SPECIES-SPECIFIC DNA PROBES FOR THE IDENTIFICATION OF *Actinobacillus actinomycetemcomitans*

MARGOT EMANUEL

Submitted in Fulfillment of the Requirements for the Degree of Master of Science in Medicine.
University of Cape Town,
May 1990

DECLARATION

I, Margot Jill Emanuel hereby declare that the work on which this thesis is based is original (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

I empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signed by candidate

10 May 1990

ACKNOWLEDGMENTS

I am indebted to Dr Lafras Steyn of the Medical Microbiology department of the University of Cape Town and to Professor Hallett of the Medical Microbiology department of the University of the Western Cape for supervising this project. Thanks also go to the following people from the University of Stellenbosch Dental Hospital: Professor Maresky for his time and guidance; Miss Mostert for her co- operation in obtaining all the specimens and also to all the patients that made this study possible.

I would also like to thank the following people:
Simon Bailee-Cooper for assisting with the photography.
Ivan Clutten for preparing the art work and for all the loyal support he has given me.
My parents for their love and support and for providing for my education.

ABSTRACT

A DNA probe was developed for the identification of the periodontal pathogen, *Actinobacillus actinomycetemcomitans*. Chromosomal DNA was extracted from *A.actinomycetemcomitans*, digested with a restriction enzyme, Sau3A, ligated to plasmid DNA (pUC18) and transformed into JM109 cells to give a partial *A.actinomycetemcomitans* library. The library was screened using Southern blot analysis. Out of the nine inserts tested, one was found to be species specific as it did not cross-hybridise to *Haemophilus aphrophilus*, a closely related organism which occurs in the normal oral microflora, nor did it cross-hybridise with 7 species of *Bacteroides* tested. A level of detection of 10^4 cells or 50ng of *A.actinomycetemcomitans* was obtained.

The probe has a length of 779bp and out of 30 restriction enzymes tested, only SspI was found to have a restriction site in the insert.

The probe was tested on clinical specimens obtained from five different periodontitis patient groups and was shown to correlate with culture results in eighteen out of twenty-two cases in detecting *A.actinomycetemcomitans*.

PREFACE

There is substantial evidence demonstrating that microorganisms play a major role in the aetiology of periodontitis. Our understanding of periodontitis is, however, limited by our ability to detect and identify the putative pathogens present. Recombinant DNA technology has been shown to overcome many of the problems associated with the previous methods for detection of periodontal pathogens.

This study was initiated in order to produce a sensitive and specific DNA probe that could be used to identify *Actinobacillus actinomycetemcomitans*, which has been strongly implicated in the aetiology of localised juvenile periodontitis. Ideally, the probe could be used directly on clinical specimens for the routine detection of *A.actinmycetemcomitans*. The probe which will be useful in determining the causative agents of certain forms of periodontitis; determining the site of active tissue destruction and monitoring the effects of treatment. The probe can also be used for monitoring *A.actinmycetemcomitans* in epidemiological studies.

This thesis is divided into four main sections. Section one is an extensive literature review that deals with the following: an introduction to periodontitis; the complexity of the oral microflora; the microorganism *A.actinomycetemcomitans* and methods used for monitoring periodontal pathogens.

Section two deals with the preparation of the *A.actinomycetemcomitans* specific probe and includes most of the methodology used and the results obtained. Section three covers the identification of *A.actinomycetemcomitans* in clinical specimens and the section four is the conclusion.

LIST OF ABBREVIATIONS

A.actinomycetemcomitans	*Actinobacillus actinomycetemcomitans*
Amp	Ampicillin
AP	Adult periodontitis
ATP	Adenosine triphosphate
B.gingivalis	*Bacteroides gingivalis*
B.intermedius	*Bacteroides intermedius*
bp	Base pairs
BSA	Bovine serum albumin
℃	Degrees celcius
CCC	Covalently Closed Circular
cpm	Counts per minute
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dTTP	Deoxythymidine triphosphate
dpm	Disintergrations per minute
EDTA	Ethylenediaminetetra-acetic acid
EtBr	Ethidium Bromide
g	Grams
H.aphrophilus	*Haemophilus aphrophilus*
IPTG	Isopropyl- ß-D-thio-galactoside
K	Kilo
kb	Kilobase
l	Litre
LB	Luria broth
LJP	Localised juvenile periodontitis
Lkt^+	Leukotoxin-producing
Lkt^-	Non-Leukotoxin-producing
M	Molar
min	Minute
ml	Millilitre
mm	Millimetre

mM	Millimolar
mg	Milligram
n	Number of
N	Normal
NCTC	National Collection of Type Cultures, Collindale, London
ng	Nanogram
nm	Nanometre
OD	Optical density
α^{32}P dCTP	Deoxy cytidine triphosphate labelled with phosphorus in the alpha position
pd	pocket depth in millimetres
pg	Picogram
PEG	Polyethylene glycol
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
rRNA	Ribosomyl ribonucleic acid
SDS	Sodium dodecyl sulphate
sec	Seconds
soln	Solution
TE	Tris (hydroxylmethyl) amino acid/EDTA Buffer
Tris	Tris (hydroxymethyl) aminomethane
TSA	Trypticase soy agar
TSBV	Trypticase soy-serum-bacitracin-vancomycin
TSBVF	Trypticase soy-serum-bacitracin-vancomycin-fluoride
μg	Microgram
μCi	Microcurie
μl	Microlitre
μm	Micrometre
μM	Micromolar
USA	United States of America
UV	Ultraviolet
V	Volts
v/v	Volume per volume
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. SECTION ONE
LITERATURE REVIEW

1.1. INTRODUCTION

Periodontal disease is comprised of a group of different disorders affecting the periodontium, each with its own aetiology and pathogenesis. The most common forms of periodontal disease are gingivitis and periodontitis. Gingivitis is characterised by inflammation of the gums and periodontitis occurs when this inflammation extends to the deeper structures of the periodontium.

The periodontium has four major components: the gingivae; the alveolar bone; the periodontal ligament and the cementum (Figure 1). The healthy tooth is anchored to the jaw by the fibres of the periodontium which attaches the cementum of the root surface, to the alveolar bone and gingiva. In a healthy mouth there is a gingival sulcus which is about 0.5mm in depth between the tooth and the gingiva (Strachan and Waite, 1978). The junctional epithelium is that part of the gingiva which, in health, attaches directly to the tooth enamel.

A clean tooth surface exposed to the oral environment rapidly acquires an amorphous protein film, called the pellicle. The pellicle is soon colonised by bacteria to form dental plaque. Plaque is a living organised community of microorganisms, usually consisting of numerous species and strains embedded in an extracellular matrix made up of products of bacterial metabolism and substances from the saliva and diet (Schluger *et al*, 1978). The microorganisms found in plaque are a part of the normal body flora. There are two types of plaque: supragingival and subgingival and they may occur separately or together on the same tooth. Supragingival plaque forms coronal to the gingival margin, that is on the exposed tooth surface and it is easily detached from the tooth. Subgingival plaque forms below the crest of the gingival margin and is not detectable without the aid of a dental probe. The cleansing activities of the mouth such as saliva, abrasion and swallowing only disturb the supragingival plaque. The subgingival plaque is usually undisturbed by the cleansing activities of the mouth and harbours many microorganisms which give rise to various products which may in turn initiate gingivitis and periodontitis.

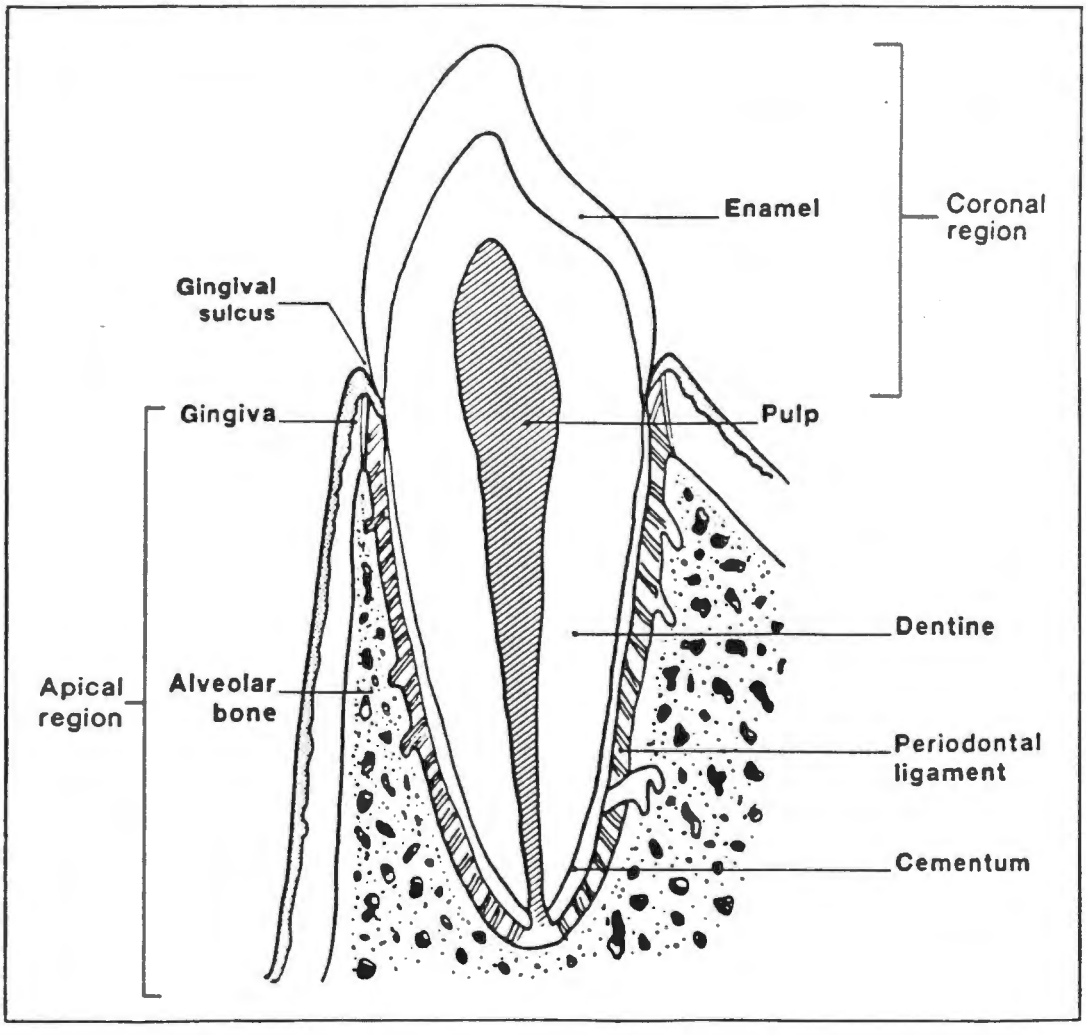


***Figure 1**: A tooth and its periodontium.*

1.1.1. Pathogenesis of plaque associated periodontal disease

The epithelium of the sulcus is in close contact with the subgingival plaque and some of the metabolites from plaque are able to diffuse through the epithelium. These metabolites from plaque initiate inflammation which may be manifested clinically by gingival swelling, redness and bleeding. This condition is known as gingivitis. A gingival pocket may form due to the detachment of the junctional epithelium from the tooth enamel. A distinguishing feature of gingivitis is that although inflammation occurs, the position of the base of the junctional epithelium remains unchanged.

When there is loss of attachment of the connective tissue between the tooth and bone and destruction of the periodontal fibres and of the alveolar bone, the condition is known as periodontitis. A periodontal pocket forms between the gingiva and tooth due to the apical migration of the base of the junctional epithelium.

The main stages in the development of chronic gingivitis and periodontitis are shown in Figure 2.

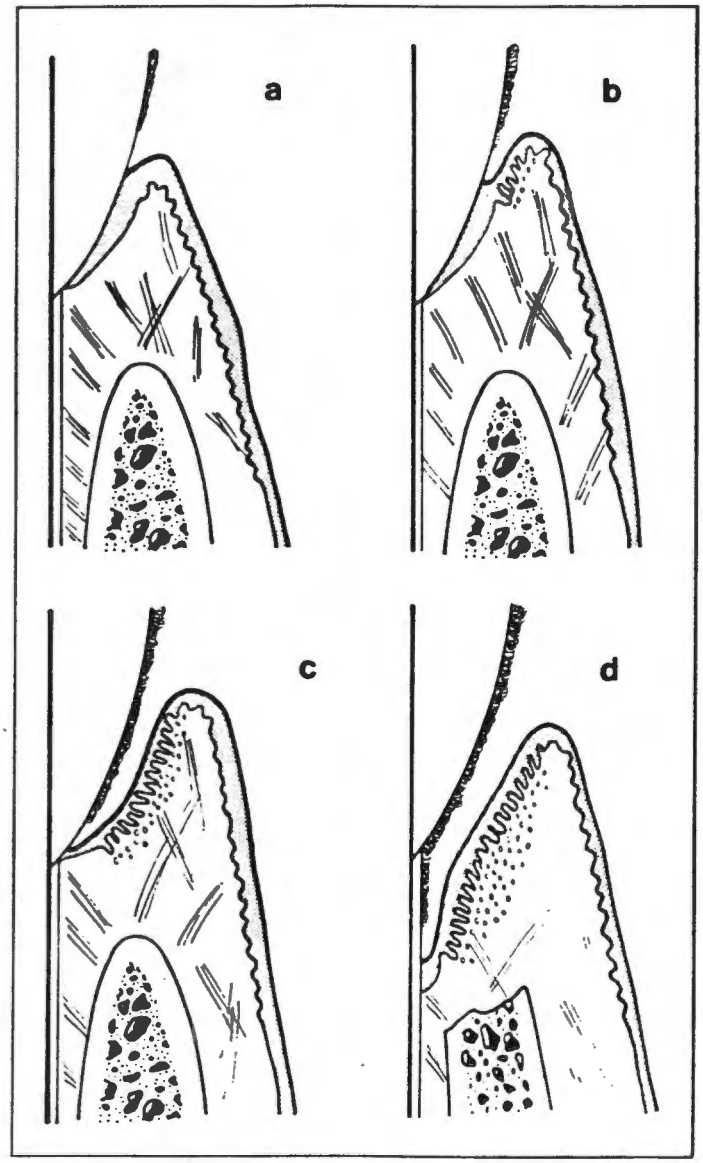


Figure2: The development of chronic gingivitis and periodontitis (Macfarlane and Samaranayake, 1989).

(a) A healthy gingival sulcus with early supragingival plaque formation.

(b) Established chronic gingivitis with minor inflammatory enlargement.

(c) Long-standing chronic gingivitis with subgingival plaque extension in a gingival pocket.

(d) Chronic periodontitis with destruction of the periodontal membrane and alveolar bone.

1.1.2. Specific and non-specific plaque hypotheses

Prior to the 1970's periodontitis was thought to be caused by an overall increase in microorganisms. This was termed the non-specific plaque hypothesis. Subsequently it was concluded that specific bacteria were associated with particular forms of periodontitis. This is called the specific plaque hypothesis. Several clinical studies have indicated that proportional increases or ecological "blooms" of a few specific microorganisms may be associated with progression of periodontitis (Holt *et al*, 1988).

Although many studies have associated various microorganisms with periodontitis, definitive

proof of the role of specific microorganisms in the aetiology of the disease remains unclear. Specific microbial changes associated with clinical signs of periodontitis may in fact be secondary ecological phenomena rather than causative associations (Holt *et al*, 1988).

The concept that only a bacteria-free tooth surface is compatible with periodontal health is giving way to the more realistic view that tooth surfaces normally harbour a resident microbial population. Some microbiotas are not only compatible with periodontal health but in fact help to maintain the healthy status of the tissues by occupying an ecological niche which is therefore no longer available to less desirable microorganisms (Savitt *et al*, 1988).

1.1.3. Classification of plaque-associated periodontal disease

Although there is little doubt that there are different periodontal diseases, their classification presents a major problem. This has an important bearing on the search for the aetiological agents of destructive periodontitis.

Six general forms of plaque-associated periodontal disease have been described: gingivitis; acute necrotizing ulcerative gingivitis (ANUG); adult periodontitis (AP); rapidly progressive periodontitis; juvenile periodontitis and prepubertal periodontitis.

1.1.3.1. Gingivitis

This is an inflammatory condition of the gingiva without apical migration of the junctional epithelium.

1.1.3.2. Acute Necrotizing Ulcerative Gingivitis (ANUG)

This disease was originally named "trench mouth" and has a rapid onset characterised by a painful, necrotic, ulcerative gingival lesion.

1.1.3.3. Adult periodontitis (AP)

This form of the disease is considered to have its onset at the age of 30 to 35 years and older. It is the most common form of periodontitis and has been investigated extensively.

1.1.3.4. Rapidly progressive periodontitis

This form of periodontitis occurs between puberty and the age of 30 years. Severe and rapid alveolar bone destruction is the prevalent feature of the disease. There is usually considerable gingival inflammation.

1.1.3.5. Juvenile periodontitis

This disease has its onset at puberty and it occurs in about 0.1% of otherwise healthy adolescents. Minimal inflammation, deep pocketing and severe destruction of the alveolar bone of incisors and first molars are typical features.

1.1.3.6. Prepubertal periodontitis

This rare condition occurs during or immediately after eruption of the primary teeth, in localised and generalised forms. These diseases are characterised by acute inflammation and rapid destruction of the periodontium.

1.2. THE ORAL MICROFLORA

1.2.1. Complexity of the oral microflora

The tooth surface and periodontal pocket harbour 10^{10} bacteria per gram wet weight (Slots and Listgarten, 1988). Culture studies have shown that approximately 300 bacterial species are present in the periodontal pocket (Moore *et al*, 1982). Only 100 of the 300 species associated with the gingival crevice have been described and many of the described species are not differentiated by most laboratories that are examining periodontal floras (Moore, 1986). It appears that only 15 to 20 of these species are actually associated with periodontitis.

1.2.2. Predominant oral microorganisms

Numerous papers have been published on the microorganisms associated with periodontitis. Slots and Listgarten (1988) summarized the basic observations that have been reported:

In the healthy gingival sulcus, a scant microflora exists consisting mainly of Gram-positive, facultative organisms (85% of the total microorganisms), usually *Streptococcus* and *Actinomyces*. Similar findings are obtained in previously diseased sites following successful treatment.

In chronic gingivitis, Gram-negative organisms are found to constitute 45% of the total microorganisms isolated and anaerobic organisms constitute 45% of the total bacteria recovered. The predominant isolates include *Streptococcus* and *Actinomyces* species, *Fusobacterium* species and *Bacteroides intermedius (B.intermedius)* as well as various non-pigmented *Bacteroides* species.

In advanced adult periodontitis, 75% of the organisms isolated are Gram-negative. The majority of these are anaerobic organisms as 90% of all the isolates have been shown to require anaerobic conditions for growth. The Gram-negative organisms include *Bacteroides intermedius, Bacteroides gingivalis (B.gingivalis), Fusobacterium* species, non-pigmenting *Bacteroides, Wolinella* species and *A.actinomycetemcomitans*.

In localized juvenile periodontitis (LJP), mainly Gram-negative rods (65%) are found and most of these are anaerobic (80%). The predominant isolates include *A.actinomycetemcomitans, B.gingivalis, B.intermedius, Capnocytophaga* species and *Eikenella corrodens (E.corrodens)*.

The proportion of Gram-negative organisms increases significantly with increasing severity of periodontitis. Certain Gram-negative species, especially *B.gingivalis, B.intermedius* and *A.actinomycetemcomitans*, appear to be closely related to periodontitis.

There have been conflicting reports about these three "indicator bacteria" in the literature. Slots *et al* (1986b) reported that at least one of these three bacteria could be identified at 99%

of the sites manifesting disease activity. In addition, Wennström *et al* (1987) found that sites from which the so-called indicator bacteria were absent, showed no clinically significant loss of probing attachment during one year of observation. Socransky (1986) stated however, that these pathogens account for only 30% of destructive lesions. Peros and Savitt (1989) claimed that diagnostic information could be gained by determining the proportion of each important organism in plaque. Levels of *A.actinomycetemcomitans* and *B.gingivalis* greater than 5% of the total cultivable microflora are, for example, consistent with a diagnosis of adult periodontitis.

In another study, Bragd *et al* (1987), defined different levels for each of the three pathogens. Levels of *A.actinomycetemcomitans*, *B.gingivalis* and *B.intermedius* greater than 0.01%, 0.1%, and 2.5% respectively of the total cultivable microflora were considered to be consistent with a diagnosis of adult periodontitis.

1.3. *ACTINOBACILLUS ACTINOMYCETEMCOMITANS*

The current study focused only on *A.actinomycetemcomitans* and all further references will concentrate on the association of *A.actinomycetemcomitans* with periodontitis.

1.3.1. Historical background

A.actinomycetemcomitans was first described by Klinger in 1912 (Phillips, 1984) and was isolated from cervicofacial actinomycosis. The original name *Bacterium actinomycetum comitans* was changed to *Actinobacillus actinomycetemcomitans*. *Actinobacillus* refers to the internal star-shaped morphology of its bacterial colonies on solid media and to the short rod or bacillary shape of individual cells. *Actinomycetemcomitans* refers to its association with *Actinomyces* in actinomycosis.

1.3.2. *A.actinomycetemcomitans* in human infection

A.actinomycetemcomitans can cause serious infections at various sites in the body including abscesses of the abdomen, brain, face, hand and thyroid gland.

1.3.3. Classification

There is some controversy about the genus to which *A.actinomycetemcomitans* should be assigned. Some workers have assigned it to the genus *Haemophilus* and others to the genus *Actinobacillus*. The latest trend has been to assign it to the latter genus. Potts *et al* (1985) determined a DNA relatedness of 28% or greater between *A.actinomycetemcomitans* and *Haemophilus segnis*, *Haemophilus paraphrophilus* and *H.aphrophilus*.

1.3.4. Features of *A.actinomycetemcomitans*

A.actinomycetemcomitans forms small (0.5-1mm in diameter), smooth, circular, convex and translucent colonies with slightly irregular edges and a star-shaped morphology (Figure 3). The morphology has also been described as "crossed cigars" (Phillips, 1984). The bacterial cells are non-motile, Gram-negative coccobacilli which occur singly, in pairs or in small clumps. The cells have also been reported to be rod shaped (Phillips, 1984).

The organism has the following biochemical characteristics: it is fermentive; non-haemolytic; indole negative and catalase positive. The bacterium is X factor (haemin) and V factor (nicotinamide adenine dinucleotide) independent. *A.actinomycetemcomitans* produces acid from glucose, fructose and mannose but not from adonitol, amygdalin, arabinose, cellobiose, dulcitol, esculin, galactose, glycerol, inositol, inulin, lactose, melibiose, rhamnose, ribose, salicin, sorbitol or sucrose. Variable results have been obtained with dextrin, maltose, mannitol and xylose (Slots *et al*, 1980).

A.actinomycetemcomitans does not ferment lactose or sucrose. These characteristics are important for differentiating it from the closely associated species *H.aphrophilus*.

Three serotypes of *A.actinomycetemcomitans* have been identified on the basis of heat stable cell surface antigens and they are designated **a**, **b** and **c**. *A.actinomycetemcomitans* serotypes **a** and **b** occur with the highest frequency in the human oral cavity. Serotype **b** is the serotype most commonly associated with localized juvenile periodontitis (Newman and Nisengard, 1988).

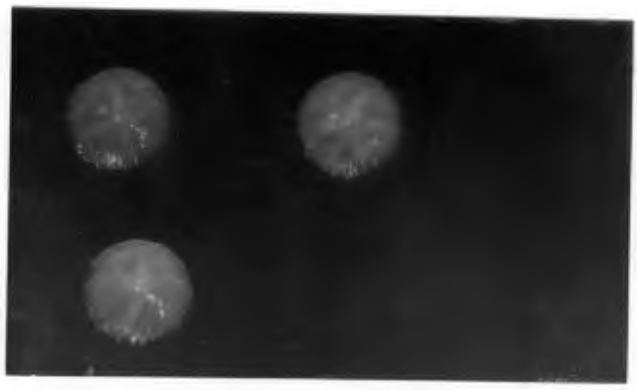


***Figure 3**: Colonial morphology of A.actinomycetemcomitans*

1.3.5. Virulence factors

A.actinomycetemcomitans produces a number of factors which may contribute to its ability to cause periodontitis. The most important of these virulence factors is a leukotoxic activity, while others include a cytotoxic activity affecting various types of cells such as fibroblasts, epithelial cells and endothelial cells. These virulence factors may permit *A.actinomycetemcomitans* to invade and colonise the gingival connective tissue (Saglie *et al*, 1986).

The most extensively studied of these virulence factors is the leukotoxin. The leukotoxin of *A.actinomycetemcomitans* is a heat labile, protease sensitive product of 115000 daltons (Rabie *et al*, 1988). Zambon *et al* (1983b) found that 55% of *A.actinomycetemcomitans* isolates from localized juvenile periodontitis lesions produced the leukotoxin, whereas only 16% of *A.actinomycetemcomitans* isolates from minimally diseased sites were shown to have leukotoxicity.

Both leukotoxin producing and non-producing *A.actinomycetemcomitans* may be present in the same periodontal site. Kolodrobetz *et al* (1989) used Southern blot analysis to show that a naturally occurring Lkt^- (non-leukotoxin producing) strain contains sequences corresponding to the leukotoxin structural gene. They also showed that the gene in a Lkt^- strain of *A.actinomycetemcomitans* is modified by having an additional HindIII restriction site which was not present in the Lkt^+ (leukotoxin-producing) strain. These authors concluded that the polymorphism reflects a strain difference rather than a mutation which gives rise to the Lkt^- phenotype.

1.3.6. Transmission of *A.actinomycetemcomitans*

Studies have been done to determine whether *A.actinomycetemcomitans* may be transmitted by contact between humans and family pets. Preus and Olsen (1987) found that individuals with *A.actinomycetemcomitans* may have obtained it from family pets and might spread it to relatives. In addition, Zambon *et al* (1983a) showed that in five families with LJP, family members harboured the same bacterial biotype and serotype of *A.actinomycetemcomitans*, indicating that there may be an intra familial spread of the organism. However, Zambon *et al* (1983a) stated that *A.actinomycetemcomitans* does not appear to be readily transmissible as only one half of the family members harboured this microorganism in spite of close daily contact.

1.3.7. Evidence for association of *A.actinomycetemcomitans* with periodontitis

Since 1977, many workers have repeatedly isolated *A.actinomycetemcomitans* from LJP lesions but only rarely from periodontally healthy or minimally diseased sites (Slots *et al,* 1980; Slots and Rosling, 1983; Zambon *et al,* 1983b; Eisenmann *et al*, 1983; Christersson *et al*, 1985b; Mandell and Socransky, 1981; Mandell, 1984; Mandell *et al*, 1986; Kornman and Robertson, 1985; Moore *et al*, 1985; Asikainen *et al*, 1986; Newman and Socransky, 1977).

Moore (1987) noted that in patients with localized juvenile periodontitis, which had been sampled microbiologically and had been reported on during the two previous years, approximately one third of the sites had *A.actinomycetemcomitans* at levels greater than 1%. This is consistent with a report by Savitt and Socransky (1984) in which *A.actinomycetemcomitans* was detected in 38% of the sites from twelve localized juvenile periodontitis patients. Other putative pathogens were isolated with equal or greater prevalence from these patients. They attributed this to the fact that a substantial dilution factor is involved in non-selective culture studies, so that 30 to 50 colonies are taken as representative of a population of millions of bacteria. If many samples are taken, the population may be accurately reflected. A single sample, however, is unlikely to reflect the total population unless the organisms are present in high proportions. Selective media may allow the detection of organisms in low proportions but introduces other considerations.

Genco *et al* (1986) found *A.actinomycetemcomitans* in 95% of 60 LJP patients, in 17% of 142 healthy patients and in 21% of 134 adult periodontitis patients. This suggests that *A.actinomycetemcomitans* is associated with LJP. Since not all of the sites were culture positive for *A.actinomycetemcomitans* it may be necessary to culture more than one site per patient, otherwise there is a chance that the infection may be missed. Genco *et al* (1986) suggests that half of the sites showing periodontal destruction should be sampled in order to make a diagnosis.

Genco *et al* (1986) also found a direct correlation between the levels of subgingival *A.actinomycetemcomitans* and the change in depth of the periodontal pockets at the same site. This clearly demonstrates the need to suppress, if not eradicate, the organism to achieve clinical success. He suggests that this principle can currently be used in clinical practice for assessing active and inactive forms of disease, as well as a means of monitoring therapy.

Moore *et al* (1985) found no substantial differences in the subgingival floras of patients with either LJP or AP. The only difference noted was between diseased and healthy sites. Furthermore, different sites with comparable destruction in the same patient often harboured flora which differed in composition. Frisken *et al* (1987) attempted to explain Moore's findings by suggesting that by using a spiral plating technique only a few *A.actinomycetemcomitans* would be isolated while direct plating would have resulted in large increases in the number of isolates obtained. Thus the differences in the results found may be partly explained by differences in the sensitivities of the recovery procedures.

Antibody studies almost unanimously report a dramatically higher level of serum antibodies to *A.actinomycetemcomitans* in LJP patients (Slots and Listgarten, 1988).

The different groups of teeth may have different microbiological flora. Alaluusa and Asikainen (1988) found differences in the detection rate of *A.actinomycetemcomitans* within the groups of teeth. The primary molars and especially the second molars, harboured *A.actinomycetemcomitans* most frequently. *A.actinomycetemcomitans* was rarely detected in the incisors.

1.4. MONITORING OF PERIODONTAL PATHOGENS

"The assessment and diagnosis of periodontitis has become one of the most critical and controversial areas in periodontology" (Kornman, 1987). Unfortunately, identification of all periodontal pathogens and accompanying diagnostic methodology has not progressed sufficiently to establish guidelines to differentiate pathogenic from non-pathogenic plaque nor to distinguish active from arrested disease. As microbiologic advances are made, they will translate into improved diagnostic testing methodology (Ciancio *et al*, 1988).

The major stumbling block to a better understanding of the microbial aetiology of periodontitis and to the development of clinically useful diagnostic tests is the lack of an absolute criterion of disease activity. A means of identifying all episodes of disease activity including their exact points of onset and termination is required.

1.4.1. Clinical indicators of periodontitis

Diagnosis has previously relied on standard clinical measurements such as the quantification of plaque, redness of the gingiva, swelling of the soft tissues, bleeding on probing with examination instruments, suppuration from the tissues, pocket formation, crevicular fluid volume as well as radiographic data to indicate progressive disease (Peros and Savitt, 1989). Techniques were developed for the assessment of these parameters. However, there are problems associated with each of these and none of these clinical parameters demonstrates a high degree of sensitivity or specificity when used alone or in combination with each other. They are thus not the ideal predictors of active advancing periodontitis (Haffajee *et al*, 1983).

1.4.2. Microbiological monitoring of periodontitis

Microbiological monitoring has been shown to overcome some of the limitations of the standard clinical diagnostic methods. In one study, clinical measurements were made with and without microbiologic data to evaluate the therapeutic success of a treatment plan. Results showed that one out of three patients were evaluated incorrectly when only clinical criteria were used (Peros and Savitt, 1989).

Until recently, microbiological monitoring was not available to the clinician because of the lack of reliable and practical methods of bacterial identification. Microbiological monitoring, however, has reached the stage of development where it should be incorporated into clinical practice. This is an excellent example of rapid progression of basic findings from laboratories around the world and implementation into clinical practice (Ciancio *et al*, 1988).

Several methods such as direct culture, immunological techniques, enzyme assays, phase contrast microscopy, dark field microscopy, electron microscopy and DNA probes are used. None of these methods are ideal on their own.

1.4.2.1. Culture

Culture is the reference method for determining the microbial composition of plaque samples. This is not practical on a routine basis, however, and it is prone to errors since the primary oral pathogens are fastidious anaerobes and often die during transport to the laboratory. Organisms are affected by sampling, dispersion, incubation and selection of media for isolation. This often results in diverse data from different laboratories. Identification of bacteria is labour intensive and commonly requires 20 or more culture plates for the study of one sample.

1.4.2.2. Immunological methods

Immunological methods are based on the clinical observation that periodontitis is associated with inflammation. Inflammation is triggered by the persistent bacterial presence within supragingival and subgingival plaque or possibly within the gingival tissues. The gingiva contain the cellular and humoral elements necessary to elicit an immunological response and polymorphonuclear leukocytes and antibodies have become the target of research.

A disadvantage of these techniques is that some bacteria, even in small numbers, are highly antigenic while others, even when present in abundance, elicit little antibody response. Studies by Genco *et al* (1985) on serum antibodies to specific serotypes of *A.actinomycetemcomitans* have shown that the sensitivity and the specificity of the serum IgG test to be 71% and 89% respectively.

1.4.2.3. Enzymes

Changes in the quality and quantity of microbial enzymes, including the trypsin-like enzyme of *B.gingivalis* and the sialidase activity associated with many putative periodontal pathogens, have been used to detect the presence of periodontopathic organisms (Moncla *et al*, 1990). Enzymatic assays must still undergo extensive testing before they can be used to identify periodontally infected patients.

Using the API-ZYM system Slots (1981) was able to distinguish *A.actinomycetemcomitans* from *H.aphrophilus* as *H.aphrophilus* differs from *A.actinomycetemcomitans* by producing ß-galactosidase and α-glucosidase. This is important as these two microorganisms are closely related and are difficult to distinguish using standard biochemical tests.

1.4.2.4. Microscopy

1.4.2.4.1 Phase and dark-field microscopy

Direct microscopy of periodontal fluid can theoretically assess the number and gross morphological features of the microorganisms present in the specimen. However, Moore (1987) stated that phase and dark-field microscopy often underestimate the numbers of small cells and cocci. Microscopic monitoring detects organisms by morphotype, which precludes identification of specific bacteria. Variable results have been obtained using these techniques and these may be attributed to various factors such as sampling as well as problems associated with dispersion of the specimens. The lack of precision of microscopic monitoring prevents it from being effectively used as a reliable microbiological assay. Advantages of phase and darkfield microscopy include rapidity as well as economy because less equipment and personnel are required (Greenstein and Polson, 1985).

1.4.2.4.2 Electron microscopy

The equipment required for electron microscopy is expensive and the preparation and sectioning of the specimens is time consuming and requires special techniques. Electron microscopy can not be used to differentiate between microorganisms with similar

morphologies. Advantages of this technique are that additional morphological features, such as fimbriae or flagella, may be studied.

1.4.2.4.3 Immunofluorescence microscopy

Using indirect immunofluorescence microscopy with serodiagnostic reagents for *A.actinomycetemcomitans* in subgingival plaque, Bonta *et al* (1985) found that the sensitivity ranged from 82% to 100% compared with culture. One disadvantage of immunofluorescence microscopy is that the monoclonal antibodies used may be so specific that they may not recognise slightly different antigenic moieties presented by microorganisms of the same species. Another problem with this technique is that the quantitation of fluorescence is subjective.

1.4.2.5. DNA probe technology

Developments in molecular biology during the last 20 years have made it possible to detect bacteria on the basis of DNA-DNA hybridisation between a labelled DNA probe and the target DNA, immobilised on nylon or nitrocellulose filters. The technology has been applied to the detection of periodontal pathogens directly in clinical samples with a high degree of sensitivity and specificity (90 to 100%).

Three major advantages of DNA probes over conventional bacteriological procedures are: (1) the organisms do not have to be viable in cell culture to be identified, as DNA is a stable molecule (Peros and Savitt, 1989); (2) the organism is identified at the molecular level, thus organisms can be distinguished despite phenotypical similarities; (3) it should be possible to measure the number of periodontal pathogens directly in a sample obtained by a dentist without the need for growth in culture (Savitt *et al*, 1988; Tenover, 1985). This reduces the time and special equipment needed for the cultivation of the organisms.

1.4.2.5.1 Problems associated with the use of DNA probes

Technical problems associated with the use of DNA probes include self hybridisation of the probe and non-specific binding of the probe to the membrane. Two types of probes are used: radioactive probes and non-radioactive. Radioactive probes are expensive, as ^{32}P, the isotope

most commonly used for radiolabelling DNA, has a short half-life. The use of radioactive probes creates safety and disposal problems. Non-radioactive probes are being used, but at present do not have the sensitivity of the radioactive probe.

One problem with using probes directly on the specimens is that the organism of interest may be present in numbers that are too low to be detected. French *et al* (1986) found that in a mixed culture, *Bacteroides* species had to account for at least 10% of the mixture in order to be detected.

The relatively new technique, Polymerase chain reaction (PCR) allows the specific amplification of discrete fragments of the DNA and facilitates detection of nucleic acid fragments that are initially present in picogram quantities. PCR has been shown to produce an increase greater than 10^5-fold in the amount of target DNA (Saiki *et al*, 1986). The PCR amplification procedure uses two oligonucleotide primers of known sequence, positioned 10 to 300 base pairs apart and complementary to the plus and minus strands of target DNA. The procedure involves repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences and extension of the annealed primers with DNA polymerase. The extension products of one primer can serve as a template for the other, so each cycle doubles the amount of DNA synthesized on the previous cycle. This results in the exponential accumulation of the specific target fragment. PCR has been successfully used for many applications but as yet has not been used to detect oral microorganisms. This could be due to the fact that unique target sequences have only recently been identified (Dix *et al*, 1990).

1.4.2.5.2 Review of relevant studies

A limited number of studies have used DNA probes in assessing mixed plaque samples. The most significant studies to date will be discussed.

Roberts *et al* (1985) stated that DNA probes have proved more reliable and reproducible than currently available biochemical tests for the detection of *Mobiluncus* species. These authors found that the average length of time for bacterial isolation by culture was 30 days with a range of 7 to 100 days. Speciation generally required an additional seven days to complete.

The average time of completion of the DNA probe assay was five days with a range of 2-14 days. These authors obtained 100% correlation between DNA-probe species identification and conventional biochemical identification.

The cost per specimen using a DNA probe is less than that of culture techniques. Perine *et al* (1985) found that 1 litre of broth culture yielded 50-100μg of purified DNA, which is stable and sufficient for the preparation of probes for 50000 tests. The major cost is that of the radioactive label. They found that the cost of testing each specimen in their study, including labour, was under $2.00 per specimen. Cultures for the same species cost $3.50 each.

French *et al* (1986) developed probes which are claimed to be sensitive, specific and rapid for the identification of *A.actinomycetemcomitans, B.intermedius* and *B.gingivalis*. These workers used an "enriched" *A.actinomycetemcomitans* specific probe to screen their *A.actinomycetemcomitans* library for *A.actinomycetemcomitans* specific sequences. This "enriched" probe was made by hybridising *A.actinomycetemcomitans* genomic DNA to a filter containing genomic *H.aphrophilus* DNA and then removing the unhybridised probe which they called the "enriched non-cross-reactive *A.actinomycetemcomitans* genomic probe". They found 5 recombinant clones in their first round of screening and of these, 4 were shown to have no cross-reactivity with *H.aphrophilus*. These clones were then pooled and used as the specific *A.actinomycetemcomitans* DNA probe. The 4 clones showed a 10-fold decrease in sensitivity when compared to the whole genomic *A.actinomycetemcomitans* DNA probe. The level of detection in 18 hours was found to be between 10^3 and 10^4 cells compared to the 10^2 level obtained from the whole genomic probe hybridisation. They claim that by obtaining at least 10 more *A.actinomycetemcomitans* specific clones, the sensitivity could be reduced to 10^2 cells.

Savitt *et al* (1988) found that probe analysis was 100% effective in detecting *A.actinomycetemcomitans* and *B.intermedius* compared to culture analysis. *B.gingivalis* was detected by the probe in 10 of the 11 culture positive specimens. The only sample that was culture positive and probe negative for *B.gingivalis* came from a patient with mild gingivitis but no history of periodontitis. These authors attributed the failure of the probe to detect the *B.gingivalis* to sampling variation. Savitt *et al* (1988) maintained that the DNA probe assay was more sensitive than the culture method. For example, *A.actinomycetemcomitans* was detected by probe analysis in 70% of the LJP patient samples but only in 10% of these samples

by culture analysis. These authors stated that the probe results are more consistent with the clinical status of the patient than the culture results. In addition, statistical analyses indicated a significant difference between probe and culture positive results. This is not suprising as the percentage of DNA probe detectable pathogens is elevated in these patient samples compared with culture. This is because DNA probe technology allows detection of pathogens in a sample that may not contain viable microorganisms at the time of sample processing. Furthermore, culture may not always accurately reflect true cell populations, as many of the cells from the pocket are not viable *in vitro*, due to their fastidious nature.

Strzempko *et al* (1987) found that their *B.intermedius* probe cross-reacted with other *Bacteroides* strains at a level of 1%. Therefore to detect these organisms in a specimen, 10^5 cells would be required to generate a signal comparable with that generated by 10^3 cells of the probe species. The sampling technique using paper points only collects an average of 10^6 cells. An extremely high proportion of cross-reacting organisms would therefore have to be present in a sample to be detected.

Smith *et al* (1989b) examined the value of using non-isotopic DNA probes to identify subgingival organisms. These authors used 2 types of non-isotopic probes: horseradish peroxidase-conjugated probes and biotin labelled probes. Instead of using a cloned DNA fragment as the probe for *A.actinomycetemcomitans*, they used whole genomic DNA, despite having found that the whole genomic DNA probe cross-hybridised to *H.aphrophilus* DNA. They scanned filters containing *A.actinomycetemcomitans* DNA hybridised to an *A.actinomycetemcomitans* probe and to a *H.aphrophilus* probe using a laser densitometer. Signal intensities for the strains of *A.actinomycetemcomitans* hybridised to the *A.actinomycetemcomitans* probe were found to be twice that obtained for the strains of *H.aphrophilus* hybridised to the *A.actinomycetemcomitans* probe.

One nanogram of target DNA was detected with the horseradish peroxidase labelled probe while 10 to 100pg was detected with the biotinylated probe. Differences of 100 to 1000 fold in sensitivity were obtained with the horseradish peroxidase probe in different experiments. Using the biotin probes, 10^4-10^5 cells of all isolates of homologous species were detected. Six isolates of *A.actinomycetemcomitans* were tested and all 6 were positive when probed with the *A.actinomycetemcomitans* probe. The *A.actinomycetemcomitans* probe, however, also

hybridised to the cross-reacting target strain, *H.aphrophilus*. From these results the authors claim that these probes were 100% sensitive and that the probe specificities ranges from 98 to 100%.

Smith *et al* (1989a) described a "reverse" DNA hybridisation method in which whole genomic DNA was extracted from "unknown" strains, labelled with digoxigenin by a random primer technique and used as probes against DNA from reference strains which had been applied to filters. It was found that the labelled probes prepared from 10 to 20 colonies consistently detected 100pg of purified homologous DNA. Because some of the probes prepared, were not species specific, cross-hybridisations occurred, and thus much caution must be taken in interpreting such results. The above workers have not as yet tested the potential of these methods for the direct identification of suspected pathogens in subgingival plaque samples.

Just before completion of this thesis, Dix *et al* (1990) published a paper describing the use of oligonucleotide probes for the identification of periodontal bacteria. These authors sequenced the 16S rRNA for each of 10 species of oral bacteria and identified the hypervariable regions. Oligonucleotide probes were designed to target hypervariable regions. To determine whether each synthetic probe was specific for the appropriate microbe, each probe was tested against total nucleic acids isolated from 20 oral microorganisms. They also tested whole genomic DNA probes against the total nucleic acids of the same 20 species. The results showed that cross-hybridisations between *A.actinomycetemcomitans* and *H.aphrophilus* were obtained using the whole genomic probes but not with the oligonucleotide probes. The *A.actinomycetemcomitans* oligonucleotide probe differed from the *H.aphrophilus* probe by 6 mismatches in a 24bp region but they state that this is sufficient to prevent hybridisation to an inappropriate target. Out of the 10 species that these authors tested, only the *Fusobacterium nucleatum* oligonucleotide probe was not specific. The detection limits obtained for *Fusobacterium nucleatum* was 200 bacteria and for all the other species tested was 10^3 bacteria.

"Although a powerful technique, DNA probes are not a litmus paper type of chairside test for the status of periodontal lesions" (Dickinson, 1986). No matter how elegant or how precise these procedures may become for the enumeration of individual species, they are completely dependent on the recognition of candidate organisms by cultural studies (Socransky *et al*, 1987).

1.5. TREATMENT

The 3 main goals of treatment for periodontitis are the elimination of inflammatory lesions, reduction of pocket depth, and the improvement of gingival attachment levels. In essence the objective of periodontal therapy is to eliminate the pathogenic subgingival microbiota, resulting in the resolution of the inflammatory lesions (Peros and Savitt, 1989).

The major treatment of periodontitis is local, consisting of chemical and physical plaque control. The systemic use of antibiotics is of great importance in the management of certain types of periodontitis. Various periodontal surgical procedures designed to repair or regenerate damaged tissues are used extensively.

Infections associated with *A.actinomycetemcomitans* may be difficult to treat by mechanical means. In LJP, subgingival scaling and root planing can not eradicate *A.actinomycetemcomitans* (Slots and Rosling, 1983; Christersson *et al*, 1985a; Kornman and Robertson, 1985) . Surgical removal of periodontal tissues using a modified Widman flap procedure or soft tissue curretage also has had limited success in eradicating *A.actinomycetemcomitans* (Christersson *et al*, 1985a). This may be because the organism can survive in the deeper parts of the pocket or in the gingival connective tissue (Saglie *et al*, 1986; Christersson, 1987). Electron microscopy studies have shown Gram-negative microorganisms within the gingiva (Saglie *et al*, 1982) which were later identified as *A.actinomycetemcomitans*. *A.actinomycetemcomitans* and black pigmented *Bacteroides* species have also been identified in sites which did not respond to either local therapy alone, or to local therapy plus systemic tetracycline (Kornman and Robertson, 1985).

The studies mentioned above all demonstrate the importance of monitoring *A.actinomycetemcomitans* in assessing the effectiveness of treatment.

2. SECTION TWO
PREPARATION OF AN
A.ACTINOMYCETEMCOMITANS SPECIFIC PROBE

2.1. CULTURE OF TYPE STRAINS AND GENOMIC DNA EXTRACTIONS

2.1.1. Introduction

The first phase of this study was to extract DNA from type culture strains of *A.actinomycetemcomitans* and to evaluate the usefulness of a whole genomic probe for the detection of *A.actinomycetemcomitans* in clinical specimens.

2.1.2. Materials and methods

2.1.2.1. Culture of type strains

A.actinomycetemcomitans strains NCTC 9710 and NCTC 9709, and *H.aphrophilus* strains NCTC 5906 and NCTC 5886, were obtained from the National Collection of Type Cultures, Collindale, London. The microorganisms were plated onto a non-selective medium consisting of Trypticase soy agar supplemented with vit K:Haemin and horse blood, as well as onto a selective medium, TSBV for *A.actinomycetemcomitans* (Slots, 1982a) and TSBVF for *H.aphrophilus* (Tempro and Slots, 1986). Prior to use, the plates were stored under aerobic conditions at 4°C and used within 7 days of preparation. All cultures were incubated at 37°C in an atmosphere of 90% air-10% CO_2 for 2-3 days, cultures on selective media plates requiring up to a week for bacterial growth.

The following strains of *Bacteroides* were also cultured and the DNA of each strain was extracted: *B.asaccharolyticus; B.buccae; B.corporis; B.denticola; B.intermedius; B.gingivalis* and *B.melaninogenicus*. These strains were isolates that had been identified by standard biochemical tests. The non-selective medium described above was used and all the plating out of the bacteria was done anaerobically in an anaerobic chamber.

2.1.2.2. Harvesting of bacteria

One millilitre of TE buffer [10mM Tris HCl (pH8) and 1mM EDTA] was added to each plate and the colonies were scraped off with a sterile, bent glass rod. The bacterial suspension (1ml) was collected in a disposable centrifuge tube (Falcon). The cells were centrifuged in a MSE centrifuge at 2000 rpm and washed 3 times in TE buffer before the DNA was extracted.

2.1.2.3. Preparation of Chromosomal DNA

Chromosomal DNA was extracted from the type strains of bacteria using the method of So *et al* (1975) as modified by Spiegel and Roberts (1984). One-quarter of a cell pellet from 10 plates was suspended in 40ml of TE buffer. EDTA (present in the TE) is a chelating agent which binds the divalent metal ions which are essential for the activity of certain nucleases and thus protects DNA from digestion by nucleases (Dillon *et al*, 1985). EDTA also weakens the cell walls.

Forty milligrams of dry lysozyme powder were added and the cell suspension was incubated at 37°C for 1 hour. Pronase (1ml of a 10mg/ml solution) was added and the suspension was incubated at 55°C for 30 minutes. Sodium dodecyl sulphate (10%) was added to a final concentration of 0.4% and the suspension was heated (55°C) until the cells had lysed; normally 1 hour was needed for complete lysis. The solution was diluted 1:1 (v/v) with TE buffer, 200μl of proteinase K (10mg/ml) was added and the lysate was incubated at 37°C overnight. The lysate was mixed with an equal volume of Tris buffered phenol (pH 8.0), and centrifuged at 6000rpm in a J.A.20 rotor on a Beckman J-21B centrifuge for 5 minutes at 4°C in siliconized 30ml Corex tubes. The aqueous phase (which was very cloudy at this stage) was mixed with an equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged as before. The aqueous phase was re-extracted with chloroform:isoamyl alcohol. The salt concentration was adjusted to 0.3M using 3M sodium acetate and the DNA was precipitated with 2 volumes of ice-cold ethanol. The addition of the monovalent cations, Na^+, $NH4^+$, or K^+ as acetate or chloride salts (to final concentrations of 0.3M, 3.7M, and 0.1M, respectively) selectively alters the solubility of the DNA and thus increases the efficiency of alcohol precipitations.

The solution was poured into disposable 50ml tubes (Falcon), kept at -70°C for 15 minutes and then centrifuged in Corex tubes in a JA.20 rotor at 13000rpm (14000g) for 20 minutes at 4°C.

The DNA pellet was dissolved in 500μl of 0.1X SSC (15mM NaCl + 1.5mM sodium citrate). NaCl (12.5μl of a 4M solution), EDTA (50μl of a 0.5M solution) and Tris-HCl [(pH 7.4) 12.5μl of a 1M solution] were added. The purpose of this high salt buffer was to minimise nuclease digestion. Fifty micrograms of DNAse-free RNAse A was added and the mixture was incubated at 60°C for 1 hour. The DNA was extracted twice with chloroform:isoamyl alcohol and precipitated with 0.3M sodium acetate and 2 volumes of cold ethanol. Following centrifugation, the DNA pellet was washed in 70% ethanol and dried in a Savant Speed Vac concentrator. The pellet was dissolved in 0.1X SSC and dialysed against 500 volumes of 0.42M NaCl overnight. The DNA was concentrated by ethanol precipitation and dissolved in 0.1X SSC.

The DNA was quantitated and the purity of the DNA was determined by scanning the sample at a wavelength of 200nm-300nm in a Beckman DU-65 spectrophotometer. A DNA concentration program (Beckman, see Figure 4) was used to calculate the purity and concentration of the DNA. The program calculated the ratio at 260 to 280nm and DNA with ratios of 1.8-2.0 were considered as being pure. The absorbance at 260nm was measured in order to calculate the DNA concentration as double stranded DNA has a concentration of 50μg/ml if the optical density at 260nm is 1.

In order to further evaluate the DNA, an aliquot was electrophoresed on an agarose gel. The gel was then stained with ethidium bromide and the DNA was visualised under UV transillumination.

2.1.3. Results

The yields of DNA obtained using this method ranged from 100-600μg per 10 culture plates which was sufficient for several experiments. Occasionally there was RNA contamination and an additional RNase digestion was performed. The *A.actinomycetemcomitans* and *H.aphrophilus* DNA migrated with a mobility of less than that of the 21kb marker on an agarose gel and showed no evidence of degradation by nucleases. In some of the *Bacteroides* preparations there was degradation of the DNA. This was minimized by using a high salt buffer to prevent nuclease digestion. To prevent mechanical shearing, care was taken not to vortex the DNA too vigorously during the preparation of the DNA.

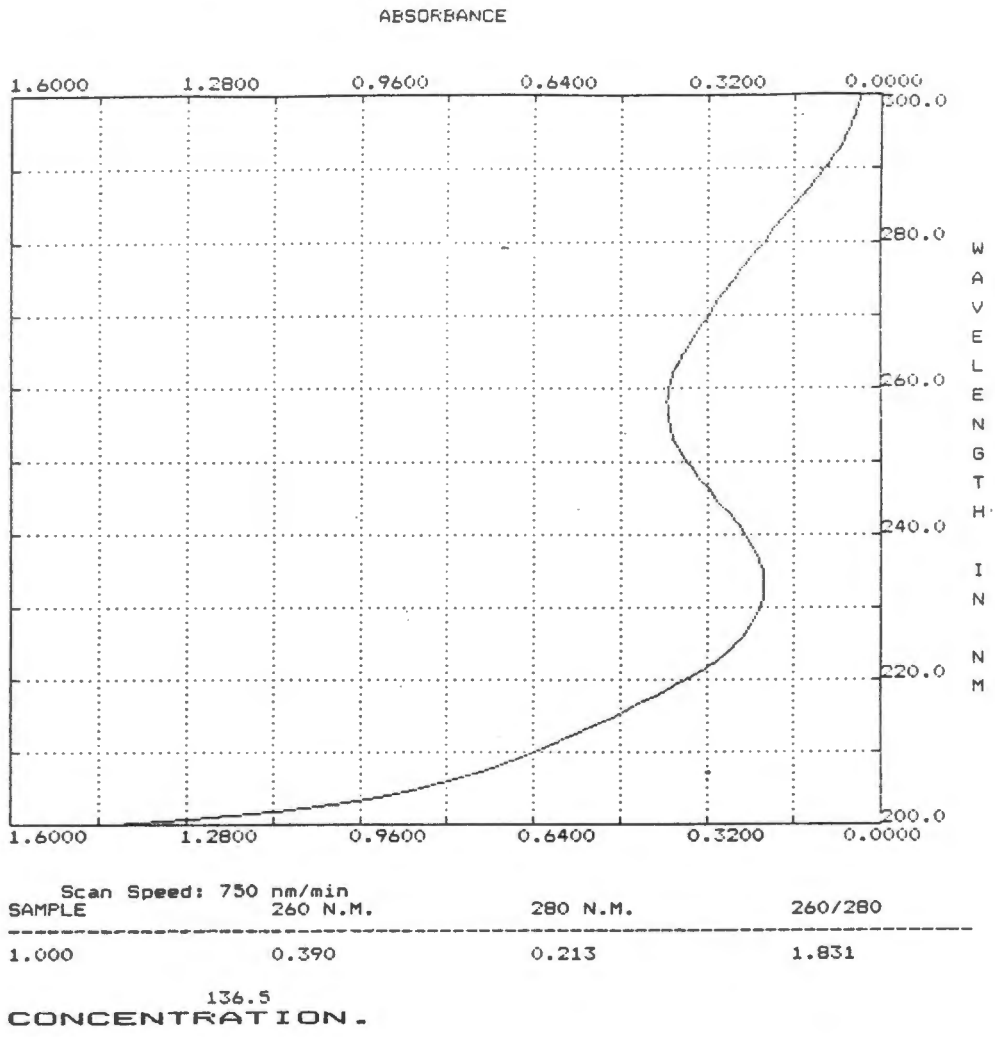


***Figure 4**: Scan of A.actinomycetemcomitans DNA*

Computer printout of a typical scan of an aliquot of A.actinomycetemcomitans DNA, indicating the purity (1.831) and concentration of the DNA (136.5µg/ml). These values were determined using the factors described on p 23.

2.2. HYBRIDISATION

2.2.1. Introduction

DNA hybridisation is the process by which complementary strands of DNA interact to form duplex molecules. The interaction is based on hydrogen bond formation between complementary base pairs present in the individual DNA strands (Dillon *et al*, 1985).

One strand (the probe) is usually labelled with a reporter molecule and can be used to detect its complementary strand in the target DNA by colony hybridisation, slot blots and Southern

hybridisation. In the latter case, the DNA was first digested with a restriction enzyme and the fragments are transferred to a nylon or nitrocellulose membrane following agarose gel electrophoresis.

2.2.2. Materials and methods

2.2.2.1. Agarose gel electrophoresis

This is a standard method used to separate; to identify; to purify and to determine the size of the DNA fragments. The mobility of a DNA molecule during gel electrophoresis depends on its mass and its molecular configuration. Duplex molecules such as covalently closed circular (CCC) plasmid DNA move more rapidly through the gel matrix towards the anode than less compact molecules such as open circular or linear DNA. The resolving power of the gel depends on the potential difference across the electrodes and on the concentration of the agarose, which determines the pore size of the gel.

For rapid analyses of the DNA digests, minigels (Minnie submarine unit, Hoefer) were used at high voltages. For preparative work where higher resolution was required, the Max submarine unit (Hoefer) was used at lower voltages per unit length of the gel. The percentage agarose used, ranged from 0.7-1% according to the requirements for each experiment. The electrophoresis buffer was TBE (0.089M Tris-borate; 0.089M boric acid; 0.002M EDTA). Occasionally a Tris-acetate buffer (0.4M Tris-acetate, 0.001M EDTA) was used. It is interesting to note that the mobility of linear and open-circular DNA in agarose gels is different in the 2 buffers. In Tris-acetate buffer, linear DNA runs before open circular DNA, while with Tris-borate the running order is reversed (Dillon *et al*, 1985).

The gel loading dye was 0.25% bromophenol blue with 40% (w/v) sucrose in distilled water. The purpose of the sucrose is to increase the density of the sample to greater than that of the electrophoresis buffer and the bromophenol blue is used to monitor the progress of the electrophoresis.

The agarose to which 1X TBE buffer had been added, was dissolved in a microwave oven and then cooled to 50°C before being poured. The gels were stained with the fluorescent, intercalating dye, ethidium bromide (Smith, 1971) after electrophoresis. For the minigels,

however, the ethidium bromide was normally incorporated into the gel. The reason for staining the preparative gels after electrophoresis was completed, is that the intercalation of ethidium bromide alters the conformation of the DNA molecule and thereby changes its mobility. The gels were placed on a UV transilluminator (UVP) and photographed using a Polaroid camera (Model CU5 88-49) with an ultra-violet filter and Polaroid Type 667 (positive) or 665 (positive\negative) black and white Land film.

Visualization by transillumination rather than by incident UV illumination was used because faint bands may not be seen by incident light.

2.2.2.2. Southern blotting

The transfer of DNA from an agarose gel to a nylon membrane was according to the technique of Southern (1975). In this procedure, the transfer of denatured DNA from the gel to the filter is achieved by capillary action. A buffer (SSC) in which nucleic acids are highly soluble, is drawn up through the gel into the membrane, taking with it single stranded DNA which becomes "trapped" in the membrane matrix.

Nylon membranes were used because they are less brittle than nitrocellulose and can be reused several times. Unlike nitrocellulose, they also do not need to be baked; cross linking of the DNA to the filter is promoted by UV transillumination.

DNA transfers were done as follows: after the ethidium bromide stained gel had been photographed it was placed in denaturing solution (1.5M NaCl; 0.5M NaOH) for 15 minutes. Complete denaturation was ensured by placing the gel in fresh denaturing solution for 30 minutes. This step converts the DNA to a single stranded form, which facilitates its binding to the filter. When high molecular weight DNA was present, it was fragmented prior to denaturation by acid depurination and base cleavage in 0.25N HCl for 15 minutes. This improves the transfer as large DNA fragments transfer less efficiently than small DNA fragments.

Following denaturation, the gel was placed in neutralizing solution (1.5M NaCl; 0.5M Tris-HCl (pH 7.2); 0.001M Na_2EDTA) for at least 30 minutes but it was normally left for 3 hours. The actual blotting procedure was as follows (Figure 5): 3 sheets of Whatman 3MM paper

were soaked in 20X SSC and placed on a sheet of Glad-wrap on a level surface. The gel was then placed upside down on the moistened filter papers and a piece of Hybond-N was carefully placed over the gel. All air bubbles were removed by rolling a sterile pipette over the surface. One sheet of Whatman 3MM paper was placed on top of the membrane and then a stack of tissues (10-12cm high) was placed above this. A glass plate was placed on the tissues. A brick (2.7kg) was placed on top of the glass plate and the transfer was allowed to take place overnight at room temperature. The following day the position of the wells was marked on the membrane and the membrane was washed in 2X SSC buffer. The membrane was air-dried, wrapped in Glad-wrap and placed on the UV transilluminator for 2-5 minutes to cross-link the DNA to the filter.

Figure 5: Southern Blot
Diagrammatic representation of a Southern transfer of DNA to a nylon membrane

2.2.2.3. Radio-isotope labelling of DNA probes.

Each probe was labelled with α^{32}PdCTP (Amersham or Du Pont; specific activity 3000 Ci/mmol) to a specific activity of 1-4 X 10^8 cpm/μg of DNA. The multiprime DNA labelling system (Amersham RPN.1601Y) was used. This method introduced by Feinberg and Vogelstein (1983), is based on the use of random sequence hexanucleotides priming DNA synthesis on the denatured template DNA.

The linear, double stranded DNA to be used as probe, normally 25ng in a volume of 10μl, was denatured at 95°C-100°C for 2 minutes and then chilled on ice for 10 minutes. The following was added: multiprime buffer solution (10μl); primer solution (5μl); distilled water (18μl); α^{32}PdCTP (5μl ie. 5μCi); and DNA Polymerase 1 "Klenow Fragment" (2μl). The advantage of using the Klenow fragment is that this enzyme carries the 5'-3' polymerase activity and the 3'-5' exonuclease activity of intact DNA polymerase I, but lacks the 5'-3' exonuclease activity. This ensures that labelled nucleotides incorporated by the polymerase are not subsequently removed as monophosphates. The solution was pipetted up and down gently, centrifuged in an Eppendorf centrifuge for a few seconds and then incubated at room temperature, overnight. The following day the percentage incorporation was determined by precipitation with trichloroacetic acid (TCA). The precipitated DNA was collected on a Whatman GF\C glass microfibre filter disc that had been placed on a 3-piece filter funnel (Whatman). The scintillation cocktail used was Beckman Ready-Safe™. The samples were counted in a liquid scintillation counter (Packard).

2.2.2.3.1 Calculation of specific activity of probe

The efficiency of counting of TCA precipitates varies, but the percentage incorporation can be used to calculate the specific activity of the probe. Multiprime labelling leads to net DNA synthesis, and so the total amount of DNA at the end of the reaction must be calculated. This was done according to the Amersham handbook, "Multiprime DNA labelling systems".

The unincorporated label was separated from the incorporated label by the spun-column procedure according to Maniatis *et al* (1982). This method involved plugging a 1ml disposable syringe with a small amount of siliconized sterile glass wool. A 0.9ml bed volume of Sephadex G-50 that had been equilibrated in STE buffer (TE buffer (pH 8.0) containing 0.1M NaCl) was prepared by packing the column and centrifuging it in a disposable tube at 1600g for 4 minutes in a MSE bench centrifuge. Sephadex was continually added until the packed volume was constant at 0.9ml. STE buffer (100μl) was added and the tube containing the column was centrifuged at exactly the same speed and time as before. After repeating this step the DNA sample was added in a total volume of 100μl. This was then centrifuged at exactly the same speed and time as before and the 100μl eluent was collected in a 1.5ml Eppendorf tube that had been placed at the bottom of the centrifuge tube. This eluent was then used for hybridisation.

2.2.2.4. Prehybridisation and hybridisation

The prehybridisation step is designed to block all the sites on the membrane that would bind the probe non-specifically, and thereby reduces the background (Dillon *et al*, 1985). Two different hybridisation methods were used, that of Roberts *et al* (1987); and that of Mason and Williams (1985). The major differences between the methods are in the composition of the solutions and the duration of incubations. The latter method was preferred for the colony blots since the dextran sulphate used in the former method resulted in a higher background. For the slot blots and Southern blots, however, the 2 methods gave similar results.

The method of Mason and Williams (1985) will be described. The damp filters were placed in plastic filing sleeves and the prehybridisation solution was added. The bags were heat sealed using an impulse sealer. The filters were prehybridised for 4 hours and the hybridisation was carried out overnight. Prehybridisation and hybridisation were both performed at 68°C in a shaking water bath (Techne).

The prehybridisation solution consisted of: 4X SET buffer; 10X Denhardts solution; 0.1% SDS; 0.1% sodium pyrophosphate and 50μg/ml denatured salmon sperm DNA, [20X SET= 3M NaCl; 20mM EDTA and 0.4M Tris-HCl (pH 7.8)]; [100X Denhardts= 2% bovine serum albumin (Fraction 5); 2% Ficoll and 2% polyvinyl pyrrolidone]. The denatured salmon sperm DNA was prepared by boiling the sonicated salmon sperm DNA for 10 minutes to produce single stranded DNA and by immediately placing it on ice to prevent reannealing. The hybridisation solution was the same as the prehybridisation solution except that single stranded, labelled probe was added at a specific activity of $5x10^7$ -$5x10^8$ cpm/μg. The probe was made single stranded by heating to 100°C for 5 minutes and cooled on ice.

The method of Roberts *et al* (1987) uses dextran sulphate in the hybridisation mixture. Dextran sulphate increases the rate of hybridisation. This has been attributed to the exclusion of the DNA from the volume occupied by the dextran sulphate, that is, the dextran sulphate effectively increased the concentration of the DNA. The other difference in the latter method is that formamide was used. Formamide decreases the T_m of nucleic acid hybrids, which allows a lower hybridisation temperature (42°C) to be used (Anderson and Young, 1985).

2.2.2.5. Post hybridisation washes

The aim of the post hybridisation washes is to remove non-specifically bound probe from the membrane. The procedure consists of washing the filters in the following solutions:

3 X 20min in 3X SET; 0.1% SDS; 0.1% sodium pyrophosphate at 68°C
2 X 20min in 1X SET; 0.1% SDS; 0.1% sodium pyrophosphate at 68°C
1 X 20min in 0.1X SET;0.1% SDS; 0.1% sodium pyrophosphate at 68°C
1 X 20min in 4X SET at room temperature.
1 X 20min in 0.1X SET; 0.1% SDS at 68°C.

The last wash was only included when more stringent conditions were needed, that is, when the target DNA sequence was closely related to the probe.

2.2.2.6. Autoradiography

The filters were air dried and exposed (16 hours to 1 week) to X-ray film (Agfa, Curix) in a X-ray cassette (Okamoto 10 X 12) with 2 Du Pont Cronex intensifying screens at -70°C. The principle of this technique is that radioactive decay products strike the film and interact with the silver halide contained in the emulsion, thereby producing an image. Intensifying screens reduce exposure times by bouncing detectable light back to the film (Dillon *et al*, 1985). They have been shown to increase the sensitivity of detection of ^{32}P, 8 to 10 fold (Maniatis *et al*, 1982). If only 1 screen is used then the enhancement factor is only half that of the above. The screens contain gamma-activated, calcium-tungstate-phoshorus.

The autoradiographs were developed for 4 minutes (Ilford Phenisol X-ray developer) and then placed in a stop bath (3% acetic acid) for 1 minute before being placed in the fixer [Amfix hi-speed fixer plus S-type hardener (Maybaker)] until the film had cleared. The film was washed under running tap-water, air dried and analyzed.

2.2.2.7. Removal of hybridised probe and reuse of blots

Nylon filters were used throughout this study and could be reprobed. To strip the filter of the hybridised probe, the filters were incubated at 45°C for 30 minutes in 0.4M NaOH. They were then transferred to 0.1X SSC; 0.1% SDS; 0.2M Tris-HCl (pH 7.5) and re-incubated at 45°C

for 30 minutes. The filters were then exposed to X-ray film overnight at -70°C to determine whether all the probe had been removed.

2.2.3. Results

2.2.3.1. Non-radioactive labelling of DNA probes

A non-radioactive DNA labelling and detection kit (Boehringer-Mannheim) was evaluated. The method used was as per described in the kit. The complete procedure from labelling the DNA and hybridisation, to detection of the signal, was accomplished within 24 hours. The procedure is also much safer than using radioactivity. However, non-specific hybridisation was found to occur and the results differed substantially from those obtained using a radioactive probe. The nylon membranes that were used for the hybridisation, had previously been probed with a radioactive probe. It is thought that the salmon-sperm DNA used in the prehybridisation step for the radioactive probe, interferes in some way with the colour reaction. Because a highly specific and sensitive probe was needed in this study, it was decided to only use radioactive probes.

2.3. SPECIFICITY OF *A.ACTINOMYCETEMCOMITANS* WHOLE GENOMIC DNA PROBES

2.3.1. Introduction

The specificity of whole genomic DNA probes from *A.actinomycetemcomitans* and *H.aphrophilus* was investigated. The whole genomic DNA from the above 2 species was labelled and probed against *A.actinomycetemcomitans* DNA and *H.aphrophilus* DNA as well as DNA from 7 species of *Bacteroides*.

2.3.2. Materials and methods

DNA and bacterial cells of the above mentioned species were applied to nylon membranes using a slot blot apparatus (BRL Hybri-Slot™ Manifold). Two different methods of treatment

of the filters were used, one for bacterial cells and the other for DNA.

2.3.2.1. Bacterial slot blots

Aliquots of bacterial cells, obtained either from overnight cultures or directly from specimens taken by the paper point method (section 3.2.2), were applied to a nylon filter (Amersham, Hybond-N) using the slot blot apparatus. Vacuum was applied to filter the samples onto the membrane and was followed by a 100μl rinse with loading buffer (1:1 3M NaCl:0.3M NaOH-2M NH_4OAc).

The filters were treated according to Fennel *et al* (1984). Briefly, they were layed on Whatman 3MM paper soaked with the following solutions: 0.5N NaOH (10 minutes); 1.0M Tris-HCl (pH 7.4; 10 minutes); and pronase (10mg\ml; 30 minutes). The filters were then air-dried for 15 minutes and washed twice for 1 minute per wash in chloroform. After drying again for another 15 minutes they were washed in a solution of 1.5M NaCl and 1.0M Tris-HCl (pH 7.4). The filters were then blotted dry, wrapped in Glad-wrap and placed DNA side down on a UV transilluminator (U.V.P.; wavelength 302nm) for 2-5 minutes, to fix the DNA to the filter. Filters were kept wrapped up and stored in the dark at room temperature.

2.3.2.2. DNA slot blots

The method used for treating the membranes containing filtered DNA, was according to Amersham, "Membrane transfer and detection methods". Samples were heated to 95°C for 5 minutes and then chilled on ice for 2 minutes. They were then filtered onto a nylon membrane. The membranes were wet in a denaturing solution (1.5M NaCl; 0.5M NaOH) for 1 minute and transferred to neutralizing solution (1.5M NaCl; 0.5M Tris-HCl (pH 7.2); 0.001M Na_2EDTA) for 1 minute. They were blotted with filter paper to remove excess buffer and air dried. The filters were wrapped in Glad-wrap and irradiated, DNA side down on a UV transilluminator for 2-5 minutes.

2.3.3. Results

The *A.actinomycetemcomitans* whole genomic probe was found not to cross-hybridise to DNA extracted from seven species of *Bacteroides* (Figure 6).

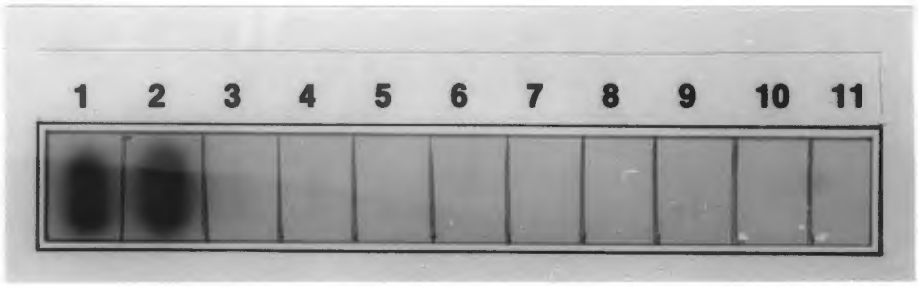


***Figure 6**: Autoradiograph of 7 Bacteroides species hybridised to a whole genomic DNA probe of A.actinomycetemcomitans strain NCTC 9709*

(1) and (2) A.actinomycetemcomitans DNA used as a positive control, 0.5µg and 0.4µg respectively; (3) and (4) B.gingivalis DNA, 0.5µg and 0.4µg respectively; (5) and (6) B.intermedius DNA, 0.5µg and 0.4µg respectively; (7) B.melaninogenicus DNA (0.4µg); (8) B.denticola DNA (0.4µg); (9) B.corporis DNA (0.4µg); (10) B.buccae DNA (0.4µg); (11) B.asaccharolyticus DNA (0.4µg).

It was found that there was cross-reactivity between the *H.aphrophilus* and *A.actinomycetemcomitans* DNA and that a more specific *A.actinomycetemcomitans* probe was required. A technique used by French *et al* (1986), to generate an enriched *A.actinomycetemcomitans* specific probe was attempted. These workers hybridised an *A.actinomycetemcomitans* genomic probe to a filter containing genomic *H.aphrophilus* DNA and then removed the unhybridised probe which they called the "enriched non-cross-reactive *A.actinomycetemcomitans* genomic probe". They then used this probe to screen their *A.actinomycetemcomitans* DNA library for *A.actinomycetemcomitans* specific sequences. An attempt to duplicate these findings was unsuccessful and it was found that after the second hybridisation, the cross-hybridisations were still extensive (Figure 7).

***Figure 7**: Autoradiograph of H.aphrophilus DNA probed with the "enriched" A.actinomycetemcomitans DNA.*

(1)-(5), each contain 1µg of H.aphrophilus (NCTC 5886) DNA;
(6)-(10), each contain 1µg of H.aphrophilus (NCTC 5906) DNA.

A possible reason for clones containing *A.actinomycetemcomitans* DNA being negative when probing with whole genomic DNA could be that the random primer method of labelling was used and it is possible that certain areas do not label well with this method. At least 300bp are needed for a positive hybridisation signal. Also, there could be small non-contiguous fragments which have been ligated together.

2.4. GENERATION OF AN *A.ACTINOMYCETEMCOMITANS* GENOMIC LIBRARY

2.4.1. Introduction

It was decided to clone the *A.actinomycetemcomitans* DNA because pilot experiments had shown that the whole genomic *A.actinomycetemcomitans* probe was highly cross-reactive with the *H.aphrophilus* DNA (section 2.3.3). This indicated that a refined or *A.actinomycetemcomitans* specific, cloned subset of probes would be required for the specific detection of the presence of *A.actinomycetemcomitans* cells. This finding has also been reported by French *et al* (1986) and Smith *et al* (1989b).

An advantage of using a cloned probe is that it provides a reproducible source of the DNA. The disadvantage of using cloned chromosomal fragments, however, is that probes which detect only certain regions of the chromosome, are not as sensitive as probes which detect multicopy plasmids or rRNA.

2.4.1.1. Vectors used for cloning

To properly serve as a vector for molecular cloning, a genetic element must meet certain requirements: 1) it must be capable of autonomous replication within the host cell so that isolation and amplification of a specific DNA fragment can be achieved; 2) it must possess (non-essential) regions within which foreign DNA may be inserted without affecting the replication of the vector or the ability to select recombinant molecules; 3) it must carry genes that confer an easily recognized phenotype so that vector-carrying cells can be easily selected; 4) it must possess a single cleavage site for at least one restriction enzyme located outside its essential regions at which foreign DNA may be inserted; 5) it should allow for regeneration

of the site during recombination or carry additional sites closely adjacent to the ends of the inserted DNA in order to recover an inserted fragment (Dillon *et al*, 1985).

A large variety of plasmid vectors are currently available. The vector used in this work was pUC18. A map of this plasmid is shown in appendix D. This plasmid was constructed from pBR322 as described by Yanisch-Perron *et al* (1985) and it contains a multiple cloning site, a portion of the ß-galactosidase **lacZ'** gene, as well as a ß-lactamase gene which confers ampicillin resistance. Cloning with pUC18 involves insertional inactivation of the **lacZ'**gene and recombinants are distinguished by their inability to synthesize ß-galactosidase. The enzyme is coded for by the gene, **lacZ'**, which is on the *E.coli* chromosome. Some *E.coli* strains have a mutated **lacZ'** gene that is defective and does not encode the α-peptide portion of the ß-galactosidase. These mutants are only able to hydrolyse galactose when they harbour a plasmid that carries the complementary **lacZ'** segment of the gene.

To detect recombinants, a test is done for ß-galactosidase enzyme activity. The lactose analogue, X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside) is hydrolysed by ß-galactosidase to a product which is deep blue. If X-gal [plus an inducer of the enzyme such as IPTG (isopropyl-thiogalactoside)] in addition to ampicillin are added to the agar, then non-recombinants, which produce ß-galactosidase, will be coloured blue. Colonies containing recombinant plasmids with a disrupted **lacZ'** gene are unable to produce ß-galactosidase and will be white.

2.4.1.2. Host cells

The bacterial host, JM109, is a derivative of *E.coli* K12. It contains the complementary part of the **lacZ'** gene on a F' plasmid, as discussed above. The genotype is recA1; endA1; gyrA96; thi; hsdR17; supE44; relA1; λ-; Δ(lac-proAB);[F', traD36, proAB, lacI^{q}ZΔM15] (Yanisch-Perron *et al*, 1985).

2.4.2. Materials and methods

2.4.2.1. Preparation of vector and insert DNA

The plasmid DNA was isolated by Triton X-100 lysis of the host cells followed by caesium chloride/ethidium bromide ultracentrifugation. This procedure is used when high yields of plasmid DNA are required which are free of contaminating material, chromosomal DNA, high molecular weight RNA and proteins.

The procedure was as follows: 10ml of an overnight culture was added to 2 X 250ml Luria broth. The cells were grown with aeration at 37°C for 24 hours and were then harvested by centrifugation in 250ml centrifuge tubes in a Beckman J.A. 14 rotor in a J-21B centrifuge (Beckman) for 10 minutes at 10000 rpm at 4°C. The cells were suspended in TE buffer (pH 8) and centrifuged again for 10 minutes at 10000 rpm. They were suspended in 2mls of 25% sucrose; 50mM Tris-HCl (pH 8). Lysozyme (0.4ml of a 10mg/ml solution in TE buffer) was added. The cells were kept on ice for 10 minutes. EDTA [1ml of a 0.25M solution (pH 8)] was added and the cells were kept on ice for a further 10 minutes. A Triton X-100 lysis solution [1% Triton X-100; 0.4% sodium deoxy-cholate; 50mM EDTA; 50mM Tris-HCl (pH 8)] was added (3.2ml) and the lysate returned to ice for 10 minutes. The solution was centrifuged in 30ml Corex tubes at 15000rpm in a J.A 20 rotor for 90 minutes.

The supernatent was removed and 1g of caesium chloride was added per 1ml of supernatent. Ethidium bromide (10mg/ml) was added (0.3 ml per 4ml of supernatent). The ethidium bromide intercalates with the DNA and thus decreases its density in CsCl. Covalently closed circular (CCC) DNA binds much less ethidium bromide than open circular and linear DNA and thus has a higher density than open circular or linear DNA. Therefore CCC DNA can be readily separated from other types of DNA on the gradient.

This solution was added to Beckman Quick seal tubes and centrifuged for 18 hours at 50000 rpm in a 70.1Ti rotor on a Beckman L8-70 ultracentrifuge. The bands were visualized under long wave UV light. They appeared as reddish bands in an orange pink background (ethidium-bromide-DNA complexes fluoresce if illuminated by UV light). The plasmid band was removed using a syringe with the needle inserted just below the band. The ethidium bromide was removed by extraction with isoamyl alcohol equilibrated with a saturated solution of

caesium chloride. The plasmid DNA was then precipitated, washed, dried and suspended in distilled water using standard procedures. This step, however, caused the CsCl to precipitate as well and therefore the DNA had to be dialysed for 4 hours against 200 volumes of STE buffer and then overnight against 200 volumes of TE buffer. It is advisable to dialyse the DNA first and only then to precipitate it with ethanol in order to prevent the CsCl from precipitating as well.

For the ligation, 1μg of pUC18 DNA was digested with BamHI to give a linear double stranded molecule with GATC cohesive ends. The plasmid DNA was then extracted once with phenol/chloroform and once with chloroform:isoamyl alcohol, precipitated with LiCl and ethanol, washed with 70% ethanol, dried and finally suspended in TE buffer. Purified *A.actinomycetemcomitans* DNA, prepared as already described, was partially digested with Sau3A yielding fragments with cohesive ends complementary to the pUC18 cohesive ends.

2.4.2.2. Ligation

Plasmid DNA (0.1μg) was mixed with the restricted *A.actinomycetemcomitans* DNA (0.2μg) and the mixture was ligated using 0.2 units of T_4 Ligase at 15°C overnight in a refrigerated water bath. The 10X ligation buffer used was the following: 0.5M Tris-HCl (pH 7.4); 0.1M $MgCl_2$; 0.1M dithiothreitol; 10mM spermidine; 10mM ATP; 1 mg/ml BSA.

The following day, an aliquot of the ligation mixture was run on a minigel to ensure that there was an increase in the molecular weight of the ligated DNA, compared to the control pUC18 DNA. This was found to be the case and thus ligation had occurred.

2.4.2.3. Competent cells

Most methods for bacterial transformation are based upon the observation by Mandel and Higa (1970) that the uptake of bacteriophage lambda DNA is enhanced by treatment of the bacterial cells with calcium chloride. Several methods of preparing competent cells were evaluated and the competence of the cells produced by each method was determined. This was done by calculating the number of colonies produced per microgram of pUC18 DNA used for transformation. The only method that gave a satisfactory transformation efficiency was that of Chung and Miller (1988). The transformation efficiencies obtained using the 3 different

methods are shown in Table 1.

The method of Chung and Miller (1988) will be described. The cells were grown to early log phase (OD_{600}= 0.3-0.6) in Luria broth. They were then centrifuged at 1000g using a J.A. 14 rotor in a J-21B centrifuge for 10 minutes. The cells were suspended in 1/10th volume of transformation and storage buffer (TSB), i.e. LB broth (pH 6.1) containing 10% PEG (MW 3350); 5% DMSO and 20mM Mg^{++} (10mM $MgCl_2$ + 10mM $MgSO_4$) at 4°C. They were then incubated on ice for 10 minutes. For the transformation, 0.1ml aliquots of the cells were mixed with 100pg of plasmid DNA. The cells were returned to ice for 5-30 minutes. The transformation and storage buffer (see above) with 20mM glucose (0.9ml) was added and the cells were incubated at 37°C with shaking (225rpm) for 60 minutes. The cells were plated onto LB/Amp/X-gal/IPTG plates and grown overnight at 37°C. Competent cells prepared by this method could not be stored and had to be prepared freshly for each transformation.

Another method that was evaluated was that described by Maniatis *et al* (1982). Ten millilitres of Luria broth was inoculated with the JM109 cells and grown overnight with aeration. The following day 1ml of this culture was inoculated into 100 mls of broth and grown to an $O.D_{550}$ of 0.2-0.4, i.e. $5x10^7$ cells/ml, which normally required 2 to 4 hours. The cells were chilled on ice for 10 minutes before being centrifuged at 4000g for 5 minutes at 4°C. The pellet was suspended in half the original volume of ice cold 50mM $CaCl_2$ and 10mM Hepes buffer. This solution was placed in an ice-bath for 15 minutes before being centrifuged under the same conditions as the previously. The pellet was suspended in 1/15 volume of ice cold 50mM $CaCl_2$ and 10mM Hepes buffer. The competent cells were dispensed into 0.2ml aliquots into pre-chilled tubes and stored at 4°C overnight. The following day sterile glycerol was added to 15% and the tubes were gently vortexed before being frozen and kept at -70°C.

The method of Dillon *et al*,(1985) was also attempted. The main difference here is the use of $MgCl_2$ instead of $CaCl_2$. The efficiency obtained using this method was found to be even lower than that obtained using the method of Maniatis *et al* (1982) (Table 1).

Table 1. Comparison of transformation efficiencies obtained by 3 different methods of competent cell preparation.

Method	Tranformation Efficiency
1) Chung and Miller (1988)	> 10^9 colonies/μg
2) Maniatis *et al* (1982)	1.2 x 10^6 colonies/μg
3) Dillon *et al* (1985)	1 x 10^4 colonies/μg

2.4.2.4. Transformation

The ligation mixture was used to transform freshly prepared competent JM109 cells and the transformed cells were plated onto LB/Amp/X-gal/IPTG plates and incubated overnight at 37°C. The following day, the white colonies (recombinants) were plated onto LB plates containing ampicillin and stored at 4°C before being screened.

2.4.2.5. Results

Eighty white recombinant colonies were obtained per transformation. The number of blue colonies obtained was high. This is due to the recircularisation of the plasmid as the alkaline phosphatase step had been omitted. The ligation was not repeated because only a partial library was required for the purposes of this study.

2.5. SCREENING OF THE PARTIAL *A.ACTINOMYCETEMCOMITANS* RECOMBINANT DNA LIBRARY

2.5.1. Introduction

The *A.actinomycetemcomitans* recombinant DNA library was screened to find a clone that would hybridise to *A.actinomycetemcomitans* DNA but not to *H.aphrophilus* DNA. Two techniques of screening the colonies were used: colony hybridisation and Southern hybridisations.

2.5.2. Materials and methods

2.5.2.1. Colony hybridisations

This technique was developed by Grunstein and Hogness (1975) as a modification of the Southern blot procedure for use in screening bacterial colonies for the presence of a particular insert of DNA.

The method used was that described in the Amersham booklet, "Membrane transfer and detection methods". The Hybond-N filter was carefully placed on the surface of the agar plate containing the bacterial colonies and then removed after 1 minute. Another replica filter was made in the same way. It was, however, left on the plate containing the bacterial colonies for 2 minutes. The filters were incubated on fresh agar at 37°C until colonies of 0.5-1mm in diameter were obtained. The filters were removed and placed colony side up, on a pad of absorbent filter paper soaked in a denaturing solution for 7 minutes. (The denaturing and neutralising solutions were the same as those used for the Southern blot as described on pg 26). The filters were then soaked on a pad of filter paper soaked in neutralizing solution for 3 minutes. This neutralizing step was repeated.

The filters were washed in 2X SSC, transferred to dry filter paper and allowed to dry, colony side up. The filters were then placed colony side down on a UV transilluminator in order to cross-link the DNA to the nylon filter.

2.5.2.2. Small scale isolation of plasmid

The partial *A.actinomycetemcomitans* library was analyzed by growing up 20 clones chosen randomly off agar plates and purifying the plasmids. An alkaline lysis procedure (Birnboim and Doly, 1979) was used to purify the plasmid DNA. The recombinant colony was scraped off the plate, inoculated into 10ml of LB containing ampicillin (50μg/ml) and grown with aeration at 37°C overnight. An aliquot of this culture (1.5ml) was centrifuged in an Eppendorf tube in an Eppendorf centrifuge for 10 minutes. The pellet was suspended in 180μl of a solution containing 50mM glucose; 10mM EDTA and 25mM Tris-HCl (pH 8.0). Lysozyme (8μl of a 5mg/ml solution) was added, and the mixture was placed on ice for 5 minutes. Four hundred microlitres of an alkaline-SDS solution (0.2N NaOH, 1% SDS) was added and gently vortexed

before being placed on ice for 5 minutes. It is important that fresh alkaline-SDS solution be prepared as the solution absorbs CO_2 from the air upon standing, causing the pH to drop. At a pH of 12.0-12.5, linear plasmid and chromosomal DNA are irreversibly denatured. The covalently closed circular (CCC) DNA also denatures but because the strands are so tightly coiled they can not separate from each other. When the pH is reduced the 2 strands reanneal immediately. However, at greater pH values, CCC DNA may become irreversibly denatured. Proteins are also denatured at pH 12.5 which reduces the possibility of enzymatic degradation of plasmid DNA. The SDS lyses the cells and denatures the proteins.

Three hundred microlitres of a high salt buffer (3M KOAc) was added and mixed with the lysate by gentle vortexing. The solution was put on ice for 1 hour and then centrifuged for 15 minutes. Lowering the pH causes the chromosomal DNA to form an insoluble complex which together with protein-SDS complexes, is precipitated by the high salt concentration. The supernatent fluid (750μl), containing the plasmid DNA, was removed and placed into an Eppendorf tube and 450μl of isopropanol was added. This was mixed well and then centrifuged for 5 minutes. The pellet was washed with 70% ethanol, dried and dissolved in 200μl of TE containing 0.15M NaCl. A phenol/chloroform extraction was done and 500μl ethanol was added to the aqueous phase. The solution was chilled at -70°C for 15 minutes, centrifuged, washed with 70% ethanol and dried. The pellet was dissolved in 100μl distilled water. The plasmid DNA was digested with EcoRI and HindIII to release the inserts which were then electrophoresed on an agarose gel, stained with ethidium bromide and visualized on a UV transilluminator. There is only a 1/16 chance of regenerating the original BamHI site and thus other sites that are close to the original BamHI site in the multiple cloning site were chosen, namely, EcoRI and HindIII. The sizes of the insert fragments ranged from 252 to 2621bp as determined using the method described in section 2.6.2.2.

The above method for rapid analysis of plasmid DNA gave good results and was quick, however, as one would expect, the preparations were contaminated to a small extent with chromosomal DNA. Thus when pure, uncontaminated preparations were required, for example, for cloning and for restriction mapping, the DNA was prepared by caesium chloride/ethidium bromide ultracentrifugation. The method used for this is discussed in section 2.4.2.1.

2.5.2.3. Isolation of insert DNA from agarose gel

The reason for separating the insert from the vector DNA is that the vector DNA cross-hybridised with the *H.aphrophilus* DNA extensively and also to a lesser extent to the *A.actinomycetemcomitans* DNA (section 2.5.3.2).

The methods of Seth (1984) and that of Maniatis *et al* (1982) were used to isolate DNA from the agarose gel following electrophoresis. In the first method, the gel band containing the DNA was excised under UV light and the agarose was cut into very small pieces. This was then mixed with an equal volume of phenol and kept at -70°C for 10 minutes. The frozen sample was then centrifuged in an Eppendorf centrifuge for 10 minutes at 4°C. The supernatent containing DNA was then further extracted with phenol (2x), and then with chloroform:isoamyl alcohol (2x). The DNA was then concentrated by ethanol precipitation. To purify the DNA further, it was adsorbed to a Nacs Prepac column (BRL). The DNA was loaded on the column which had been previously equilibrated in 0.2 M NaCl in TE buffer. The bound nucleic acid was washed with the same buffer to remove any gel impurities. The bound fragment was then eluted with 1M NaCl in TE buffer. The effluent was reconcentrated by ethanol precipitation.

The method of Maniatis *et al* (1982) involving electroelution of the bands into dialysis bags was also attempted. The slice of agarose containing the DNA was excised from the gel and placed into a dialysis bag containing 0.2X TBE buffer. The bag was immersed in a shallow layer of 0.5X TBE buffer in an electrophoresis tank and a current was passed through the bag, normally 100V for 2-3 hours. The progress was monitored by using a hand held UV lamp in a darkroom to observe how much DNA had moved out of the gel and onto the side of the bag. The polarity was then reversed for 2-3 minutes to release the DNA from the side of the bag. The buffer was recovered from the bag and the DNA purified by using a Nacs Prepac column as described above.

2.5.3. Results

2.5.3.1. Isolation of insert from agarose gel

The yields using both methods were low and thus the DNA from several isolations were pooled. Using the phenol extraction/freeze squeeze method of Seth (1984), a recovery of 21% was obtained, while the electroelution method of Maniatis *et al* (1982) gave a recovery of 36%.

2.5.3.2. Colony hybridisations

When replica filters of the *A.actinomycetemcomitans* library were probed with either *A.actinomycetemcomitans* chromosomal DNA or *H.aphrophilus* chromosomal DNA, similar results were obtained (Figure 8). It would be expected that the *H.aphrophilus* chromosomal DNA would not hybridise to every *A.actinomycetemcomitans* recombinant colony, however, the labelled *H.aphrophilus* chromosomal DNA seemed to hybridise to the clones to an even greater extent than the labelled *A.actinomycetemcomitans* chromosomal DNA.

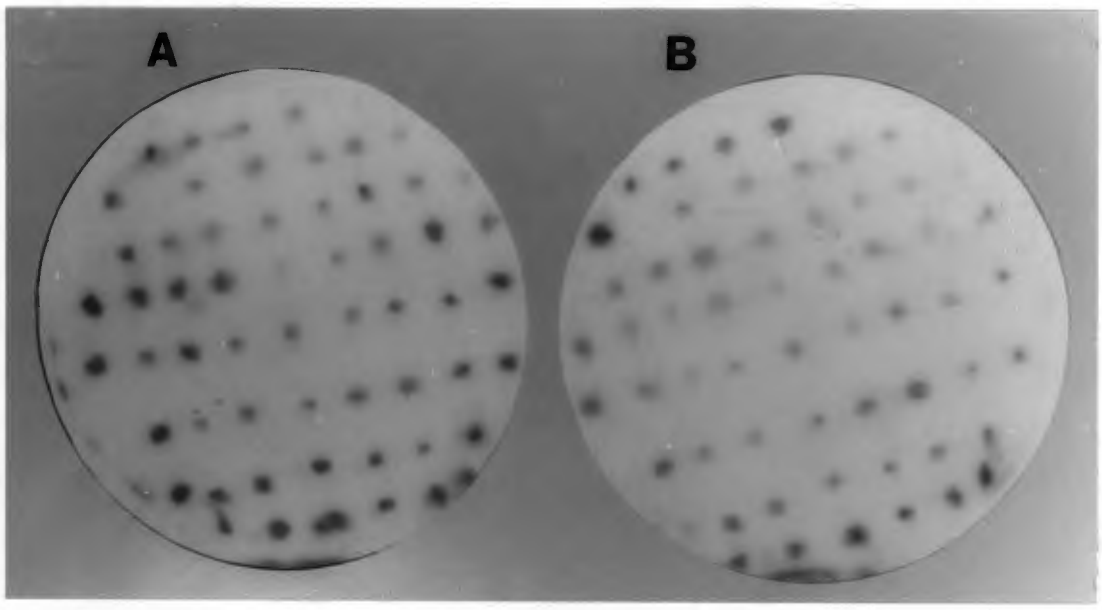


Figure 8: *Autoradiograph of colony blots of an A.actinomycetemcomitans library probed with: A) labelled H.aphrophilus whole chromosomal DNA; B) labelled A.actinomycetemcomitans whole chromosomal DNA*

An experiment was done to investigate whether this finding could be explained by the pUC18 DNA and/or the JM109 cells cross-hybridising with the *A.actinomycetemcomitans* and *H.aphrophilus* chromosomal DNA. Dilutions of pUC18 DNA and of JM109 cells were applied to slot blots and these were probed with *A.actinomycetemcomitans* and *H.aphrophilus* chromosomal DNA probes. The results showed that at concentrations of 500ng and greater of pUC18 DNA, there was a significant hybridisation with the *H.aphrophilus* chromosomal DNA. This cross-hybridisation was less evident with the *A.actinomycetemcomitans* chromosomal DNA (Figure 9).

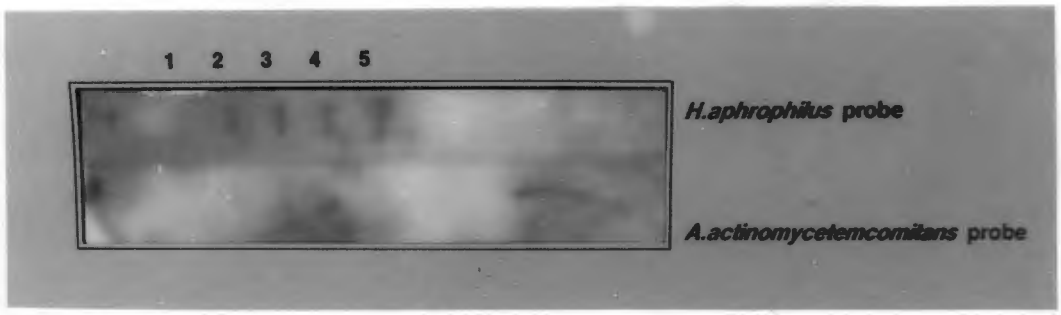


Figure 9: *Autoradiograph of slot blots of pUC18 DNA probed with A.actinomycetemcomitans and H.aphrophilus whole chromosomal DNA.*
(1)-(5) 100ng; 500ng; 1µg; 2µg and 5µg of pUC18 DNA respectively.

At high concentrations of JM109 cells there was a significant cross-hybridisation with the *A.actinomycetemcomitans* chromosomal probe, which was not at all apparent with the *H.aphrophilus* chromosomal probe (results not shown).

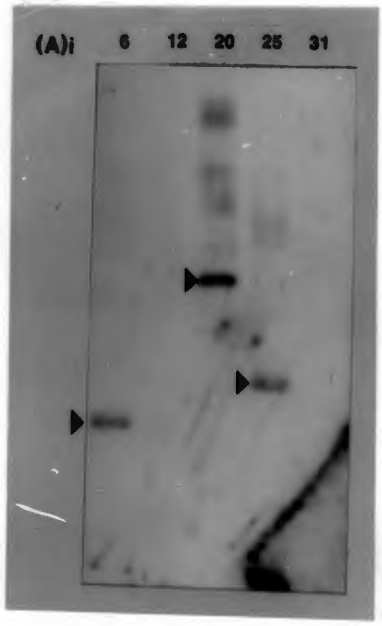
Due to the apparent cross-hybridisation between the vector and the *H.aphrophilus* DNA it was decided not to make use of the colony hybridisation technique in further experiments.

2.5.3.3. Screening for *A.actinomycetemcomitans* specific probe using purified insert fragments

An experiment was done to determine which of 9 plasmid preparations chosen were *A.actinomycetemcomitans* specific. Equal amounts of digested plasmid preparation were run on an agarose gel in duplicate and then transferred to a nylon filter by means of a Southern blot. Two identical filters were produced and one was probed with a whole genomic probe of *A.actinomycetemcomitans* DNA, while the other was probed with a whole genomic probe produced from *H.aphrophilus* DNA.

Visualization of the ethidium bromide stained gel, showed both the plasmid DNA bands and the insert DNA bands. On the autoradiograph of the blot probed with the *A.actinomycetemcomitans* probe, the insert DNA can be seen clearly [Figures 10 A (i) and (ii)] although it is interesting to note that the plasmid bands are visible when probed with the *H.aphrophilus* probe (Figure 10 B). This is because the pUC18 DNA cross-reacts with the *H.aphrophilus* DNA as has been already discussed (section 2.5.3.2).

The results of these experiments are shown in Figure 10 and in Table 2.



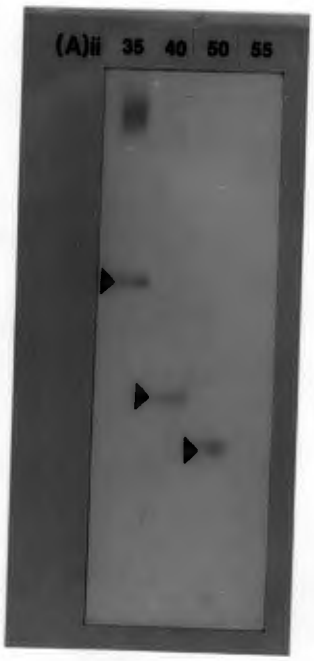


Figure 10:
Autoradiographs of a Southern blot of DNA of 9 recombinant plasmids, hybridised to either:

(A) (i) and (ii) *A.actinomycetemcomitans probe (Specific activity: 1.44 x 10^8cpm/µg) or*

(B) *H.aphrophilus probe (Specific activity: 1.23 x 10^8cpm/µg). Each plasmid was digested with both EcoRI and HindIII. The number above each lane is the plasmid number.*

In Figure A (i) and (ii), the arrows shows the positions of the insert bands.

In Figure B, the arrow shows the position of the plasmid band.

Table 2. **Hybridisation of *A.actinomycetemcomitans* and *H.aphrophilus* genomic probes to recombinant plasmids**

	PLASMID NUMBER	
A.actinomycetemcomitans probe	6, 20, 25, 35, 40, 50	12, 31, 55
H.aphrophilus probe	none	6, 12, 20, 25, 31, 35, 40, 50, 55

It is evident from Table 2 that the *A.actinomycetemcomitans* probe hybridises to inserts 6; 20; 25; 35; 40 and 50 and the *H.aphrophilus* probe does not hybridise with these inserts.

The next step was to label each of the inserts and to probe each of these against *A.actinomycetemcomitans* and *H.aphrophilus* DNA. The inserts were removed from each of the plasmids (section 2.5.2.3). Each of the inserts that appeared to be *A.actinomycetemcomitans* specific was radioactively labelled in turn and hybridised to the following: *A.actinomycetemcomitans* genomic DNA which had been digested with 3 different restriction enzymes; undigested *A.actinomycetemcomitans* genomic DNA; and a filter containing *H.aphrophilus* DNA digested with 3 restriction enzymes and undigested *H.aphrophilus* DNA.

In this experiment, only one of the inserts was found to be *A.actinomycetemcomitans* specific in all cases, and not to cross-hybridise to *H.aphrophilus* DNA at all concentrations tested. This is the probe that was used in all future work and is called probe 6. In Figure 11, a hybridisation signal can be seen in each lane containing the *A.actinomycetemcomitans* DNA when probed with probe 6. When the same probe was used on the filter containing the *H.aphrophilus* DNA, no signal at all was seen. The hybridisation signal with the BamHI digested DNA (lane 3) appears to be much stronger than that obtained in the other lanes. This is difficult to explain but it may be due to poor transfer of the DNA or perhaps some of the DNA has run off the gel. It appears from the photograph of the ethidium bromide stained gel (Figure 12) that equal amounts of DNA were applied to the gel.

Figure 11: Autoradiograph of probe 6, probed against digested and undigested A.actinomycetemcomitans DNA.

Lanes: (1) Bacteriophage lambda DNA digested with HindIII and EcoRI; (2) A.actinomycetemcomitans DNA, undigested (4µg); (3)-(5) A.actinomycetemcomitans DNA (4µg), digested with BamHI, EcoRI and Sau3A respectively.

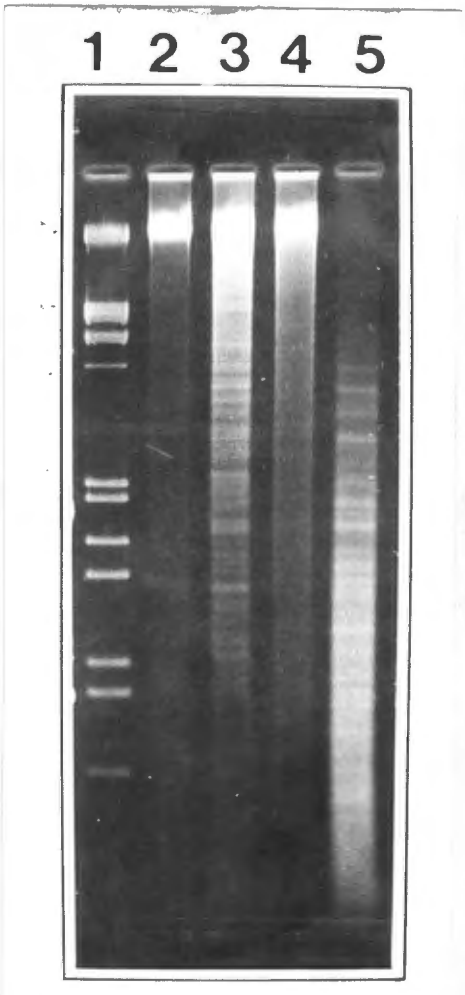


Figure 12: Agarose gel electrophoresis of undigested and digested A.actinomycetemcomitans DNA

Lanes: (1) Bacteriophage lambda DNA digested with HindIII and EcoRI; (2) A.actinomycetemcomitans DNA, undigested (4µg); (3)-(5) A.actinomycetemcomitans DNA (4µg), digested with BamHI, EcoRI and Sau3A respectively.

Slot blots containing different concentrations of *A.actinomycetemcomitans* and *H.aphrophilus* DNA, were made and the identical blots were hybridised to the different insert probes. In each of these, probe 6 hybridised with the *A.actinomycetemcomitans* DNA, but not with the *H.aphrophilus* DNA, at all concentrations tested (Figure 13C). The other 4 probes all hybridised to some extent with the *H.aphrophilus* DNA (Figures 13A and B).

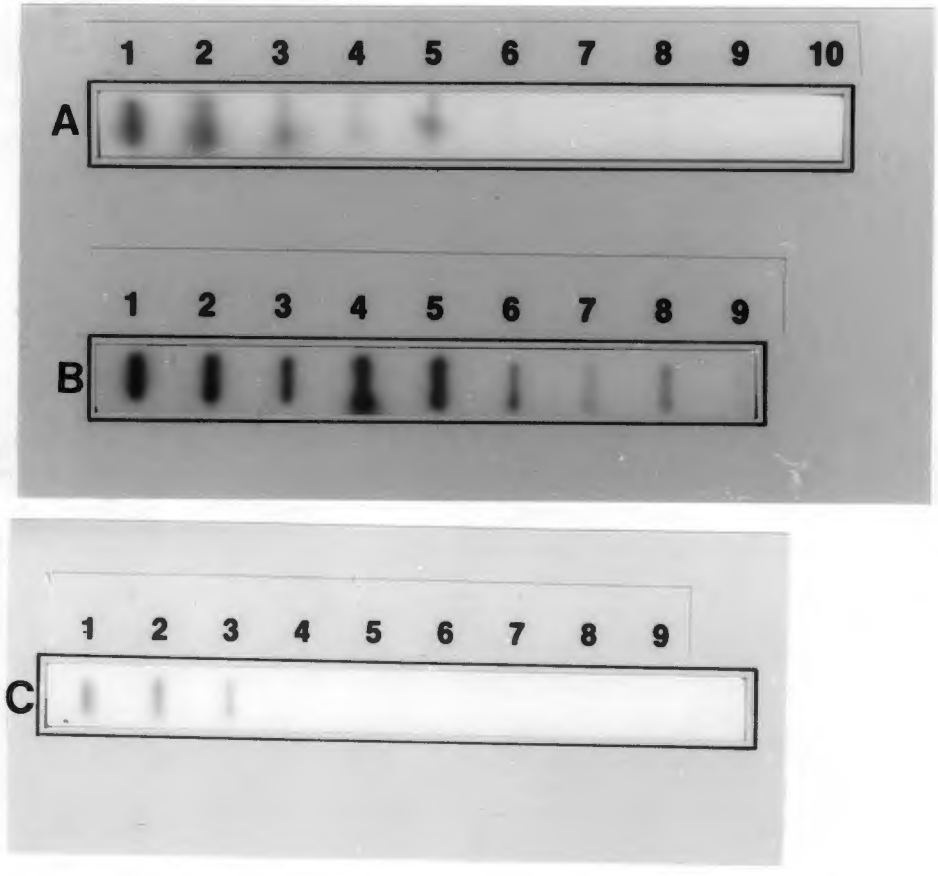


Figure 13. *Autoradiograph of the slot blot of different concentrations of A.actinomycetemcomitans and H.aphrophilus DNA probed with: A) Probe 40; B) Probe 25; C) Probe 6*

The slots for the above figures are all the same:
(1)-(3) A.actinomycetemcomitans DNA, NCTC 9710, 2µg; 1µg and 0.5µg; (4)-(6) H.aphrophilus DNA, NCTC 5886, 2µg; 1µg and 0.5µg; (7)-(9) H.aphrophilus DNA, NCTC 5906, 2µg, 1µg and 0.5µg respectively.

2.5.3.4. Specificity studies

To determine whether the probe was strain specific it was hybridised to DNA from another strain of *A.actinomycetemcomitans*, NCTC 9709. The probe was shown to hybridise to this strain as well, although the signal obtained was less intense than that obtained with the homologous strain (Figure 14). The probe was found to be species specific, however, as it was also tested against a filter containing 7 strains of *Bacteroides* which are found in the oral

cavity, and no cross-hybridisation was found (Figure 15). The strains tested were: *B.assacarolyticus; B.buccae; B.corporis; B.denticola; B.melaninogenicus; B.intermedius* and *B.gingivalis*.

Figure 14: *Probe 6 probed against DNA of A.actinomycetemcomitans, strains NCTC 9710 and NCTC 9709. (1)-(3) A.actinomycetemcomitans, NCTC 9710, 1µg; 0.5µg and 0.4µg; (4)-(6) A.actinomycetemcomitans, NCTC 9709, 1µg; 0.5µg; and 0.4µg respectively.*

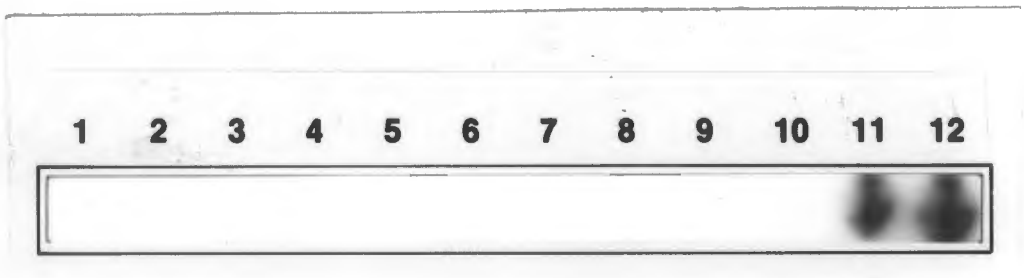


Figure 15. *Autoradiograph of probe 6 probed against 7 Bacteroides species (1) no specimen ; (2) B.asaccharolyticus DNA (0.4µg) ; (3) B.buccae DNA (0.4µg); (4) B.corporis DNA (0.4µg); (5) B.denticola DNA (0.4µg); (6) B.melaninogenicus DNA (0.4µg); (7) B.intermedius DNA (0.4µg); (8) B.intermedius DNA (0.5µg); (9) and (10) B.gingivalis DNA, 0.4µg and 0.5µg respectively; (11) and (12) A.actinomycetemcomitans DNA used as a positive control, 0.4µg and 0.5µg respectively.*

2.5.3.5. Sensitivity studies

Sensitivity studies were then done to determine the minimum detectable amount of DNA and also the minimum number of bacterial cells that could be detected using the probe. The range of DNA concentrations tested was from 20ng to 1μg (Figure 16) and the numbers of bacterial cells tested ranged from 10^2-10^8cells (Figure 17). The minimum concentration of DNA detectable was found to be 50ng and the lowest number of *A.actinomycetemcomitans* detectable was found to be 10^4 cells.

Figure 16: *Autoradiograph of varying concentrations of A.actinomycetemcomitans DNA probed against probe 6. (1)-(9) A.actinomycetemcomitans, NCTC 9710, 1µg; 750ng; 500ng; 400ng; 300ng; 200ng; 100ng; 50ng and 20ng respectively.*

Figure 17. *Autoradiograph of probe 6 hybridised to varying amounts of A.actinomycetemcomitans cells. (1)-(6), A.actinomycetemcomitans, NCTC 9710, 10^7; 10^6; 10^5; 10^4; 10^3 and 10^2 cells respectively.*

2.6. CHARACTERISATION OF *A.ACTINOMYCETEMCOMITANS* INSERT DNA

2.6.1. Introduction

Restriction enzymes are endonucleases that cleave both strands of the DNA at specific nucleotide sequences on the molecule. Although, there are 3 types of restriction enzymes, Type II restriction endonucleases are most frequently used as they recognize specific palindromic sequences and cleave the DNA at specific sites within the sequence (Dillon *et al*, 1985).

Most of the restriction enzymes used in this study were obtained from Boehringer Mannheim and were used according to the manufacturer's instructions. Digestions were normally carried out at 37°C for 2-3 hours, however, when large amounts of DNA were digested, the reactions were left to proceed overnight. Bacteriophage lambda DNA was used as a molecular weight marker after digestion with HindIII and EcoRI (see Appendix E for the sizes of the fragments).

Factors which affect the efficiency of the digests are: choice of the correct incubation buffer and the purity of the DNA. The DNA must be free of agents such as EDTA; SDS; phenol and ethidium bromide, which may interfere with or inhibit the activity of the enzyme. Compounds

such as ethanol and glycerol could change the dielectric constant of the restriction buffer, thus the volume of enzyme used should always be less than 10% of the total reaction volume. Digestions with 2 different restriction enzymes were done sequentially. If the enzymes had different buffer requirements, the lower ionic strength buffer was used first, and then the ionic strength was adjusted to that required for the second enzyme.

2.6.2. Materials and methods

2.6.2.1. Construction of a restriction map of clone no. 6

A restriction map is a map showing the position of the sites at which the restriction enzymes cut the DNA. The insert DNA of clone 6 was mapped in order to characterise it. DNA obtained by large scale isolation of plasmid DNA by caesium chloride/ethidium bromide ultracentrifugation, was digested with several restriction enzymes in order to prepare a map of the insert. The following enzymes were used in the digestions: ApaI; Asp700 (Xmn718); Asp718; BamHI; BclI; BglII; BstEII; ClaI; DraI; DraII; EcoRI; EcoRV; HindIII; HinfI; HpaI; KpnI; MspI; NcoI; NdeI; NsiI; PstI; PvuII; SacI; SalI; ScaI; SmaI; SspI; StuI; StyI and XbaI. All the digestions were carried out overnight and the gels were also run overnight at low voltages in order to obtain good resolution. Results showed that only SspI had a recognition site in the insert (Figure 18).

Experiments were then done to determine whether this result was because the reaction conditions were incorrect, or because the DNA was contaminated with some compound which inhibited the enzyme, or even if the enzymes were inactive. It was found that on repeating the digestions, the same results were obtained. Several different preparations of DNA were used and no differences were found, and finally the enzymes were found to digest lambda DNA to completion, thus the enzymes were not inactive. When one considers that the insert is 779bp long it is not surprising that very few enzymes have a recognition sequence in the fragment. An enzyme that recognises a 6bp palindrome and cuts a 50% GC site, will cut DNA with a 50% GC content every 4^6bp, i.e. 4096bp. The mol% G+C content of *A.actinomycetemcomitans* is known to be 42.7% (Kilian, 1976), thus enzymes which have a recognition site with a higher AT content were chosen.

2.6.2.2. Determination of fragment sizes

Two methods were used to determine the molecular weights of the fragments obtained after restriction enzyme digestion. One method is to construct a standard curve on semi-log paper using a DNA molecular weight marker and then read the molecular weights of the unknown fragments (using the mobilities obtained on the gel) from the graph. The other method is based on that of Southern (1979) who showed that the reciprocal of mobility plotted against fragment length is linear. Using this relationship, Schaffer and Sederoff (1981) have developed a least squares analysis to determine the sizes of unknown fragments whose mobilities are known. A computer programme distributed by the MBCRR (Molecular Biology Computer Research Resource), Harvard School of Public Health, Dana-Farber Cancer Institute-D1154, 44 Binney Street, Boston, Massachusetts, 02115 has been developed to do this analysis (Fristensky, 1987). The programme used to determine the sizes of the fragments is called "gel". An example of a typical output is shown (Table 3). This method was found to be preferable to the semi-log method as it is more convenient to use and the semi-log method has a large margin of error associated with it.

2.6.3. Results

2.6.3.1. Mapping of SspI site

The site was mapped using the program, "gel", as discussed above. The recombinant plasmid was digested with EcoRI and HindIII to release the insert fragment. (The reason for using HindIII and EcoRI is discussed in section 2.5.2.2.). Two fragments were obtained, one of 2672bp and the other of 830bp (Figure 19). The output from "gel" is shown in Table 3. The 2672bp fragment is the vector fragment, as pUC18 has a molecular weight of 2686bp and the 830bp fragment is the HindIII-EcoRI fragment containing the insert. The original BamHI site is actually 30bp away from the HindIII site and 21bp away from the EcoRI site (see Appendix D). Thus a total of 51bp must be subtracted from the 830bp fragment to leave an insert fragment of 779bp.

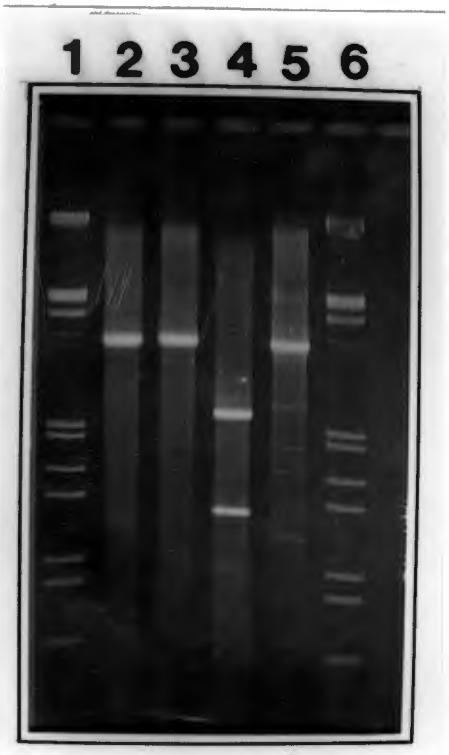


Figure 18: Agarose gel electrophoresis of restriction fragments produced by digestion of recombinant plasmid 6 with several restriction enzymes. Lanes: (1) and (6) Bacteriophage lambda DNA cut with EcoRI + HindIII; (2); (3); (4) and (5) Plasmid 6 digested with NdeI; ScaI; SspI and AspI respectively.

Table 3. Output obtained by computer program "gel" showing the sizes of the HindIII-EcoRI restriction fragments.

Determination of molecular weight of insert of plasmid 6

STD LEN	DIST	PRED LEN	DEVIATION	%DEV	C[I]
21226.00	6.500	21175.58	50.416	0.238117647	.400
5148.00	17.500	5829.53	-681.531	-13.239106347	.800
4973.00	20.000	4860.30	112.702	2.266119492	.500
4268.00	23.500	3870.19	397.814	9.321126215	.100
2027.00	35.000	2065.18	-38.179	-1.884116099	.000
1904.00	36.500	1916.71	-12.706	-0.667116938	.300
1709.00	40.500	1576.33	132.666	7.763122585	.300
1375.00	43.500	1363.40	11.595	0.843117874	.700
947.00	50.500	967.56	-20.562	-2.171116373	.900
831.00	53.000	852.17	-21.169	-2.547116291	.100
564.00	59.500	598.55	-34.547	-6.125115373	.000
MO= 1.30460400E+00			LO=-1.418545000E+03		
SC= 4.895887000E+03			SD=2.868859000E+02		
CCAP=1.173855000E+05					

UNKNOWN FRAGMENTS		
FRAGMENT	DISTANCE	PREDICTED LENGTH
insert 6	53.50	830.417

The column STD LEN refers to the actual lengths of the molecular weight marker used. DIST refers to the migration distance of each of the relevant marker fragments. PRED LEN is the predicted length that the marker fragment should have based on the actual distance migrated, as determined by linear regression, using all the standards. DEVIATION is the difference between the predicted length and the actual length and %DEV is the same figure expressed as a percentage of the actual length. The migration distances of the unknown fragments are then entered and "gel" calculates the predicted length of these fragments based on the molecular weight data of the standards.

A SspI site is present in the vector and from the map in Appendix D, it is clear that the SspI-HindIII fragment of the vector has a molecular weight of 582bp. A digestion of plasmid 6 with SspI generated fragments of 2194 and 1306bp (Figure 19).

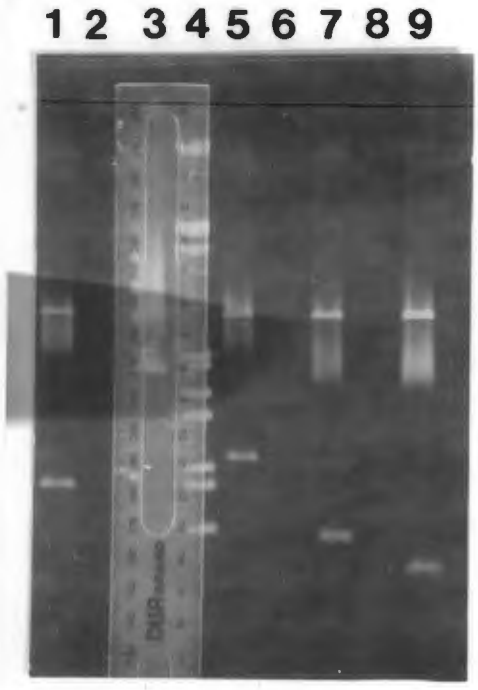


***Figure 19**: Agarose gel electrophoresis of HindIII-EcoRI digestions of recombinant plasmids 6, 20, 25,40 and 50.*
Lanes: (1) Plasmid 6; (3) Plasmid 20; (4) Bacteriophage lambda DNA digested with EcoRI and HindIII; (5) Plasmid 25; (7) Plasmid 40; (9) Plasmid 50. All the plasmids were digested with EcoRI and HindIII.

If it is assumed that the 2194 fragment is the vector fragment, then the distance of the SspI site from the HindIII site can be calculated by subtracting the 582bp fragment from the 1306bp fragment to give a fragment of 724bp.

The size of the SspI-EcoRI fragment can be determined by subtracting the 724bp HindIII-SspI fragment from the 830bp HindIII-EcoRI fragment to give a fragment of 106bp. Thus the SspI site is 724bp from the HindIII site and 106bp from the EcoRI site. From the above data, the restriction map of the insert (Figure 20) was produced.

***Figure 20**: Restriction map of insert, showing position of SspI site relative to the EcoRI and HindIII sites. A.actinomycetemcomitans DNA is indicated by the shaded area. The thick, single line is part of the pUC18 DNA.*

To confirm this provisional map, double digestions of plasmid 6 with EcoRI + SspI or HindIII + SspI were performed (Figure 21). The differences between the actual fragment sizes obtained and the predicted values are very small (Table 4). The greatest difference between the 2 values obtained was 45bp which is within the limits of experimental error. Thus the restriction map above (Figure 20) appears to be correct. It is possible that the 106bp EcoRI-SspI fragment has either run off the gel, or is too small to be detected.

It appears from Figure 21 that the BamH1 site in the multiple cloning site was regenerated, thus 3 fragments were produced by a Ssp1 + BamH1 digestion of plasmid 6. The sizes of the 3 fragments are consistent with this. (The probability of the BamH1 site being regenerated is 1/16.)

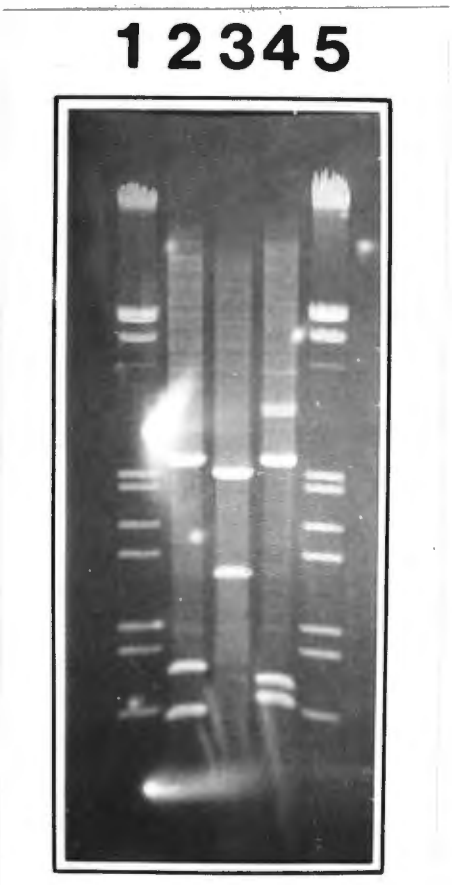


***Figure 21**: Electrophoresis of restriction fragments produced by double digestions of recombinant plasmid 6 on a 0.7% agarose gel.*
Lanes: (1) and (5) Bacteriophage lambda DNA cut with EcoRI + HindIII; (2); (3) and (4) Plasmid 6 digested with: BamHI + SspI; EcoRI + SspI; HindIII + SspI respectively

Table 4: Sizes of restriction fragments produced by digestion of recombinant plasmid 6 with SspI + EcoRI and SspI + HindIII. The sizes of the fragments were determined using "gel".

Restriction fragments produced by double digestion of plasmid 6 with SspI + HindIII and SspI + EcoRI			
Fragment	Predicted length	Actual length	Difference
SspI + HindIII (1)	2194	2239	45
SspI + HindIII (2)	724	699	25
SspI + HindIII (3)	582	601	19
SspI + EcoRI (1)	2088	2119	31
SspI + EcoRI (2)	1306	1280	26
SspI + EcoRI (3)	106	Fragment not detected on gel.	

3. SECTION THREE
IDENTIFICATION OF PERIODONTAL PATHOGENS IN CLINICAL SPECIMENS

3.1. INTRODUCTION

The complexity of the oral flora, the cyclical nature of periodontitis and the relatively large variance in the data obtained within and between subjects have combined to hamper the identification of the causative agents of periodontitis in humans.

The following section deals with some of the difficulties associated with the identification of periodontal pathogens in clinical specimens.

3.1.1. Technical difficulties

3.1.1.1. Sampling method

It is very difficult to obtain a sample of the right size from the right place at the right time. The sample must adequately reflect the number of the organisms at a site (Socransky *et al*, 1987). If the sample obtained is too large then the pathogen may be diluted by non-contributory "contaminating" species. If it is too small or taken from the wrong place then the pathogen may be missed altogether. The diameter of an average periodontal pocket at its orifice is in the order of 100-300μm while sampling devices are considerably larger (300-1200μm) in diameter (Socransky *et al*, 1987).

Tanner and Goodson (1986) compared the efficiencies of the most commonly used sampling devices, namely, scaler or curette; barbed broach cannula; pocket irrigating devices and paper points. They found that there is no method that is ideal for every type of study. In the current study, the paper point technique was used. The paper point technique is readily carried out, it does not appear to compromise the viability of periodontal anaerobes and it has been shown to constitute a relatively reproducible sampling method (Christersson *et al* (1985b). The technique is described in section 3.2.2.

3.1.1.2. Dispersion

Adherent microorganisms have to be separated from one another without loss of viability. The various species in plaque have different cell wall fragilities, thus vigorous dispersion techniques may select for those microbial species with robust cell walls, while gentler procedures may leave more tightly adherent species in clumps. Dispersion procedures often compromise between these extremes and thus may introduce artifacts in the final analysis (Socransky *et al*, 1987). Dispersion of samples may be obtained with a vortex mixer at the maximal setting for 30-60 seconds and a 10ml Hungate tube. In order to facilitate breaking up of bacterial aggregates, 6-8 glass beads of 3mm in diameter should be included in the sampling vial. Vortex mixing has been shown to exert little or no damage on individual bacterial cells (Slots, 1986a).

3.1.1.3. Transport media

Transport of periodontal specimens to the laboratory should take place in a viability-preserving, non-supportive, anaerobic medium (Slots, 1986). Generally 2 types of transport media are used: one that contains bacterial nutritional factors as well as growth inhibitory agents and one which is a buffered salt solution containing no nutrients. In this study the latter type was used, namely, Ringers solution.

3.1.1.4. Selective and non-selective media

A non-selective medium should ideally grow all bacteria in the same proportions as present in a sample, but unfortunately, none of the available isolation media fulfill this requirement (Slots, 1986). Important periodontal species can be missed or seriously underestimated if only non-selective media are used. This is because organisms can occur in numbers too low to be detected on non-selective media, however, even in low proportions, the organisms may play a crucial role in disease development. *A.actinomycetemcomitans* exemplifies a bacterium which in low proportions may be clinically significant (Slots, 1986). Another problem with the use of non-selective media, is that it may allow growth of organisms inhibitory to potential periodontal pathogens such as *A.actinomycetemcomitans* (Slots, 1982a).

3.1.2. Data analysis

Care must be taken not to combine inappropriate data as this will mask real differences which may occur at different locations within the same oral cavity.

3.1.3. Problems associated with the complexity of the oral microflora

Opportunistic microbial species may take advantage of the conditions produced by the true pathogen. Changes in environment, for example, the release of required substrates from damaged tissues or deepening of the periodontal pocket could select for certain opportunistic species (Socransky *et al*, 1987).

Generally, periodontitis appears to be chronic with episodic periods of exacerbation and remission (Goodson, 1986). The investigator may miss the peak of disease activity if measurements are not made regularly and this may lead to an underestimate of the contribution of a pathogen to a lesion. Another problem may be the continued presence of a pathogen at a site after the disease has gone into remission.

3.2. MATERIALS AND METHODS

3.2.1. Patient groups

Clinical specimens were obtained from patients in the groups described in Table 5.

Table 5: Patient groups

Patient group	Age	n	p.d. (millimetres)	Clinical data
Localised juvenile Periodontitis	10-23	3	>5	Bleeding on probing
Rapidly Progressive Periodontitis	20-46	2	>5	Rapid bone loss Bleeding on probing
Adult Periodontitis	27-66	16	>5	Bleeding on probing
Adult-Normal	24	1	<3	No mild or gingival inflammation

Specimens were collected over a period of one year but unfortunately, the number of specimens obtained in each patient group was low. There were only 3 localized juvenile periodontitis patients due to the low occurrence of localized juvenile periodontitis (0.1% of otherwise healthy adolescents).

Patients were excluded from the study if they had had their teeth cleaned within the last 4 months prior to the initial visit and if they had been on antibiotic treatment in the last 4 months.

3.2.2. Sampling procedure

The mesial, buccal aspect of the 1st molars and incisors were sampled using the paper point method. The procedure was as follows: the sample sites were isolated with cotton rolls and dried; the supragingival plaque was removed with sterile cotton pellets and two paper points (Johnson and Johnson), were inserted to the depth of the pocket. These paper points were left there for 10 seconds and then removed and placed into tubes.

One paper point was placed into an Eppendorf tube containing 200μl of TE buffer and 2 glass beads (2-3mm in diameter) for the probe analysis. The tubes were then placed on ice and transported to the laboratory where they were processed as soon as possible (section 3.2.4).

The other paper point was used for culture analysis and was placed into a sterile Hungate tube containing 0.9ml of sterile Ringers solution and 3 glass beads. Serial dilutions were made in Ringers solution and then plated out onto non-selective medium, TSA; and 2 selective media, TSBV and TSBVF. The plates were incubated at 37°C in 10% CO_2 for 2-4 days. The colonies on the selective media plates were subcultured and characterised.

3.2.3. Biochemical characterisation of isolates

The following tests were performed: Gram-stain (Kopeloff's modification); catalase test; oxidase test and fermentation of glucose; galactose; fructose; sucrose; mannose; mannitol; raffinose; trehalose; arabinose and lactose. The results were compared to that of a known culture of *A.actinomycetemcomitans* which was used as a positive control. Colonies that were Gram-negative coccobacilli; catalase positive; oxidase negative and fermented glucose;

mannose; galactose; maltose and fructose but did not ferment arabinose; trehalose; raffinose; lactose or sucrose were scored as *A.actinomycetemcomitans*.

The catalase test was performed by adding 3% H_2O_2 to colonies that had been grown on TSBV plates and by assessing bubble formation. The oxidase test was performed by placing a few drops of a tetra-ethyl-paraphenylene-diamine-dihydrochloride solution on a piece of filter paper and by smearing the growth across the surface of the impregnated paper. A positive reaction was shown by the development of a dark purple colour within 15 seconds (Slots, 1982b).

3.2.4. DNA probe analysis

The samples for the DNA probe analysis were applied onto a nylon membrane using the slot-blot apparatus. Control samples of known quantities of *A.actinomycetemcomitans* were also applied to the filters in order to estimate the number of cells in each test sample. The control concentrations used were 10^3-10^7 cells. Known amounts of *H.aphrophilus* cells were also applied to the filters to act as negative controls.

In an attempt to amplify the bacterial DNA, an alternative method was used. In this method, the samples were applied with the slot-blot on duplicate membranes. The filters were removed and one was placed on a non-selective agar plate (TSA), while the other was placed on a selective agar plate for *A.actinomycetemcomitans* (TSBV). The plates were incubated in a 10% CO_2 incubator overnight. The following day the filters were removed from the plates and treated as previously described for colony hybridisations.

3.3. RESULTS AND DISCUSSION

3.3.1. Probe analysis

The filters containing the specimens were probed in 3 batches. In the first batch, the level of detection of the positive control cells obtained was 10^4. In an attempt to obtain greater sensitivity, the specimens on the filters were incubated on selective and non-selective media overnight as discussed in section 3.2.4. It was found that no hybridisation signal was produced

on the filters that had been incubated on the non-selective media plates. However, a very clear signal was often produced from the filters that had been incubated on the TSBV plates (Figure 22). This is possibly because the *A.actinomycetemcomitans* cells present in the specimen, were diluted or inhibited by all the other microorganisms which would have grown on the non-selective plates.

An experiment was done to determine whether there was a significant difference between the results obtained by incubating the filter on the plates overnight and by processing the filters immediately. Duplicate aliquots of the specimens were applied to the nylon filters and the membrane was divided into 2 halves. One half was treated immediately, while the other half was incubated on a TSBV plate, overnight, before being treated. The results obtained using these 2 methods were similar and the overnight incubation does not appear to significantly improve the hybridisation signal obtained.

The results obtained from a few patients are shown in Figures 22A, B and C. It can be seen from Figure 22A that isolate 11 was negative and isolate 12 was positive for *A.actinomycetemcomitans*. Both of these patients presented with localised juvenile periodontitis. The signal obtained in lanes 10-12 of Figure 22A is faint but is detectable and is roughly equivalent to that obtained from between 10^3 and 10^4 cells of the positive control. Isolate 12 was the only probe positive result obtained from the localised juvenile periodontitis patient group. The culture results were also positive for this patient. It would be expected that a higher percentage of the localised juvenile periodontitis patients would be positive for *A.actinomycetemcomitans*.

From Figure 22B it can be seen that isolate 13 (healthy adult) and 14 (adult periodontitis) are probe positive. Culture results from isolate 14 confirmed these results but were negative for isolate 13. This is possibly because the specimen obtained was not fresh and no growth was obtained.

Most of the culture positive isolates were applied to filters and probed. Figure 22C shows the strong hybridisation signal obtained from one of these isolates which was taken off a TSBV plate from isolate 21 (adult periodontitis).

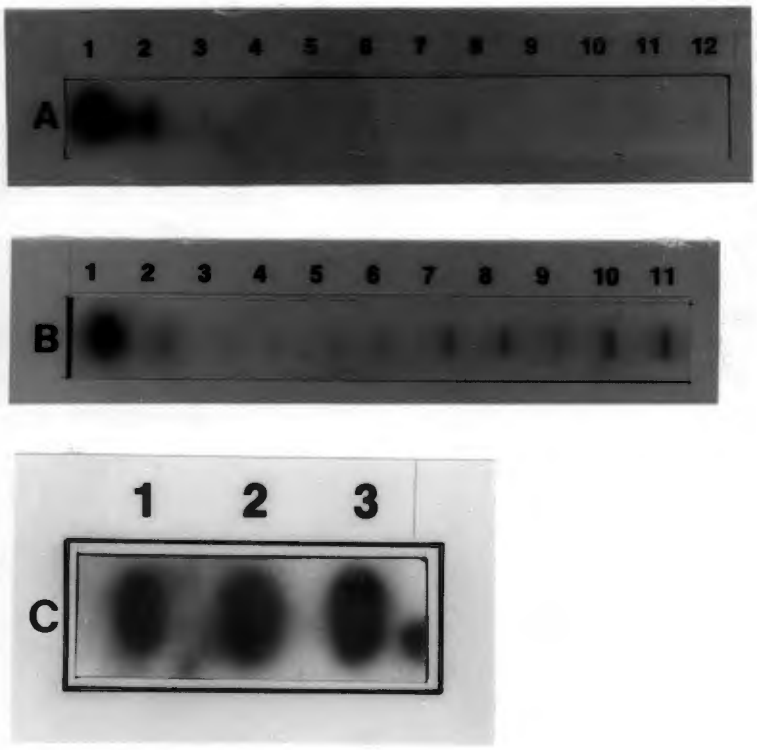


Figure 22. *Autoradiographs of slot blots of DNA obtained from clinical isolates. These filters were not first incubated before being treated.*

(A): (1)-(5) A.actinomycetemcomitans, NCTC 9710, 10^7; 10^6; 10^5; 10^4 and 10^3 cells respectively; (6) and (7) Isolates no. 11, site 26; (8) and (9) Isolates no. 11, site 31; (10)-(12) Isolate no. 12 site 16.

(B): (1)-(5) A.actinomycetemcomitans, NCTC 9710, 10^7; 10^6; 10^5; 10^4 and 10^3 cells respectively; (6) and (7) Isolate no.13, site 16; (8) and (9) Isolate no. 13, site 26; (10) and (11) Isolate 14, site 26

(C): (1-3) Isolate from a culture positive specimen (isolate 21)

Table 6. Results of DNA probe assay and of culture for the detection of *A.actinomycetemcomitans*.

	Probe Positive	Culture Positive	Probe and Culture Positive
Localised juvenile periodontitis (n=3)	1	1	1
Adult Periodontitis (n=16)	4	2	2
Rapidly progressing periodontitis (n=2)	1	0	0
Healthy adult (n=1)	1	0	0

The number of cases in which probe and culture results correlated, either both positive or both negative was in eighteen out of twenty-two cases. The number of cases positive by probe was seven out of twenty-two, i.e. 31,8% and three out of twenty-two cases were positive by culture, i.e. 13,6% (Table 6). The only case in which the probe results were positive and culture results, negative came from a specimen that had been left at 4°C for the weekend. No growth was obtained on the selective media and only a few colonies were found on the non-selective media but none of them resembled *A.actinomycetemcomitans*.

Unfortunately, problems were encountered in obtaining a high specific activity probe to be used in screening the third batch of specimens. After reprobing the filters six times, the signal was so low that the results of a further 24 patients had to be rejected.

Possible reasons why problems were encountered in obtaining a probe with a high specific activity include the fact that the amount of DNA used for labelling is difficult to quantitate accurately. This is due to such a small amount of DNA being obtained after purification on the Nacs Prepac column (section 2.5.2.3). The DNA concentration calculated using the OD_{260} reading was probably inaccurate as the reading was so low. Possibly a more accurate concentration could be obtained by running an aliquot of the DNA on an agarose gel and comparing the DNA to known amounts of DNA. It was found that by using more DNA, higher specific activities could be obtained. However, in future experiments the amount of DNA needs to be increased even more to detect smaller amounts of bacteria.

It is possible that from repeated freezing and thawing, the DNA might have become degraded and the same volume of probe might have contained less DNA. A new batch of DNA was prepared and labelled but the specific activity of the labelled probe obtained was still low. The fragment that was labelled was relatively small and could account for the low incorporation obtained.

It was found that there is a limit to how many times the nylon membranes can be stripped of probe and reprobed. The low signal obtained for the last batch of specimens could be due to the fact that the membranes had been reprobed six times. The Amersham Membrane detection method booklet, however, shows the results of the fifth reprobing of the same filters and there did not appear to be a substantial decrease in signal. It is possible that the DNA was not sufficiently cross-linked to the filter.

In order to check if the low incorporation of label obtained was due to the labelling kit, a new kit was used but the specific activity obtained was still low.

The availability of specimens was another major problem. Some of the specimens were not supplied for DNA analysis, others were not supplied for culturing and specimens were not always supplied fresh. This resulted in not being able to compare the results obtained for both methods in many cases.

3.3.2. Culture analysis

In the culture positive specimens, the star-shaped morphology was usually very obvious on the TSBV plates but not so obvious on the TSA plates. When the Type strain NCTC 9710 was plated onto TSBV and TSA plates, the Gram-stain showed that the microorganisms were Gram-negative coccobacilli, but the colonies often did not have a star-shaped morphology. The literature also states that the cells are very sticky and difficult to break up. However, this was not found to be the case with the type strain. This might be because the cells had been subcultured repeatedly and that the reported features in the literature apply to primary isolations alone. This observation was confirmed by Slots (1982b) where he found that on repeated subculture, the ability of the colonies to adhere to the medium and also the inner structure of the colony altered.

Savitt *et al* (1988), only performed the catalase and oxidase tests and classified all colonies on the *A.actinomycetemcomitans* selective media which were both catalase and oxidase positive, as *A.actinomycetemcomitans*. The validity of this assumption is highly questionable as there are many reports which state that *A.actinomycetemcomitans* is oxidase negative and the type strain used in this study was oxidase negative.

In some cases the sugar results differed by one or two sugars from the documented results, but because the literature reports are conflicting, the organism was recorded as being culture positive if it was positive by all the other biochemical tests. Slots *et al* (1988) have shown that the present species of *A.actinomycetemcomitans* includes groups of organisms with different biochemical and antigenic properties, indicating that the taxonomic position of these organisms is still not sufficiently clear.

4. SECTION FOUR

CONCLUSION

The DNA probe produced appears to be specific for *A.actinomycetemcomitans* and does not cross hybridise to *H.aphrophilus*, a closely related species which is also found in the oral cavity. The level of detection obtained using the probe was 50ng of *A.actinomycetemcomitans* DNA and 10^4 *A.actinomycetemcomitans* cells. Other workers have attained greater sensitivity using DNA probes, but only Dix *et al* (199O) have attained the same specificity.

Of the ten oligonucleotide probes that they developed, Dix *et al* (1990) only tested the *B.gingivalis*, *B.intermedius* and *F.nucleatum* oligonucleotide probes on plaque samples and they did not attempt to confirm the results by culture. The specimens that they examined did not come from specific patient groups. It remains to be seen whether their *A.actinomycetemcomitans* oligonucleotide probe can be used directly on clinical specimens.

Savitt *et al* (1988) used their DNA probes on clinical specimens but the specificity of their *A.actinomycetemcomitans* enriched probe is questionable (see section 2.3.3). In their DNA probe assay, the 10^3 cell control was the lowest control run that gave a consistently detectable signal. Thus, this level was chosen as a marker for detectability for both the probe and the culture analyses. Clearly samples containing less than 1000 cells can have these levels detected by culture analysis, but the clinical significance of detection of pathogens representing a minute proportion of the cultivable microflora is questionable (Savitt *et al* (1988). These workers reported results as $<10^3$, low risk; 10^4 or 10^5, moderate risk; and $>10^5$, high risk for periodontitis. As the level of detection in the current study was 10^4 cells, any signal less than 10^4 was regarded as being negative.

A problem that was encountered which has not been reported by other workers, was that cross-hybridisation occurred between the plasmid DNA and the *H.aphrophilus* DNA (section 2.5.3.2) and false positive results were obtained. This was circumvented by using the cloned insert alone as the probe.

If screening for periodontal pathogens is to be incorporated into routine practice, it would be advantageous to make use of non-radioactive labelling of the DNA in order to make the test safer for laboratory workers. Some of the new non-radioactive labelling kits available have been shown to approach the sensitivity obtained using radioactive labelling and this technique appears to be the method of the future.

In order to make the probe more sensitive, the PCR technique could be used. A unique target sequence has been now found and the next step would be to sequence the fragment that has been used in this study (probe 6) and then to make primers to the specific target sequences. Another approach that could be used is to investigate further the oligonucleotide probe for *A.actinomycetemcomitans* described by Dix *et al* (1990) and perhaps use this as a primer for PCR.

A problem with using PCR on oral microorganisms is that microorganisms such as *A.actinomycetemcomitans* are present in low numbers even in healthy mouths and the detection of small numbers of cells does not necessarily indicate a diseased site. Normally the presence of less than 10^3 *A.actinomycetemcomitans* cells is regarded as being a low risk site for periodontitis. It would be necessary to some how quantitate the amount of amplification obtained in order to relate the results to the amount of *A.actinomycetemcomitans* actually present.

Unfortunately, the results obtained using the probe for *A.actinomycetemcomitans* are not always reproducible and more controls are needed to standardise the results. Controls for the specific activity of the probe are needed as well as for standardising the length and stringency of the washes. As the number of specimens obtained was so low it is impossible to say at this stage if the DNA probe is more sensitive than culture.

In conclusion, the DNA probe that has been developed to identify *A.actinomycetemcomitans* appears to be highly specific and suitable for screening clinical specimens, but further work remains to be done in making the probe more sensitive.

5. REFERENCES

Alaluusa S and Asikainen S. (1988) Detection and Distribution of *Actinobacillus actinomycetemcomitans* in the Primary Dentition; J.Periodontol **59** (8): 504-507

Anderson ML and Young BD. (1985) Quantitative Filter Hybridization, In: Nucleic Acid Hybridization: a practical approach. Eds Hames B and Higgins S. IRL Press Oxford, Washington DC: p106

Asikainen S, Jousimies-Somer H, Kanervo A and Saxèn L. (1986) *Actinobacillus actinomycetemcomitans* and Clinical Periodontal Status in Finnish Juvenile Periodontitis Patients; J.Periodontol **57**: 91-93

Birnboim HC and Doly J. (1979) A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA; Nucl Acids Res **7**: 1513-1523

Bonta Y, Zambon JJ, Genco RJ and Neiders ME. (1985) Rapid Identification of Periodontal Pathogens in Subgingival Plaque; J.Dent Res **64**: 793-798

Bragd L, Dahlèn G, Wikström M and Slots J. (1987) The Capability of *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis* and *Bacteroides intermedius* to Indicate Progressive Periodontitis; A Retrospective Study; J Clin Periodontol **14**: 95-99

Christersson LA, Slots J, Rosling BG and Genco RJ. (1985a) Microbiological and Clinical Effects of Surgical Treatment of Localized Juvenile Periodontitis; J.Clin Periodontol **12**: 465-476

Christersson LA, Slots J, Zambon JJ and Genco RJ. (1985b) Transmission and Colonization of *Actinobacillus actinomycetemcomitans* in Localized Juvenile Periodontitis Patients; J.Periodontol **56**: 127-131

Christersson LA, Wikesjö U, Albini B, Zambon JJ and Genco RJ. (1987) Tissue localization of *Actinobacillus actinomycetemcomitans* in Human Periodontitis. II. Correlation between Immunofluorescence and Culture Techniques; J.Periodontol **58**: 540-545

Chung C and Miller R. (1988) A Rapid and Convenient Method for the Preparation of Competent Bacterial Cells; Nucl Acid Res **16** (8): 3580

Ciancio SG, Genco RJ, Goodson JM and Schallhorn RG. (1988) Nonsurgical Antibacterial Approaches to Periodontal Treatment; JADA **116**: 22-32

Dickinson D. (1986) Reaction: DNA Probe Detection of Periodontal Pathogens; Oral Microbiol Immunol **1**: 63-64

Dillon JR, Nasim A and Nestmann E. (1985) Recombinant DNA Methodology, John Wiley and Sons, New York

Dix K, Watanabe SM, McArdle S, *et al* (1990) Species-Specific Oligonucleotide Probes for the Identification of Periodontal Bacteria; J.Clin Microbiol **28** (2): 319-323

Eisenmann AC, Eisenmann R, Sousa O and Slots J. (1983) Microbiological Study of Localized Juvenile Periodontitis in Pananma; J.Periodontol **54**: 712-713

Feinberg AP and Vogelstein B. (1983) A Technique for Radiolabelling DNA Restriction Endonuclease Fragments to High Specific Activity; Anal Biochem **132**: 6-13

Fennel CL, Totten P, Quinn T, Patton D, Holmes K and Stamm W. (1984) Characterization of *Campylobacter*-Like Organisms from Homosexual Men; J.Infect Dis **149** (1): 58-65

French CK, Savitt ED, Simon SL, *et al* (1986) DNA Probe Detection of Periodontal Pathogens; Oral Microbiol Immunol **1**: 58-62

Friskin KW, Tagg JR, Laws AJ and Orr MB (1987) Suspected Periodontopathic Microorganisms and their Oral Habitats in Young Children; Oral Microbiol Immunol **2**: 60-64

Fristensky B. (1987) Cornell Sequence Analysis Package distributed by MBCRR, Harvard School of Public Health, Dana-Farber Cancer Institute-D1154, 44 Binney Street, Boston, Massachusetts 02115

Genco RJ, Zambon JJ and Christersson LA. (1986) Use and Interpretation of Microbiological Assays in Periodontal Diseases; Oral Microbiol Immunol **1**: 73-79

Genco RJ, Zambon JJ and Murray PA. (1985) Serum and Gingival Fluid Antibodies as Adjuncts in the Diagnosis of *Actinobacillus actinomycetemcomitans*-Associated Periodontal Disease; J.Periodontol **56** (special issue): 41-50

Goodson JM. (1986) Clinical Measurements of Periodontitis; J.Clin Periodontol **13**: 446-455

Greenstein G and Polson A. (1985) Microscopic Monitoring of Pathogens Associated with Periodontal Diseases, A Review; J.Periodontol **56** (12): 740-747

Grunstein M and Hogness D. (1975) Colony Hybridization: A Method for the Isolation of Cloned DNAs that Contain a Specific Gene; Proc. Natl. Acad. Sci. USA **72**: 3961-3965

Haffajee AD, Socransky SS and Goodson JM. (1983) Clinical Parameters as Predictors of Destructive Periodontal Disease Activity; J.Clin Periodontol **10**: 257-265

Holdeman LV, Cato EP and Moore WEC. (1977) Anaerobe Laboratory Manual. 4th ed. Blacksburg: VPI Anaerobe Laboratory

Holt SC, Ebersole J, Felton J, Brunsvold M and Kornman K. (1988) Implantation of *Bacteroides gingivalis* in Nonhuman Primates Initiates Progression of Periodontitis; Science **239**: 55-57

Kilian M. (1976) A Taxonomic Study of the Genus *Haemophilus* with the Proposal of a New Species; J.Gen Microbiol **93**: 9-62

Kolodrobetz D, Dailey T, Ebersole J and Kraig E. (1989) Cloning and Expression of the Leukotoxin Gene from *Actinobacillus actinomycetemcomitans*; Infect.Immun **57**(5): 1465-1469

Kornman KS. (1987) Nature of Periodontal Diseases: Assessment and Diagnosis; J.Periodont Res **22**: 192-204

Kornman KS and Robertson PB. (1985) Clinical and Microbiological Evaluation of Therapy for Juvenile Periodontitis; J.Periodontol **56**: 443-446

Macfarlane TW and Samaranayake LP. (1989) Microbiology of Periodontal Diseases; In Clinical Oral Microbiology, Wright London: p51

Mandel M and Higa A. (1970) Calcium-dependant Bacteriophage DNA Infection; J.Mol Biol **53**: 159-162

Mandell RL. (1984) A Longitudinal Investigation of *Actinobacillus actinomycetemcomitans* and *Eikenella corrodens* in Juvenile Periodontitis; Infect Immum **45**: 778-780

Mandell RL and Socransky SS. (1981) A Selective Medium for *Actinobacillus actinomycetemcomitans* and the Incidence of the Organism in Juvenile Periodontitis; J.Periodontol **52**: 593-598

Mandell RL, Tripodi LS, Savitt E and Goodson JM. (1986) The Effect of Treatment on *Actinobacillus actinomycetemcomitans* in Localized Juvenile Periodontitis; J.Periodontol **57**: 94-99

Maniatis T, Fritsch EF and Sambrook J. (1982) Molecular Cloning. A laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Mason P and Williams J. (1985) Hybridization in the Analysis of Recombinant DNA. In: Nucleic Acid Hybridization: a practical approach. Eds Hames B and Higgins S. IRL Press Oxford, Washington DC

Moncla BJ, Braham P, Dix K, Watanabe S and Schwartz D. (1990) Use of Synthetic Oligonucleotide DNA Probes for the Identification of *Bacteroides gingivalis*; J.Clin Microbiol **28** (2): 324-327

Moncla BJ, Strockbine P, Braham P, Karlinsey J and Roberts MC. (1988) The Use of Whole-cell DNA Probes for the Identification of *Bacteroides intermedius* Isolates in a Dot Blot Assay; J.Dent Res **67** (10): 1267-1270

Moore WC. (1986) Rapid Identification of Important Periodontal Micro-organisms by Cultivation: Oral Microbiol Immunol **1**: 56-57

Moore WC. (1987) Microbiology of Periodontal Disease; J.Periodont Res **22**: 335-341

Moore WC, Holdeman LV, Cato EP, *et al* (1985) Comparative Bacteriology of Juvenile Periodontitis; Infect Immun **48**: 507-519

Moore WC, Ranney RR and Holdeman LV. (1982) Subgingival Microflora in Periodontal Disease: Cultural studies: In Host-parasite interactions in Periodontal Diseases, pp.13-26. Washington D.C., American Society for Microbiology

Newman MG and Nisengard R. (1988) *Actinobacillus actinomycetemcomitans*. In Oral Microbiology and Immunology, p252. WB Saunders Company, Harcourt Brace Jovanovich, Inc

Newman MG and Socransky SS. (1977) Predominant Cultivable Microflora in Periodontosis; J.Periodont Res **12**: 120-128

Perine PL, Totten PA, Holmes KK, *et al* (1985) Evaluation of a DNA-Hybridization Method for Detection of African and Asian Stains of *Neisseria gonorrhoeae* in Men with Urethritis; J.Infect Dis **152** (1): 59-63

Phillips JE. (1984) Facultative Anaerobic Gram-negative Rods, In: Bergey's Manual of Systematic Bacteriology, Vol 1. Eds Krieg N and Holt J, Williams and Williams, Baltimore/London p570-573.

Peros WJ and Savitt ED. (1989) The Microbiology of Periodontal Disease; Clin Microbiol. Newsl **11** (7): 49-51

Potts TV, Zambon JJ and Genco RJ. (1985) Reassignment of *Actinobacillus actinomycetemcomitans* to the Genus *Haemophilus* as *Haemophilus actinomycetemcomitans* comb. nov.; Int. J System Bacteriol **35**: 337-341

Preus HR and Olsen I. (1988) Possible Transmittance of *Actinobacillus actinomycetemcomitans* From a Dog to a Child with Rapidly Destructive Periodontitis; J Periodont Res **23**: 68-71

Rabie G, Lally E and Shenker B. (1988) Immunosuppressive Properties of *Actinobacillus actinomycetemcomitans's* Leukotoxin; Infect Immun **56** (1): 122-127

Roberts MC, Hillier SC, Schoenknecht FD and Holmes KK. (1985) Comparison of Gram-stain, DNA Probe and Culture for the Identification of *Mobiluncus* Species in Female Genital Specimens; J.Infect Dis **152**: 74-77

Roberts MC, Moncla B and Kenny GE. (1987) Chromosomal DNA Probes for the Identification of *Bacteroides* Species; J.Gen Microbiol **133**: 1423-1430

Saglie FR, Carranza FA, Newman MG, Cheng L and Lewin KJ. (1982) Identification of Tissue-invading Bacteria in Human Periodontal Disease; J.Periodontol Res **17**: 452-455

Saglie FR, Smith CT, Newman MG, *et al* (1986) The Presence of Bacteria in the Oral Epithelium in Periodontal Disease. II. Immumohistochemical Identification of Bacteria; J.Periodontol **57**: 492-500

Saiki R, Bugawan TL, Horn GT, Mullis KB and Erlich HA. (1986) Analysis of Enzymatically Amplified ß-globin and HLA-DQα DNA with Allele-specific Oligonucleotide probes; Nature **324**: 163-166

Savitt ED and Socransky SS. (1984) Distribution of Certain Subgingival Microbial Species in Selected Periodontal Conditions; J.Perio Res **19**: 111-123

Savitt ED, Strzempko MN, Vacccaro KK, Peros WJ and French CK. (1988) Comparison of Cultural Methods and DNA Probe Analyses for the Detection of *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis* and *Bacteroides intermedius* in Subgingival Plaque Samples; J.Periodontol **59** (7): 431-438

Schaffer HE and Sederoff RR (1981) Improved Estimation of DNA Fragment Lengths from Agarose Gels; Anal Biochem **115**: 113-122

Schluger S, Youddelis RA and Page RC. (1978) Dental Deposits; Periodontal Disease, Henry Kimpton Publishers, London, p137

Seth A. (1984) A New Method for Linker Ligation; Gene Anal Techn **1**: 99-103

Slots J. (1981) Enzymatic Characterization of Some Oral and Nonoral Gram-negative Bacteria with the API-ZYM System; J Clin Microbiol **14**: 288-294

Slots J (1982a) Selective Medium for the Isolation of *Actinobacillus actinomycetemcomitans*; J.Clin Microbiol **15**: 606-609

Slots J. (1982b) Salient Biochemical Characters of *Actinobacillus actinomycetemcomitans*; Arch Microbiol **131**: 60-67

Slots J. (1986a) Rapid Identification of Important Periodontal Microorganisms by Cultivation; Oral Microbiol Immunol **1**: 48-55

Slots J, Bragd L, Wikstrom M and Dahlen G (1986b) The occurence of *Actinobacillus actinomycetemcomitans, Bacteroides gingivalis* and *Bacteroides intermedius* in Destructive Periodontal Disease in Adults; J.Clin Periodontol **13**: 570-577

Slots J and Listgarten MA. (1988) *Bacteroides gingivalis, Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* in Human Periodontal Diseases; .Clin Periodontol **15**: 85-93

Slots J, Reynolds HS and Genco RJ. (1980) *Actinobacillus actinomycetemcomitans* in Human Periodontal Disease: A Cross-sectional Microbiological Investigation; Infect Immunol **29**: 1013-1020

Slots J and Rosling BG. (1983) Suppression of the Periodontopathic Microflora in Localized Juvenile Periodontitis by Systemic Tetracycline; J.Clin Periodontol **10**: 465-486

Smith AC. (1971) In Vivo Effects of Intercalating Drugs on the Superhelix Density of Mitochondrial DNA Isolated from Human and Mouse Cells in Culture; J.Mol Biol **59**: 272-295

Smith GLF, Socransky SS and Sansone C. (1989a) "Reverse" DNA Hybridization Method for the Rapid Identification of Subgingival Microrganisms; Oral Microbiol Immunol **4**: 141-145

Smith GLF, Socransky SS and Smith CM. (1989b) Non-isotopic DNA Probes for the Identification of Subgingival Microorganisms; Oral Microbiol Immunol **4**: 41-46

So M, Crosa J and Falkow S. (1975) Polynucleotide Sequence Relationships Among Ent Plasmids and the Relationship Between Ent and Other Plasmids; J.Bacteriol **121**: 234-238

Socransky SS. (1986) Reaction: Use and Interpretation of Microbiological Assays in Periodontal Disease; Oral Microbiol Immunol **1**: 80

Socransky SS, Haffajee, Smith AD and Dzink JL. (1987) Difficulties Encountered in the Search for the Etiologic Agents of Destructive Periodontal Diseases; J.Clin Periodontol **14**: 588-593

Southern EM. (1975) Detection of Specific Sequences Among DNA Fragments Separated by Gel Electrophoresis; J.Mol Biol **98**: 503-517

Southern EM. (1979) Measurement of DNA Length by Gel Electrophoresis; Anal Biochem **100**: 319-323

Spiegel CA and Roberts M. (1984) *Mobiluncus* gen.nov., *Mobiluncus curtisii* subsp.*curtisii* sp.nov., *Mobiluncus curtisii* subsp. *holmessi* subsp.nov., and *Mobiluncus mulierus* sp nov., Curved Rods from the Human Vagina; Int. J Syst. Bacteriol **34** (2): 177-184

Strahan JD and Waite IM. (1978) A Colour Atlas of Periodontology, Wolfe Medical Publications Ltd, p10

Strzempko MN, Simon SL, French CK, *et al* (1987) A Cross-reactivity Study of Whole Genomic DNA Probes for *Haemophilus actinomycetemcomitans, Bacteroides gingivalis* and *Bacteroides intermedius;* J.Dent Res **66** (10): 1543-1546

Tanner AC and Goodson JM. (1986) Sampling of Microorganisms Associated with Periodontal Disease; Oral Microbiol Immunol **1**: 15-20

Tempro P and Slots J. (1986) Selective Medium for the Isolation of *Haemophilus aphrophilus* From the Human Periodontium and Other Oral Sites and the Low Proportion of the Organism in the Oral Flora; J.Clin Microbiol **23** (4): 777-782

Tenover FC. (1985) DNA Probes for Infectious Diseases; Clin Microbiol Newsl **7**: 105-112

Wennström JL, Dahlèn G, Svensson J and Nyman S. (1987) *Actinobacillus actinomycetemcomitans, Bacteroides gingivalis* and *Bacteroides intermedius*: Predictors of Attachment Loss? Oral Microbiol Immunol **2**: 158-163

Yanisch-Perron C, Vierira J and Messing J. (1985) Improved M13 Phage Cloning Vectors and Host Strains: Nucleotide Sequences of the M13mp18 and pUC19 Vectors; Gene **33**: 103-119

Zambon JJ, Christersson LA and Slots J. (1983a) *Actinobacillus actinomycetemcomitans* in Human Periodontal Disease: Prevalence in Patient Groups and Distribution of Biotypes and Serotypes Within Families; J.Periodontol **54**: 707-711

Zambon JJ, DeLuca C, Slots J and Genco RJ. (1983b) Studies of Leukotoxin from *Actinobacillus actinomycetemcomitans* Using the Promyelocytic HL-60 Cell Line; Infect Immunol **40**: 205-212

APPENDIX A. MEDIA AND SOLUTIONS

All media and solutions were autoclaved at 121°C for 20 minutes with the exception of solutions containing heat labile constituents which were either steamed for 30 minutes on 3 consecutive days or filtered using disposable 0.45μm filters (Schleicher and Schuell).
The amounts are for 1 litre final volume

1.TSBV (A.actinomycetemcomitans selective medium)

Trypticase soy agar (BBL)	40g
Yeast extract (Difco)	1g
Distilled water	860ml

Heat in a microwave oven until dissolved
Allow to cool to 56°C and then adjust the pH to 7.2 with 1N NaOH
Autoclave
Allow to cool to 50°C and add:

100 ml horse serum
20ml bacitracin (187.5mg/50ml)
20 ml vancomycin (12.5mg/50ml)

2. TSBVF (Selective medium for H.aphrophilus)

Trypticase soy agar	40g
Sodium Fluoride	0.05g
Distilled water	860 ml

Heat in a microwave oven until dissolved
Autoclave
Allow to cool to 50°C and add:

100 ml horse serum
20 ml bacitracin (187.5mg/50ml)
20 ml vancomycin (12.5mg/50ml)

3. Luria Broth

Bacto-tryptone	10g
Yeast extract	5g
NaCl	5g

Adjust the pH to 7.2 with NaOH

Make up to 1l with distilled water

4. M9 Broth

Na_2HPO_4	6g
KH_2PO_4	3g
NaCl	0.5g
NH_4Cl	1g

Adjust the pH to 7.4, autoclave and add:

1M $MgSO_4$	2ml
20% Glucose	10ml
1M $CaCl_2$	0.1ml

5. Trypticase Soy Agar (Cy:Dithio) (Non-selective medium)

Trypticase Soy agar (BBL)	40g
Yeast extract	5g
Salts solution	40ml
Resazurin solution	4ml
Distilled water	900ml

Heat in a microwave oven until dissolved

Add Cysteine:dithio soln 10 ml

Autoclave

Allow to cool to 50°C and add Vit K:Haemin 10 ml

Horse blood 50 ml

Cysteine:dithio solution:	Cysteine HCl 0.5g
	Dithiothreitol 0.1g
	Distilled water 10 ml

Vitamin K_3:haemin solution:

Vit K_3 =5mg\ml in 95% ethanol

Haemin =50mg+ 1ml 1N NaOH, made up to 100ml with distilled water. Add 1ml stock Vit K_3 to 100ml haemin.

The salts solution and resazurin solution were prepared according to the method of Holdeman, Cato and Moore (1977).

6. Luria broth/Amp/X-gal/IPTG plates

Autoclave 500ml Luria broth agar

Cool to 50°C

Add:	Amp (25mg/ml)	2ml
	X-gal	1ml
	IPTG	2.5 ml

X-gal- 2% (w/v) in dimethylformamide

IPTG stock solution is 0.1M

7. Serum water sugars

Peptone	4g
Na_2HPO_4	0.8g
Distilled water	800ml
Sterile serum	200ml
Bromocresol purple, 0.2% soln	10 ml

Dissolve the peptone and the phosphate in the water. Add the serum and the indicator. Add the appropriate sugar to a final concentration of 1% and then adjust the pH to 7.6-7.8. Place in a steam bath for 30 minutes on 3 consecutive days.

The following sugars were made up: glucose; lactose; galactose; maltose; mannose; sucrose; arabinose; trehalose; fructose and raffinose.

8. Ringers solution

Sodium chloride	2.25g
Potassium chloride	0.105g
Calcium chloride	0.09g
Sodium hydrogen carbonate	0.05g
Distilled water	1 litre

The solution was dispensed into 0.9 ml aliquots in Hungate tubes and autoclaved.

9. Horse blood

Horse blood was obtained from Delft Provincial Animal Farm. The blood was collected into sterile Transfuso Vac containers (Baxter), containing citrate, phosphate, and dextrose solution.

10. Buffers and Solutions

Most of the buffers used in this study were made up as described by Maniatis *et al* (1982). All solutions were stored at 4°C.

Preparation of phenol

Phenol was melted at 68°C and aliquots (250ml) were stored at -20°C. When required, a frozen aliquot was melted, and 8-hydroxyquinoline was added to a final concentration of 0.1%. The 8-hydroxyquinoline acts as an antioxidant and as a partial inhibitor of RNase. It is also a weak chelator of metal ions. The phenol was mixed 2-4 times with 1M Tris-HCl (pH 8.0), and then mixed 1-4 times with 0.1M Tris-HCl (pH 8) and 0.2% ß-m
ercaptoethanol until the pH of the supernatent was greater than 7.6. The phenol was saturated with an aqueous solvent to reduce losses from the solutions during phenol extractions. The phenol was stored at 4°C under 1M Tris-HCl (pH 8.0).

Phenol denatures proteins and selectively removes denatured DNA from aqueous solutions.

Chloroform:isoamyl alcohol

The mixture was used as a solution of 24:1 (v/v) of chloroform and isoamyl alcohol. The chloroform denatures the proteins, while isoamyl alcohol reduces foaming during the extraction and facilitates the separation of the aqueous and organic phase (Maniatis *et al*, 1982). The solution was stored at room temperature.

APPENDIX B. MANUFACTURERS AND/OR SUPPLIERS

1. Air Products SA (Pty Ltd), P.O. Box 366, Kuils River. RSA

Anaerobic gas mixture (Hydrogen-10%; Carbon dioxide-10%; Nitrogen-80%); Carbon dioxide gas; Nitrogen gas (high purity)

2. Amersham Lincoln Place, Green End, Aylesbury, Bucks HP20 2TP

α-^{32}P[dCTP]; Hybond-N; Hyperfilm-MP

3. Baltimore Biological Laboratory (BBL), (Division of Becton, Dickinson and Company), Cockeysville, Maryland 21030, USA

Trypticase soy agar

4. BDH Chemicals LTD, Broom Rd, Poole BH 12 4NN, England

Acetic acid (glacial); Boric acid; L-Cysteine hydrochloride; Ethanol; Hydrochloric acid; Isopropanol; ß-Mercaptoethanol; Phenol; Resazurin; Sodium acetate trihydrate; Sodium chloride; Sodium fluoride; Sodium hydroxide; Sarkosyl; Sucrose; Tri-sodium citrate; Triton X-100

5. Boehringer Mannheim GmbH-Biochemica, P.O. Box 310 120, D 6800 Mannheim 31, W. Germany

Ampicillin; Caesium chloride; dATP; dCTP; dGTP; DNA ligase; IPTG; Proteinase K; RNase A; Tris; X-gal

Restriction Enzymes:

ApaI; Asp700 (Xmn718); Asp718; BamHI; BclI; BglII; BstEII; ClaI; DraI; DraII; EcoRI; HindIII; HinfI; HpaI; KpnI; MspI; NcoI; NdeI; PstI; PvuII; SacI; SalI; ScaI; SmaI; SspI; StuI; XbaI

6. Difco Laboratories, Detroit, Michigan,48201, USA

Arabinose; Bacto-agar; Glucose; Mannitol; Mannose; Peptone; Sucrose; Todd Hewitt broth; Tryptone; Yeast Extract

7. Gibco BRL GmbH, Postfach 1212, Disselstrasse 5,7514
Eggenstein, West Germany

Hybri-Slot Manifold; Nacs Prepac columns

8. Merck, E.Merck, Frankfurter Strasse 250, D-6100, Darmstadt,
Federal Republic of Germany

Calcium chloride; Crystal violet; Bromophenol blue; Calcium chloride; Chloroform; EDTA; Formamide; Glycerol; 8-hydroxyquinoline; Isoamyl alcohol; Magnesium sulphate; Sodium hydrogen carbonate; TCA; Tris

9. Promega, 2800 South Fish Hatchery Rd, Madison, WI 53711
5305 USA

Restriction Enzymes:
EcoRV; NsiI; StyI

10.Saarchem Pty Ltd, P.O. Box 144, Muldersdrift, 1747 RSA

Potassium acetate

11. Sigma, P.O. Box 14508, St Louis, Missouri, 63178, USA

Agarose; Bacitracin; BSA; Haemin; Dithiothreitol; Ethidium bromide; Ficoll; Lysozyme; Polyvinyl pyrrolidone; Pronase; SDS; Sephadex G-50; Vancomycin; Vitamin K3

APPENDIX C. COUNTING OF BACTERIAL CELLS

In order to be able to apply known amounts of bacterial cells to the slot blots, the cell count was determined. This was done using a Gallenkamp counting chamber.

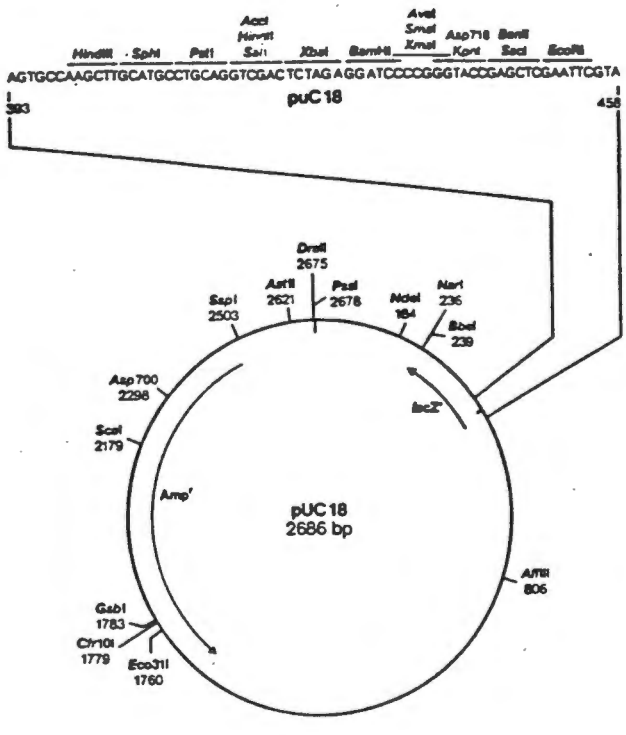
Dilutions of the cells were made and then 1 drop of the bacterial suspension was placed in the middle of the chamber. The coverslip was worked to and fro across the surface of the slide until interference patterns, "Newtons rings" were observed. The chamber was set up and counted at least 4 times because the estimated concentration depends on how closely the coverslip fits to the slide. At least 150 organisms were counted each time.

Each little square in the chamber has a depth of 0.02mm and an area of $1/400mm^2$. Thus the volume of a small square is $1/20000mm^3$.

$\therefore$The number of cells/ml= $2n \times 10^7$, where n is the mean number of organisms per small square.

APPENDIX D. RESTRICTION MAP OF PUC18

(Figure modified from Biochemicals for Molecular Biology, Biochemicals Catalogue, Boehringer Mannheim, 1987, pg 126)



APPENDIX E. SIZES OF MOLECULAR WEIGHT MARKER FRAGMENTS

The sizes of the fragments (in base pairs) generated after digestion of bacteriophage lambda DNA with EcoRI and HindIII are the following:

21226

5148

4973

4268

3530

2027

1904

1709

1375

947

831

564