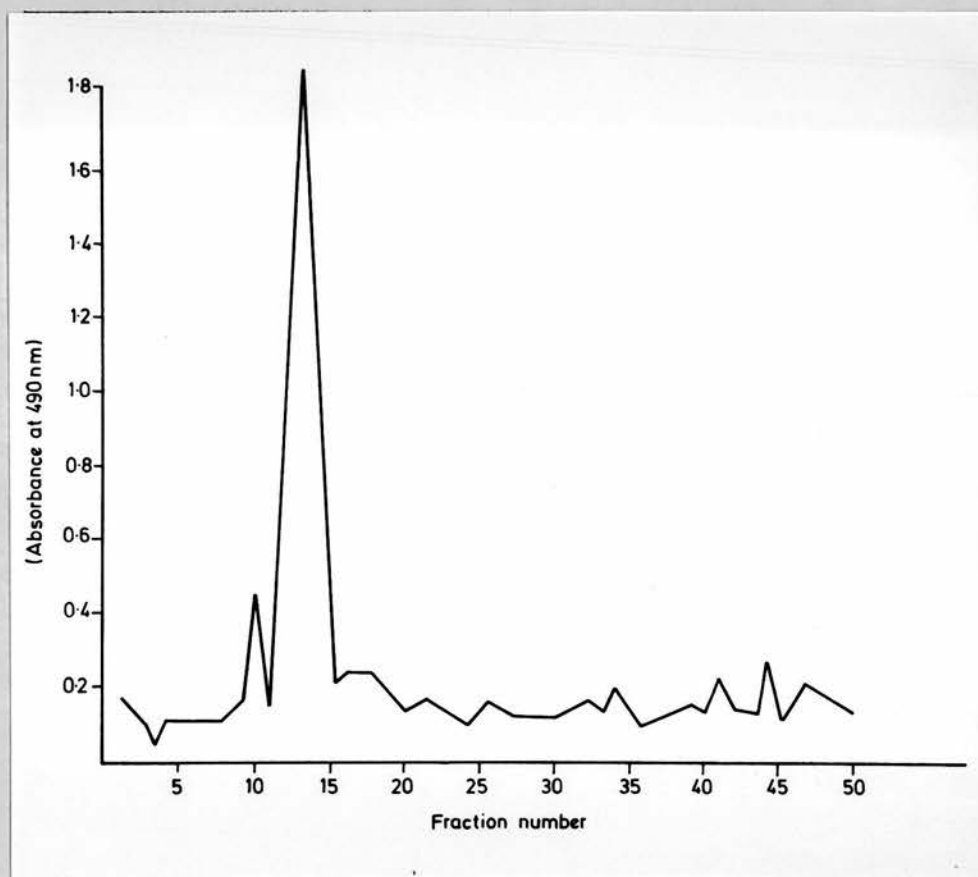


FIGURE 6.2:

Purification of an NaOH extract of SDS-treated cell walls of *Ps. anaerobius* by ion-exchange chromatography on a DEAE-cellulose column.  $A_{490}$  represents carbohydrate content of fractions.

Cell membrane preparation: the yield of freeze-dried membrane containing material from 10 litres of an 18h PPY culture was 1.3g. The phenol extraction procedures resulted in 56 mg of crude lipoteichoic acids.

Cell membrane antigen: The elution profile of the crude phenol extract applied to Sepharose 6B is shown in Figure 6.3. Readings in the first 12 fractions correspond to blue dextran applied to the column to measure the void volume. Two peaks were apparent and fractions corresponding to these and the remaining column fractions were pooled and freeze-dried as already described. Both peak I and peak II were shown to contain antigen by ELISA and dot-immunobinding (Figure 6.4), although peak II gave a less intense reaction in the latter procedure. RIE of these peaks was not attempted, since it had proved negative with the crude phenol extract. To reduce nucleic acid contamination prior to chemical analysis, each peak was applied to an immunoabsorbent column containing IgG raised against whole cells of GPAC 56393. The column was equilibrated in borate-buffered saline (BBS: 0.1M NaCl, 0.2M di-sodium tetraborate, pH 7.3) to avoid phosphate in the storage buffer contaminating the eluent. After freeze-drying each peak was dissolved in 1 ml BBS to give a carbohydrate concentration of approximately  $1 \text{ mg ml}^{-1}$  and applied separately with a downward flow of 20 ml BBS. To elute the antigens, sodium carbonate

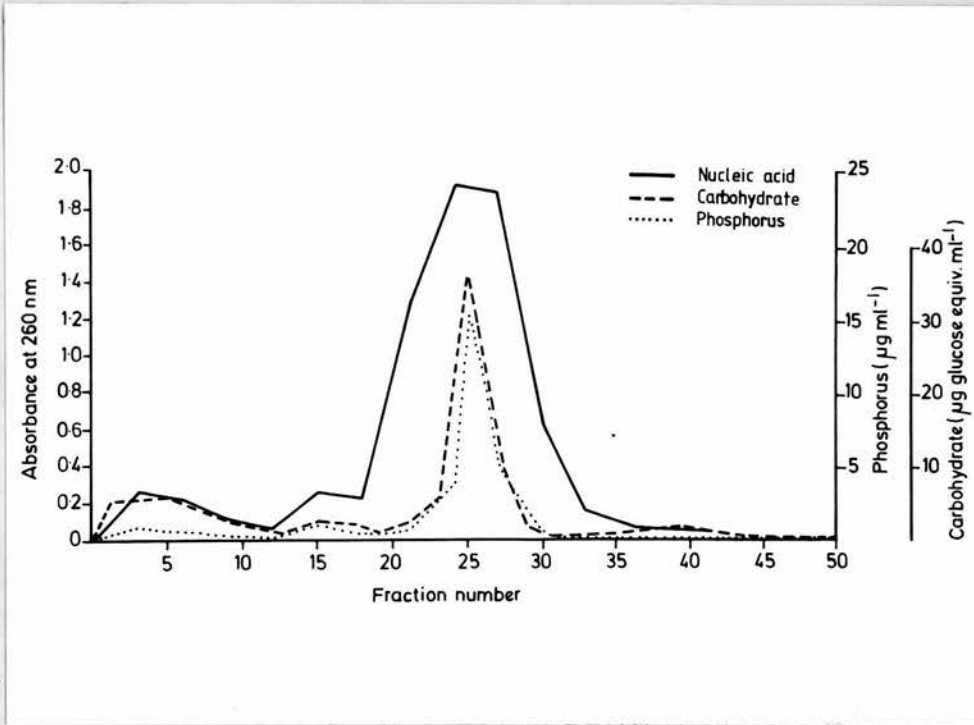


FIGURE 6.3:

Purification of a phenol extract of the cell membranes of Ps. anaerobius on a Sepharose 6B column.  $A_{260}$  represents nucleic acid content of fractions. Peak I = fractions 14-17; peak II = fractions 20-27.

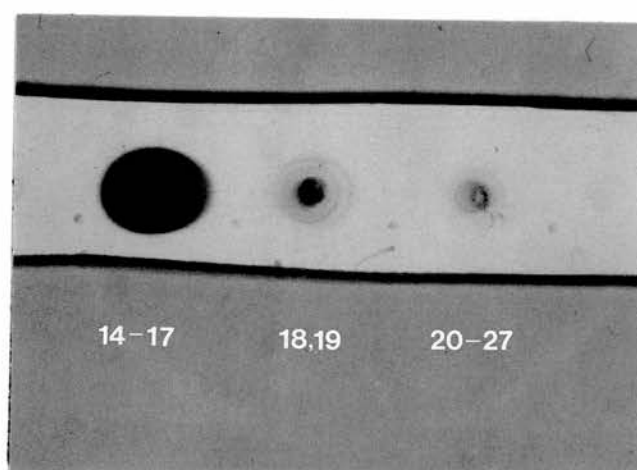


FIGURE 6.4: Dot-immunobinding assay of pooled fractions of phenol extract from Sepharose 6B column.



buffer, pH 9.6/ethylene glycol (1:1, v/v), was applied in the reverse direction. Ten 4 ml fractions were collected and, following dialysis, each was assayed for its carbohydrate content. For each peak, this assay revealed a single peak from the IgG column. ELISA and dot-immunobinding confirmed that both these IgG-purified peaks were still antigenic.

GLC analysis revealed each purified peak to contain only glucose. Peak I material was methanolysed for analysis of its fatty acid content (Table 6.1). No sialic acid was detected in this antigen, but it did contain glycerol phosphate. The antigen contained  $10 \mu\text{g ml}^{-1}$  phosphorus.

Comparison of membrane and EDTA extracts: PAGE of the EDTA extract, the membrane-containing extract and the crude phenol extract followed by immunoblot transfer to nitrocellulose and probing with Ps. anaerobius antiserum revealed several antigens common to the first two of these preparations (Figure 6.5, tracks 1 and 2); two of these were the common antigens described in Chapter 5. However, neither of these were present in the crude phenol extract (track 3), suggesting that they had been denatured by phenol extraction. The antigenic content of this extract was concentrated at the gel front and corresponded in position to antigens present in both the EDTA extract and membrane-containing fraction. Peak I material collected from the Sepharose 6B column



TABLE 6.1:

LIPID COMPOSITION OF PEAK I FROM SEPHAROSE 6B CHROMATOGRAPHY OF  
COLD PHENOL EXTRACT OF PS. ANAEROBIUS CELL MEMBRANES

Component	Percentage of total lipid
C 16:0	11.8
C 16:1	4.5
C 18:0	1.8
C 18:1	8.2
C 18:2	2.4
C 21:0	71.3

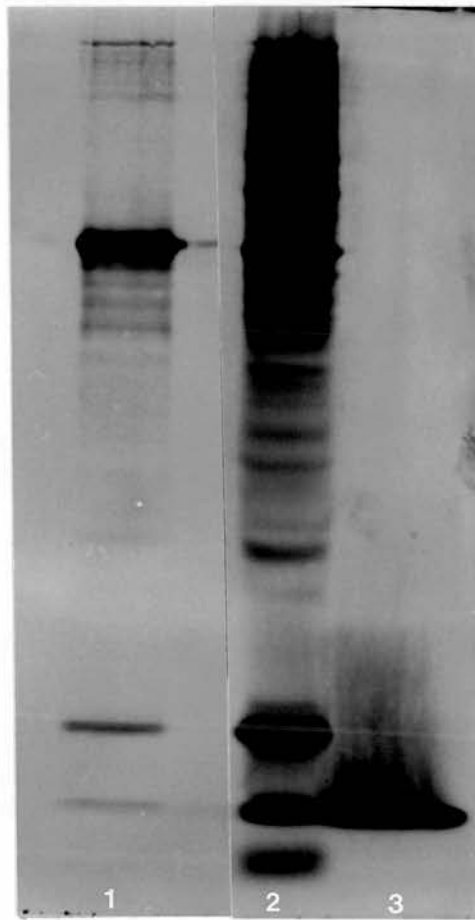


FIGURE 6.5:

Immunoblot transfer from a polyacrylamide gel of EDTA extract (track 1), membrane-containing fraction (track 2) and crude phenol extract (track 3) from Ps. anaerobius.

and purified with IgG appeared identical to the crude phenol extract after PAGE and immunoblot transfer.

Figure 6.6 shows fused rocket and rocket immunoelectrophoresis of purified phenol extracts from the Sepharose 6B column (wells 1-8), the EDTA extract (well 9) and DEAE-cellulose-purified cell wall antigens (wells 10-12). Each well received 6  $\mu$ l of antigen solutions at a concentration of 5 mg ml<sup>-1</sup> protein (EDTA extract) or carbohydrate (wall and membrane extracts). Only the EDTA extract formed a visible precipitin arc when stained with Coomassie blue; subsequent crossed immunoelectrophoresis of this extract resulted in a single precipitin arc.

Treatment of EDTA-extracted antigens: the use of PAGE and immunoblot transfer to visualise the effects of various treatments on the two common antigens contained in an EDTA extract from Ps. anaerobius is illustrated in Figure 6.7; this shows the nitrocellulose membrane incubated with specific antiserum. Track 1 contains an untreated control and demonstrates the high and low molecular weight antigens; track 2 contains the heat-treated extract from which the low molecular weight antigen has disappeared; track 3 contains the formalin-treated extract from which both antigens have disappeared; track 4 contains the periodate-treated extract which lacks the low molecular weight antigen, similar to track 2; track 5 contains the periodate control, in which both antigens are still present. The reactions

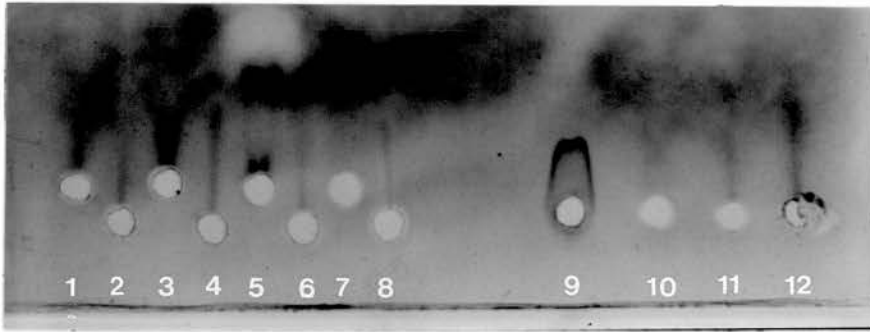


FIGURE 6.6:

Fused rocket (wells 1-8) and rocket (9-12) immunoelectrophoresis of Ps. anaerobius extracts.

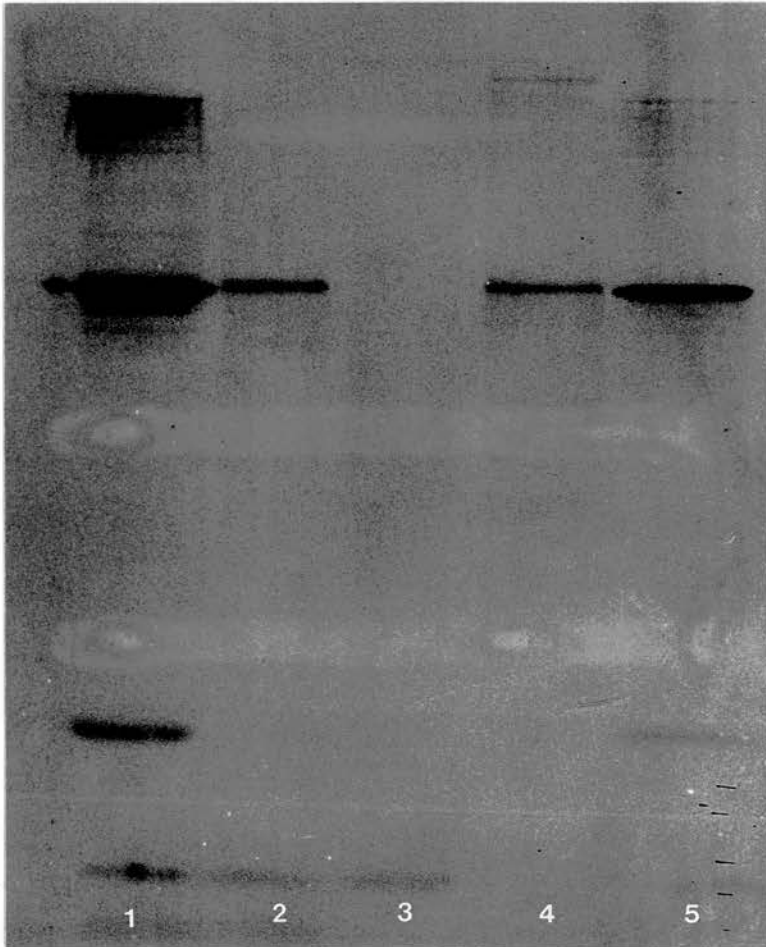


FIGURE 6.7:

Immunoblot transfer from a polyacrylamide gel of control and treated EDTA extracts from Ps. anaerobius.

of the high molecular weight antigen after heat and periodate treatment (tracks 2 and 4) are reduced in intensity relative to untreated controls; this suggests some degree of denaturation, since all tracks received the same amount of protein.

## 6.7 Discussion

The present study has demonstrated two cell surface-associated antigens belonging to Ps. anaerobius, one of which may be isolated from purified cell walls and another which resides in the cytoplasmic membrane. This is the first description of such components in GPAC, although similar antigens have been demonstrated in other Gram-positive bacteria, including Clostridium difficile (Poxton and Cartmill, 1982) and group B streptococcus, type II (Cumming et al., 1983). Cumming's group obtained 950 mg of freeze-dried cell walls from 10 litres of Todd-Hewitt culture medium incubated overnight, approximately three times the amount obtained from Ps. anaerobius in this study. The total carbohydrate extracted from streptococcal cell walls by Cumming et al. represented 1% (10 mg) of this preparation, whereas it accounted for 10% (285 mg) of the cell wall of Ps. anaerobius. The use of SDS ensures the removal of all non-covalently bound material, such as that contained in an EDTA extract, from the cell wall preparation.

Two alternative teichoic acid extraction procedures were tested with purified cell walls in a pilot study: 0.2M HCl at 100°C for 30 min, and 10% w/v TCA stirred with cell walls at 4°C for 48h. Neither of these resulted in higher yields of

carbohydrate than NaOH, nor were the extracts any more reactive in immunoelectrophoresis or ELISA. Extraction with hot HCl or prolonged treatment with cold TCA may hydrolyse teichoic acid phosphodiester bonds, thus denaturing certain antigenic determinants.

The predominance of glucose in the cell wall antigen and the absence of glycerol and ribitol phosphate are in agreement with the findings of Bahn et al. (1966); the secondary wall polymers of Ps. anaerobius are therefore more correctly referred to as teichoic acid analogues. The cell membrane antigen also possesses glucose, but in common with many Gram-positive bacteria it contains glycerol phosphate and may be termed a lipoteichoic acid.

Considerable difficulties were encountered in demonstrating the antigenicity of the cell wall carbohydrate. This extract did not form precipitating complexes with specific antibody in immunoelectrophoresis and did not react in an ELISA procedure. The former may have occurred through an imbalance in antigen-antibody proportions causing the formation of small soluble complexes, rather than larger insoluble precipitates. In an attempt to overcome this problem, a range of antiserum and antigen concentrations was tested, but no combination resulted in visible precipitation. A further possibility is that the teichoic acid conformation in the intact cells used to raise antisera was such that the molecules were not adequately exposed to immunocompetent cells; the resulting antisera may contain a low titre of



antibodies, possibly with little affinity for teichoic acid determinants. The greater sensitivity of the dot-immunobinding assay proved invaluable in this case and was the only test in which the cell wall extract reacted with specific antiserum. This indicated that extraction with NaOH had not, as initially suspected, completely denatured these antigens; although it is a relatively mild extraction procedure, some intra-teichoic acid phosphodiester bonds may be alkali-labile. Negative ELISA results may have been due to the polysaccharide antigen failing to adsorb to polystyrene. Gray (1979) suggested that this problem can be overcome by coupling polysaccharides to poly-L-lysine before coating polystyrene tubes; an alternative method, cited by Anthony et al. (1985) is to conjugate the antigen with tyramine. Neither method was attempted in the present study.

Extraction of lipoteichoic acids from cell membranes with cold 80% phenol causes less damage than TCA, which degrades these polymers and results in teichoic acid chains free from lipid (Wicken and Knox, 1970). However, phenol extraction results in contamination of membrane preparations with proteins and cell wall material as shown in Figure 6.3. For this reason the membrane extracts (peaks I and II) were further purified by affinity chromatography using specific IgG to facilitate chemical and immunological analysis. Unlike the cell wall antigen, each of these peaks gave positive reactions in ELISA as well as dot-immunobinding. The presence of wall antigen in peak II,

suggested by Coley et al. (1975) and confirmed by Poxton and Cartmill (1982) for C. difficile by immunoelectrophoresis, could not be ascertained in the present study. Since the antigens did not precipitate with antibody in agarose gels, an ELISA inhibition procedure was conducted which involved incubating antiserum to GPAC 56393 with purified cell wall antigen prior to its addition to microtitre plate wells already coated with IgG-purified peak II. However, no inhibition of reaction between antiserum and peak II was observed although a range of concentrations of each component was tested.

An EDTA extract from Ps. anaerobius produced a single precipitin arc in both rocket and crossed immunoelectrophoresis, indicating the presence of at least one non-covalently bound antigen that the purified LTA (peak I) from the membrane did not possess, since it did not precipitate. As indicated in Figure 6.5, the EDTA extract contains numerous antigens, some of which are also present in the unpurified membrane-containing fraction, but which are subsequently denatured by phenol extraction. These may be the precipitating antigens demonstrated in the EDTA extract. Thus the purified LTA may contain additional non-precipitating antigenic determinants present in much lower concentrations, if at all, in the EDTA extract. Figure 6.5 indicates low molecular weight antigens in both the EDTA and crude phenol extracts present at the front of the original polyacrylamide gel. Immunoblot transfer of IgG-purified LTA (peak I) from a polyacrylamide gel

revealed an identical pattern to the crude phenol extract, suggesting that LTA may also be present in the EDTA extract. Thus it appears that EDTA removes membrane-associated antigens from whole cells of Ps. anaerobius. The significance of LTA antigens in whole cells was discussed by Ward (1981); he commented that the appearance of a membrane component on the surface of an intact cell and therefore its ability to act as an antigen depended on the cell wall thickness in relation to LTA length and conformation, as well as the arrangement of peptidoglycan chains. If wall or membrane teichoic acids are not exposed on the surface of whole cells and therefore not strongly immunogenic during the course of an infection, they may be of little diagnostic value for detecting antibodies in patients' sera. This does not, however, prohibit their use in purified forms as antigens for serological investigations to aid the taxonomy of GPAC. Ekstedt's theory (Ekstedt, 1974) that different growth conditions altered the structure of antigens expressed at the cell surface of Staphylococcus aureus could also be applied to commensal GPAC: as components of the normal flora, their surface antigens have been 'processed' by the host. Alterations in their normal environment, occasioned for example by hormonal changes, or access to other sites with different ecological pressures, facilitated by trauma to local tissues, may result in the expression of different antigens requiring a new immune response by the host to control the

infection. This may be one factor in the pathogenicity of commensal bacteria, including GPAC, for humans.

The effects of various treatments on the two EDTA-extracted antigens common to isolates of Ps. anaerobius were observed in an immunoblot transfer from a polyacrylamide gel. The high molecular weight antigen was denatured by formalin, but not by heat or periodate which suggests that its determinant sites consist of protein. Heat, periodate and formalin denatured the low molecular weight antigen, suggesting that it contains both carbohydrate and protein moieties. Sensitivity to proteolytic enzymes, but resistance to heat and periodate are characteristics of a recently described receptor for human serum albumin (HSA) on the surface of Pc. magnus (Myhre, 1984), but no indication of its molecular weight was given. The capacity to bind HSA had also been observed in groups A, C and G streptococci (Kronvall et al., 1979; Myhre and Kronvall, 1980) and the authors of these reports speculated that such a coating of bacteria with host protein might serve to protect them against an immune response. The possession of similar receptors suggested to Myhre a relationship between Pc. magnus and these streptococci. This may also explain the serological cross-reactivity observed between GPAC and certain streptococci (Stone, 1940). The receptor on Pc. magnus was highly specific for HSA and did not bind human polyclonal IgG or fibrinogen, but this does not preclude its activity as an antigen. Serological cross-reactions between Pc. magnus and Ps. anaerobius have been

observed (Chapter 5) and it would be of interest to determine whether these involve a molecule on the surface of Ps. anaerobius capable of binding HSA. Myhre tested 36 strains of Pc. magnus, all of which bound HSA, but there was a considerable range of percentage uptake; only three strains of Ps. anaerobius were tested and none of these bound significant quantities of HSA, but investigation of a larger number of isolates may highlight intra-species variation, as displayed by Pc. magnus.

In conclusion, it is impossible to assess the suitability of the wall and membrane antigens examined in this study for classification purposes, since only a single strain was investigated. Equivalent antigens from a large number of GPAC should be studied to determine any significant differences between strains that might aid their identification. Comparisons should be made between the immunogenicity of these purified extracts in animals and whole cells containing the antigens in a less concentrated and possibly less exposed form. The development of antisera containing a high titre of antibodies to wall and membrane teichoic acids warrants investigation as a potential benefit to diagnoses and taxonomic studies.

CHAPTER 7

CONCLUSION

"Honour to those tireless souls who sacrifice their sleep to the advancement or the healing of their fellow men!.... What fanaticism once promised its elect, science now achieves for all mankind!"

Monsieur Homais; Madame Bovary,  
Gustave Flaubert.

The investigations undertaken for this thesis were as follows: the development of a modified identification scheme for Gram-positive anaerobic cocci; the recovery of Gram-positive anaerobic cocci from bacteriological swabs; the survival of these bacteria in air; the isolation of Gram-positive anaerobic cocci from the oral cavity and vagina; examination of cell surface antigens to assess their suitability as the basis of a future serological classification scheme for Gram-positive anaerobic cocci.

A satisfactory classification scheme for GPAC does not exist at the present time. Current schemes, which involve the identification of many of these bacteria by a series of negative results from numerous tests, continue to be used despite their shortcomings. It seemed prudent, therefore, to reduce the number of tests required to place isolates in currently defined species. A scheme was developed, based on presently accepted morphological and biochemical criteria that involved a minimum number of tests. The efficacy of this scheme was confirmed by comparing results with those of other workers subjecting the same strains to greater numbers of tests. The sole fermentation test in the final scheme



utilised glucose; further distinction of species was possible on the basis of liquoid sensitivity, which identifies Ps. anaerobius, and GLC analysis of metabolic acid end-products. Many asaccharolytic strains of GPAC did not grow well in pre-reduced culture media and therefore correspondingly low amounts of acids were produced for analysis. Although this was not the cause of failure to identify any isolates in the present study, it may compound the problems of end-product variation associated with different culture media which contributes to inconsistent speciation.

The identification of GPAC to currently accepted species level by this scheme is still time-consuming and relies heavily on GLC, which makes it impractical for many service laboratories. However, if attempts to identify isolates to species level, which may after all prove to be invalid, were abandoned, susceptibility to vancomycin, novobiocin and liquoid, fermentation of glucose and failure to grow in air plus 10% CO<sub>2</sub> at 37°C within seven days could easily be incorporated in service laboratory procedures, along with routine antibiograms. A similar scheme was proposed recently by Watt et al. (1984) and is a practical alternative to current classification procedures until such times as an equivalent scheme based on definitive taxonomic criteria is available.

Results obtained during the development of this scheme suggest that the NCTC collection of anaerobic cocci should be revised to exclude those strains proven to be microaerophilic and



expanded to include a greater number of fully-characterised obligately anaerobic cocci.

Although swabs are considered less than ideal for the collection of specimens likely to contain anaerobes, clinicians continue to submit such specimens to service laboratories. Aspirated pus or exudate and tissue are regarded as the specimens of choice from anaerobic infections, but they may be impossible to obtain, therefore a swab is the only alternative for isolating bacteria from spontaneously discharging abscesses, superficial wound infections or infections producing very little exudate. The survival of GPAC on swabs has not been adequately studied therefore an investigation was conducted to determine the most suitable swab for the collection of GPAC to be used in future studies of the mouth and vagina and whether or not a transport medium might be beneficial in this respect. Data were also accumulated on the survival of GPAC on blood agar exposed to atmospheric oxygen over a prolonged period. Results obtained were interpreted with some caution, since in vitro experiments of this nature cannot be directly compared with in vivo situations.

Recoveries of GPAC from both cotton-wool and albumin-coated swabs plated out immediately after loading were much higher than those reported previously for other anaerobes. None of the transport media tested markedly enhanced recovery rates and it was concluded that plain cotton-wool or albumin-coated swabs, replaced in their containers after use and kept at room temperature, yielded

the highest numbers of GPAC if plated out within 2h of loading. The loss of approximately 90% of the original inoculum, even from swabs used to seed plates immediately, is clearly a cause for concern if, in a clinical situation, few GPAC cells are present in the sample. However, similar losses have been recorded for other bacteria, e.g. beta-haemolytic streptococci.

Attempts were made to obtain fresh clinical isolates of GPAC to ascertain their sensitivity to atmospheric oxygen and to assess the degree of care with which samples likely to contain GPAC should be treated. It is impossible to call the strains in this investigation 'fresh', since several subcultures were necessary to ensure purity. Indeed, it was unlikely that any strains that were extremely sensitive to oxygen would be included, since the service laboratory from which they were obtained does not utilise pre-reduced media. Results suggested that these clinical isolates were not particularly sensitive to air, since all survived 24h exposure following primary isolation and subculture. This confirmed previous evidence that 'pathogenic' anaerobes tolerate greater exposure to air and higher Eh values than 'non-pathogenic' anaerobes. In a clinical situation, GPAC are usually present with other bacteria which may be more oxygen sensitive or CO<sub>2</sub>-dependent than GPAC. Unless adequate precautions are taken to ensure the recovery of more fastidious organisms, GPAC may be recovered in greater proportions than were present at infected sites. This would create a false impression of the significance of GPAC in such

cases and one can speculate that this may have occurred before the addition of CO<sub>2</sub> to anaerobic incubation environments became standard practice. However, different strains of GPAC may vary in oxygen tolerance, therefore all samples should be processed and transferred to an anaerobic environment as quickly as possible. If anaerobic growth conditions are adequate, e.g. pre-reduced media, BTL jars and fresh catalysts, subculture procedures can be undertaken in air with impunity. Evidence of blood agar plates offering higher recoveries of GPAC than swabs stored over similar periods suggests that direct plating of specimens immediately after they have been obtained from patients provides a greater chance of isolating GPAC. This practice may not, however, be feasible in a clinical situation.

Low numbers of GPAC were recovered from the oral cavity, despite using pre-reduced selective media and an anaerobic cabinet for primary cultures and initial subcultures. An explanation for this finding may be the high standard of oral hygiene possessed by the subjects examined; consequently there was no established dental plaque or periodontal pocketing at the sites sampled which would provide a suitable environment for GPAC to proliferate. A similar study of periodontally diseased individuals would be of interest. The presence of high numbers of GPAC in the oral cavity may be a predisposing factor to their involvement in local and disseminated infections, e.g. orofacial abscesses, CNS infections, aspiration pneumonia, bacteraemia and endocarditis. Thus the

maintenance of good oral health has both local and systemic implications.

By virtue of its inhibitory effect on Bacteroides spp, bicozamycin incorporated in selective media is undoubtedly valuable when GPAC are sought: Bacteroides spp are commonly found in periodontal pockets and the female genital tract and often display similar colonial morphologies to GPAC. The finding of larger numbers of GPAC in the vagina supports existing evidence that they are often present in the posterior fornix. The identification of Ps. anaerobius, Pc. magnus and Pc. prevotii as predominant species confirms the findings of previous investigators. A greater proportion of symptomatic women (80%) carried GPAC in the vagina than asymptomatic (55%) which lends weight to the opinion that "anaerobic vaginosis" is a significant clinical entity. A higher proportion of swabs positive for GPAC may have been recorded if females at a later stage of pregnancy had been sampled, since the number of GPAC in the vagina has been observed to increase in the third trimester. The possibility of sexual transmission of GPAC and the existence of a carrier state in the female and male genito-urinary tract should be investigated by longitudinal studies. These may clarify the role of GPAC in genito-urinary pathology and identify susceptible individuals.

The classification of GPAC is constantly under review and it is of considerable importance that an identification scheme for these organisms that is both taxonomically valid and practical for

diagnostic laboratories is developed. The technique of SDS-PAGE has proven taxonomic value and in this study was used to examine the protein content of non-covalently bound cell surface material extracted from GPAC with EDTA. Visual assessment of protein profiles obtained indicated homogeneity within the species Ps. anaerobius, but heterogeneity within other biotypes. Nonetheless, distinct groups displaying virtually identical patterns within these individual species were discernible. This suggests that classification of GPAC may be possible, based on a reference collection of patterns from a large number of strains obtained under rigorously standardised conditions. However, such a method would be feasible only for taxonomic rather than diagnostic purposes. Of more practical use in the latter case may be the development of a serological classification scheme for GPAC. Since the components of an EDTA extract are likely to be exposed on intact cell surfaces during the course of an infection, they may be targets for the host's immune response. The antigenic content of EDTA extracts was examined by ELISA and immunoblot transfer to nitrocellulose following SDS-PAGE; antisera were raised in rabbits against live cells injected intravenously. The predominant feature to emerge from immunoblotting procedures was the possession of two common antigens by strains of Ps. anaerobius. This confirmed previous evidence of shared antigens within this species. Various treatments suggested that the determinant site of the high molecular weight antigen contained protein, whereas that of the low

molecular weight antigen had both protein and carbohydrate components. Earlier reports suggested that antigens extracted from Ps. anaerobius whole cells by sonication or autoclaving were species-specific; evidence accumulated in the present study indicates that EDTA extracts contain antigens common to other species of GPAC, e.g. Pc. magnus, Pc. asaccharolyticus and Pc. prevotii, as detected by antisera to whole cells. In a number of cases, serological tests suggested alternative identifications to those arrived at by biochemical tests; some strains reacted strongly with more than one antiserum, whereas others failed to react with any antiserum available. Ps. anaerobius appeared to be strongly immunogenic, whereas Pc. prevotii produced very low antibody titres in rabbits. The overall conclusions from these results are that currently defined species of GPAC may possess several common antigens, non-covalently bound to the cell surface. Further investigations using ELISA, dot immunobinding assays and immunoblot transfer from polyacrylamide gels should distinguish specific and cross-reacting antigens and aid their separation and purification. By determining which antigens are most immunologically potent, more efficient antisera or monoclonal antibodies could be developed for taxonomic and diagnostic purposes.

Differences in the composition, and thus the antibody specificity, of secondary wall polymers in beta-haemolytic streptococci from the basis of the classification of these



bacteria. With a similar aim, a preliminary investigation into the nature of these compounds, as well as the lipocarbohydrate associated with the cytoplasmic membrane, was undertaken. This is the first study to describe these components of GPAC and, although confined to a single strain, it served to highlight difficulties that should be considered in investigations of larger numbers of GPAC. One problem was the small amounts of material obtained after purification procedures, necessitating bulk cultures. A major difficulty was the repeated failure of immunoelectrophoresis and ELISA to detect antigenic activity in cell wall preparations, despite testing a range of concentrations of each component; only a dot-immunobinding assay yielded success. Membrane-associated lipoteichoic acids gave a positive reaction in ELISA as well as a dot-immunobinding assay, but failed to precipitate in antibody-containing agarose gels. It seems that certain membrane-associated antigens may be removed from whole cells with EDTA.

In addition to assessing differences in composition of secondary wall polymers and lipoteichoic acids for taxonomic purposes, consideration should be given to whether or not these molecules are exposed at the surface of intact cells. Both composition and exposure may be influenced by growth conditions in vivo; these factors determine the immunogenicity of wall and membrane components during an infection and ultimately their value in serological diagnoses; if the determinant sites of these components are masked in vivo, patients' sera will not contain

specific antibodies. In purified form, however, such components may be taxonomically useful.

Wall and membrane teichoic acids from various bacteria have immunomodulatory effects. Future studies in this area may offer evidence of the pathogenic potential of GPAC.



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APPENDICES

## APPENDIX I

### Thioglycollate medium:

#### Thioglycollate medium without dextrose or indicator (BBL)

0.25% yeast extract (Oxoid L21)  
0.25% sodium succinate

Dissolve ingredients by boiling for 1 min; dispense into required amounts and autoclave.

Add 1% of sugar solution prior to inoculation.

#### BM enrichment broth: (Deacon et al., 1978)

Trypticase (BBL)	1%
Proteose peptone (Oxoid)	1%
Yeast extract (Difco)	0.5%
Sodium chloride	0.5%
Sodium succinate	0.25%

Dissolve in distilled water and adjust pH to 7.4; dispense into required amounts and autoclave.

To pre-reduced medium (200 ml) add:

4.0 ml 3.75% L-cysteine hydrochloride  
(final concentration 0.075%)

### Standard solutions:

for GLC:

#### Volatile fatty acids:

0.01M acetic acid  
0.01M propionic acid  
0.01M iso-butyric acid  
0.01M n-butyric acid  
0.01M iso-valeric acid  
0.01M n-valeric acid  
0.01M iso-caproic acid  
0.01M n-caproic acid



Non-volatile fatty acids:

0.04M lactic acid  
0.01M succinic acid

Store at 4°C.

APPENDIX 2

SDS-PAGE Electrode Buffer (final strength)

0.025M Tris  
0.192 M glycine  
0.1% sodium dodecyl sulphate (SDS)

Method:

Weigh out 6.057g Tris (hydroxymethyl) methylamine (BDH Analar), 28.827g glycine (BDH chromatographically homogeneous) and 2.0g SDS (BDH specially pure) into separate containers.

Dissolve Tris and glycine in approximately 1000 ml distilled water; adjust pH to 8.3 with 1N NaOH (BDH Analar).

Add SDS after it dissolves; make volume up to 2000 ml with distilled water.

Store at room temperature.

Stacking gel buffer : (double strength)

0.25M Tris-HCl, pH 6.8  
0.2% SDS

Method:

Into separate containers, weight out 15.143g Tris (hydroxymethyl) methylamine (BDH Analar) and 1.0g SDS (BDH specially pure).

Dissolve Tris in approximately 250 ml distilled water; adjust to pH 6.8 with 1N HCl (BDH Analar).

Add SDS and when dissolved make up to 500 ml with distilled water.

Store at room temperature.

## Separating gel buffer (double strength)

0.75M Tris-HCl, pH 8.8  
0.2% SDS

## Method:

Weigh out 90.855g Tris (hydroxymethyl) methylamine (BDH Analar) and dissolve in approximately 500 ml distilled water; adjust to pH 8.8 with 1N HCl (BDH Analar).

Add 2.0g SDS (BDH specially pure), dissolve and make up volume to 1000 ml with distilled water. Filter through Whatman No. 1 paper. Store at room temperature.

## Buffer for immunoelectrophoresis:

Solution 1:	barbital sodium	26.0g
	barbital (barbitone)	4.24g
	distilled water	2000 ml

Solution 2:	glycine	112.4g
	Tris (not Analar grade)	90.4g
	distilled water	2000 ml

## Method:

Mix equal volumes of solutions 1 and 2 (final molarity is 0.187M).

Check that pH is 8.8; store at 4°C.

## 1% Agarose for immunoelectrophoresis:

25 ml	buffer (see above)
75 ml	distilled water
1.0g	agarose (BDH)

## Method:

Mix ingredients and dissolve by boiling; stir continuously. Add Triton X 100 (scintillation grade) to 1% v/v.

Dispense in 15 ml volumes into Universals; store at 4°C.