MOLECULAR AND LEUCOTOXICITY STUDIES OF *Actinobacillus actinomycetemcomitans*

Evelyn Anne Downes

B.Sc. (Glasgow) M.Sc. (Strathclyde)

Thesis submitted for the Degree of Doctor of Philosophy
(Oral Sciences/ Bacteriology)

Faculty of Medicine.
Department of Oral Sciences,
Glasgow Dental Hospital and School,
378, Sauchiehall Street,
Glasgow.

October 1995

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

SUMMARY

Actinobacillus actinomycetemcomitans is a Gram-negative rod, which has been implicated in the aetiology of periodontitis especially, juvenile periodontitis and also with non-periodontal diseases e.g. endocarditis, brain abscesses and soft tissue abscesses.

The aims of the study described in this thesis, were to (i) investigate the strain diversity of a collection of *A.actinomycetemcomitans* held at Glasgow Dental Hospital and School and (ii) develop a rapid and accurate method to screen *A.actinomycetemcomitans* strains for the production of leucotoxin which is regarded as an important virulence factor.

The *A.actinomycetemcomitans* collection consisted of 96 *A.actinomycetemcomitans* isolates (85 clinical strains and 11 reference strains). The clinical isolates were collected between 1985 and 1990 and included multiple strains from a number of individuals and reflect a variety of clinical conditions, including juvenile periodontitis, chronic periodontitis and prepubertal periodontitis. The majority of these strains were stored as freeze dried ampoules and had been used in experimental studies including adhesion, hydrophobicity and leucotoxicity activity.

Strain diversity was investigated in two ways. The entire collection was screened for the presence of plasmids and demonstrated that 47% (40 out of 84) of clinical isolates and 9% (1 out of 9) of reference strains harboured plasmids. The number of plasmids found in plasmid positive strains varied between 1 and 3; 31% of all strains contained only a single plasmid. These results contrast with several published studies which have reported that very few strains of *A.actinomycetemcomitans* harbour plasmids.

A subset of 17 strains was selected, on the basis of leucotoxic status, single or multiple isolates from single individuals and plasmid profile and investigated in detail.

The strain diversity of the 17 strains of *A.actinomycetemcomitans* was further studied by the determination of whole cell DNA restriction endonuclease

fragmentation patterns (REFPs) with 3 different 4 base restriction enzymes - *Hin*fI, *Sau*3AI and *Hae*III.

Type strains isolated in America were shown to be genetically very similar to each other. Additionally, these American Type strains showed very little genetic similarity to the Danish Type strain and to the clinical strains isolated in Glasgow, which suggested a different clonal origin for the American and European strains. In addition, the REFP results showed that it was possible for an individual to be infected by at least two different strains of *A.actinomycetemcomitans.*

The second aim of the investigation was to produce a rapid method to screen strains of *A.actinomycetemcomitans* for leucotoxin production. Two methods were compared, one of which had not been previously used to investigate the toxicity of *A.actinomycetemcomitans.* The first involved the exposure of HL-60 cells (derived from human monocytes), to whole and sonicated cells of *A.actinomycetemcomitans.* Cell death was measured by trypan blue exclusion. The second, novel, method was a chemiluminescence assay, which had been used to previously investigate the activity of the adenylate cyclase toxin of *Bordetella pertussis.* The toxins from both *A.actinomycetemcomitans* and *B.pertussis* are known to disrupt the cell membranes of human polymorphonuclear leucocytes (PMNLs). Healthy PMNLs will normally phagocytose bacteria with the production of a burst of light, which can be enhanced and measured. However, when exposed to toxin producing bacteria, which kills or disrupts the PMNL membrane, the cell can no longer phagocytose and therefore little chemiluminescent activity results. The chemiluminescence and cytotoxicity assays were compared and in both cases, the same five strains of *A.actinomycetemcomitans* were shown to be toxic. However, variation was seen in the rank order of toxicity.

When outer membrane protein preparations of *A.actinomycetemcomitans* were probed with a monoclonal antibody (MCA 9D4) to the related toxin, adenylate cyclase toxin produced by *B.pertussis,* the presence of a protein with a molecular weight approximately equivalent to that of the *A.actinomycetemcomitans* toxin was

found.

The overall conclusions of the thesis are :

(i) there was no association of plasmid status with either the production of a toxin or a particular form of periodontal disease.

(ii) analysis of whole cell DNA REFPs, suggested a distinct clonal origin for American Type strains, compared to European Type and wild strains.

(ii) the novel chemiluminescent assay is as accurate a method of determining toxicity status as the older method of HL-60/trypan blue exclusion assay. Although the two methods are very similar, when large numbers of strains have to be screened, the chemiluminescence method is faster than and the results obtained comparable to the trypan blue exclusion assay

(iii) the monoclonal antibody to the *B.pertussis* toxin, recognises an outer membrane protein with approximately the same molecular weight as the *A.actinomycetemcomitans* leucotoxin despite the low (less than 50%) homology between the two toxins. This result suggests the view that the leucotoxin is present in the outer membrane and supports the findings of other groups.

Future work to be carried out on the collection of *A.actinomycetemcomitans* strains held at the Glasgow Dental Hospital and School could include further investigation into the clonal structure of the collection - using both whole cell and plasmid DNA. The use of techniques such as polymerase chain reaction could be used as an alternative method to restriction enzyme analysis. Additionally, the leucotoxic status of the whole collection could now be found out rapidly, using the chemiluminescence assay and the hypothesis that the majority of the remainder of the strains are non-toxic, confirmed or rejected.

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ABBREVIATIONS

ATCC : American Type Culture Collection

AktA : leucotoxin from *Pasturella haemolytica*

AppA : haemolysin from *Actinobacillus pneumoniae*

B cell : B lymphocyte

BDH : British Drug Houses

BHI : brain heart infusion

BSA : bovine serum albumin

C : cytosine

°C : degrees centrigrade

$CaCl_2$: calcium chloride

Ca^{2+} : calcium ions

CBA : Columbia Blood Agar

CCC : covalently-closed circle

cm : centimetres

CO_2 : carbon dioxide

$CoCl_2$: Cobalt cholride

Cp : Chronic periodontitis

^{51}Cr : Chromium 51

CTAB : cetyl trimethyl ammonium bromide

CycA : Adenylate cyclase

DAB : 3,3'-diaminobenzidine

DMSO : dimethylsulphoxide

DNA : Deoxyribonucleic acid

EDTA : Ethylenediaminetetraacetic Acid

e.g. : for example

et al : and others

FCS : Foetal Calf Serum

FMLP : N-formyl-methionyl-leucyl-phenylalanine

g : grams

G : guanine

GDH : Glasgow Dental Hospital

HEPES : N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HIV or : HIV oral rinse

hly : haemolysin

HlyA : haemolysin A

HPBL : human peripheral blood lymphocytes

H_2O_2 : hydrogen peroxide

i.e. : that is to say

in vitro : in glass

in vivo : in life

IgA : Immunoglobulin A

IgG : Immunoglobulin G

IgM : Immunoglobulin M

IL-1 : interleukin-1

Jp : Juvenile periodontitis

K : potassium

kb : kilobases

kDa : kilo Daltons

LDH : lactate dehydrogenase

Lkt A : *A.actinomycetemcomitans* leucotoxin

LPS : Lipopolysaccharides

luminol : 5-amino-2, 3-hydro-1, 4-phthalazinedione

M : molar

mA : milliamps

MCA : monoclonal antibody

MEE : multilocus enzyme electrophoresis

$mgml^{-1}$: milligrams per millilitre

ml : millilitres

mm : millimeter

mM : millimolar

mRNA : messenger ribonucleic acid

mV : millivolts

μgml^{-1} : micrograms per millilitre

µm : micrometer

Na : sodium

NAD : β-Nicotinamide Adenine Dinucleotide

NCTC : National Collection of Type Cultures

nm : nanometer

O_2 : oxygen

O_2^- : oxygen ions

OC: Open circle

OD : optical density

OMP : outer membrane protein

PAGE : polyacrylamide gel electrophoresis

PBS : phosphate buffered saline

PCR : polymerase chain reaction

pH : negative decimal log of molar hydrogen ion concentration

pIs : isoelectric point

PMA : phorbol-myristate-acetate

PMNL : polymorphonuclear leucocytes

Pp : Prepubertal periodontitis

^{86}Rb : Rubidium 86

REFP : restriction enzyme fragmentation pattern

rpm : revolutions per minute

rRNA : ribosomal Ribonucleic acid

RTX toxin : Repeats in ToXins

SAPU : Scottish antibody production unit

Sarkosyl : N-Lauroylsarcosine, sodium salt

S_D : Dice coefficient

S.D. : Standard deviation

SDS : sodium dodecyl sulphate

SEM : scanning electron microscopy

T cell : T lymphocyte

TBE : tris borate edta

TE : tris edta

TE_{10} : 10 mM Tris EDTA

TE_{50} : 50 mM Tris EDTA

TEM : transmission electron microscopy

TEMED : N,N.N',N'- tetra methyl-ethylenediamine

TES : Tris/ EDTA/ Salt

TGE : tris glucose edta

TNF : tumour necrosis factor

Trisma-base : hydroxymethyl aminomethane

Tris-HCl : hydroxymethyl aminomethane hydrochloride

TSB : Trypic Soy Broth

Tween-20 : polyoxyethylenesorbitan - monolaurate

UV : ultraviolet

V factor : β-Nicotinamide Adenine Dinucleotide

V : voltage

v/v : volume per volume

w/v : weight per volume

X factor : haemin

Zn^{2+} : zinc ions

ACKNOWLEDGEMENTS

I would like to thank the following people for all their help, advice and friendship over last 5 years, without whom, this thesis would not have been completed.

To Professor T.W. MacFarlane, for all his help, advice, patience and understanding, without which this thesis would not have been finished

To Dr. David Platt for his friendship, help and encouragement, again without which this thesis would not have been completed.

To Drs. Roger Parton, John Coote, Robert Davies, Gareth Westrobe and Sala Chettibi at the University Department of Microbiology for all their help and advice in the preparation of SDS-PAGE gels, Western blots and luminometer experiments.

To Mr. Marshall, Mr. MacKenzie and Mr Alan Lennon for their technical help and advice at Glasgow Dental Hospital and School. I would also like to thank all the members of staff and the research students (especially Eva Adonogionaki and Aileen Bell) in both the Oral Sciences and Bacteriology Departments of the University of Glasgow, for their friendship and encouragement.

Finally, thank you to the staff of the Identification Bureau, Grampian Police, for never failing to ask (daily) "Well, is it finished yet?"!!

The work described in this thesis was funded by a Medical Research Council Research Studentship.

DEDICATION

This thesis is dedicated to my parents, John and Una Downes,

my sisters Hilary and Melanie, and to

my husband Hugh Gillies, for understanding.

" The outstanding feature of this organism, is the tenacity with which the individuals cling to one another."

L. Colebrook, 1920

DECLARATION

This thesis is the original work of the author

CHAPTER 1
LITERATURE REVIEW

CHAPTER 1

LITERATURE REVIEW

1.1 TAXONOMY

The genus *Actinobacillus* was first described by Lignieres and Spitz in 1902 and the species *Actinobacillus actinomycetemcomitans* in 1912 by Klinger (Topley and Wilson, 1990). *Actinobacillus*, with the genera *Pasteurella* and *Haemophilus*, form the family *Pasteurellaceae* (Sneath and Johnson, 1973), all of which are taxonomically similar to each other but difficult to classify. With the exception of some strict anaerobes, the *Pasteurellaceae* family comprises the majority of the endogenous parasites among the nonenteric, fermentative Gram-negative rods associated with animals and man (Kilian, Frederiksen and Biberstein, 1981). They are very important pathogens both in human and veterinary medicine. Some species are responsible for life-threatening infections while others are commensals of the mucosal membranes of both man and animals.

A.actinomycetemcomitans has been shown to have more similarities to the genus *Haemophilus* than to *Actinobacillus* (Potts, Zambon and Genco, 1985) and it was proposed by Potts *et al* (1985) that *A.actinomycetemcomitans* should be reclassified as *Haemophilus actinomycetemcomitans* on the basis of DNA-DNA hybridisation studies. Further support for this reclassification was later presented by Chuba *et al* (1988) who partially sequenced the 16S rRNAs of seven members of the *Pasteurellaceae*, from which the approximate phlyogenic relationships of these species was inferred. *A.actinomycetemcomitans* clustered with *Haemophilus*. Potts *et al* (1985) also used serological techniques to classify *A.actinomycetemcomitans* and determined that it could be associated with either the genera *Haemophilus* or *Actinobacillus*, as it does not fulfil all the criteria for inclusion into either genus completely. Biochemically it is urease and beta galactosidase negative, both of which are requirements for inclusion in the *Actinobacillus* genus. In addition it does not

require either haemin (X factor) or NAD (V factor) for growth, which are requirements for inclusion in the *Haemophilus* genus. This is not an absolute requirement, as *Haemophilus aphrophilus* requires neither of these factors (Potts *et al*, 1985).

A.actinomycetemcomitans and *H.aphrophilus* were shown by King and Tatum (1962) to be related to each other by biochemical and morphological analysis. Jantzen, Berdal and Omland (1980) studied the cellular fatty acid composition of *A.actinomycetemcomitans* and *Haemophilus* species and were unable to distinguish between them. Subsequently, however, different authors have used various parameters and techniques e.g. composition of bound fatty acids (Brondz and Olsen, 1984a) and the lipid and carbohydrate content of lipopolysaccharides (LPS - Brondz and Olsen 1984b, 1989) to characterize *A.actinomycetemcomitans* and it has been shown that *A.actinomycetemcomitans* differs from other *Actinobacillus* and *Haemophilus* species - even those which have morphological similarities, e.g. *H.aphrophilus*.

A.actinomycetemcomitans and *H.aphrophilus* were further investigated by Brondz, Olsen and Sjostrom (1990) using biochemical analysis, which included lysis kinetics and methylene blue reduction. Both *A.actinomycetemcomitans* and *H.aphrophilus* characterised differently from all other *Haemophilus* and *Pasteurella* species analysed.

Caugant, Selender and Olsen (1990) studied the genetic relationship of *A.actinomycetemcomitans*, *H.aphrophilus* and *H.paraphrophilus*. They demonstrated, by the use of multilocus enzyme electrophoresis, that overall, there was no genetic similarity between *A.actinomycetemcomitans* and the other members of the genus *Haemophilus*.

The DNA base ratio (G+C content) of *A.actinomycetemcomitans* and several *Haemophilus* species were determined by Potts and Berry (1983) who found *A.actinomycetemcomitans* to have a G+C content between 44.9 and 46.6%. This range was greater than and distinct from the ranges obtained for other *Actinobacillus* and *Haemophilus* species, which had G+C contents of below 42%. Coykendall,

Setterfield and Slots (1983) showed that the G+C content of *A.actinomycetemcomitans* was between 46.7 and 48.7%. Likewise. the G+C values obtained for *H.aphrophilus* was higher (44.5 - 46.5%) with other *Actinobacillus* species having a G+C content between 43.2 and 44%.

DNA probes from *A.actinomycetemcomitans* strains Y4 and NCTC 9710 had homology to *H.aphrophilus* and *H.paraphrophilus* of only 25 to 37%, while a DNA probe from *H.aphrophilus* had homology to *A.actinomycetemcomitans* of between 30 to 39% and homology to *H.paraphrophilus*, of between 73 to 77% (Potts and Berry, 1983). Potts and Berry (1983) suggested that this indicated a very close relationship between the two *Haemophilus* species which does not exist between *A.actinomycetemcomitans* and *H.aphrophilus*. Other haemophili and actinobacilli showed homology to the two test probes of 10% or less (Potts and Berry, 1983).

The degree of homology between two regions of DNA, from the same or a different genome, is determined by the target sequence of DNA. The origin of the probe sequence of DNA is important and it would be of interest to know if it was obtained from a plasmid, a ribosomal gene, structural gene, or from either a conserved or divergent region? Each of these parameters would have an effect upon the percentage homology seen between the probe and the target. The authors did not give any indication as to the origin of the probe, other than the strain designation of the *A.actinomycetemcomitans* used.

Hybridisation studies by Coykendall, Setterfield and Slots (1983) and Potts *et al* (1986) found *A.actinomycetemcomitans* formed a group distinct from *H.aphrophilus* and other *Actinobacillus* species.

Thus, the current taxonomic position of *A.actinomycetemcomitans* remains unclear and although it is justifiably classified as a species within *Actinobacillus*, it shares few characteristics with other members of the genus. It has many of the biochemical characteristics of the genus *Haemophilus*, but on the basis of DNA-DNA homology, it remains distinct from this genus as well.

1.2 MORPHOLOGY

1.2.1 Colony morphology

When freshly isolated *A.actinomycetemcomitans* strains are cultured upon a solid medium, they tend to form small, circular colonies, 0.5 to 1.0 mm in diameter (Scannapieco *et al*, 1987) with a rough or smooth appearance. The smooth colonies usually have an internal morphology which has been described as "stellate" (Colebrooke, 1920) or "crossed-cigars" (Heinrich and Pulverer, 1954). Rough colonies have ellipsoidal structures distributed randomly throughout, are firmly attached to the surface of the agar and when removed leave behind an imprint of both colony and internal structures (Blix *et al* 1990). Stereo-microscopy, scanning and transmission electron-microsopy have demonstrated that, in cross-section, the colony structure resembled a "jelly-fish" with pseudopod-like protrusions of various sizes on the lower surface of the colony. These become embedded in the agar (Blix *et al* 1990) and at the colony-agar interface a large number of "vesicles" have been identified : the centre of the protrusions appears to be composed of cells which are devoid of cell contents. No explanation for either the large number of vesicles or "empty cells" has been given.

Blix *et al* (1990) suggested that the pseudopods may have been responsible for the attachment of *A.actinomycetemcomitans* to agar, a hypothesis which was supported by the observation that, on repeated subculture pseudopods and fimbriae (section 1.2.3 Fimbriae) were lost as colonies of *A.actinomycetemcomitans* became less firmly attached to the agar. This corresponded with a change in colony morphology, from translucent to opaque and from rough to smooth.

Cultures of *A.actinomycetemcomitans* grown under anaerobic conditions appeared to be more adhesive to the surface of the agar than those grown aerobically. In addition, colonies from anaerobic cultures were of a firmer consistency and when grown in a fluid medium, had a greater tendency to clump, compared with colonies grown, under aerobic conditions (Scannapieco *et al*, 1987).

A summary of the morphological characteristics is give in Table 1.1.

1.2.2 Light microscopy

When viewed by light microscopy, *A.actinomycetemcomitans* was shown to be a nonmotile, Gram negative coccobacillus which occurred as single cells (0.7 ± 0.1 by 1.0 ± 0.4 μm) in pairs or in clumps (Zambon, 1985). Extended incubation (up to 16 days) led to shortening and rounding of the cells (Holt *et al*, 1980).

1.2.3 Electron microscopy

There have been several studies reported in the literature concerning the surface morphology of *A.actinomycetemcomitans* using transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

Transmission electron microscopy (TEM)

The cell wall and membrane of *A.actinomycetemcomitans* was described by Holt *et al* (1980) and Lai *et al* (1981) as typically gram-negative. The periplasm was poorly defined and in some cases could not be distinguished from either the outer or cytoplasmic membranes. Both groups cultured *A.actinomycetemcomitans* on similar media, although Holt *et al* (1980) used Ruthenium red to stain the samples, while Lai *et al* (1981) stained the samples with uranyl acetate and lead citrate.

Surface structures

Lai *et al* (1981), in a TEM study of 14 strains of *A.actinomycetemcomitans*, found a correlation between vesicle production and strains previously designated as toxic to PMNs. In addition, cells grown on agar media were covered with adherent exopolymeric material and demonstrated more outer membrane associated vesicles, both attached to and separate from the outer membrane, than cells grown in a fluid medium Lai *et al* (1981).

MORPHOLOGY OF *A.actinomycetemcomitans* ON INITIAL ISOLATION	Rough colonies Starshaped inner structure
MORPHOLOGY OF *A.actinomycetemcomitans* ON CONTINUED SUBCULTURE	Smooth colonies Inner structure disappears
SIZE OF *A.actinomycetemcomitans* RODS	Small, gram negative rods 1.5 - 1.7 μm by 0.55 μm
SURFACE	Membrane vesicles "Blebs" attached to surface

Table 1.1 Morphological characteristics of *A.actinomycetemcomitans*

(After Hammond and Stevens, 1982)

The vesicles appeared to have been shed from the outer membrane surface. It has since been demonstrated that the leucotoxin from *A.actinomycetemcomitans* is located in either the cell envelope and/or in the membranous vesicles on the bacterial cell surface (DiRienzo *et al*, 1985. Berthold *et al*, 1992).

It is known that some Gram negative bacteria elaborate vesicles associated with the outer membrane e.g. *Neisseria meningitidis* (DeVoe and Gilchrist, 1973). During normal log phase growth of *N.meningitidis*, a large numbers of vesicles or "blebs" are released and it was suggested by DeVoe and Gilchrist (1973) that this could be the mechanism by which the endotoxin of *N.meningitidis* is released into the environment.

Fimbriae

Holt *et al* (1980) and Inouye *et al* (1990) reported the presence of surface associated fimbriae, which appeared as long hair-like appendages which originated from the outer membrane and appeared to link cells in close proximity to one another. Rosan *et al* (1988) estimated fimbriae to be 5 nm in width and reaching several μm in length as demonstrated by TEM.

It has been reported that some forms of bacterial adhesion are mediated by fimbriae (Beachey, 1981). McGee *et al* (1979) suggested that the fimbriae of *N.meningitidis* play a role in the attachment of these bacteria to host cells. They demonstrated that the loss of membrane associated fimbriae after repeated subculture corresponded to a change in morphology and a reduced ability to adhere.

Variations in fimbrial expression in *Neisseria* have been shown to be subject to phase variation in which the fimbria (and certain outer membrane proteins) are reversibly or irreversibly switched on or off. (Saunders, 1986). Phase variation in *Neisseria gonorrhoeae* is recognised by alterations in colonial morphology, when cells are grown on a clear agar. Colonies of fimbriated gonococci are recognized by the small, dense colonies they produce, while the unfimbriated gonococcal colonies are larger and more diffuse (Swanson, 1984). It was suggested by Saunders (1986) that the

ability to modulate between different forms (fimbriated and unfimbriated) may be advantageous to *N.gonorrheae* in population terms, enabling the organism to adapt and survive within a variety of different environments.

Both Rosan *et al* (1988) and Preus *et al* (1988) have postulated that fimbriae are an important factor in the colonisation and survival of *A.actinomycetemcomitans* in anaerobic periodontal pockets perhaps by mediating adherence to epithelial cell surfaces (Preus *et al*, 1988. See section 1.7.1).

Rosan *et al* (1988) reported that *A.actinomycetemcomitans* cultured on solid media possessed fimbriae which were produced under both aerobic or anaerobic conditions. This is at variance with the findings of Scannapieco *et al* (1987) who demonstrated that anaerobic conditions, favoured the production of fimbriae. However, since only a small number of strains were investigated by Scannnapieco *et al* (1987) and the number studied by Rosan *et al* (1988) was not recorded, the effect of atmospheric conditions on fimbriae remains uncertain.

Inouye *et al* (1990) described three colonial forms of *A.actinomycetemcomitans* namely transparent-smooth, transparent-rough and opaque-smooth. Both types of transparent colonies were shown to be both adhesive to the agar surface and highly fimbriated, while opaque colonies were neither adhesive nor fimbriated. These workers suggested that the transparent-rough colonies were intermediate between transparent-smooth and opaque-smooth, as colonies upon repeated subculture, eventually changed from transparent-smooth to opaque-smooth, in a process which appeared to be irreversible.

Scanning electron microscopy

There has been only one reported SEM study of *A.actinomycetemcomitans* (Holt *et al*, 1980). *A.actinomycetemcomitans* strains possessed a thick amorphous layer that appeared to cover and join neighbouring cells. Holt *et al* (1980) suggested that this was due to an artefact in the preparation of the cells for examination. Fimbriae, which

were seen under transmission electron microscopy were not observed by scanning electron microscopy. Thick fibrous strands which appeared to join cells which were separated by large distances, were seen. These appeared to be associated with the outer membrane of the cell and were demonstrated to be morphologically different to the short, thick fimbria which connect adjacent cells.

1.3 BIOCHEMICAL CHARACTERISATION

There have been several investigations into the biochemistry of *A.actinomycetemcomitans*. Slots (1982) examined 136 strains and Sneath and Stevens (1985) 3 *A.actinomycetemcomitans* strains (in a much wider study of 89 *Actinobacillus* strains, in a total of 261 different strains of *Actinobacillus*, *Pasteurella* and *Yersinia*). The results are summarised in Table 1.2.

All *A.actinomycetemcomitans* strains investigated were catalase positive, oxidase negative and required neither X nor V factors. Growth in all cases was favoured by CO_2. All strains reduced nitrate to nitrite and produced strong alkaline and acid phosphatases. *A.actinomycetemcomitans* fermented glucose, fructose and mannose with variable fermentation of dextrin, galactose, maltose, mannitol and xylose. Conflicting results have been reported for the fermentation of galactose. King and Tatum (1962) and Page and King (1966) described it as positive, whereas Slots (1982) reported it as negative.

1.4 INTERACTIONS WITH THE IMMUNE SYSTEM

1.4.1 Neutrophil chemotaxis

There is conflict in the literature with regard to defects in neutrophil chemotaxis. Early work suggested that neutrophil chemotaxis dysfunction was involved in the pathogenesis of juvenile periodontitis and rapidly progressive periodontitis (Clark, Page and Wilde, 1977. Lavine *et al*, 1979. Van Dyke *et al*, 1980). Later work described the defect as either elevated levels of chemotaxis - seen in patients with

(A)	**SUGAR FERMENTATION**		
POSITIVE	Glucose	Fructose	Mannose
NEGATIVE	Adonitol Cellobiose Lactose Ribose Sucrose	Amygdalin Glycerol Melibiose Salicin	Arabinose Inositol Rhamnose Sorbitol
VARIABLE	Dextrin Xylose	Maltose	Mannitol
(B)	**OTHER**		
POSITIVE	Peroxidase breakdown Nitrate reduction NaF resistance		
NEGATIVE	Oxidase reduction Proteolysis reduction Haemin requirement NAD requirement		
(C)	**GASEOUS REQUIREMENT**		
Aerobic plus 5% CO_2	Good growth		
Anaerobic (N_2 only)	Little or no growth		
Anaerobic plus 5% CO_2	Growth variable		

Table 1.2 Biochemical identification tests for *A.actinomycetemcomitans*
After Hammond and Stevens (1982)

rapidly progressive periodontitis or as depressed levels of chemotaxis, seen most frequently in patients with juvenile periodontitis (Van Dyke *et al*, 1980). During episodes of bacterial infection an increase in the level of chemotaxis was seen (Hill *et al*, 1974). Van Dyke *et al* (1980) reported a decrease in chemotaxis but did not collect or report any microbiological data.

Kinane *et al* (1988a, 1988b) used different techniques (e.g. a Boyden chamber) to assess PMNL locomotion. They failed to show any evidence for depressed PMNL locomotion and when the PMNLs were exposed to different doses of N-formyl-methionyl-leucyl-phenylalanine (FMLP - a chemoattractant) an enhancement in the level of PMNL locomotion was seen (Kinane *et al*, 1988a). Further more, it was suggested that although PMNs from patients with early-onset forms of periodontal disease may have abnormal PNM locomotion (either enhanced or inhibited) the differences may in part be explained by the differences in the laboratory techniques used (Kinane *et al*, 1988b). With the use of time-lapse video analysis, single-cells examined with the under-agarose technique showed no differences in locomotion between PMNLs from control or experimental groups.

Van Dyke *et al* (1982c) suggested two basic causes of PMNL dysfunction; either the PMNL was inherently defective or an extra-cellular factor was present which acted as an inhibitor. Van Dyke *et al* (1982a) demonstrated that neutrophil chemotaxis could be inhibited by a soluble product of certain oral bacteria including *A.actinomycetemcomitans*, *Fusobacterium nucleatum* and certain *Bacteroides* species. Although not characterised, the bacterial factor was shown to be a non-chemotactic, soluble product which competed for the chemotactic receptors on the neutrophil.

The ability of *A.actinomycetemcomitans* to evoke *in vivo* chemotaxis of rabbit PMNLs was investigated by Holm, Kalfas and Holm (1989). They demonstrated a factor responsible for the increase in chemotaxis, found in both viable and heat-killed *A.actinomycetemcomitans*. The factor was not fully characterized but certain

properties (e.g. the active factor was found in heat killed bacteria) distinguished it from leucotoxin.

Holm *et al* (1989) were able to demonstrate the effect of the chemotactic enhancement factor from *A.actinomycetemcomitans* on rabbit PMNLs, while Van Dyke *et al* (1982a) demonstrated a chemotactic inhibitory factor from *A.actinomycetemcomitans* on human PMNLs. The normal environment for *A.actinomycetemcomitans* is the sub-gingival plaque in man, which may partially explain the contradiction between the results of Van Dyke *et al* (1982a) and Holm *et al* (1989) who worked on rabbit PMNLs.

The work of Van Dyke *et al* (1982a) was supported by Ashkenazi, White and Dennison (1992) who demonstrated a bacterial extract which was a non-endotoxin, heat-labile protein, that was chemotactic for neutrophils. However, when PMNLs were preincubated with the bacterial extract, chemotaxis was inhibited.

Van Dyke, Horoszewicz and Genco (1982b) suggested that chemotaxis inhibition could be the result of a decrease in the rate of migration of the population of neutrophils and not a decrease in the number of PMNLs. Van Dyke *et al* (1982c) suggested that individuals with normal neutrophil function may be affected by bacterial products which interfered with the binding of chemotactic factors, linked to the neutrophil surface by a mechanism of high affinity.

The reduction in the number of chemotaxic receptors on PMNLs in individuals with juvenile periodontitis was further demonstrated by Van Dyke *et al* (1990). They suggested that the reduction in FMLP receptors was specific in cases of localised juvenile periodontitis which exhibit neutrophil chemotaxic abnormalities.

Ashkenazi *et al* (1992) investigated different mechanisms which could be responsible for the inhibition of neutrophil chemotaxis when exposed to an extract from *A.actinomycetemcomitans* e.g. the binding of FMLP and cytoskeletal changes caused by F-actin polymerisation. The bacterial extract itself was shown to be chemotactic for PMNLs, but when PMNLs were incubated with the extract, chemotaxis towards

various stimuli, in different assays, was inhibited. None of the mechanisms investigated was shown to cause an inhibition in chemotaxis.

It was suggested by Ashkenazi *et al* (1992) that discrepancies between their results and those of Van Dyke *et al* (1982) could be attributed to differences in (i) the preparation of the bacterial extract, (ii) the *A.actinomycetemcomitans* strains used and (iii) the method of calculation of the bacterial extract protein concentration.

1.4.2 Immune suppression and mitogenic effects of *A.actinomycetemcomitans*

Shenker, Tsai and Taichman (1982a, 1982b) characterised an immunosuppressive effect of *A.actinomycetemcomitans* on the proliferation of human peripheral blood lymphocytes (HPBL), following exposure to mitogens and antigens. In addition, they demonstrated that the exposure of HPBL to *A.actinomycetemcomitans* sonicated extracts caused suppression of both T and B-cell functions. They suggested that this could be mediated through a common mechanism e.g. the activation of T-suppressor cells. This could cause a reduction in the response of an individual's immune system, which in turn could lead to an augmentation in the pathogenicity of *A.actinomycetemcomitans.*

When HPBLs from healthy individuals were exposed to purified leucotoxin from *A.actinomycetemcomitans*, for 90 minutes, the response of the HPBLs to mitogens was significantly reduced (Rabie, Lally and Shenker, 1988). They demonstrated a decrease in the number of viable monocytes, however it would appear that the leucotoxin also had non-lethal effects upon lymphocytes. Rabie *et al* (1988) suggested that the leucotoxin did not directly affect all lymphocytes but only a subset. If this subset included T regulator cells, which were then activated, as suggested by Shenker *et al* (1982b), this could contribute to the pathogenicity of *A.actinomycetemcomitans.*

Yoshie *et al* (1985) demonstrated that B cells proliferated *in vitro* in response to whole *A.actinomycetemcomitans* cells. This proliferation was observed at days 1 and 2 of culture and was shown to be of equal magnitude in cells from immunised normal,

naive normal and nude rats. It was later determined by Eastcott *et al* (1990) that this proliferation was due to Lipopolysaccharides (LPS). It was suggested by Eastcott *et al* (1990) that LPS could have an *in vivo* effect of inducing local B cell proliferation which was independent of any specific immune response to either *A.actinomycetemcomitans* or its products.

1.4.3 *In vivo* killing of *A.actinomycetemcomitans*

Miyasaki *et al* (1986) showed that although *A.actinomycetemcomitans* was resistant to the bactericidal action of serum and oxidant injury, opsonized *A.actinomycetemcomitans* could be killed by neutrophils under both aerobic and anaerobic conditions. Oxidative killing, which is dependent on O_2, H_2O_2 and O_2^-, was shown to be cyanide sensitive, which indicates a myeloperoxidase mechanism (Weiss, Lampert and Test, 1983). Non-oxidative killing was dependent upon a variety of lysosomal enzymes, peptides and proteins Miyasaki *et al* (1986), but the precise mechanism of killing of *A.actinomycetemcomitans* is uncertain.

Miyasaki, Bodeau and Fleming (1991) and Miyasaki and Bodeau (1991) demonstrated that *A.actinomycetemcomitans* was sensitive to the bactericidal effects of different neutrophil granule components and the bactericidal effects of neutral serine proteases. Neutrophil granule extracts were gel filtered and seven fractions were obtained. The killing of *A.actinomycetemcomitans* by the fraction which contained cathepsin, was shown to be independent of oxygen and neutral serine protease activity.

1.4.4 Immunological response to *A.actinomycetemcomitans*

Individuals with juvenile periodontitis have been shown to have elevated levels of IgG, IgA1 and IgA2 antibodies to antigens of *A.actinomycetemcomitans* (Ebersole *et al*, 1982).

Zambon (1985) compiled data from several studies and concluded that 69 to 90% of localised juvenile periodontitis patients had higher serum antibody titres against

A.actinomycetemcomitans compared with lower levels (3 to 13%) in healthy subjects or people with different forms of periodontitis. Wilson (1991) demonstrated that sera from localised juvenile periodontitis patients had IgG antibodies directed against the major outer membrane protein (29kDa) of *A.actinomycetemcomitans*, a protein found in all strains of *A.actinomycetemcomitans* examined.

IgA has two subclasses IgA1 and IgA2 in both monomeric and polymeric forms (Brown *et al*, 1991). IgA1 is susceptible to protease cleavage by microorganisms which may have compromised the host immune system (Kilian and Reinholt, 1986). Serum from juvenile periodontitis patients taken prior to, during and after treatment showed both forms of IgA antibody to *A.actinomycetemcomitans* were present, although the IgA1 form was more common (Brown *et al*, 1991). High levels of IgA1 antibodies could be neutralised by *A.actinomycetemcomitans* proteolytic enzymes which negated the protective effect offered by IgA1.

1.4.5 Serotypes of *A.actinomycetemcomitans*

There have been at least five serotypes of *A.actinomycetemcomitans* described : **a** to **e**. Saarela *et al* (1993) studied a collection of 515 clinical isolates from a Finnish population. The most common serotype present was serotype **c** (41% of the sample population). Serotypes **a** and **b** were found in 25% of the population, with the remaining isolates being characterised as serotypes **d**, **e** or unclassified. Saarela *et al* (1992) showed that it was possible for multiple infection by different serotypes of *A.actinomycetemcomitans* within the same individual.

Slots and Genco (1983) isolated serotype **b** more frequently than serotypes **a** and **c** in North American localised juvenile periodontitis patients: Asikainen *et al* (1991) found serotype **b** predominated in Finnish periodontal patients.

1.5 PLASMIDS

Bacterial plasmids are covalently closed circular DNA molecules, significantly smaller

than (but co-existing with) the bacterial genome. Although not essential for the cell's survival, they may confer ancillary phenotypic functions that are advantageous to the host cell, e.g. antibiotic and heavy metal resistance (Watanabe, 1963. Schottel, 1987) and virulence (Hovi *et al*, 1988. Woodward, McLaren and Wray, 1989). They replicate independently of the host genome and some are capable of cell to cell transfer within a population.

Since bacterial populations are generally regarded as having a clonal structure (Selender & Levin, 1980. Levin, 1981. Orskov & Orskov, 1983. Miller & Hartl, 1986) the plasmid profile (number and size of plasmids harboured by a given strain) is often taken to reflect its history. Thus, generally :

(i) Strains of the same organism with different histories may have accumulated different combinations of plasmids.

(ii) Conversely, identical plasmid profiles among strains is often taken as evidence of epidemiological relatedness.

There are, however, exceptions to this. Some *Salmonella* species have plasmids which are exceptionally stable (Brown, Munro and Platt., 1986). There are, conversely, plasmids which display a high degree of rearrangement (Chart *et al*, 1989).

1.5.1 History

Plasmids were first discovered in *Shigella* species by Japanese researchers in 1959 (cited by Watanabe, 1963). Ochiai *et al* (1959) had shown that it was possible to transfer multiple drug resistance between *Shigella* strains *in vitro*. Watanabe called the transferable genetic elements "episomes".

Previously, Lederberg (1952) had used the term "plasmid" to describe any extrachromosomal hereditary determinant which included chloroplasts in plants and mitochondria in eukaryotes. The term was eventually used exclusively to describe extrachromosomal DNA elements.

1.5.2 Structure

Plasmids are double stranded circular molecules of DNA, usually in the form of a covalently-closed circle (CCC), which is then twisted to form supercoiled DNA. Open circle (OC) is a form of plasmid molecule with a single-stranded nick and is thus in a relaxed state. Other conformations of plasmid molecules include di- and tri-mers.

Plasmids can be classified by many criteria e.g. size; failure to coexist with related plasmids (incompatibility - Datta, 1979; Timmis, 1979); specification of phenotypic traits such as antibiotic resistance genes (Holmberg *et al*, 1984), virulence factors (Hovi *et al*, 1988. Woodward, *et al*, 1989) and metabolic capacities.

Plasmids with a molecular weight less than approximately 15 kb are dependent upon the host cell for most of the enzymes involved in replication, but encode the housekeeping genes which govern their segregation and copy number. Plasmid size and incompatibility is related to the regulation of copy number of each plasmid found in the host cell. For example, large plasmids may have a copy number of only 1 or 2 per genome equivalent whereas smaller plasmids are typically multicopy (15 - 50 per cell). The latter plasmids are under relaxed replication control, which means they accumulate in large amounts (upto 1000 copies per cells) when the bacteria stop growing (Lewin, 1985).

Plasmids have been found in almost every bacterial species in which they have been sought including *A.actinomycetemcomitans* (Olsvik & Preus, 1989; LeBlanc *et al*, 1993; Poulsen *et al*, 1994) and are described in more detail in Chapter 3. Some have a narrow or limited host range and are able to replicate only within a single species : others have a broad range of genera in which they are stably maintained.

1.6 HABITAT AND TRANSMISSION

1.6.1 Habitat

A.actinomycetemcomitans is found predominantly in subgingival dental plaque and periodontal pockets (Slots and Genco, 1984) although it will colonise the oral

mucosal surfaces (Slots *et al*, 1980. Van der Velden, 1986. Christersson *et al*, 1987). Mandell and Socransky (1981) reported the recovery of *A.actinomycetemcomitans* from patients with juvenile periodontitis only, while Slots, Reynolds and Genco (1980) recovered *A.actinomycetemcomitans* from healthy individuals. However, Van der Velden *et al* (1986) reported that in periodontally healthy young adults, who abstained from oral hygiene in one quadrant of their mouths for 23 days, no *A.actinomycetemcomitans* was recovered.

Asikainen, Alaluusua and Saxen (1991) recovered *A.actinomycetemcomitans* from saliva, tongue and subgingival sites of both healthy individuals and those with periodontal disease. Such sites could thus act as a potential reservoir for the recolonisation of subgingival areas.

1.6.2 Transmission of *A.actinomycetemcomitans*

The precise mode of transmission of *A.actinomycetemcomitans* is unknown. Christersson *et al* (1985) showed that it was possible to transfer *A.actinomycetemcomitans* from diseased periodontal sites to healthy ones, within an individual mouth, by means of a periodontal probe. These newly infected sites were sampled for three weeks before colonisation and up to twelve weeks after. However, after two weeks the newly infected sites were found to be negative for *A.actinomycetemcomitans*. This short period of colonisation (1 to 2 weeks) could be due to (i) antagonistic interactions among the resident plaque microflora and *A.actinomycetemcomitans*, (ii) the development of an inhibitory immune response or (iii) competition for available nutrients. Muller, Lange and Muller (1989) examined the toothbrushes of individuals infected with *A.actinomycetemcomitans*, and demonstrated immediately after tooth cleaning the presence of *A.actinomycetemcomitans* on 62% of the brushes. The number of *A.actinomycetemcomitans* isolated correlated weakly with the degree of periodontal destruction. In addition, when *A.actinomycetemcomitans* could be recovered from

saliva and mucosal membranes, the number of *A.actinomycetemcomitans* isolated on toothbrushes was significantly increased.

Studies by Zambon, Christersson and Slots (1983) and Alaluusua, Asikainen and Lai (1991) suggested that *A.actinomycetemcomitans* could be transmitted within families. Zambon, Christersson & Slots (1983) studied 5 families and claimed that each member of the same family had the same strain of *A.actinomycetemcomitans* on the basis of sero- and biotype. It was also suggested by Gunsolley *et al* (1990) that *A.actinomycetemcomitans* might be transmitted between members of the same family in which one or more has an early-onset form of periodontitis. However the sample populations used in all these experiments were very small and therefore no great reliance can be placed upon these results. Saarela *et al* (1993) studied the transmission of oral bacteria, including *A.actinomycetemcomitans*, between spouses, all of whom suffered from advanced periodontitis. They demonstrated that 2 of the 4 couples studied shared *A.actinomycetemcomitans* and *Streptococcus mutans* strains with the same sero- and ribotypes and suggested that transmission of oral bacteria between spouses was possible.

Familial transmission of *A.actinomycetemcomitans* and *Porphyromonas gingivalis* in 4 families was investigated by Pettit *et al* (1993). Most of the adults in the study and all the children suffered from some form of periodontal disease and on the basis of restriction endonuclease restriction patterns, Pettit *et al* (1993) suggested that both *A.actinomycetemcomitans* and *P.gingivalis* were transmitted between parents and children.

Preus and Olsen (1988) isolated *A.actinomycetemcomitans* from 2 members of the same family with Papillon-Lefevre syndrome, as well as from their dog and an unrelated (healthy) 12 year old. On the basis of restriction endonuclease analysis, they stated the strains were identical. The only connection between the two families were that the dogs they owned had come from the same litter. Preus and Olsen (1988) stated that in both cases the dogs were the source of the *A.actinomycetemcomitans*.

This has been the only reported incidence of this potential source of *A.actinomycetemcomitans.*

1.6.3 Association between *A.actinomycetemcomitans* and other bacteria.

A.actinomycetemcomitans appears to be found most often in active periodontal sites and there have been several theories to explain the relationship. Zambon, Christersson, & Slots (1983b) hypothesised that a host response occurred at the sites which are first colonised by *A.actinomycetemcomitans*, but as the immune response develops *A.actinomycetemcomitans* is inhibited from colonising other sites. Alternatively, the colonisation of *A.actinomycetemcomitans* may be inhibited by other bacteria present in the site of potential disease. Hillman and Socransky (1982) demonstrated unnamed oral bacteria, from healthy individuals, which were inhibitory *in vitro*, to the growth of *A.actinomycetemcomitans* strain Y4. Patients, however, with periodontitis were shown to lack these inhibitory organisms.

Hammond *et al* (1984) demonstrated *in vitro* killing of *A.actinomycetemcomitans* by a bacteriocin produced by *Streptococcus sanguis*. Hillman, Socransky and Shivers (1985) described bacterial interactions in plaque from periodontitis patients: the higher the proportion of *S.sanguis* found in a site, the less chance of also locating *A.actinomycetemcomitans*. Later, Hillman and Shivers (1988) found that *S.sanguis* produced hydrogen peroxide, which was inhibitory to the growth of *A.actinomycetemcomitans*. Thus the presence or absence of bacteria capable of inhibiting *A.actinomycetemcomitans* in certain areas of the mouth could help explain the localised nature of some forms of the disease.

1.7 POTENTIAL VIRULENCE FACTORS OF *A.actinomycetemcomitans*

A number of potential virulence factors have been described for *A.actinomycetemcomitans*, which are thought to (i) enable it to colonise the mouth and periodontium (e.g. adhesion - Winkler *et al*, 1988); (ii) adversely affect the host

defence mechanisms (e.g. lipopolysaccharide - Saglie *et al*, 1990) and (iii) cause tissue destruction (e.g. collagenase activity - Robertson *et al*, 1982. Uitto, Suomalainen and Sorsa, 1991). The factors are listed in Table 1.3. Another important factor is a leucotoxin which will be discussed in detail later (1.8).

It is possible that the virulence factors act either alone or in conjunction with one or more of the other factors, which would allow *A.actinomycetemcomitans* to survive and grow in the mouth by evading the host defence mechanisms.

With the exception of the leucotoxin, which has been the most widely studied of the virulence factors, relatively little work has been done on the others.

1.7.1 Adhesion of *A.actinomycetemcomitans*

The ability of *A.actinomycetemcomitans* to adhere to and invade the tissues of the mouth is (obviously) important in the colonisation of this environment.

A.actinomycetemcomitans appears to have the ability to adhere to different oral surfaces - buccal epithelial and tissue culture cells, as well as tooth enamel and hydroxyapatite. There have been several studies into the adhesion of *A.actinomycetemcomitans*, however, different experimental methods have been used, which make accurate comparisons difficult.

Winkler *et al* (1988) demonstrated, *in vitro*, that certain Gram-negative periodontopathic, anaerobes bind to periodontal surfaces in larger numbers than Gram-positive organisms. They suggested that the initial step in binding could be the adhesion of the bacterial fimbriae to the cell membrane. *A.actinomycetemcomitans* has been shown to possess fimbriae (Blix *et al*, 1990) which would enable it to adhere to the periodontal surfaces (see section 1.2.3).

A preliminary study by Rosan *et al* (1988) showed that two fimbriated *A.actinomycetemcomitans* strains adhered to hydroxyapatite in higher numbers (3 - 4 fold differences) than non-fimbriated strains.

VIRULENCE FACTORS	REFERENCE
ADHESION	Winkler *et al*, 1988 Rosan *et al*, 1988 Winkler *et al*, 1988 Taher and MacFarlane, 1991a Taher and MacFarlane, 1991b
PENETRATION	Saglie *et al*, 1982 Gillett & Johnson, 1988
ALVEOLAR BONE RESORPTION	Iino & Hopps, 1984 Wilson, Kamin & Harvey, 1985 Yoshie *et al*, 1987 Kamin, Wilson & Harvey, 1985
COLLAGENASE	Robertson *et al*, 1984 Kamin *et al, 1986* Uitto, Suomalainen & Sorsa, 1990
LIPOPOLYSACCHARIDE	Sveen & Skang, 1980 Iino & Hopps, 1984 Nishihara *et al*, 1989 Saglie *et al*, 1990
BACTERIOCIN	Hammond, Lillard & Stevens, 1987 Stevens, Lillard & Stevens, 1987

Table 1.3 Potential virulence factors (excluding leucotoxicity) produced by *A.actinomycetemcomitans*

Further investigation by Rosan *et al* (1988), however showed no variation in adhesion between strains.

Continued subculture would appear to have more effect upon the adherence of *A.actinomycetemcomitans* to hydroxyapatite *in vitro* than other factors, such as the composition of growth medium (Kagermier and London, 1985). There is evidence that continued subculture can produce non-fimbriated strains with a consequent reduction in adherence. Kagermier and London (1985) further demonstrated that when hydroxyapatite was coated with saliva the ability of *A.actinomycetemcomitans* to adhere was greatly reduced. Rosan *et al* (1988), however suggested that this was not in fact the case. Taher and MacFarlane (1991a) studied the ability of 33 strains of *A.actinomycetemcomitans* (both Type and clinical strains) to adhere to buccal epithelial cells. The adhesion of *A.actinomycetemcomitans* was not altered by the addition of saliva (confirming the results of Rosan *et al*, 1988) however the addition of human serum did have a significant inhibitory effect. It was suggested that human serum had an effect upon the bacterial cell surface and not the host surface, as this effect was seen on a variety of surfaces. The same strains were further examined for their hydrophobic effect (Taher and MacFarlane, 1991b). They demonstrated a hydrophobic effect in all strains studied which was unaltered by repeated subculture, variation in the suspension medium or time of incubation.

Very little has been published on the colonisation of the oral epithelium by *A.actinomycetemcomitans*. Cell surface carbohydrates which are present on the surface of *A.actinomycetemcomitans* as a microcapsule may aid colonisation of the periodontal surfaces (Holt *et al*, 1980). Taher and MacFarlane (1991a) suggested that *A.actinomycetemcomitans* has the ability to adhere to the buccal mucosa before colonisation of the gingival area.

1.7.2 Penetration of the oral epithelium by *A.actinomycetemcomitans*

In 1982 Saglie *et al* and Gillett and Johnson (1982) reported finding

A.actinomycetemcomitans in the gingival tissue and periodontium. Immuno-stained bacteria were found in the soft tissue and it was suggested that these bacteria were *A.actinomycetemcomitans*. No traumatic damage was observed in the tissue surrounding the bacteria, which suggested to Gillett and Johnson (1982) that this was not an artefact, i.e. the bacteria were present in the periodontal tissue before it was sectioned for staining. Later Saglie *et al* (1985) demonstrated the presence of *A.actinomycetemcomitans* and other oral bacteria in the oral epithelium in tissue from juvenile periodontitis patients, with immunoperoxidase techniques. Saglie *et al* (1985) demonstrated different staining patterns of the bacteria which included "spots" and more diffuse areas. They suggested that the diffuse regions were areas where bacterial products were deposited, while the size and shape of the spots were consistent with individual bacteria. The possibility that the presence of bacteria in the epithelium was an artefact was discounted by Saglie et al (1985) since they demonstrated a pattern of bacterial invasion i.e. the bacteria were found in "chains" and wider intracellular spaces. Although the bacteria were found in both healthy and diseased tissue, periodontal diseased tissue showed bacteria in greater than 99% of the sections, while 56% of normal tissues demonstrated bacteria.

Meyer *et al* (1991) reported the ability of *A.actinomycetemcomitans* to invade a human oral cell line (KB - derived from a human oral epidermoid carcinoma). Thirty nine different *A.actinomycetemcomitans* strains were investigated and several factors were thought to have an effect upon the ability of *A.actinomycetemcomitans* to invade tissue. These included (i) the effect of temperature, (ii) the presence of either cytochalasin D (an inhibitor of microfilament formation and phagocytic like activity) and (iii) cycloheximide (an inhibitor of mammalian cell protein synthesis - but not of prokaryotic protein synthesis). A reduction in the invasion of *A.actinomycetemcomitans* was seen after incubation with Cycloheximide and at a reduced temperature. Meyer *et al* (1991) concluded from these results that metabolically active cells (both bacterial and host cells) were needed for invasion to

occur. The addition of cytochalasin D inhibited invasion of the monolayer by up to 99%, which suggested that invasion by *A.actinomycetemcomitans* occurs through a microfilament-dependent, phagocytic process. A negative control consisting of *H.aphrophilus* was used and neither the addition of cytochalasin D nor cycloheximide reduced the percentage invasion of *A.actinomycetemcomitans* to the level recorded for this negative control. Differences in the ability of rough and smooth colonies of *A.actinomycetemcomitans* to invade the KB monolayer was also investigated; smooth variants derived from rough strains invaded the cell line to a greater degree than did the original rough strains.

1.7.3 Collagenase activity of *A.actinomycetemcomitans*

The collagenolytic activity of *Porphorymonas gingivalis* has been studied in some detail, but very little has been reported for *A.actinomycetemcomitans*. Robertson *et al* (1982) investigated *A.actinomycetemcomitans* and a number of other oral bacterial species for their collagenolytic status. They found that only *A.actinomycetemcomitans* and *Bacteroides* species showed any collagenase activity. This activity was found in the supernatant of the cultures in which the strains were grown and in the supernatant of cell sonicates. It was inhibited by the addition of EDTA and by boiling for 3 minutes and characterised as being heat-labile and partially inhibited by serum. In addition the collagenase activity could be enhanced by growth in a peptide-depleted medium.

There are two potential pathways for collagen loss in periodontal disease (i) a decrease in production (Page and Schroder 1973) or (ii) a reduction due to an increase in collagenase activity by bacterial components (Robertson and Simpson, 1976. Uitto, Suomalainen and Sorsa, 1990). Suomalainen and Sorsa (1991) studied 10 freshly isolated strains of *A.actinomycetemcomitans* and found collagenase was released from normal human PMNLs when exposed to the *A.actinomycetemcomitans* in vitro.

1.7.4 Cellular effects of capsular material and lipopolysaccharides (LPS) from *A.actinomycetemcomitans*

Capsular material and LPS from *A.actinomycetemcomitans* were isolated by Kamin *et al* (1986) and examined for their effect upon gingival fibroblasts. These workers demonstrated that capsular material had a potent inhibitory effect upon fibroblast proliferation, whereas LPS had very little effect. The capsular material inhibited both DNA and collagen synthesis - and increased cell size. This result agreed with the previous work of Shenker, Kusher and Tsai (1982c) and Stevens, Gatewood and Hammond (1983) who concluded that the fibroblast-inhibitory activity was due to neither an endotoxin nor a leucotoxin. Stevens *et al* (1983) demonstrated that the fibroblast-inhibitory material irreversibly inhibited cell proliferation in the presence of serum. Also DNA synthesis was decreased, but RNA and protein synthesis were unaffected. Although the cells appeared to be intact and viable, no proliferation of fibroblasts was seen.

It was reported by Saglie *et al* (1990) that low concentrations of LPS from *A.actinomycetemcomitans* stimulated human macrophages to produce interleukin-1 (IL-1) and tumour necrosis factor (TNF) mRNA. Protein levels of IL-1 and TNF also increased, both of which are potent monokines that mediate inflammation and resorption of bone. It was suggested by Saglie *et al* (1990) that macrophages which migrate to the site of periodontal infection are stimulated by the LPS of *A.actinomycetemcomitans* to produce IL-1 and TNF, which mediate gingival inflammation and alveolar bone resorption. It was suggested by Rifkind and Hiejl (1979) that macrophages were involved in bone destruction characteristic of periodontal disease.

Bacterial products (e.g. LPS) which may enter the periodontium via the gingival crevice have been related to bone resorption (Iino and Hopps, 1984). The loss of bone could be due to mediators of bone resorption e.g. lymphokine and prostaglandins released during an immune response or may have been the direct effect of bacterial

products e.g. LPS.

Capsular material, extracted from *A.actinomycetemcomitans* was demonstrated to be a potent bone resorption mediator (Wilson, Kamin and Harvey, 1985) active at concentrations one thousand-fold lower than the minimum concentration of lipopolysaccharide found to stimulate bone resorption (Wilson *et al* 1985. Iino and Hopps 1984). The capsular material, although not completely defined, was shown to be a mixture of protein and carbohydrate.

Lipopolysaccharides from other oral bacteria are known to resorb bone (Sveen and Skang, 1980). Iino and Hopps (1984) demonstrated the *in vitro* release of calcium from cultivated living bones, by the application of LPS from *A.actinomycetemcomitans*, while there was no release of calcium from dead bone. They suggested that bone-resorption mediated by LPS was dependent upon mechanisms in the living cell. Iino and Hopps (1984) noted that although the chemical composition of the LPS from oral bacteria differed from that of the enterobacteria, the potency of the different LPSs were about the same.

The effect of antibodies, directed against LPS of *A.actinomycetemcomitans*, upon the mitogenic responses of murine spleen cells to LPS and lipid-A of *A.actinomycetemcomitans* was studied by Nishihara *et al* (1989). This antiserum was shown to inhibit the mitogenic effect - as did normal rabbit serum, but a negative reaction was found with normal rabbit immunoglobulin. Nishihara *et al* (1989) speculated that the effect of serum from humans would be inhibitory but no evidence was presented. They also suggested that antibodies against *A.actinomycetemcomitans* LPS could modify the immune response of lymphocytes at periodontally diseased sites.

1.7.5 Bacterocin activity of *A.actinomycetemcomitans*

The virulence factors of *A.actinomycetemcomitans* which have been most fully studied are those which have an effect upon the human host defence mechanisms. However, a

bacteriocin, active *in vitro* against certain *Streptococci*, *Actinomyces* and other *A.actinomycetemcomitans* strains was isolated from *A.actinomycetemcomitans* strain Y4, by Hammond, Lillard and Stevens (1987). This was purified by Stevens, Lillard and Hammond (1987) and shown to be a protein comprising of 2 subunits, with molecular weights of 50 000 and 70 000 kDa respectively. The bacteriocin from *A.actinomycetemcomitans* interfered with the colonisation and growth of certain *Streptococcal* species e.g. *S.sanguis* and was also antagonistic to the growth of other *A.actinomycetemcomitans* strains (Hammond *et al*, 1987). The bacteriocin appeared to share some similarities with leucotoxin; the molecular weight of each is approximately the same and both consist of two subunits (the subunits have approximately the same molecular weights). As with the leucotoxin, the bacteriocin is heat inactivated and sensitive to proteolysis (Hammond *et al*, 1987). However, Hammond *et al* (1987) concluded that the bacteriocin and leucotoxin were different from each other because of the different target cells involved.

1.8 LEUCOTOXIC ACTIVITY OF *A.actinomycetemcomitans*

Baehni *et al* (1979) demonstrated that a strain of *A.actinomycetemcomitans* (designated Y4) which was derived from a patient with juvenile periodontitis was toxic to human polymorphonuclear leucocytes *in vitro*. This was later shown by Tsai *et al* (1979) to be due to a heat and protease sensitive, soluble product, toxic only to human PMNs and monocytes. Human lymphocytes, platelets and fibroblasts as well as rabbit, rat, chicken and mouse leucocytes were unaffected by this substance (Baehni *et al*, 1979., Tsai *et al*, 1979.). The toxic effect of the material was shown to be heat labile, (Baehni *et al*, 1979), and dose and time dependent (Tsai *et al*, 1979., Taichman, Dean and Sanderson, 1980).

Taichman *et al* (1980) stated that as the leucotoxin was inhibited by heat, it was not a lipopolysaccharide. Studies of *A.actinomycetemcomitans* by Taichman *et al* (1980) also demonstrated that the leucotoxin bound to the target cell membranes, which

resulted in morphological and biochemical changes and ultimately in cell death. This led Taichman *et al* (1980) to propose 3 stages in monocyte-killing by the leucotoxin. The first, occurred within the first five minutes of exposure of the cells to the leucotoxin and resulted in the cessation of normal cell movement. After approximately 15 minutes, cells which had been labelled previously with ^{86}Rb and ^{51}Cr, were shown to have released all ^{86}Rb activity which was accepted as an early indicator of cell damage. The second phase, which lasted for up to an hour, was characterised by the lack of appreciable cytoplasmic leakage, cell movement and the formation of cytoplasmic threads which emanated from the cell. The final phase was characterised by the release of the cytoplasmic contents - measured by LDH, lysozyme and ^{51}Cr release. It was postulated that the early release of ^{86}Rb, indicated that damage had occurred in the Na/K transport system. (Taichman *et al*, 1980). Toxin was also shown to bind to cells at +4°C and after extended incubation, cell death was observed. Endocytosis was suppressed by the addition of cytochalasin B and incubation at +4°C which indicated that the toxin may not require to be internalised for cell lysis to occur, although small amounts of toxin could still have entered the cell by micropinocytosis (Taichman *et al*, 1980).

Taichman *et al* (1991) showed that the likely site for the action of the leucotoxin was the plasmalemma, where its action resulted in the formation of pores (approximately 1 nm) with subsequent colloid lysis. The finite pore size would allow only small molecular weight solutes to equilibrate between the cytoplasm and medium (Iwase *et al*, 1990), which is the typical action of an RTX toxin (Repeats in ToXins - see 1.8.7).

The toxic effect of the Y4 leucotoxin was altered by the addition of heated or unheated sera from either healthy or juvenile periodontitis patients, (McArthur *et al*, 1981). The presence of heated/ unheated healthy human and rabbit sera enhanced the toxic effect, but sera from juvenile periodontitis patients and immune rabbit serum resulted in neutralisation of the toxin.

The degree of enhancement or inhibition of the toxin varied with the concentration of

serum used. Certain juvenile periodontitis sera at concentrations below 2.5% (v/v) enhanced the toxic effect, but above this level toxicity was inhibited (McArthur *et al*, 1981). The inhibitory activity in juvenile periodontitis sera was shown to be due to an IgG antibody whereas the factor which was responsible for enhancement of activity was found to be a large molecular weight protein. No further work on this factor has been reported to date.

Baehni *et al* (1981) tested whole cell and sonicates of Type cultures and fresh isolates of *A.actinomycetemcomitans* (14 strains in total), for their toxic activity on PMNs, as determined by LDH release. They demonstrated that leucotoxicity could be enhanced or inhibited by the presence of normal or juvenile periodontitis/ rabbit anti-*A.actinomycetemcomitans* sera respectively. They also demonstrated that strains of *A.actinomycetemcomitans*, which were leucotoxin positive, possessed antigens which were not identified in non-leucotoxic strains. Also, the majority (ten out of fourteen) of their isolates were toxic and suggested that the variation seen could be due either to the presence of plasmids or to bacteriophage infection although they offered no evidence for either. The presence of bacteriophage in *A.actinomycetemcomitans* strains was later shown by Preus, Olsen and Namork (1987), in a very small sample but was not correlated to the toxic activity of the infected strains of *A.actinomycetemcomitans*.

Zambon *et al*, (1981) used the promyelocytic HL-60 cell line, derived from a 36 year-old Caucasian woman, with acute promyelocytic leukaemia (Collins, Gallo & Gallagher, 1977., Gallagher *et al* 1979) as an alternative to human PMNs to demonstrate the toxicity of *A.actinomycetemcomitans*, as determined by the release of LDH. With the tissue culture cell line the effect of the leucotoxin was shown to be dose and temperature dependent and inhibited by the addition of anti-*A.actinomycetemcomitans* rabbit serum. However, enhancement of the toxic effect was not seen in HL-60 cells, when human serum was added. Taichman *et al* (1980) speculated that the enhancement of the toxic effect seen in human PMNs was due to

the presence of host cell receptors. It was suggested by Zambon *et al* (1983) that these surface receptors were not present on HL-60 cells, which may have become altered when the cell line became established. Alternatively, one of the growth factors in the medium used for the cultivation of the HL-60 cells may have inhibited enhancement of toxicity by, for example, binding to the neutrophil receptors proposed by Taichman *et al* (1980).

1.8.1 The frequency of isolation of toxic stains of *A.actinomycetemcomitans*

The presence of leucotoxic strains of *A.actinomycetemcomitans* in plaque samples from healthy gingival sites suggested that the role of the organism in periodontal disease is not a simple cause and effect relationship. Zambon *et al* (1983) demonstrated leucotoxicity in 16% of *A.actinomycetemcomitans* isolated from healthy subjects. No age was given for these subjects. This contrasts with Tervahartiala *et al* (1988) who reported that 50% of the *A.actinomycetemcomitans* strains isolated from healthy children, were toxic. A decrease in the prevalence of leucotoxic strains with age was demonstrated by Tsai and Taichman (1986), who showed that younger juvenile periodontitis patients (age 6 - 12 years of age) had more leucotoxic strains, than older patients (13 - 25 years of age. Tervahartiala *et al*, 1988). All patients, however, had antibodies to the leucotoxin. The overall sample population used by Tervahartiala *et al* (1988) was smaller than the population of Zambon *et al* (1983) - only 19 isolates (6 from healthy children) compared with 100 isolates (11 from healthy subjects).

Seventy-five percent of *A.actinomycetemcomitans* strains isolated from insulin dependent diabetes mellitus periodontal patients, were shown to be toxic to healthy PMNLs (Zambon *et al*, 1983). However, the population size used by Zambon *et al* (1983) was only 4 patients. Mashimo *et al* (1988) demonstrated *A.actinomycetemcomitans* infrequently in diabetics.

1.8.2 Extraction of leucotoxin from *A.actinomycetemcomitans*

The toxin from *A.actinomycetemcomitans* was isolated by Tsai *et al* (1984) who demonstrated that it had a molecular weight of 115 kDa, by SDS PAGE analysis. It was extracted using polymyxin B, in an adaptation of a method to solubilize the enterotoxin of *E.coli*. Polymyxin B is a cyclic decapeptide that disrupts membranes, which indicated that the toxin was associated with the cell surface. This was further demonstrated by the work of Ohta *et al* (1991) who used both RNAase and DNAase to release the toxin bound to the bacterial cell surface. Here the nucleases probably had the same function as the polymyxin B, as nucleases do not work exclusively upon nucleic acid but also recognise and disrupt basic bonds. Thus it is likely that basic bonds on the bacterial cell surface are broken during extraction, releasing the toxin, rather than by disruption of leucotoxin complexes in the nucleic acid on the cell surface as suggested by Ohta *et al* (1991). Furthermore, there is no evidence that the latter complexes occur on the surface of *A.actinomycetemcomitans* and the evidence for their presence in other bacterial species is slight. Whereas DNA has been found internalised in membrane vesicles from *Haemophilus influenzae* - these were resistant to digestion by external nucleases (Kahn, Barany and Smith, 1983). The vesicles produced by *N.gonorrhoeae* are associated with DNA and are hydrolyzed by external DNAase. However these vesicles are not associated with leucotoxin, but with the inter cell transfer of genetic material (Dorward, Garon and Judd, 1989).

1.8.3 Effect of bacterial and colonial morphology upon leucotoxicity of *A.actinomycetemcomitans*

Ohta *et al* (1987) studied *A.actinomycetemcomitans* strains with different colonial morphology (e.g. rough-surfaced, umbonate-shaped colonies and smooth-surfaced, convex-shaped colonies) to determine any correlation between morphology and leucotoxin production. None was found. Tervahartiala *et al* (1989) found a similar lack of correlation between the leucotoxic activity of *A.actinomycetemcomitans*

strains and the numbers of outer membrane vesicles present or the original source of the strain (e.g. juvenile or adult periodontitis or healthy children).

1.8.4 The effect of *A.actinomycetemcomitans* leucotoxin on T cells.

The *in vitro* effect of the leucotoxin on PMNs has been well studied, but the period of exposure of PMNs to the leucotoxin, *in vitro*, has generally been limited to a few hours. Mangan *et al* (1991) studied the *in vitro* effect of the leucotoxin on T lymphocytes over a 2 day period. Cell death of 70% was demonstrated and occurred in a time and concentration dependent manner.

1.8.5 Localisation of leucotoxin on *A.actinomycetemcomitans.*

Berthold *et al* (1992) localised the leucotoxin of *A.actinomycetemcomitans* to either the cell envelope and/ or membrane vesicles on the outer surface of the bacterial cell, using monospecific polyclonal antibody with electron microscopy and immunocytochemical techniques. Lai *et al* (1981) had previously noted that vesicles were more common on the cell membrane of toxic strains, than non-toxic.

1.8.6 Effect of *A.actinomycetemcomitans* on *Macaca fascicularis* - animal model

Taichman *et al* (1984) reported that monkeys (*Macaca fascicularis*) with no evidence of periodontal disease harboured *A.actinomycetemcomitans* in subgingival plaque samples. However no information was presented concerning the population size, the percentage of monkeys positive for *A.actinomycetemcomitans* or the number of *A.actinomycetemcomitans* isolates found. Ebersole *et al* (1990) found *A.actinomycetemcomitans* in approximately 40% of the monkey population they examined.

Monkey PMNs were killed when exposed to sonic extracts of *A.actinomycetemcomitans* from leucotoxic positive isolates. Furthermore, sera from human juvenile periodontitis patients were also shown to be able to inhibit the

leucotoxic killing of monkey PMNs. The ability of monkey sera to inhibit leucotoxic activity was shown to be due to IgG antibodies (Taichman *et al*, 1984). As a result it was suggested that these monkeys could act as an animal model for studying the host-*A.actinomycetemcomitans* relationship. Taichman *et al* (1987), later studied twenty one different non-human primate species (one hundred and twelve individual monkeys) which encompassed New and Old World monkeys and Greater and Lesser Apes. *A.actinomycetemcomitans* was recovered from both New and Old World monkeys (no statistics were given). All *A.actinomycetemcomitans* isolated from the non-human primates were shown to provoke a dose-dependent release of ^{51}Cr from labelled human PMNs and HL-60. The leucotoxin had no effect upon PMNs from the New World monkeys and Lesser Apes, however it was toxic to PMNs from the Great Apes and most Old World Monkeys. With the exception of lemurs and cebus monkeys, all the primates possessed neutralising antibodies against the leucotoxin.

1.8.7 *A.actinomycetemcomitans* leucotoxin and other pore forming toxins

The *A.actinomycetemcomitans* leucotoxin belongs to a group of toxins called RTX (Repeats in Toxin) (Welch, 1991) characterised by a glycine-rich C-terminal repeat unit at the end of each protein (Strathdee & Lo, 1989). Further features include the creation of pores in the cell membrane of target cells (Iwase *et al*, 1990), although this is not an exclusive feature as the haemolysins of *Proteus mirabilis* and *Serratia marcescens* are both pore-forming cytolysins but do not belong to the RTX group (Uphoff and Welch, 1990). The operon of the genes involved in all aspects of synthesis, activation and toxin secretion, are located together on the chromosome (Kraig, Dailey and Kolodrubetz, 1990). *A.actinomycetemcomitans* leucotoxin differs from the other RTX toxins, as, unlike the other RTX toxins, it remains associated with the bacterial cell (Lally *et al*, 1991b). The mechanism for secretion has not yet been determined (Brogan *et al*, 1994).

The toxins which have been shown to belong to this family are produced by several

pathogenic Gram negative bacteria, and cover a wide range of organisms and toxins e.g. *E.coli* - haemolysin (Felmlee and Welch, 1988), *Pasturella haemolytica* - leucotoxin (Strathdee and Lo, 1989), *Actinobacillus pleuropneumoniae* - haemolysin (Frey and Nicolet, 1988) and *Bordetella pertussis* - adenylate cyclase (Glasser *et al*, 1988). These bacteria produce cytotoxins of 102 - 177 kDa, which are major virulence factors for each organism - see Table 1.4.

Despite the high degree of DNA homology seen between the different RTX genes, the LktA protein from *A.actinomycetemcomitans* is very much more basic than the *E.coli* HktA protein. This may be due to the evolutionary divergence of *A.actinomycetemcomitans*, from the other bacteria which form the RTX group. The differences may have a role in the pathogenicity of *A.actinomycetemcomitans* in the gingival crevice (Ebersole *et al*, 1990., Kraig *et al*, 1990). Possible differences may be related to target cell specificity, mode of secretion of the toxin (Kraig *et al*, 1990) or localisation of the leucotoxin in the periplasm (DiRienzo *et al*, 1985).

1.8.8 Structure of leucotoxin operon

The original classification of the RTX toxins was on the basis of the glycine-rich repeat units at the C-terminal end of the protein (Strathdee, 1989). The glycine-rich repeat unit sequences are a conserved feature of RTX toxins, although the number of repeat units varies between toxins e.g. *A.actinomycetemcomitans* has 14 repeat units, *E.coli* has 13 repeat units and *P.haemolytica* has 8 repeat units (Lally *et al*, 1991a). Each repeat unit, of nine amino acids, has been represented by the overall consensus sequence of L-X-G-G-X-G-N-D-X (Lally *et al*, 1991a. Forestier and Welch, 1991. Welch, 1991), when arranged in a tandem manner with other units in the protein. At the amino acid level, the consensus sequence is highly conserved (Lally *et al*, 1991a). This region is thought to be important in the toxicity of the *A.actinomycetemcomitans* leucotoxin - a view strengthened by the observation that a panel of seventeen monoclonal antibodies, selected on the basis of inhibition of leucotoxin activity

Bacterium	Toxin	Number of amino acids	Molecular Weight (kDa)	Total number of Glycine Repeats
(a) Aa	Leucotoxin (LktA)	1055	116	14
(b) Bp	Adenylate cyclase (CycA)	1706	177	41
(c) Ec	Haemolysin (HlyA)	1023	110	16
(d) Ph	Leucotoxin (AktA)	953	102	12
(e) Ap	Haemolysin (AppA)	956	103	9

Table 1.4 Features of the Cytotoxins of the RTX Group

(a) *A.actinomycetemcomitans* : Lally *et al*, 1989
Kraig, Dailey & Kolobetz, 1990
Lally *et al*, 1991

(b) *B.pertussis* : Glasser *et al*, 1988
Lausch, Paulaitis & Friedman , 1990

(c) *E.coli* : Femlee, Pellet & Welch, 1986
Mackman *et al*, 1986
Femlee & Welch, 1988

(d) *P.haemolytica* : Strathdee & Lo, 1987
Strathdee & Lo, 1989

(e) *A.pleuropneumoniae* : Frey & Nicolet, 1988
Chang, Young & Stuck, 1989
Lalonde *et al*, 1989

(Direnzo *et al*, 1985) all recognise this region in the homologous protein as well as in deletion mutations of the *E.coli* -haemolysin (Felmlee & Welch, 1988). It was suggested by Ludwig *et al* (1988) that the repeat domain of the *E.coli* -haemolysin was responsible for the Ca^{2+} dependent binding of the toxin to erythrocytes. Lally *et al* (1981) showed the repeat units from *A.actinomycetemcomitans* bound Ca^{2+} in a dose dependent manner, which could be inhibited by heat or the addition of SDS or mercaptoethanol.

The role of the repeat unit and Ca^{2+} ions for the haemolytic activation of the toxin from *E.coli* has been investigated more thoroughly. The removal of one unit resulted in a requirement for an elevated Ca^{2+} ion concentration, while the removal of three units resulted in a protein with no haemolytic activity, even in the presence of very high Ca^{2+} ion concentrations.

The most widely studied toxin of the family is the haemolysin (Hly A) of *E.coli*. This is a 110 kDa protein, the product of the *hlyA* gene, which is secreted across the cytoplasm and outer membranes, and which required the product of two further genes, *hlyB* and *hlyD*. The gene *hlyC* produced a protein which has been shown to be responsible for post-translational conversion of the protein from the inactive to the active form (Welch and Pellet, 1988).

All members of the RTX family show a high degree of sequence homology to the *hly* operon of *E.coli*- Figure 1.1 - with *B.pertussis* showing variation in the *cycA* and *cycC* gene arrangements. It also has an additional gene, *cycE* contiguous to *cycABD* (Coote, 1992). The gene for the leucotoxin was cloned first by Kolodrubetz *et al* (1989) then by Lally *et al* (1990). Kraig, Dailey and Kolodrubetz (1990) sequenced the *lktA* gene and showed it was linked to the *lktC* gene. The homology between the proteins from different RTX toxins was found and these are listed in Table 1.5.

Chang, Young and Struck (1991) demonstrated that *A.pleuropneumoniae* haemolysin

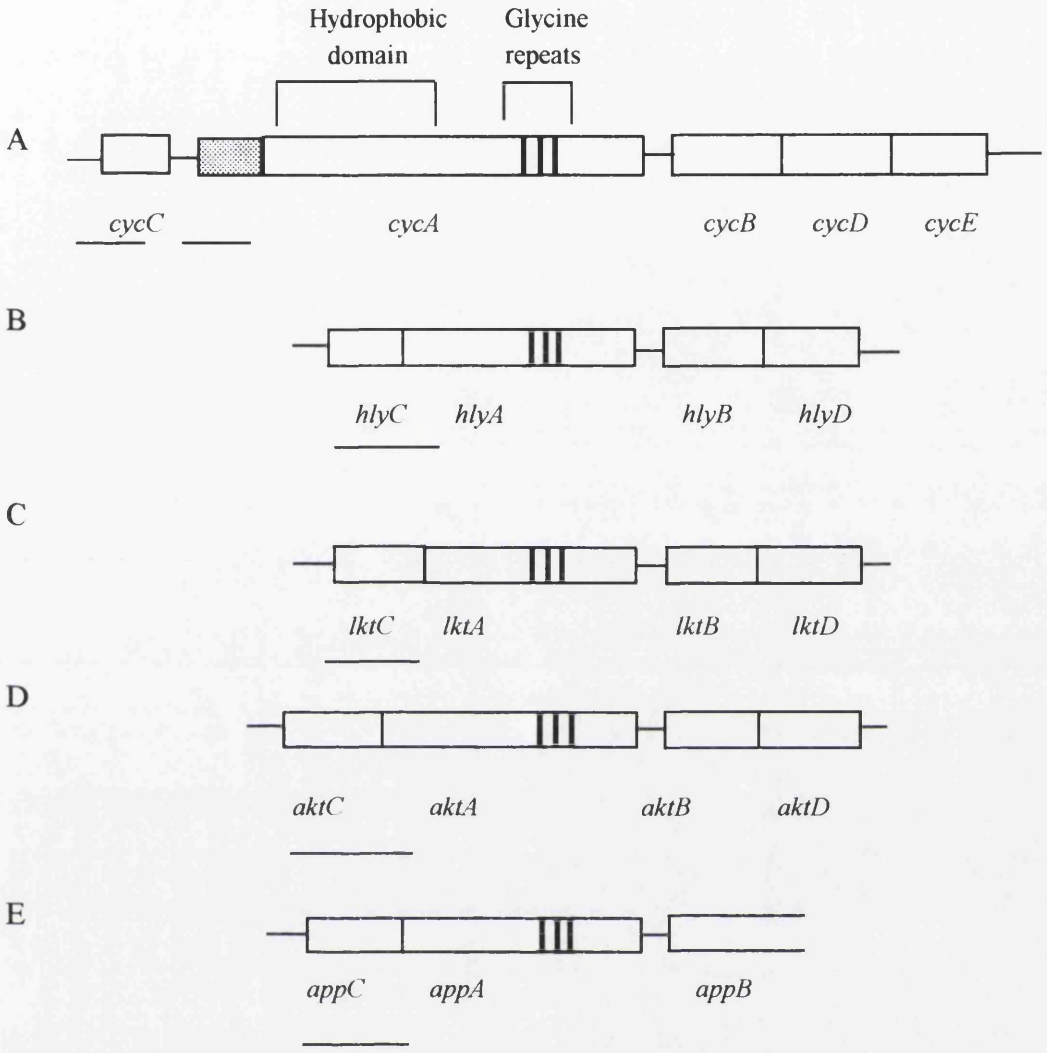

Figure 1.1 Gene arrangements of the RTX cytotoxin determinants

A : *B.pertussis* - adenylate cyclase
B : *E.coli* - haemolysin
C : *P.haemolytica* - leucotoxin
D : *A.actinomycetemcomitans* - leucotoxin
E : *A.pleuropneumoniae* - haemolysin

In each case, the *A* gene is the toxin structural gene; *C* is required for toxin activation and the *BDE* gene products are required for toxin secretion

Arrows indicate direction of transcription.

After Coote, 1992

Protein A	Ec	Ap	Ph
Aa	51%	42%	43%

Protein B	Ec
Aa	67%

Protein C	Ec	Ap	Ph
Aa	70%	55%	55%

Protein D	Ec
Aa	72%

Table 1.5 Homology between the cytotoxin proteins

From : Ebersole *et al*, 1990
Kraig, Dailey & Kolodrubetz, 1990

Aa : *A.actinomycetemcomitans*
Ec : *E.coli*
Ap : *A.pleuropneumoniae*
Ph : *P.haemolytica*

genes *appBD* do not lie downstream from the haemolysin structural genes *appA*, although there is a high degree of homology at the DNA and amino acid sequence level of these genes and the characterised *BD* genes from other RTX toxins.

1.8.9 Transcription

Variation seen in leucotoxin production from *A.actinomycetemcomitans* may be partially explained due to the lack of expression of one of the genes *lktB*, or *lktC* responsible for the secretion of the toxin (Spitznagel *et al* 1991). Leucotoxin negative variants appear to be very common amongst *A.actinomycetemcomitans* strains (greater than 80% ; Zambon, 1985).

Spitznagel *et al* (1991) examined a total of 5 leucotoxin positive and negative strains and all were shown to possess the entire operon. Leucotoxin positive strains, by RNA blot hybridisation analysis, were shown to produce two RNA transcripts, one of 9.3 kb in length, the other 4.3 kb, which encodes *lktCABD* and *lktCA*, respectively. The predominant mRNA of 4.3 kb encode *lktC* and *lktA* and terminates at a rho-independent transcriptional terminus, situated between *lktA* and *lktB* (Lally *et al*, 1991b. Spitznagel *et al*, 1991). The less abundant 9.3 kb mRNA encodes for *lktCABD*, results when transcription fails to terminate this at the rho-independent transcriptional terminator and continues to transcribe through *lktB* and *lktD*. This results in a lower level of *lktB* and *lktD*. When leucotoxic negative strains were examined by RNA blot hybridisation, they demonstrated a 50-fold lower level of the 4.3 kb *lktCA* transcripts and no 9.3kb *lktCABD* transcripts.

Brogan *et al* (1994) compared the *lkt* promoters of JP_2, a highly toxic strain, to a strain which demonstrated little toxicity, and demonstrated variation in the promoters. They found that the JP_2 *lkt* operon was transcribed from two promoters, whereas the non-toxic strain only had one promoter. Additionally, there appeared to be a domain of 530 base pairs in the non-toxic promoter, not found in the JP_2 *lkt* operon. They suggested that the JP_2 *lkt* promoter could have arisen from the deletion of a 530 base

pair sequence found in the non-toxic strain. Brogan *et al* (1994) suggested that the level of toxicity appeared to be related to the *lkt* promoter structure, but they could not rule out the effect of other factors, such as *trans*-acting proteins, from having an effect upon the expression of the *lkt* operon.

The leucotoxic effect of *A.actinomycetemcomitans* strains was shown by Spitznagel *et al*, (1991) to be related to the growth phase. High levels of the RNA transcript were demonstrated in positive strains during the mid- to late log phase, which corresponded to high levels of toxin activity. Both levels decreased during stationary phase, which could suggest that an environmental factor is also involved in the expression of the toxin. This is similar to the situation in *E.coli* where the haemolysin toxin peaks in late exponential growth, then declines (Nicaud *et al*, 1985).

Spitznagel *et al* (1991) suggested that the growth phase is important in determining the toxicity of *A.actinomycetemcomitans*. ATCC 29523, previously reported as variable for toxin production (Baehni *et al*, 1981., Zambon *et al*, 1983., Zambon, 1985) demonstrated low levels of toxic activity when the strain was grown to late log phase. The leucotoxin, when compared to JP_2 leucotoxin (a very toxic strain) (Spitznagel *et al*, 1991) was shown to be 30-fold less active than the most active JP_2 extract.

The maximal levels of *lkt* mRNA in ATCC 29523 were found in the same sample that had leucotoxic activity.

A previously designated leucotoxic negative strain, ATCC 33384 (Zambon, Slots and Genco, 1983b) was shown to have at most 1/90th of the leucotoxic activity of the most active JP_2 sample. The maximal level of *lkt* mRNA from ATCC 29523 is slightly higher than the level of *lkt* mRNA from ATCC 33384, which could explain why ATCC 29523 had detectable levels of leucotoxic activity while ATCC 33384 did not. These results led Spitznagel *et al* (1991) to conclude that different levels of leucotoxin activity found in different strains of *A.actinomycetemcomitans* are due to differences in the levels of *lkt* mRNAs.

The *P.haemolytica* leucotoxin gene has been shown to be regulated at the transcriptional level by changes in temperature, pH and iron concentration (Strathdee and Lo, 1989b). All of these could contribute to the variable toxicity *in vitro* by differences in growth medium. There is no published data to suggest that this is the method of regulation of the leucotoxin from *A.actinomycetemcomitans.*

1.8.10 Activation

The mechanism for activation of leucotoxin in *A.actinomycetemcomitans* has not been widely studied, but information is available about other RTX toxins.

However, the precise mechanism for activation of the toxins is unknown. The toxins are synthesised as inactive products from the respective genes, which are then modified by the **C** gene product, to the active form. The **C** gene products are highly conserved (Kraig *et al,* 1990) and it is possible for the **C**, **B** and **D** gene products from *E.coli* to activate and export the inactive toxins of *P.haemolytica* and *A.pleuropneumoniae in vivo* (Forestier and Welch, 1991,. Gyri *et al,* 1990., Highlander, Engler and Weinstock, 1990).

The activation of the *E.coli* haemolysin has been studied most fully and an understanding of this process has been achieved. Hardie *et al* (1991) showed *in vitro* activation of the *E.coli* haemolysin to be post transcriptional, cytoplasmic and requiring a cellular factor. The latter could be removed by dialysis and was identified as an acyl carrier protein (Issartel, Koroakis and Hughes, 1991) which donates an acyl fatty acid to the prohaemolysin. Although the activating factor was specific for the enterobacteria only, it is most probable that the process of activation is the same with the other RTX toxins.

Without modification, the toxins from *E.coli*, *P.haemolytica* and *B.pertussis*, are unable to bind to the target cell membranes. (Cruz *et al,* 1990., Boehm, Welch and Snyder, 1990., Oropeza-Wekerle *et al,* 1989). Pellet *et al* (1990) showed the change from the inactive to active form to be a conformational one. Modified HlyA protein

was bound by a specific monoclonal antibody, in the region of the Ca^{2+} binding repeat domains. Pellet *et al* (1991) suggested that an interaction may have occurred at the Ca^{2+} binding repeat domains, which may have created the final conformation required for binding and membrane insertion of the toxin.

1.8.11 Toxin secretion

One major difference between the *A.actinomycetemcomitans* leucotoxin and the other RTX toxins is in the method of secretion. *A.actinomycetemcomitans* leucotoxin (LktA) is located in the periplasm (DiRienzo *et al*, 1985. Berthold *et al*, 1992). Many of the basic amino acids which give LktA the pI value 9.7 (Ebersole *et al*, 1990) - compared to pIs of approximately 6.2 for the *lktA* protein from other bacteria - are at the C-terminal end (Kraig *et al*, 1990). The carboxy terminal 250 amino acids of the haemolysin from *E.coli* are essential for the secretion and lytic activity of the haemolysin (Felmlee and Welch, 1988). The least conserved region between the *E.coli* haemolysin and *A.actinomycetemcomitans* leucotoxin extends over the last 200 amino acids of LktA, which may be responsible for the different properties that LktA has been shown to possess e.g. target cell specificity and localisation to the periplasm (Tsai et al, 1984. Simpson et al, 1988).

The export of the *lktA* protein from E.coli has been studied in some detail. In the two step process, translocation of the protein to the periplasm is accompanied by loss of the leader protein followed by the transfer of the protein across the outer membrane by a specific mechanism (Pugsley and Schwartz, 1985., Hirst and Welch, 1988., Holland, Blight and Kenny, 1990).

The products of *hlyB* and *hlyD* genes and the *tolC* (the *tolC* gene is unlinked to the operon) are involved in secretion of HlyA (Welch, 1991) from *E.coli*. *hlyB*, *hlyD* and *tolC* are thought to form a pore in the bacterial cell membrane for the export of the toxin.

1.8.12 Mode of action

Iwase *et al* (1990) demonstrated that the association of the toxin with the target cell membrane was insufficient for cell lysis to occur, as it could be inhibited by the addition of monoclonal antibodies directed against the toxin, or by washing the toxin from the cells, during the first five minutes of toxin interaction with the plasma membrane. The addition of cations e.g. Ca^{2+} and Zn^{2+} appeared to minimise the extent of the toxin-mediated membrane damage. Certain saccharides, dependent upon size, were able to act as osmotic protectants e.g. maltose almost completely inhibited cytoplasmic release. This led to the theory that the leucotoxin of *A.actinomycetemcomitans* had a membranolytic activity, capable of producing pores in the target cell membrane with a diameter approximately that of maltose - 0.96 nm. Other RTX toxins produce pores with diameters of 0.6 - 3.0 nm (Ehrmann *et al*, 1991,. Clinkbeard, Mosier and Confer, 1989., Lalonde *et al*, 1989., Bhakdi *et al*, 1986).

1.8.13 Evolution of RTX toxins

On the basis of similarity between the toxins of *A.actinomycetemcomitans* and *E.coli* - (Table 1.5) Kraig *et al* (1990) suggested that a recent transfer of the leucotoxin gene must have occurred, as *A.actinomycetemcomitans* belongs to the same family as *P.haemolytica* and *A.pleuropneumoniae*. Some strains of *E.coli* carry a plasmid copy of the haemolysin gene, but this does not resemble the *A.actinomycetemcomitans* leucotoxin (Kraig *et al*, 1990., Hess *et al*, 1986). The G+C value of the ***hkl*** determinant on plasmids has a value of 39% which contrasts with that of 50% for the rest of the genome (Femlee, Pellet and Welch, 1986).

More recently, Welch (1991) suggested that the RTX family as a whole evolved as a fusion of a number of different elements. The adenylate cyclase toxin of *B.pertussis*, however, may have undergone a unique rearrangement, separating the C and A genes. The pseudogenes found in *A.pleuropneumoniae* (Chang *et al*, 1991) show homologies

to different RTX toxins. The RTX A pseudogene upstream from *appB* most closely resembles the *hlyB* gene from *E.coli*. Chang *et al* (1991) suggested that the *appCA* and *appBD* operons were derived from different ancestral RTX determinants.

The genes which encode the Ca^{2+} independent-haemolysin of *P.mirabilis* have been shown to have a similar operon structure and close amino acid sequence with *S.marcescens*, despite a large difference in the G+C value of each organism, which suggested the genes diverged a long time ago (Uphoff and Welch, 1990). While *P.mirabilis* and *S.marcescens* are not members of the RTX family, they may represent another family of virulence factors (Welch, 1991). The reason why the RTX toxins should have different target cell specificities from each other is unknown. It may be due to protein folding which could influence its interaction with cell receptors or alternatively the protein shape may affect its ability to create pores in cell membranes of different compositions. The type of receptors involved in the target cell membrane for the toxin recognition/ binding are not known.

1.8.14 Leucotoxicity assays

A number of different assays, have been developed to investigate toxin production by *A.actinomycetemcomitans*. Whole bacterial cells, sonicates of whole cells or purified leucotoxin have been used with freshly isolated human polymorphonuclear cells (Baehni *et al*, 1979., Baehni *et al*, 1981) or cultivated human tissue cell lines e.g. HL-60 (Zambon *et al*, 1983., Simpson, Berthold and Taichman, 1988). Cell death has been determined by trypan blue exclusion (Ohta *et al*, 1987); lactate dehydrogenase release (Zambon *et al*, 1983); lysosomal elastase release (Tervahartiala *et al*, 1989); release of ^{51}Cr (Simpson *et al*, 1988) and fluorescent spectroscopy (Ohta *et al*, 1991). Comparison of the methods is difficult, as the level of cell death at which a strain is designated toxic, varies between the different methods.

1.9 INFECTION DUE TO *A.actinomycetemcomitans.*

A.actinomycetemcomitans can cause a range of infections in humans e.g. periodontal disease (1.10), endocarditis, brain abscesses, soft tissue abscesses, septicaemia, urinary tract infection, chest abscesses and pneumonia (King and Tatum, 1962., Page and King, 1966., Vandepitte, De Geest and Jousten, 1977., Kaplan *et al*, 1989., Bowker, Connellan and Freeth, 1992.) - see Table 1.6

The most common non-periodontal infection reported was bacterial endocarditis, with 58 cases in the literature since 1962 (Kaplan *et al*, 1989., Bowker *et al*, 1992). Infection due to *A.actinomycetemcomitans* may go undetected as it is an unusual organism which may not grow on non-selective media, or may be undetected due to its small size.

1.10 PERIODONTAL DISEASE

The periodontium is the collective term given to the tissues that support the teeth in the jaws. It consists of the gingiva, periodontal ligament, cementum and alveolar bone. The periodontium has several functions which include

(a) the attachment of the teeth to the alveolar bone;

(b) the resistance and resolution of the forces generated by the movement of the teeth during eating and speech;

(c) the continual adjustment of structural changes associated with wear and ageing by continuous regeneration of the tissue (Schulger *et al*, 1990).

Schulger *et al* (1990) classified the diseases of the periodontium (periodontitis) into 4 distinct entities, on the basis of age of onset, rate of progression, microbial flora, distribution of lesions and history, as follows :

(a) Prepubertal periodontitis

(b) Juvenile periodontitis

(c) Adult or Chronic periodontitis

Disease	Number of cases	Reference
Infective	23	Page & King, 1966
Endocarditis	1	Vandepitte *et al*, 1977
	1	Schack *et al*, 1984
	1	Bowker *et al*, 1992
Actinomycosis	4	Page & King, 1966
Meningitis	1	Page & King, 1966
Septicaemia	1	Page & King, 1966
Soft tissue abscess	2	Kaplan *et al*, 1989
Facial abscess	2	Page & King, 1966
Sinusitis	1	Page & King, 1966
Brain abscess	1	Martin *et al*, 1967
Empyema	1	Kaplan *et al*, 1989
Chestwall abscess	1	Kaplan *et al*, 1989
Urinary tract infection	1	Kaplan *et al*, 1989

Table 1.6 Human infection due to *A.actinomycetemcomitans*

(d) Rapidly progressive periodontitis

Many studies have tried to relate microbial (see section 1.10.1), immunological (see section 1.4.1) and biochemical factors (e.g. hormonal changes associated with puberty and pregnancy) to active disease with a view to obtaining markers to identify individuals at high risk of developing chronic inflammatory periodontitis (See Reviews by Johnson *et al*, 1988., Wilton *et al*, 1988., Maiden *et al*, 1990).

A number of oral bacterial species have been associated with the different forms of periodontal disease e.g. *Prevotella intermedius (B.intermedius)*, *Porphorymonas gingivalis (B.gingivalis)*, *Capnocytophaga* species and *A.actinomycetemcomitans* (Tanner *et al*, 1979., Mashimo *et al*, 1983., Moore *et al* , 1982., Page *et al*, 1985., (review) Slots and Listgarten, 1988. Slots and Rams, 1990. Slots, Feik and Rams, 1990.). While these bacteria maybe important in the progression of the disease, this review will be concerned only with *A.actinomycetemcomitans*.

1.10.1 Involvement of *A.actinomycetemcomitans* in periodontal disease.

Asikainen, Alaluusua and Saxen (1991) recovered *A.actinomycetemcomitans* from the saliva, tongue and subgingival sites of both healthy individuals and those with periodontal disease. Zambon *et al* (1983) argued that active periodontal sites might represent the first site colonised by *A.actinomycetemcomitans*. As the host defence system is stimulated, *A.actinomycetemcomitans* may be inhibited from colonising other sites. Alternatively, *A.actinomycetemcomitans* may be inhibited by other bacteria present in the sites of potential lesions. The loss or reduced expression of virulence factors from *A.actinomycetemcomitans* may result in sites, which although colonised by *A.actinomycetemcomitans* do not become diseased (Zambon *et al*, 1983).

It has been reported that *A.actinomycetemcomitans* is difficult to eliminate from periodontal pockets (Christersson *et al*, 1985a., Kornman and Robertson, 1985., Rodenburg *et al*, 1990). Indeed, Rodenburg *et al*, reported an increase in the numbers of *A.actinomycetemcomitans* recovered after treatment consisting of supra- and

subgingival debridement. It has been suggested that the apparent ability of *A.actinomycetemcomitans* to survive *in vivo* maybe related to its reported ability to invade host tissue (Slots and Genco, 1984., Christersson *et al*, 1984). However, although the appearance of *A.actinomycetemcomitans* in tissues may be due to invasion, the possibility that it is an artefact in the preparation of the tissue for both light and electron microscopy cannot be excluded.

1.10.2 Prepubertal periodontitis

Prepubertal periodontitis has its onset during or immediately after the eruption of the primary dentition. It has two forms - localised and generalised - which differ in progression and features, as summarised in Table 1.7.

1.10.3 Involvement of *A.actinomycetemcomitans* in prepubertal periodontitis.

There is very little published evidence for any involvement of *A.actinomycetemcomitans* in prepubertal periodontitis. Crossner *et al* (1990) studied periodontitis in the primary dentition, associated with *A.actinomycetemcomitans* infection, in two siblings (male and female). They suggested that *A.actinomycetemcomitans* might trigger a phagocytic dysfunction of PMNLs since the eradication of *A.actinomycetemcomitans* from the subgingival region, restored the phagocytic activity of PMNLs to normal levels. The reduced phagocytic activity of the PMNLs was measured as the capacity to ingest IgG-coated latex particles. After reinfection two and half years later, the phagocytic activity of the PMNLs was again reduced, but this reversed after antibiotic therapy.

1.10.4 Juvenile periodontitis

Juvenile periodontitis differs from the adult form of the disease by the age of onset and the absence of plaque and calculus related to tissue destruction. The features are summarised in Table 1.8. Family studies have revealed an apparent genetic

Common Features of Prepubertal Periodontitis
1. Onset during or after eruption of primary dentition
2. Swollen and inflamed gingiva, with the accumulation of gross subgingival plaque and calculus, although these clinical signs may be absent.
3. The aetiology of the disease is not fully understood, however it has been associated with Papillon-Lefevre syndrome.
4. It has also been found in clinically healthy children, which suggests that may be a clinical disease.
5. It has been associated with defects in neutrophil and monocyte function.

Table 1.7 Common features of prepubertal periodontitis

From :

1. Ngan, Tsai & Sweeney, 1985
   Page *et al*, 1983
2. Sonis, 1979
   Page *et al*, 1983
3. Fourel, 1974
   Van Dyke *et al*, 1984
   Preus & Gjermo, 1974
   Bimstein *et al*, 1990
4. Melnick, 1974
   Baer, 1984
   Page *et al*, 1983
5. Shurin *et al*, 1979
   Bowden *et al*, 1982
   Page, 1983
   Page *et al*, 1983
   Kinane *et al*, 1988a
   Kinane *et al*, 1988b

involvement, consistent with it being either an X-linked dominant or autosomal recessive trait (Saxen, 1980a., Saxen, 1980b). Hart *et al* (1992) re-examined the evidence for X-linked dominant inheritance and found that the number of females which were affected by juvenile periodontitis was greater than the number of males because a greater number of females were included in the studies. When the proportions of affected males and females were examined rather than the absolute numbers of affected individuals, the proportions were similar (Hart *et al*, 1991). Two studies have been made, where there is evidence of father to son transmissions (Boughman *et al*, 1986., Saxby, 1987). Hart *et al* (1992) concluded that some X-linked forms of the disease do occur, but that this is not the most important mode of inheritance.

1.10.5 Involvement of *A.actinomycetemcomitans* in juvenile periodontitis

There have been several epidemiological studies which have related *A.actinomycetemcomitans* infection to juvenile periodontitis in particular populations (Alauusua and Asikainen, 1988., Slots *et al*, 1980., Eisenhann, 1983., Gunsolley *et al*, 1988). Eisemann *et al*, (1988) suggested that *A.actinomycetemcomitans* was found in 60% of the young adult population of Panama, which had a high incidence of juvenile periodontitis. Alauusua and Asikainen (1988) found *A.actinomycetemcomitans* in 13% of the young Finnish population, which they claim had one of the lowest levels of juvenile periodontal disease in the world. The high incidence of juvenile periodontitis within one racial group may suggest that environmental and/ or genetic determinants maybe important factors. In a review, Slots and Listgarten (1988) concluded that three Gram negative bacterial species were important pathogens in periodontitis in man. These were *P.gingivalis*, *P.intermedius* and *A.actinomycetemcomitans*. The latter has been found in 85 - 100% of localised juvenile periodontitis (Slots, Reynolds and Genco, 1980., Slots *et al*, 1982., Zambon, Christersson and Slots, 1983., Asikainen *et al*, 1986, 1987. Slots, Feik and Rams, 1990).

Common Features of Juvenile Periodontitis
1. Age of onset between 11 and 21 years of age, but diagnosis can be made at any age beyond puberty upto mid-to-late twenties.
2. Lesions initially localised to the periodontium around the first molar and incisor, may then progress to other areas, which results in the generalised form of the disease.
3. Little plaque deposits seen, however probing of the periodontal pockets, results in bleeding.
4. Worldwide variation in the prevalence of the disease. Europe has a low prevalence of 0.1 percent, whilst the negro population has been shown to have a prevalence as high as 55 percent.
5. Clinical studies suggest that the disease is more common in females than in males.
6. Familial distribution consistent with X-linked dominant or an autosomal mode of inheritance.
7. Most affected individuals have functional defects in either neutrophils or monocytes.
8. Lesions maybe highly active immediately following puberty, but subsequent destruction maybe slow or spontaneously cease ("burn-out").

Table 1.8 Common features of juvenile periodontal disease

From :
1. Manson & Lehner, 1974
2. Liljenberg & Lindhe, 1980
3. Davies, Smith & Porter, 1985
4. Saxen, 1980a, 1980b
   Saxby, 1984
   Manson & Lehner, 1974
5. Saxby , 1984
6. Saxen, 1980a, 1980b
   Page *et al*, 1985
7. Schulger *et* , 1990
8. Schulger *et* , 1990

Several authors have either failed to find *A.actinomycetemcomitans* associated with juvenile periodontitis (Okuda *et al*, 1984. Vandesteen *et al*, 1984. Naiming *et al*, 1991) or isolated them in very small numbers (Moore *et al*, 1985. Mandell *et al*, 1986. Mashimo *et al*, 1988). Moore *et al* (1985) suggested that the prevalence of *A.actinomycetemcomitans* may differ in different geographical areas.

1.10.6 Chronic adult periodontitis

Adult periodontitis is the most common form of periodontitis - the common features of which are listed in Table 1.9.

1.10.7 Involvement of *A.actinomycetemcomitans* in adult periodontitis

Slots (1986) summarised the work of three studies into the bacterial specificity in adult periodontitis. *A.actinomycetemcomitans* was found in 50% of the progressive lesions in each of the three studies. However, when the sites were in remission, it was present in only 6% of samples collected from previously active lesions. In addition, *A.actinomycetemcomitans* was found in only 2.1% of sites which were non-progressive (Slots, 1986).

Wolff *et al* (1985) carried out a study of 284 adults (aged 20 - 40 years of age) whose periodontal status ranged from healthy to moderate chronic periodontitis. Overall, *A.actinomycetemcomitans* was found in 13% of subgingival and 4.9% of supragingival plaque samples for individuals.

When the pocket depth was greater than 5 mm, the numbers of colonies of *A.actinomycetemcomitans* recovered from the pocket were significantly reduced.

Dahlen *et al* (1989) studied a population of 20 Kenyan adults (30 to 65 years of age) and recovered *A.actinomycetemcomitans* in 40% of the sample population and in 28% of the sites sampled. However there was no evidence of periodontal disease in this group of volunteers.

Common Features of Chronic Adult Periodontitis
Age of onset is usually 30 to 35 or older.
Not confined to first molar or incisors, but these are more commonly and severely affected than are the canines and premolars.
The disease usually affects many teeth.
Conditions enhancing plaque accumulation are present and the quantity of microbial deposits are linked with the severity of the lesions
Serum, neutrophils and monocyte abnormalities generally not present.
The extent and distribution of bone loss are highly variable; both vertical and horizontal patterns maybe seen.
Acute inflammation with marginal proliferation of the gingivae is usually not seen.
Lesions are not as amenable to treatment with antibiotics as with other forms of periodontitis.
Acute destruction and exacerbation can occur at one or more sites.

Table 1.9 Features of chronic adult periodontitis

After Schulger *et al*, 1990

1.10.8 Rapidly progressive periodontitis

The term rapidly progressive periodontitis was first used by Crawford, Socransky and Brathhall (1975) to describe highly active lesions at specific sites for which there was radiographic evidence of rapid bone destruction. The common features are given in Table 1.10. Rapidly progressive periodontitis is most commonly seen in young adults in their early twenties, but has been shown to be present in individuals upto thirty-five years of age. During the active phase, the gingival tissues become extremely inflamed with haemorrhage, proliferation of the marginal gingiva and exudation. Tissue destruction is rapid and most bone loss appears to occur within a few weeks or months (Page *et al*, 1983).

The quiescent phase of the disease is characterised by the presence of clinically normal gingivae, accompanied by very advanced bone loss and deep periodontal pockets. This phase may be permanent or transient.

1.10.9 Involvement of *A.actinomycetemcomitans* in rapidly progressive periodontitis

There has been very little published data on the relationship between *A.actinomycetemcomitans* and rapidly progressive periodontitis. Page *et al* (1983) found serum antibodies to various *Actinobacillus* species in rapidly progressive periodontitis and also reported that most of the patients with this disease had defects in neutrophil or monocyte chemotaxis.

1.11 AIMS

It would appear that there is some dubiety within the literature regarding the number of plasmid harbouring strains of *A.actinomycetemcomitans*. Whilst most workers have found plasmids, variations in the frequency of occurrence of plasmid positive strains, in the different studies exist. (Olsvik & Preus, 1989; LeBlanc *et al*, 1993; Poulsen *et al*, 1994). These variations may, in part, be due to the temporal and geographical

Common Features of Chronic Periodontitis
Age of onset between puberty and 35.
Lesions affect most of the teeth.
Some patients may have had juvenile periodontitis previously.
Evidence of severe and rapid bone destruction, after which the destructive process may cease spontaneously or markedly slow down.
During the active phase, the gingival tissue is acutely inflamed with marginal proliferation : during the arrested phase, the tissues may appear free from inflammation.
The amounts of microbial deposits are highly variable.
Most of the patients have functional defects in neutrophils or monocytes.
Some individuals are remarkably responsive to treatment by curettage coupled with antibiotic administration e.g. tetracycline, while a small portion is refractory to any known form of therapy.

Table 1.10 Common features of rapidly progressive periodontitis

After Schulger *et al*, 1990

isolation of the strains under investigation.

The first aim of this thesis was to investigate the strain diversity of the collection of *A.actinomycetemcomitans* strains held at the Glasgow Dental Hospital and School. These strains were collected over a 5 year period and included Type strains which were originally isolated in America and Denmark. The initial stage of the investigation into clonality was to determine the frequency of strains of *A.actinomycetemcomitans* which harboured plasmids and to use this information (in addition to information known previously about the collection) to determine which strains would be studied in the subsequent experimental investigations.

The clonality of *A.actinomycetemcomitans* strains has been investigated previously, however, these have tended to be American studies, of American strains and therefore much information is known regarding the clonality of American isolates and relatively little about European strains. One European study (Haubek *et al,* 1995), suggested that the *A.actinomycetemcomitans* strains of Europe were less virulent than those of America, suggesting a different clonal origin. It was intended that the generation of RFLPs for a subsection of the Glasgow Dental Hospital collection would reveal more information of the clonality of European *A.actinomycetemcomitans* isolates.

There have been several studies into the toxic effects of *A.actinomycetemcomitans* on e.g. freshly isolated human polymorphonuclear cells (Baehni *et al,* 1979., Baehni *et al,* 1981) and cultivated human tissue cell lines e.g. HL-60 (Zambon *et al,* 1983., Simpson, Berthold and Taichman, 1988). Cell death has been determined by trypan blue exclusion (Ohta *et al,* 1987); lactate dehydrogenase release (Zambon *et al,* 1983); lysosomal elastase release (Tervahartiala *et al,* 1989); release of ^{51}Cr (Simpson *et al,* 1988) and fluorescent spectroscopy (Ohta *et al,* 1991). Because there are many different assaying systems, comparisons of the different methods is difficult. The second aim of this investigation, was therefore to devise a rapid, accurate method, to determine toxicity. It was hoped that this standardised protocol would enable the rapid screening of the *A.actinomycetemcomitans* collection for toxic activity and the

number of toxic strains within the collection found.

It is intended that by investigating the clonal origin and leucotoxic status of *A.actinomycetemcomitans*, the epidemiology of this important human pathogen be further understood.

CHAPTER 2
GENERAL METHODS

CHAPTER 2

GENERAL METHODS

2.1 GROWTH OF *A.actinomycetemcomitans*

All constituents and the preparation of the media are shown in Appendix 1.1

2.1.1 Solid media (Constituents and preparation in Appendix 1.1)

Throughout the study, strains of *A.actinomycetemcomitans* were first grown on Columbia Blood Agar (CBA) at 37°C, 5% CO_2 for 48 hours, and thereafter inoculated into Trypic Soya broth (TSB - Oxoid) as required.

2.1.2 Broth media (Constituents and preparation in Appendix 1.1)

In preliminary DNA fingerprinting investigations Brain Heart Infusion (BHI - Oxoid) broth was used : in later investigations this was changed to TSB .

TSB was prepared in either 100 or 400 ml volumes, autoclaved and stored at room temperature until required. Aseptic techniques were used throughout.

Twenty millilitre volumes of TSB were used routinely for growth of the organism for DNA profiling (Chapters 4 and 5) and leucotoxicity studies (Chapter 7). Volumes of 400 ml were used in the isolation of outer membrane proteins (Chapter 6).

Before inoculation, broths were aliquoted into 20 ml volumes and stored at 37°C, in 5% CO_2 in air for 4 - 5 hours, with the lids slightly loose, to saturate the media with carbon dioxide. After inoculation broths were returned to 37°C, in a 5% CO_2 incubator (LTE, Greenfield, Oldham).

Broth was inoculated with a heavy suspension of *A.actinomycetemcomitans* - typically the total growth from 1 CBA plate per 20 ml of broth or from 5 plates per 400 ml, using sterile cotton wool swabs.

2.1.3 Atmospheric requirements

Attempts to enhance growth in broth by the addition of sodium bicarbonate (BDH) as a source of carbon dioxide did not increase the yield of cells compared with those grown in a CO_2 incubator.

Candle extinction jars were used to culture *A.actinomycetemcomitans* on solid media prior to the determination of plasmid profiles. In all other investigations, to avoid spillage and contamination of liquid media, broths were incubated in a CO_2 incubator (LTE, Greenfield, Oldham), as were all agar plates.

Unless otherwise stated, all cultures of *A.actinomycetemcomitans* were grown in air supplemented with 5% CO_2, at 37°C.

2.2 STORAGE OF *A.actinomycetemcomitans*

2.2.1 Freeze-dried ampoules.

A.actinomycetemcomitans strains, which had been isolated previously, were stored in freeze-dried ampoules. Bacteria were revived, by the addition of 200 - 300 µl of BHI to the ampoules and the resultant suspension plated out for single colonies onto one CBA plate and incubated at 37°C in 5% CO_2 for 72 hours.

Single colonies were subcultured and identification was confirmed using the API 20A strip (API/Bio Merieux, England), catalase reaction (Roche, England) and stellate morphology.

2.2.2 Protect beads

After a total of 2 subcultures from revival, a heavy suspension of *A.actinomycetemcomitans* was made into "Protect beads" (Lab M, England) in duplicate. One ampoule was stored at -20°C, the other at -70°C.

Revival of the culture involved removal of beads from the frozen vial as quickly as possible, to minimise "freeze-thaw" stress on the stock culture.

A maximum of 2 beads were removed to CBA plates (two plates per strain) and

roughly spread across the agar. These were incubated in in air, supplemented with 5% CO_2, at 37°C for 48 hours before being examined for growth. A sweep was made from these plates, which was then spread for single colonies on CBA and were grown for 48 hours as before. These were then used as stock plates in subsequent studies. Stock plates were stored at 4°C. Thus the inoculum used in each investigation comprised bacteria that had been subcultured three times from revival. Plates were stored for a maximum of three weeks at 4°C before freshly revived strains were used.

2.3 ISOLATES OF *A. actinomycetemcomitans*

The Oral Microbiology Unit in Glasgow Dental Hospital and School had a collection of 96 *A.actinomycetemcomitans* isolates that comprised 85 clinical strains and 11 reference strains.

The clinical strains had been collected over a number of years (1985 - 1990) and included multiple strains from a number of individuals. The characteristics of the strains are shown in Tables 2.1a to 2.1c. The strains reflect a variety of clinical conditions, including juvenile periodontitis, chronic periodontitis and prepubertal periodontitis.

The majority of these strains were collected by I. Taher and used in experimental studies including adhesion, hydrophobicity and a leucotoxicity assay (Taher, 1989). The freeze-dried ampoules which were opened and subsequently used throughout, were prepared by I. Taher.

A subset of these strains were used subsequently in epidemiological and leucotoxicity studies, as detailed in Table 2.2.

2.4 HL-60 CELLS

HL-60 cells provided an easy and convenient substrate for the leucotoxicity assay. They were obtained from The European Collection of Animal Cell Cultures (Porton Down, Wiltshire, England). This cell line was derived from a 36 year-old caucasian

woman, with acute promyelocytic leukaemia (Collins, Gallo & Gallagher, 1977., Gallagher *et al* 1979). Cells were grown in 100 ml volumes in RPMI 1640 (Life Technologies, Scotland) supplemented with 10% Fetal Calf Serum (FCS - Globepharm England - Appendix 1.1), in 260 ml "Nunc" Tissue culture flasks (Life Technologies).

For passage, cells were transferred aseptically to sterile plastic universals, centrifuged gently (500 rpm for 10 minutes) and the supernatant discarded. The cells were then resuspended in freshly prepared media and returned to a CO_2 incubator, at 37^0C supplemented with an atmosphere of air and 5% CO_2 (Grant, England)

The method recommended by the suppliers involved decanting the RPMI 1640 medium from the sedimented cells and its replacement with fresh RPMI 1640/ FCS, which thus avoided the need to replace the tissue culture flasks at each passage. This method, however resulted in a large numbers of cells per flask, which led to the rapid utilisation of the nutrients in the media and required the cells to be passaged more frequently. This in turn led to an increase in the volume of media which was used. This method predisposed to contamination. Thus it was decided to use the techniques described previously.

Three flasks of HL-60 cells were grown at any one time, each at a slightly different stage of development. The concentration of cells was not allowed to increase beyond a density of 3 - 4 X 10^7 cells ml^{-1}, which thus ensured the availability of cells for use in the assay.

2.5 HAEMOCYTOMETER

The haemocytometer (BDH) provided a method for counting animal cells grown in culture. It consisted of a large slide with a series of lines etched upon its surface dividing it into 9 large squares. The centre square was divided further into 25 squares which were then subdivided into 16 squares each. The large squares had an area of 1 mm^2. After slight wetting of the sides of the chamber, a coverslip was placed on top,

resulting in patterns of interference. The depth of the chamber at this point was 1mm, which gave a total volume of 10^{-4} ml. The wells of the chamber were filled with HL-60 cells diluted 1 in 20 with PBS containing trypan blue (BDH - Appendix 1.5) by capillary action. All cells, in the 4 corner squares only, were counted, both stained (dead) and clear (viable). The number of cells per ml of suspension was calculated using the following formula :

$$\text{Number of cells} = \frac{10^4 \, n \, y}{4}$$

where 10^4 = multiplication factor

n = number of cells in 4 squares

y = dilution of cells (1 in 10)

4 = number of squares

HL-60 cell viability was expressed as the percentage of live cells, calculated using the following formula :

$$\text{CELL VIABILITY} = \frac{\text{TOTAL CELLS - TOTAL DEAD CELLS}}{\text{TOTAL CELLS}} \times 100\%$$

2.6 SITES FOR EXPERIMENTAL STUDIES

Due to the availability of equipment, it was not possible to carry out all the experimental work at a single location. The different University Departments are listed below, with the experimental study carried out there noted along side.

Department of Oral Sciences, Glasgow Dental Hospital and School

Within the Department of Oral Sciences at the Glasgow Dental Hospital and School, stocks of *A.actinomycetemcomitans* were maintained, for use in all the experimental work (Chapters 3 - 7). The appropriate cultures were removed to the Department

where the work was to be carried out on petri dishes and these cultures used as a stock. All petri dishes were sealed with tape prior to removal from the Dental Hospital.

HL-60 cells were also grown and maintained here (Chapter 7).

Blood, used to prepare PMNLs for use in the chemiluminescence experiments, was obtained from volunteers in the Oral Medicine Department (Chapter 7).

Department of Bacteriology, Glasgow Royal Infirmary

Within the Department of Bacteriology at Glasgow Royal Infirmary, the work described in Chapters 3 - 5 was carried out, with the exception of the use of the spectrophotometer, which was carried out in the Department of Oral Sciences.

Department of Cell Biology, Glasgow University

Within the Department of Cell Biology, PMNLs were prepared from the blood taken from volunteers at the Dental Hospital, prior to use in chemiluminescence studies within the Department of Microbiology (Chapter 7).

Department of Microbiology, Glasgow University

The experimental work described in Chapter 6 and the chemiluminescence studies described in Chapter 7 were carried out within the Department of Microbiology, Glasgow University.

Identification number	Source	Site of isolation
1	GDH R8529/85	Cp
2	GDH 143/85	Cp
3	GDH 156/85	Cp
4	GDH 216/85	Cp
5	GDH 471/85	Cp
6	GDH 1954/85	Cp
7	GDH 105/86	Cp
8	GDH 107/86	Cp
9	GDH 114/86	Cp
10	GDH 117/86	Cp
11	GDH 119/86	Cp
12	GDH 121/86	Cp
13	GDH 125/86	Cp
14	GDH 127/86	Cp
15	GDH 129/86	Cp
16	GDH 1014/86	Jp
17	GDH 1028/86	Cp
18	GDH 1114/86	Cp
19	GDH 1115/86	Cp
20	GDH 1116/86	Cp
21	GDH 1123/86	Cp
22	GDH 1125/86	Jp
23	GDH 1212/86	Jp
24	GDH 1224/86	Jp
26	GDH 2351/86	Jp
27	GDH 1214/86	Cp
28	GDH 28/87	Cp
29	GDH 33/87	Jp
30	GDH 39/87	Jp
31	GDH 110/87	Cp
32	GDH 228/87	Cp
33	GDH 310/87	Jp
34	GDH 312/87	Cp
35	GDH 510/86	Cp
36	GDH 87034/87	Pp
89	GDH 877/90	HIV or
90	GDH 589/90	HIV or
91	GDH 592/90 (1)	HIV or
92	GDH 592/90 (2)	HIV or
95	GDH 2102/91 241	HIV or
96	GDH 2212/90 213	HIV or

Table 2.1a : Single strains of *A.actinomycetemcomitans* isolated from individual patients attending the Glasgow Dental Hospital

GDH : Glasgow Dental Hospital

Cp : Chronic periodontitis Jp : Juvenile periodontitis

Pp : Prepubertal periodontitis HIV or : HIV oral rinse

Identification number	Source	Site of isolation
37µ	GDH 130376 261 C1S1	Cp
38µ	GDH 130376 261 C2S	Cp
39µ	GDH 130376 261 C1	Cp
40µ	GDH 130376 261 C3S1	Cp
41µ	GDH 130376 476 C1S	Cp
42µ	GDH 130376 476 C2S	Cp
43µ	GDH 130376 476 C3	Cp
44*f*	GDH 213705 163 C188	Cp
45*f*	GDH 213705 163 C288	Cp
46*f*	GDH 213705 163 C388	Cp
47*f*	GDH 213705 163 C488	Cp
48á¥	GDH 225843 224 1-1	Cp
49á¥	GDH 225843 224 1-2	Cp
50á¥	GDH 225843 224 1-3	Cp
51á¥	GDH 225843 224 C1-S	Cp
52á¥	GDH 225843 224 C1-S	Cp
53á¥	GDH 225843 224 C4-S	Cp
55á¢	GDH 225843 426 1	Cp
56á¢	GDH 225843 426 2	Cp
57á¢	GDH 225843 426 3	Cp
54áñ	GDH 225843 465 1	Cp
58áñ	GDH 225843 465 2	Cp
59áñ	GDH 225843 465 3	Cp
60áñ	GDH 225843 465 1-1	Cp
61áñ	GDH 225843 465 1-2	Cp
62ô	GDH 226824 166 C188	Jp
63ô	GDH 226824 166 C2S	Jp
64x	GDH 227916 116	Cp
65x	GDH 227916 263	Cp
66x	GDH 227916 334	Cp
67x	GDH 227916 366	Cp
68x	GDH 227916 456	Cp
69P	GDH 229075 126	Cp
70P	GDH 229075 144	Cp
71P	GDH 229075 273	Cp
72P	GDH 229075 314	Cp
73P	GDH 229075 344	Cp
74Ü	GDH 229473 114	Jp
75Ü	GDH 229473 164	Jp
76Ü	GDH 229473 376	Jp
77Ü	GDH 231293 124	Jp
93>	GDH 2102/90 161	HIV or
94>	GDH 2102/90 181	HIV or

Table 2.1b Multiple strains of *A.actinomycetemcomitans* isolated from individual patients attending the Glasgow Dental Hospital, including multiple strains from the same periodontal pocket

GDH : Glasgow Dental Hospital

Cp : Chronic peridontitis HIV or : HIV oral rinse

Jp : Juvenile periodontitis

The symbols µ *f* á ô x P Ü < > indicate strains from the same individual.

The symbols ¥ ¢ ñ indicate strains from the same pocket.

Identification number	Source	Country of origin	Year of isolation	Site of isolation
78	ATCC 29522	USA		Mandibular abscess
79	ATCC 29523	Denver		Blood
80	ATCC 29524	Seattle	1970	Chest aspirate
81	Y4	USA		Juvenile periodontitis
82	NCTC 9709	USA		Abscess
83	NCTC 9710	Denmark	1949	Abscess
84	NCTC 10979	USA		Mandibular abscess
85	NCTC 10980	USA		Blood
86	NCTC 10981	USA		Neck abscess
87	NCTC 10982	Seattle	1970	Chest aspirate
*88	JP_2			Juvenile periodontitis

Table 2.1c Reference and Type strains of *A.actinomycetemcomitans* held in the Glasgow Dental Hospital collection with city/ country and year of isolation, if known

ATCC : American Type Culture Collection
NCTC : National Collection of Type Cultures
* : obtained from N.S. Taichman
data from ATCC/ NCTC catalogues

Identification number	Plasmid status	Leucotoxicity status, preassay
1	+ ve	Unknown
8	+ ve	Unknown
24	+ ve	Positive *
#29	+ ve	Unknown
32	- ve	Unknown
33	- ve	Unknown
36	- ve	Unknown
55	+ ve	Unknown
56	+ ve	Unknown
57	+ ve	Unknown
74	- ve	Unknown
75	- ve	Unknown
76	- ve	Unknown
79	- ve	Positive **
80	- ve	Negative ***
81	+ ve	Variable ****
83	- ve	Unknown
88	- ve	Positive *****

Table 2.2 Leucotoxic status (prior to assay) of *A.actinomycetemcomitans* strains used in all experimental studies

* Taher, 1989

** Baehni *et al*, 1981. Spitznagel, Kraig & Kolodrubetz, 1991. Zambon *et al*, 1983.

*** Baehni *et al*, 1981. Zambon *et al*, 1983.

**** Tsai *et al*, 1979.

***** Tsai *et al*, 1983.

#29 This strain was only used in the experimental work detailed in Chapters 4 and 5.

CHAPTER 3
PREVALENCE OF PLASMIDS IN *A.actinomycetemcomitans*

CHAPTER 3

PREVALENCE OF PLASMIDS IN *A.actinomycetemcomitans*

3.1 INTRODUCTION

Plasmids have been demonstrated in a small number of *A.actinomycetemcomitans* strains (Olsvik & Preus, 1989; LeBlanc *et al*, 1993; Poulsen *et al*, 1994). These authors suggested the global expansion of very few clones on the basis of detection of similar plasmids of similar size in unrelated strains.

The molecular epidemiology of bacterial strains can be investigated in three ways. The first is to demonstrate plasmid content. Further analysis of plasmid DNA by digestion with restriction enzymes to generate a restriction enzyme fragmentation pattern (REFP) or "plasmid fingerprint", distinguishes plasmids of similar size and provides scope for the assessment of plasmid relatedness (Platt and Smith, 1991. Chapter 5). An alternative (and sometimes complementary) approach applicable to both plasmid free and plasmid carrying strains of bacteria, involves similar treatment - purification and digestion - of the entire genome to generate a REFP.

The work which is discussed in this chapter, was the demonstration of the plasmid content of the entire *A.actinomycetemcomitans* collection held at the Glasgow Dental Hospital and School, 91 strains in total, by the method of Platt, Heraghty and Taggart (1988), which has been used successfully on a number of different bacteria, (Therlfall *et al*, 1985; Platt *et al*, 1986; Coia, Nor-Hussain and Platt, 1988., Platt, Heraghty and Taggart, 1988; Platt and Smith, 1991).

3.2 MATERIALS AND METHODS

3.2.1 Crude isolation of plasmids

The determination of plasmid profiles was carried out by the method of Platt, Heraghty and Taggart (1988).

An overnight culture of each of the 95 strains of *A.actinomycetemcomitans*

investigated, grown on CBA, at 37°C, 5% CO_2, was harvested with a dry swab into 300µl Tris borate (TBE - Appendix 1.2) in an eppendorf. An equal volume of a 10% solution of sodium dodecyl sulphate (SDS - Appendix 1.2) was then added. The tube was mixed by inversion and left at 55°C for 5 minutes until the contents had become clear and viscous, which indicated that the cells had lysed. The samples were then centrifuged for 15 minutes at 13000 rpm in a microcentrifuge (Andermann, England). The pellet was removed, to leave a clear supernatant. From this clear supernatant, 100µl was removed and 5µl of gel loading buffer added (Appendix 1.2) .

This procedure was repeated on 3 separate occasions for each strain.

3.2.2 Electrophoresis

Agarose gels of 0.7% were prepared from Type II agarose (Sigma, Poole) in TBE. Samples of 100µl were loaded under TBE buffer. The gels were run at 100 V for 1 hour, then at 200 V (4 vcm^{-1}) for a further 4 hours.

3.2.3 Visualisation of plasmids

The gels were stained in TES which contained ethidium bromide (Appendix 1.2), for 15 minutes and viewed using an ultraviolet light transilluminator at 365 nm (Ultra Violet Products, Cambridge, England) and photographed using Polaroid type 665 Land film. The molecular length of the plasmids was determined by reference to a molecular length standard *E.coli* 39R861 (NCTC 50192 - Threlfall, 1986) that harboured plasmids of 147, 63, 37 and 7 kb.

3.2.4 Rationale for the interpretation of plasmid profiles

Plasmid profiles can provide a base for epidemiological studies in the following ways :

(i) As a method of assessing the diversity of bacterial populations.

(ii) As a method of comparing strains from defined epidemiological

situations.

The contribution of plasmid profiles in epidemiological investigations derives from

(i) ease of determination

(ii) the size and diversity of the plasmid pool in many bacterial genera.

The plasmid complement (or profile) generally reflects the recent history of a bacterial strain because some have the ability to be transferred between bacteria and are of variable stability, with the result that some organisms will lose plasmids, whilst others accumulate them and on this basis the source of many infections can be traced. Whereas the demonstration that different strains of bacteria which carry plasmids of different molecular length suggests that the strains may be unrelated, the demonstration that two strains harbour plasmids of the same size does not necessarily indicate that they are epidemiologically related. This requires verification by further investigation and forms the basis of molecular epidemiology and ideally knowledge of the long term stability of the plasmids.

Plasmid REFP generation (Chapter 5) not only discriminates between plasmids of different molecular lengths, but also between plasmids of the same molecular length, which have different conformations i.e. linear, OC and CCC.

Plasmids of the same molecular length can only be distinguished from each other by the generation of a REFP. Plasmids which differ only in conformation will have the same REFP. The information which is obtained from plasmid profiles is therefore limited.

3.3 RESULTS

Plasmid profiles were obtained for the clinical and Reference strains listed in Table 2.1.

Figures 3.1a and 3.b demonstrate two gels, which contain both plasmid positive and plasmid negative strains.

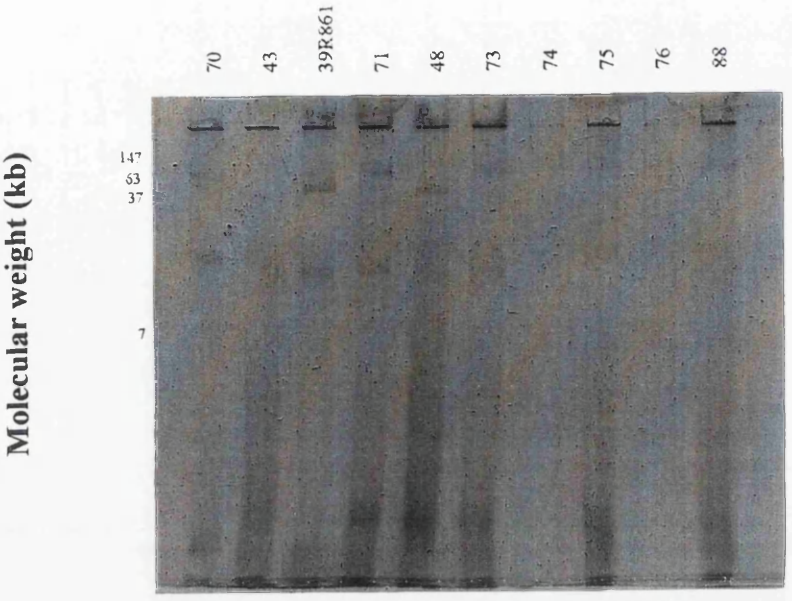

Figure 3.1a Plasmid profiles of 4 plasmid positive and 6 plasmid negative *A.actinomycetemcomitans* strains

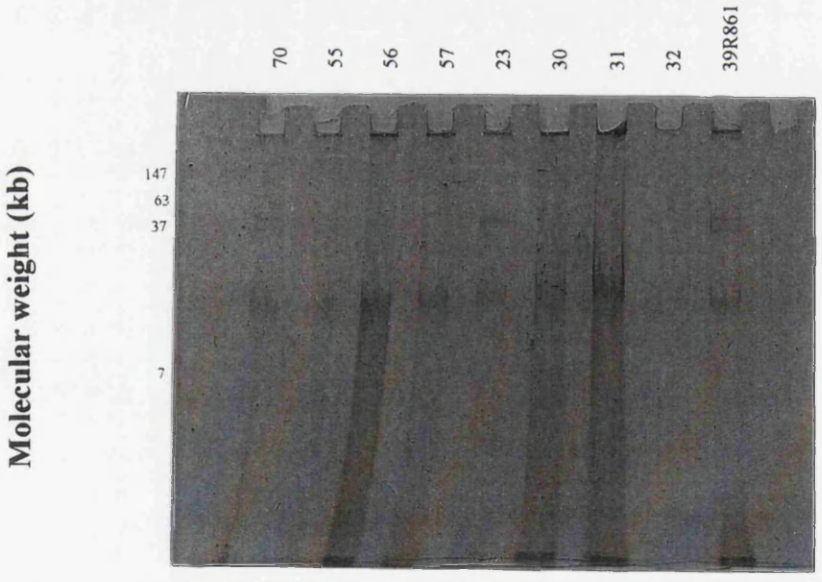

Figure 3.1b Plasmid profiles of 3 plasmid positive and 7 plasmid negative *A.actinomycetemcomitans* strains

Overall, plasmids were detected in 43.2% of the collection: 40 of the 84 clinical strains (47.6%) and 1 of the 11 Reference strains - ATCC 29523 - (9.1%) were plasmid positive (Tables 3.1a, b and c). Plasmid size ranged from approximately 5 to 160 kb and the number of differently sized plasmids seen in plasmid positive strains varied between 1 and 3. The majority of plasmid positive strains, 73.2%, harboured a single plasmid, while 2 plasmids were detected in 24.4% and 3 in the remaining 2.4%.

Whilst no single plasmid or profile predominated, the distribution of the sizes suggested 4 possible clusters at 10 kb (range 5 - 20 kb), 30 kb (range 25 - 40 kb), 60 kb (range 45 - 60 kb) and 80 kb (range 65 - 80 kb).

A total of 11 plasmid profiles were identified as shown in Tables 3.2 and 3.3. This did not include plasmid-free strains, which formed a 12th plasmid profile group. The molecular length range for each plasmid profile group is given in Table 3.2. These results indicate significant diversity of *A.actinomycetemcomitans* strains, although the incidence of plasmid-free strains would argue against the adoption of plasmid profiles as a first line technique in epidemiological investigation.

3.3.1 Single isolates

Forty-nine strains were collected from forty-nine different individuals over the period 1985 - 1991. As only one strain was isolated from each individual, these were termed single strains. Plasmid profiles of these strains, separated them into 8 of the 12 plasmid profile groups described in Table 3.2. This Table demonstrates the distribution of these single strains amongst the different plasmid profile groups. Plasmid-free isolates accounted for 68.0% of the single strains. Strains with one plasmid comprised 22.0% of the single strain group, whilst strains with two plasmids comprised the remaining 10.0%. Groups 5, 11 and 12 were not represented amongst the 49 single strains.

Id No.	Number and Size (kb) of Plasmids	Year of Isolation	Country of Origin
1	2 : 30, 60	1985	Scotland
2	0	1985	Scotland
3	0	1985	Scotland
4	0	1985	Scotland
5	0	1985	Scotland
6	0	1985	Scotland
7	1 : 10	1986	Scotland
8	2 : 10, 60	1986	Scotland
9	0	1986	Scotland
10	1 : 10	1986	Scotland
11	0	1986	Scotland
12	1 : 10	1986	Scotland
13	1 : 50	1986	Scotland
14	1 : 30	1986	Scotland
15	2 : 10, 30	1986	Scotland
16	0	1986	Scotland
17	0	1986	Scotland
18	0	1986	Scotland
19	1 : 10	1986	Scotland
20	0	1986	Scotland
21	0	1986	Scotland
22	1 : 30	1986	Scotland
23	0	1986	Scotland
24	1 : 50	1986	Scotland
26	1 : 50	1986	Scotland
27	2 : 10, 50	1986	Scotland
28	0	1987	Scotland
29	2 : 60, 140	1987	Scotland
30	0	1987	Scotland
31	0	1987	Scotland
32	0	1987	Scotland
33	0	1987	Scotland
34	0	1986	Scotland
35	0	1986	Scotland
36	0	1986	Scotland
89	0	1990	Scotland
90	1 : 20	1990	Scotland
91	0	1990	Scotland
92	0	1990	Scotland
95	0	1991	Scotland
96	0	1991	Scotland

Table 3.1a Plasmid status of single strains of *A.actinomycetemcomitans* isolated from individual patients attending the Glasgow Dental Hospital

Id No.	Number and Size (kb) of Plasmids	Year of Isolation	Country of Origin
37µ - 43µ	0	Not known	Scotland
44*f*	2 : 60, 140	Not known	Scotland
45*f*	2 : 5, 10	Not known	Scotland
46*f*	0	Not known	Scotland
47*f*	2 : 5, 10	Not known	Scotland
48á¥	1 : 30	Not known	Scotland
49á¥	1 : 50	Not known	Scotland
50à¥	1 : 50	Not known	Scotland
51à¥	1 : 80	Not known	Scotland
52à¥	1 : 80	Not known	Scotland
53à¥	1 : 80	Not known	Scotland
55á¢	1 : 60	Not known	Scotland
56á¢	1 : 60	Not known	Scotland
57á¢	1 : 80	Not known	Scotland
54áñ	1 : 80	Not known	Scotland
58áñ	1 : 80	Not known	Scotland
59áñ	1 : 10	Not known	Scotland
60áñ	1 : 80	Not known	Scotland
61áñ	1 : 80	Not known	Scotland
62ô	3 : 5, 10, 15	Not known	Scotland
63ô	1 : 5	Not known	Scotland
64x - 68x	0	Not known	Scotland
69P	1 : 30	Not known	Scotland
70P	2 : 30, 60	Not known	Scotland
71P	1 : 60	Not known	Scotland
72P	1 : 30	Not known	Scotland
73P	1 : 30	Not known	Scotland
74Ü	0	Not known	Scotland
75Ü	0	Not known	Scotland
76Ü	0	Not known	Scotland
77Ü	2 : 30, 60	Not known	Scotland
93> - 94>	0	1990	Scotland

Table 3.1b **Plasmid status of multiple strains of *A.actinomycetemcomitans* isolated from individual patients attending the Glasgow Dental Hospital, including multiple strains from the same periodontal pocket**

The symbols µ *f* á ô x P Ü < > indicate strains from the same individual.
The symbols ¥ ¢ ñ indicate strains from the same pocket.

Id No.	Number and Size (kb) of Plasmids	Country of Origin
78	0	USA
79	1 : 5	USA
80	0	USA
81	0	USA
82	0	USA
83	0	Denmark
84	0	USA
85	0	USA
86	0	USA
87	0	USA
88	0	USA

Table 3.1c Plasmid status of Reference and Type strains

GROUP IDENTIFICATION	NUMBER OF STRAINS IN GROUPS	PLASMID PROFILE	
		NUMBER OF PLASMIDS	MOLECULAR LENGTH RANGE (kb)
1	34	0	0
2	5	1	5 - 25
3	2	1	30 - 45
4	3	1	50 - 65
5	0	0	70 - 85
6	1	2	5 - 25, 30 - 45
7	2	2	5 - 25, 50 - 65
8	0	0	5 - 25, 140
9	1	2	30 - 45, 50 - 65
10	1	2	50 - 65, 140
11	0	0	5 - 25, 5 - 25
12	0	0	5 - 25, 5 - 25 5 - 25

Table 3.2 **Plasmid profiles and frequency of occurrence amongst the single strains**

The molecular length range is purely arbitory. Where two or more ranges are given, plasmids in these ranges have been found.

3.3.2 Multiple isolates from the same patient

Multiple isolates of *A.actinomycetemcomitans* from 9 individuals were obtained over the period 1985 to 1991 (Table 2.1).

Isolates from 8 of these patients could be divided into 8 groups on the basis of number and size of plasmids as shown in Table 3.3. This Table shows the approximate molecular length range of the plasmids and indicates the range of plasmids found in the same individual. Multiple isolates from 4 individuals, strains 37 - 43; 64 - 68; 74 - 77 and 93 - 94 were all shown to be plasmid-free and are included as a single group in Table 3.3. The 14 strains (numbers 48 to 61) which were found in the same individual are excluded from these results and are dealt with separately. The results for Patients A - D are summarised in Table 3.4

Strains 44 - 47 (patient A) showed three different profiles, which indicated that of the 4 isolates there were possibly 3 different strains.

Strains 62 and 63 (patient B) had different profiles, indicating perhaps the presence of two different strains.

Strains 69 - 73 (patient C) show three different profiles. The molecular lengths (Table 3.3) of these suggest that they may be mono- and di-mer forms of the same plasmid.

Strains 74 - 77 (patient D) had two different profiles; 1 plasmid-free (strains 74 - 76) and 1 plasmid positive (strain 77) with two plasmids of approximate molecular length of 30 and 60 kb. These plasmids could be represent mono- and di-mer forms of the same plasmid.

From patient E, 14 different *A.actinomycetemcomitans* strains were cultured, from 3 separate pockets. Each isolate possessed only one plasmid. Those in group 5 predominated (approximate molecular length of 80 kb) with 57.1%. Table 3.5 shows the distribution of the plasmids. These isolates were found in association with each other i.e. isolates with different plasmid profiles were found in the same pocket with each other.

GROUP IDENTIFICATION	NUMBER OF STRAINS IN GROUPS	PLASMID PROFILE	
		NUMBER OF PLASMIDS	MOLECULAR LENGTH RANGE kb)
1	20	0	0
2	1	1	5 - 25
3	3	1	30 - 45
4	1	1	50 - 65
9	2	2	30 - 45, 50 - 65
10	1	2	50 - 65, 140
11	2	2	5 - 25, 5 - 25
12	1	3	5 - 25, 5 - 25, 5 - 25

Table 3.3 Distribution of plasmids in multiple strains from 8 individuals

Patient	Number of Isolates	Number of Profiles	Number of Isolates with Molecular length (kb)
A	4	3	1 @ 0 2 @ 5, 10 1 @ 60, 140
B	3	2	1 @ 5 1 @ 5, 10, 15
C	5	3	3 @ 30 1 @ 60 1 @ 30, 60
D	4	2	1 @ 0 1 @ 30, 60

Table 3.4 Plasmid Profiles of Multiple Isolates from Four Individuals, A - D, Colonised by More Than One Distinct Isolate

GROUP IDENTIFICATION	NUMBER OF STRAINS IN GROUPS	PLASMID PROFILE	
		NUMBER OF PLASMIDS	MOLECULAR LENGTH RANGE (kb)
2	1	1	5 - 25
3	1	1	30 - 45
4	4	1	50 - 65
5	8	1	70 - 85

Table 3.5 **Distribution of plasmids among 14 isolates from 3 gingival pockets of a single patient (E)**

Strains were investigated to show the presence, if any, of large - plasmids those with a molecular length of 450 kb or more, using a method adapted by Rosenberg *et al* (1982). This did not reveal any such plasmids.

3.4 DISCUSSION

Plasmid profiling has advantages over other typing methods such as phage typing. Phage typing requires antisera, a large number of phage and indicator strains and is strain specific. Plasmid profiling is a relatively cheap and fast method. The main disadvantage of plasmid profiling is the requirement for sufficient differences in the molecular lengths of different plasmids which would allow them to separate into distinct patterns.

Plasmids have been reported previously in *A.actinomycetemcomitans* by Olsvik and Preus (1989); LeBlanc *et al*, 1993 and Poulsen *et al*, 1994. Olsvik and Preus (1989) demonstrated the presence of 4 plasmids in 11 fresh clinical isolates, which ranged in size from 6 to 30 kb. They also reported the presence of a 30 kb plasmid in 5 Reference strains they investigated, although the results of this were not shown.

These results differ greatly from those obtained in the present study. Only 1 of 4 of the Reference strains investigated by Olsvik and Preus (1989) was shown to possess a 5 kb plasmid - which was smaller than the one reported by Olsvik and Preus (1989). The three smaller plasmids seen by Olsvik and Preus (1989) had approximate molecular lengths of 10.55, 7.8 and 6 kb. They were found in all strains with the exception of the Reference strains but only when the method of Birnboim and Doly (1979) was used. The large 30 kb plasmid was detected only with the method of Kado and Liu (1986). The method used to prepare the crude plasmid preparations in this investigation was an adaptation of the method of Birnboim and Doly (1979) (Platt, Heraghty and Taggart, 1988).

Poulsen *et al* (1994) suggested that plasmids were rarely found in laboratory strains of *A.actinomycetemcomitans* as they were easily lost upon subculture. LeBlanc *et al*

(1993), demonstrated only 2 plasmid positive strains out of 39 strains investigated. The majority of *A.actinomycetemcomitans* strains investigated were shown to be plasmid negative - it is thus possible that the plasmids of *A.actinomycetemcomitans* are not stable and are easily lost during subculture or storage.

Plasmid negative strains formed the largest group of the *A.actinomycetemcomitans* collection held at the Glasgow Dental Hospital - greater than 50% (this figure included the Reference strains). It is not possible to say if this group forms a single clone without further investigation of the genome (Chapter 4). This group will not be discussed further.

Plasmid positive strains were shown to have 1 of 11 profiles which encompassed a wide range of molecular lengths and combination of plasmids in the strains.

No plasmid or profile appeared to be dominant, although there were four possible clusters of plasmids at approximately 10, 30, 60 and 80 kb.

Single strains collected in 1986 showed a large number of plasmids of 10, 30 and 50 kb, and combinations thereof, but no trends in the period 1985 to 1991 could be distinguished.

Multiple strains from individual patients were investigated and fell into a number of groups - in no case did all the strains from the same individual have the same plasmid profile, with the exception of plasmid negative strains.

Patient A (strains 44 - 47) appeared to be colonised by at least 3 different strains of *A.actinomycetemcomitans*. Two of the four strains shared a common profile, 5 and 10 kb; one had 2 plasmids of 60 and 140 kb and one had no plasmids at all. This patient may originally have been colonised by 3 different strains of *A.actinomycetemcomitans*, with the plasmid profiles as given above. Alternatively, colonisation may have occurred with only 2 different strains and the loss (at some point in the history of the strain, perhaps as recently as during subculture in the laboratory) of the plasmids from a subset of one of the strains would therefore have

resulted in the negative plasmid profile seen.

Patient B (strains 62 and 63) would appear to have been colonised by 2 strains of *A.actinomycetemcomitans*. This phenotype however could be explained by the presence of di- and tri- mer forms of the 5 kb plasmid i.e. the pocket may have originally been colonised by 1 strain of *A.actinomycetemcomitans*, which had 1 plasmid of 5 kb or by 1 strain with all 3 plasmids, 2 of which were lost.

Three different plasmid profiles were observed in Patient C (strains 69 - 71) which would suggest colonisation by 3 strains. However, the size of the plasmids (30 and 60 kb) suggests that one of two events could have occurred. Originally, the gingival pocket may have been colonised by 2 strains of *A.actinomycetemcomitans*, one with a 30 kb plasmid, the other with a 60 kb plasmid. A third strain arose by the transfer of either of the plasmids to the other strain, which created a third distinct strain. Alternatively, 1 strain initially had both plasmids and the loss of either or both plasmids would give rise to the profiles observed.

Patient D (strains 74 - 77). The two plasmid profiles seen in this individual would suggest, as before, that the site has been colonised by 2 strains of *A.actinomycetemcomitans*. Alternatively, the plasmid-free strains may have arisen due to the loss of 2 plasmids.

Patient E (strains 48 - 61) These strains were cultivated from 3 different gingival pockets.

Strains 48 - 54 : within this group from the same pocket, 3 different sizes of plasmid were seen, 30; 50/ 60 and 80 kb.

Strains 55 - 67 : within this group from the same gingival pocket, 2 different sizes of plasmids were seen, 10 and 80 kb.

These data suggest that it is possible for a patient to harbour different *A.actinomycetemcomitans* strains in different sites in the mouth and in the same gingival crevice.

There appears to be no correlation between the size of plasmid and the type of

periodontal lesion from which it was isolated - for example, strain 29, was cultivated from a juvenile periodontitis lesion and harboured 2 plasmids of approximately 60 and 140 kb, whereas 63, was cultivated from a juvenile periodontitis lesion and demonstrated 1 plasmid, of approximately 5 kb.

All the Type strains, with the exception of 79 (ATCC 29523) were plasmid free. This strain demonstrated one plasmid of 5 Kb. This was not associated with periodontal disease, but was originally isolated from blood (Table 2.1).

Plasmids which do not appear to have any known role e.g. either antibiotic resistance or toxin production are designated as "cryptic". When the antibiotic sensitivities (Taher, 1989) of the *A.actinomycetemcomitans* collection from the Glasgow Dental Hospital were compared to the plasmid profiles, no correlation was seen. As the gene for the most studied toxin of *A.actinomycetemcomitans* has been found and is not associated with a plasmid (Chapter 1 and Chapter 7) it is therefore likely that the plasmids which were found in *A.actinomycetemcomitans* are cryptic.

It is not possible to say if the *A.actinomycetemcomitans* collection held at the Glasgow Dental Hospital was derived from the same or different clones, without further investigation, as no one plasmid or profile predominated. Greater than 50% of the collection is plasmid-free - without further investigation of the genome no further information on this group can be made. However, the diversity of plasmid profiles argues that the Glasgow Dental Hospital collection of *A.actinomycetemcomitans* strains was derived from more than one ancestral clone.

CHAPTER 4
STRAIN DIVERSITY OF *A.actinomycetemcomitans* DETERMINED BY WHOLE CELL DNA RESTRICTION ENDONUCLEASE FRAGMENTATION PATTERNS (REFPS)

CHAPTER 4

STRAIN DIVERSITY OF *A.actinomycetemcomitans* DETERMINED BY WHOLE CELL DNA RESTRICTION ENDONUCLEASE FRAGMENTATION PATTERNS (REFPS).

4.1 INTRODUCTION

The cleavage of high molecular weight genomic DNA by appropriately selected restriction endonucleases can generate restriction endonuclease fragmentation patterns (REFPs) or characteristic fingerprints for individual bacterial strains. It is a rapid method which enables large numbers of bacterial isolates to be studied at the same time. Strain diversity of strains, both from the same and different individuals can easily be assessed and the epidemiology studied.

The use of REFPs in the study of the epidemiology of *A.actinomycetemcomitans* and other oral bacteria linked to periodontitis has been well documented by several authors who include Zambon, Sunday and Smukto (1990), Genco and Loos (1991), Han *et al* (1991), van Steenbergen *et al* (1991) and Poulsen *et al* (1994). They used a variety of techniques which ranged from the generation of REFPs to the hybridisation of DNA probes from characterised strains of *A.actinomycetemcomitans* to separate genomic DNA restriction enzyme generated fragments from strains under investigation.

With the exception of Han *et al* (1991) these authors agreed that the population of *A.actinomycetemcomitans* could be divided into subpopulations, which corresponded roughly to the serotypes of the strains investigated. Han *et al* (1991) suggested, however, that there were at least ten clones of *A.actinomycetemcomitans*, in a population of twelve strains.

Clonality is a subject which has provoked much debate - every discipline appears to have a different definition of "clone". Population geneticists, taxonomists and

epidemiologists approach the problem of clonality from different points of view and there can be some difficulty in determining the point at which a species becomes a clone and a clone becomes a strain.

Taxonomists deal with organisms from the order level, down to species; population geneticists deal with organisms at the species to strain level, while epidemiologists deal with organisms at the strain level. Somewhere within this framework "clones" fit. There is no clear definition of a clone. One of the most precise definitions of a bacterial clone, is a single cell, its progeny and all subsequent generations which represent a "monophyletic branch on an evolutionary tree" (Whittam, 1995). A clone is a closed genetic system where variation can only occur through mutation, recombination (internal or external, i.e. transformation, where genes from one clone can be embedded in genes from another, which results in mosaic genes), deletion etc. Because of this, a bacterial clone is not composed of genetically identical bacteria. This is the most useful definition of a clone. Orskov and Orskov (1983) defined "bacterial clones" as "bacterial cultures isolated independently from different sources, in different locations and perhaps at different times, but showing so many identical phenotypic and genetic traits that the most likely explanation for this identity is a common origin". Whittam's definition could apply equally to an entire strain as to a single clone; whilst the definition of Orskov and Orskov (1983) could be applied more appropriately at the strain level. The population geneticist considers a clone to be close to the species level, whereas the epidemiologist considers the clonality of an organism to be near the strain level.

For this study, the definition of a clone was considered to be that defined by the epidemiologist i.e. a bacterial strain and a clone are closely related to each other.

The work described in this chapter examined the diversity of a collection of strains of known origin using REFP analysis.

The *A.actinomycetemcomitans* strains investigated comprised a mixture of Type strains and freshly isolated clinical isolates from the West of Scotland. The temporal

and spatial origin was known for the majority of the Type strains and all of those isolated in Glasgow. It might be expected that strains isolated in the same location would have had a similar clonal origin to each other - and a different clonal origin to strains from a different continent. However, there are several possibilities for the clonal origin of *A.actinomycetemcomitans*. For example, the epidemic spread of a recently evolved strain of *A.actinomycetemcomitans*, i.e. worldwide distribution, could be facilitated through travel of infected individuals.

Additionally, the same individual could be colonised by different strains of *A.actinomycetemcomitans*, if the strains were very diverse, were easily spread and established i.e. the individual was easily colonised.

The West of Scotland strains may or may not belong to a common clone, this would depend upon the ease of transmission (Christersson *et al*, 1985., Preus and Olsen, 1988., Alaluusua *et al*, 1991., Zambon *et al*, 1993) and opportunities for spread e.g. the ethnic origin of the transient tourist population.

4.2 MATERIALS AND METHODS

4.2.1 Purification of whole cell DNA

On the basis of plasmid status and previously known information on the *A.actinomycetemcomitans* collection (Table 2.2), a subset of 18 strains was chosen.

All reagents were prepared as described in Appendices 1.3 and 1.4. Twenty millilitre overnight cultures of the 18 strains of *A.actinomycetemcomitans* (Table 2.2) were prepared in TSB broth, as previously described (2.1.2). These were centrifuged as before, after which the cells were resuspended in 3 ml TE_{50}. This volume was divided into 3, 1 ml aliquots, which were washed twice in TE_{50}. After the second wash, the cells were resuspended in 500 µl TE_{50}. Twenty-five microlitres of 20% SDS (Sigma) was then added individually to each tube, which were immediately mixed by inversion. After 5 minutes incubation on ice, the suspension became clear and viscous, which indicated that the cells had lysed. Fifty microlitres of proteinase K was

added and the tubes mixed, by inversion. The lysates were sheared to reduce the viscosity of the DNA, by charging and expelling the material through a syringe with a 0.5 x 25G needle (Microlance Nr. 18). The tubes were then incubated at 37°C for 2 hours.

The samples were extracted by the addition of 500 µl phenol/ chloroform (BDH), with thorough mixing followed by centrifugation at 13000 rpm for 15 minutes, after which the aqueous layer was removed to a fresh eppendorf. Five hundred microlitres of isopropanol was added and the samples left for 60 minutes at room temperature. DNA was pelleted by centrifugation as before and the isopropanol removed. Pellets were dried in a 37°C incubator and resuspended in 100 µl of TE_{10}. Triplicate tubes for each sample were pooled and to each 300 µl, 100 µl 7.5M ammonium acetate (Sigma) was added; the tubes thoroughly mixed. Six hundred microlitres of icecold 95% ethanol (BDH) was added and the tubes thoroughly mixed. After overnight incubation at -20°C the ethanol was removed and the dried pellet resuspended in 300 µl of TE_{10}. Twenty microlitres of RNase A was added and the samples were incubated at 37°C for 60 minutes. A second phenol/ chloroform extraction was performed as before, followed by a further isopropanol precipitation step. The tubes were left at room temperature for 60 minutes and the DNA pelleted and dried as before. The dried pellet was resuspended in 300 µl TE_{10} and 100 µl of 7.5M ammonium acetate added, followed by 600 µl of 95% ethanol (icecold). The tubes were left overnight at -20°C. The DNA was pelleted and dried as before, then resuspended in 100 µl TE. From this volume, 3 µl was removed and diluted in 87 µl TE (1 in 30). From the diluted fraction, 60 µl was removed and scanned in microcuvettes over the range 310 to 210 nm, in a UV-8 spectrophotometer (Pye, Unicam, Cambridge, UK.). This was then assessed for concentration and purity. The scan gave an indication of any likely type of contamination, by comparison with controls of lambda DNA spiked with different reagents considered to be the most probable cause of contamination e.g. ethanol and phenol.

DNA purity was estimated from the ratio OD_{260} : OD_{280} according to the criteria of Maniatis, Fritsch & Sambrook (1991)

Ratio = 1.8 : DNA considered to be pure.

Ratio > 1.8 : DNA contaminated with protein or phenol.

Ratio < 1.8 : DNA contaminated with ethanol.

The OD_{260} reading allowed the calculation of nucleic acid concentration in the sample, when pure. An OD_{260} of 1 corresponded roughly to 50 μgml^{-1} for double stranded DNA (Maniatis et al, 1991). The DNAs were then diluted to an approximate concentration of 10 μgml^{-1}.

4.2.2 Restriction enzymes

The generation of restriction endonuclease patterns of whole cell DNA after restriction with frequent cutter enzymes i.e. four base cutters, is not widely used (Platt, Browning & Candlish, 1996). The use of four base cutters, however, overcomes the limitations described by Owen (1989). The resolution of large fragments into an open window enables computer analysis of the pattern and gel-to-gel comparison to be made. Patterns generated in this fashion enable a degree of visual quality control to be carried out, as partial digestion products are easily recognisable. This method can be adapted for a disparate range of organisms using a range of eight enzymes. The main disadvantage of this method is the small proportion of the genome which is analysed.

The restriction enzymes used were chosen from a panel tested in initial experiments, which included *Pst*I, *Hind*III, *Sma*I, *EcoR*V, *Ava*II, *Sau*3AI, *Hinc*II and *Hae*III (New England Biolabs). From this panel three were chosen - *Sau*3AI, *Hin*fI and *Hae*III - and used exclusively. The three enzymes chosen were 4 base cutters, which give a greater frequency of large fragments. Enzymes with a recognition sequence of six bases tended to give a peak molecular weight of 5 kb and the remaining DNA has the

appearance of being smudged, whereas 4 base cutters tended to give a clean area ("open window") at the top and a smudge at the bottom. The size of the open window varied with both enzymes and organism, but generally, DNA of molecular weight between 8 and 30 kb is desirable - the enzymes used in this investigation resulted in bands with a molecular weight of less than 8 kb being detected.

The enzymes used were unaffected by methylation with the exception of *Hae*III which did not cleave GGC_mC, whereas GG_mCC was cleaved.

A 20µl aliquot of each DNA was digested with between 20 and 30 units of enzyme. To this, 5 µl of the appropriate reaction buffer (*Sau*3AI - New England buffer *Sau*3AI; *Hae*III and *Hin*fI - New England buffer 2) was added and the total volume made upto 50 µl with distilled water.

Digestion was at 37°C for 4 hours. Parallel digests of lambda DNA were carried out to control each enzyme. Additionally, lambda DNA was separately digested with *Pst*I and *Kpn*I enzymes, which were used as gel calibrators. This allowed accurate sizing of the restricted fragments (see 4.2.4).

4.2.3 Electrophoresis of whole cell DNA

Restricted whole cell DNA was electrophoresed in 0.8% agarose gels (Appendix 1.3). These were prepared using TBE buffer and cast in BRL Horizon 20.25 tanks.

To each digest volume of 50 µl, 5 µl of gel sample buffer was added and the total 55 µl was loaded under TBE buffer. The gels were run at a constant current of 20 mA, typically for 42 hours, after which they were stained in TES containing ethidium bromide, examined under ultraviolet light and photographed as described previously (3.2.3).

4.2.4 Digitised REFPs

Photographs of the restricted DNA were digitised as previously described in detail (Platt and Sullivan, 1990). This program was run on a 486 computer with Summa

Graphic II digitising pad. Fragment sizes were calculated by interpolation from the calibrators with a robust modified hyperbolic fitting routine derived from Plikaytis *et al* (1986). The Dice coefficient of similarity (Dice, 1945) was used to calculate similarities between different REFPs.

The Dice coefficient (S_D) was calculated by the equation

$$S_D \quad \frac{2m}{a+b} \quad X\ 100\%$$

m is the number of matching fragments between the two tracks.
a is the total number of fragments in track a.
b is the total number of fragments in track b.

The results generated by Molmatch enabled the number of bands which were different between the two compared strains to be found. The total number of fragments in each REFP varied, and therefore the percentage value which each contributed to the overall Dice coefficient of similarity altered for each comparison. It was important to assess the value for each matching band as this would affect the placing of a strain within a particular clone.

For example, if a + b equals 100 and m equals 50, then each band contributes 2% to the overall similarity coefficient (100%). If m = 45, then the Dice coefficient is 90%. However, if a + b equals 40 and m equals 20, then each fragment contributes 5%. If 5 bands are different then the Dice coefficient is reduced to 75%. Therefore not only is the Dice coefficient important, but the total number of different bands is also important.

Thus Dice coefficients are not ideal but provide a means of assessment of large amounts of data, provided the limitations are recognised and incorporated into the overall interpretation. For example, two strains are compared, one of which has a large molecular weight band equivalent to 50% of the total genome. If this represents the only difference between the two strains, the Dice coefficient will be high, although in reality the two strains are not at all closely related.

Fragment size variation was set at 5% - in preliminary experiments this degree of variation was shown to accurately reflect the degree of operator input error.

4.3 RESULTS

The REFPs from the three enzymes were examined both visually and numerically using Dice coefficients.

Visual assessment of the REFPs obtained with each enzyme (Figures 4.1 - 4.4) allowed a number of different preliminary conclusions to be drawn.

(i) Irrespective of restriction enzyme, there were fragments common to all strains.

(ii) There was clear evidence of considerable strain diversity in the REFPs from each enzyme.

(iii) The maximum number of common fragments and least discrimination was seen in *Hin*fI REFPs.

(iv) On visual inspection both *Hae*III and *Sau*3AI REFPs indicated that certain strains were clustered, whereas other strains were quite distinct and visual clusters were the same for both enzymes.

These preliminary observations argued in favour of numerical analysis using Dice coefficients of similarity.

4.3.1 Dice coefficients

***Hae*III**

Figure 4.1 demonstrates the REFPs for the 18 strains under investigation, generated after digestion with *Hae*III. It was not possible to produce a REFP for strain 79, using this enzyme, despite repeated attempts. This image was digitised and the results are shown in Figure 4.2. The Dice coefficients of similarity were found and are shown in Table 4.1a. An open window was seen which extended down to approximately 4.0 kb but exceptionally to 3.36 kb (strain 83).

Variation between the strains ranged from 56.4 (strain 55 compared to strain 33) to 100% (strain 55 compared to strain 57).

On the basis of *Hae*III digestion alone, only strains 55 and 57 were completely identical with a Dice coefficient of 100%. However, strains 55, 56 and 57 formed a distinct cluster of S_D greater than 97%. Several other clusters were seen, including, 32 and 33 (S_D 96.8%) and 74, 75 and 76 (S_D greater than 90%) formed a distinct cluster and strain 81 was also related to these (S_D greater than 87%).

***Hin*fI**

Figure 4.3 demonstrates the digitised image of the REFP for the strains under investigation, generated after digestion with *Hin*fI. Dice coefficients for the strains under investigation are shown in Table 4.1b.

The Dice coefficients of similarity varied between 72.0 (strain 33 compared to 83) and 100% (e.g. strain 75 compared with both 74 and 76) - seven strains demonstrated Dice coefficients of 100% (see Table 4b). Other strains notably similar were 32 and 33 (S_D 93.6%); 8, 74, 75, 76 and 81 (SD greater than 96.6%); 24, 81 and 88 (SD greater than 96.3%); 29, 74, 75, 76 and 80 (S_D greater than 93.3%) and 80, 74, 75 (SD greater than 96.6%) all of which formed distinct clusters.

The open window for this enzyme was very narrow and ranged from approximately 5.6 to 2.1 kb.

The discriminating powers of the enzymes varied - *Hin*fI did not discriminate well, whilst both *Sau*3AI and *Hae*III were highly discriminating. This reflects deeper elements of the clonal structure.

***Sau*3AI**

Figure 4.4 demonstrates the digitised image of the REFP for the strains under investigation. Of these, only 3 pairings of strains were shown to have Dice coefficients of 100% - strains 32 and 33; strains 55 and 57; 29 and 75 (Table 4.1a).

Other strains notably similar were 55 and 56; 56 and 57 (S_D 95.2%); 29, 74 and 76 (S_D greater than 87%); 74, 75 and 76 (S_D greater than 88%); 80 and 81 (S_D greater than 90%) and 83 and 88 (S_D greater than 94%) which all formed distinct clusters.

The open window for this enzyme, with these strains, ranged between 6.6 and approximately 1.7 kb. Dice coefficients varied between a low of 46.7 (32/33 compared to 83) and a high of 100% (as described in Table 4.1a).

4.3.2 Hierarchical assessment

The Dice coefficients of similarities for each of the enzymes revealed the following clusters of *A.actinomycetemcomitans*, seen irrespective of the enzyme used.

(i) Strains 29, 74, 75 and 76

(ii) Strains 55, 56 and 57

The following clusters were seen when 2 enzymes (*Hae*III and *Hin*fI) were used

(i) Strains 74 and 81

The following clusters were seen with 2 enzymes *Sau*3AI and *HaeIII*

(ii) Strains 32 and 33

The following clusters were seen with 1 enzyme only (*Hin*fI)

(i) 8 with 29, 74, 75 and 76

The following clusters were seen with 1 enzyme only (*Sau*3AI)

(i) 24 and 29

(ii) 80 and 81

(iii) 83 and 88

Figure 4.5 demonstrates a proposed hierarchical structure for the strains of *A.actinomycetemcomitans* investigated.

4.3.3 Common bands

Molmatch generated molecular weights for the digitised bands, which could then be examined and comparison between the different strains made, to show which bands, if

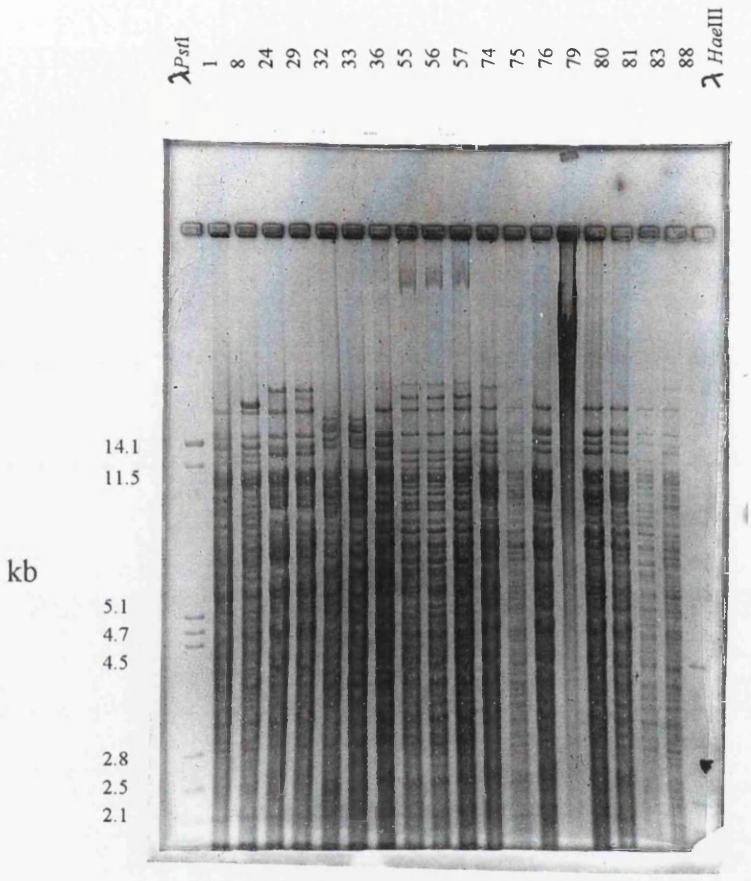

Figure 4.1 REFP library for 18 strains of *A.actinomycetemcomitans* after digestion with *Hae* III

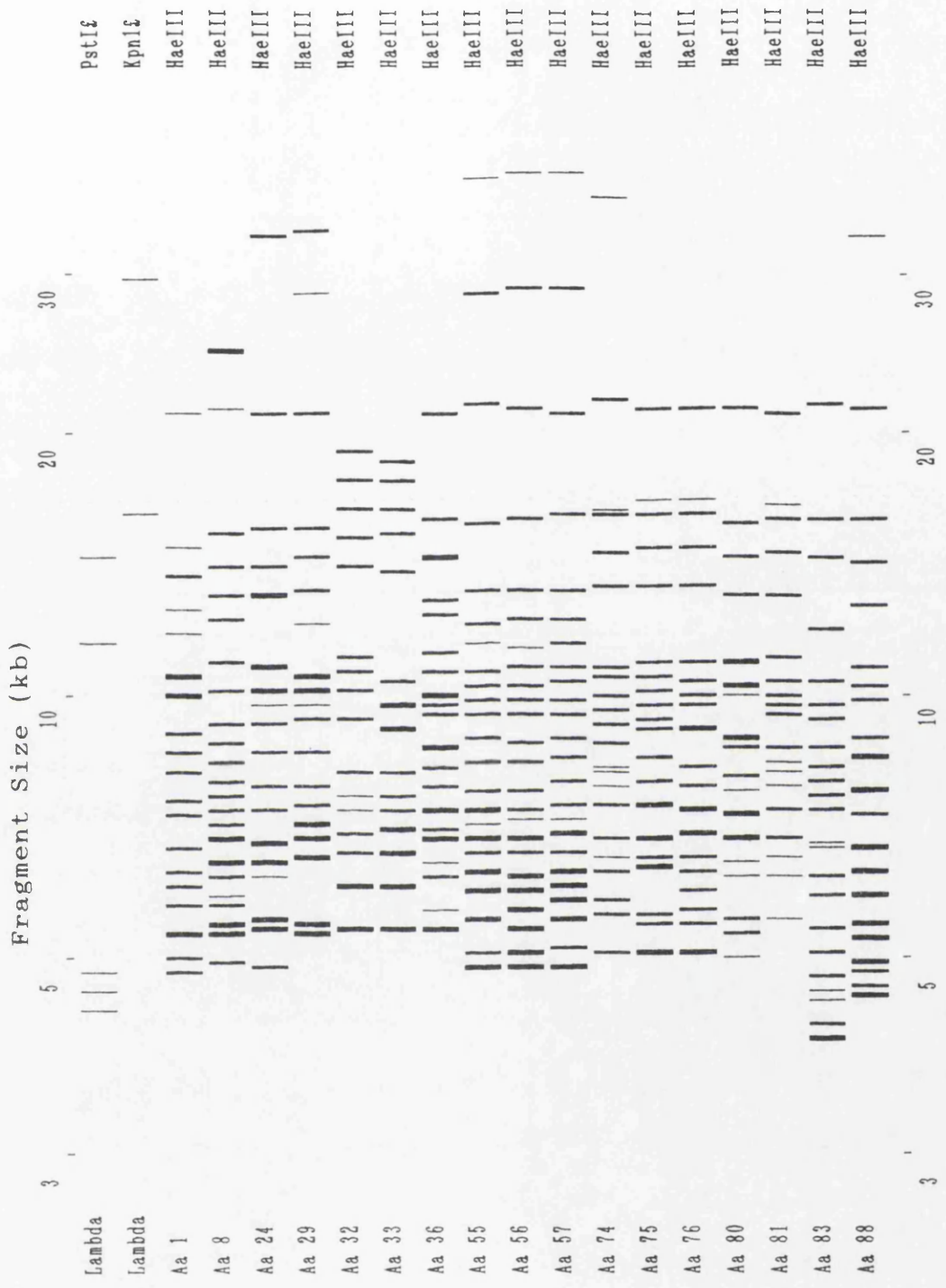

Figure 4.2 Graphical output from digitised images of *A.actinomycetemcomitans* strains, digested with *Hae* III

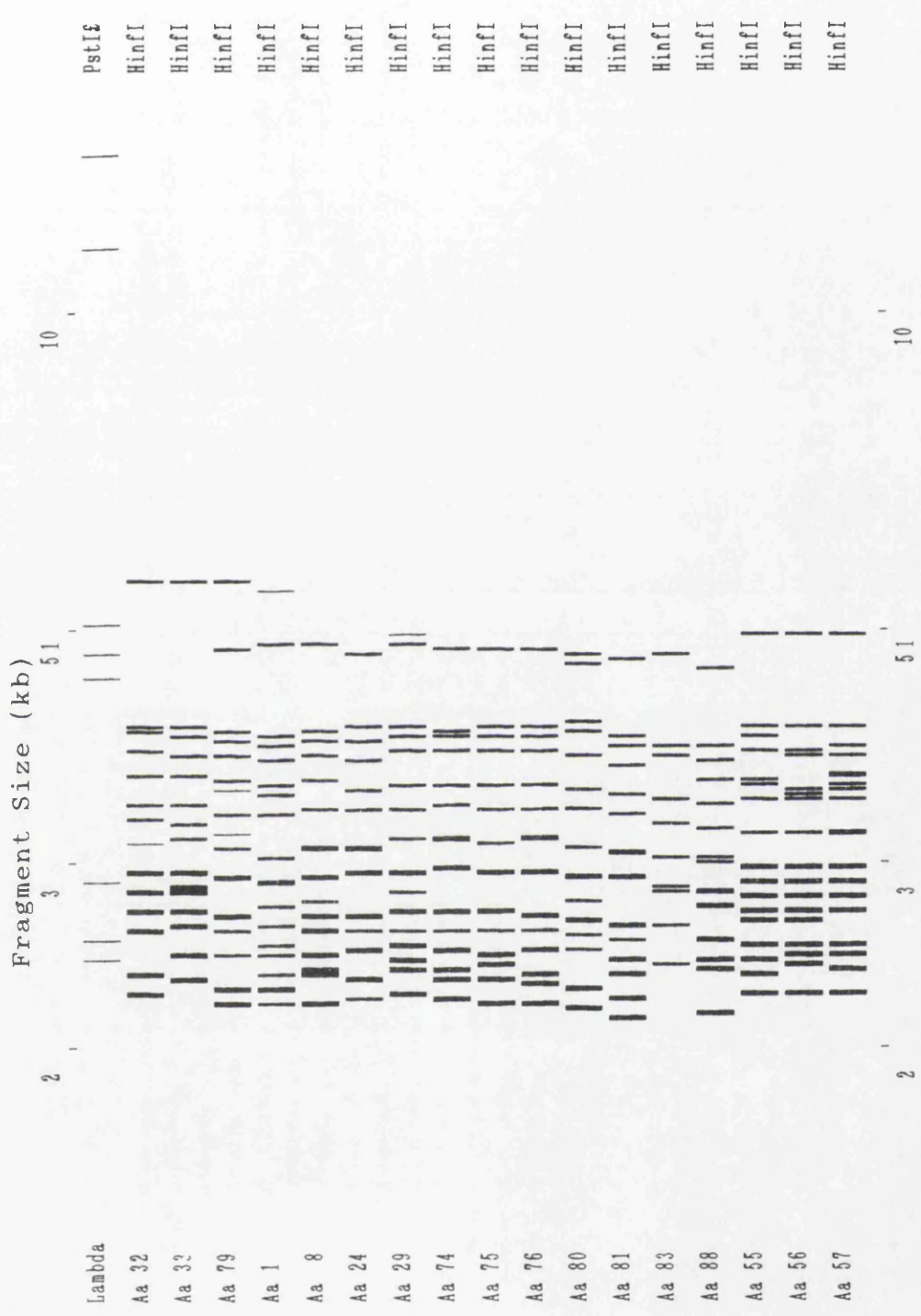

Figure 4.3 Graphical output from digitised images of *A.actinomycetemcomitans* strains, digested with *Hin* fI

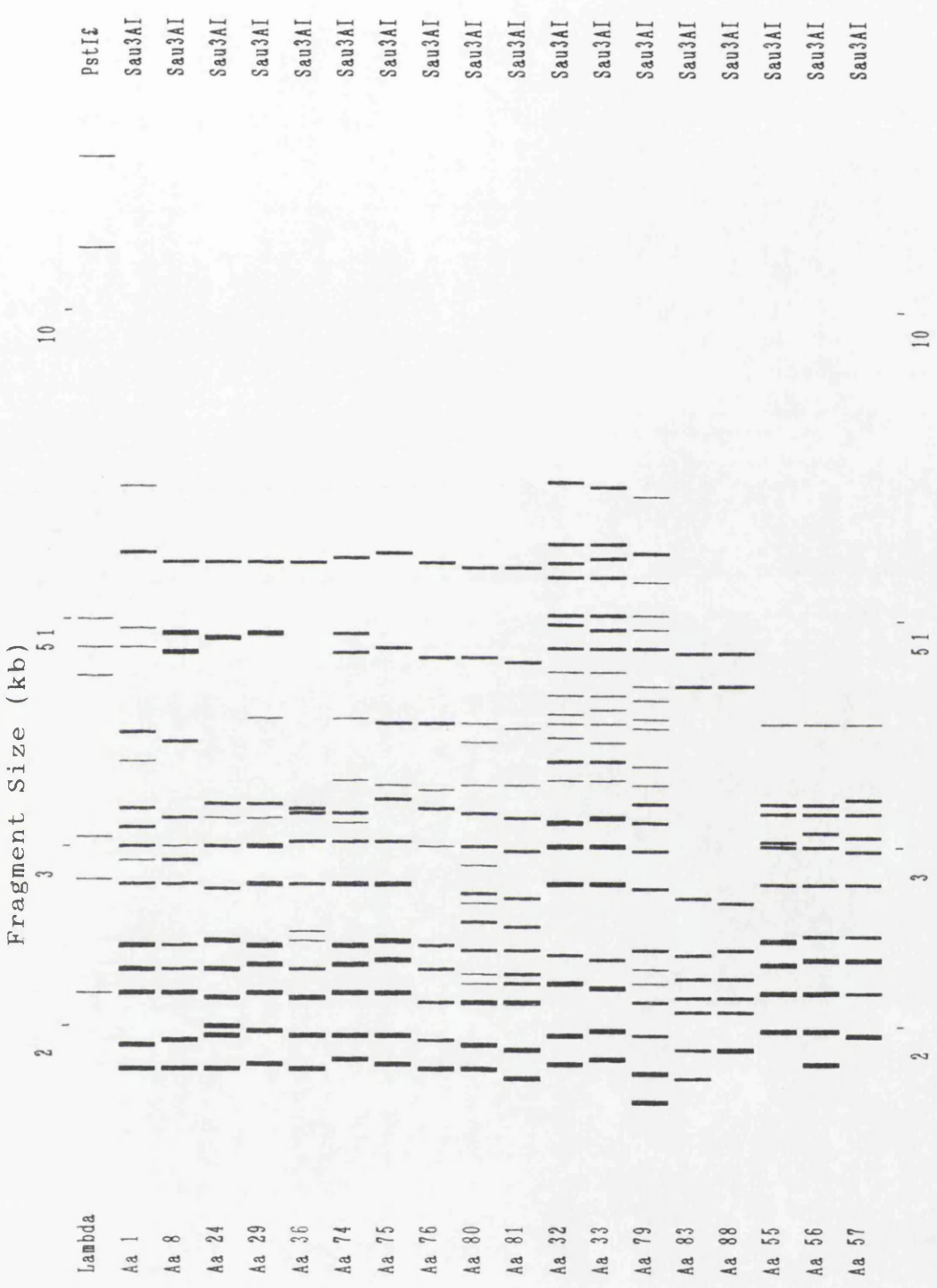

Figure 4.4 Graphical output from digitised images of *A.actinomycetemcomitans* strains, digested with *Sau* 3AI

*Strain No.*1	8	24	29	32	33	36	55	56	57	74	75	76	79	80	81	83	88
1	89.7	78.6	85.7	70.3	70.3	74.1	76.9	81.5	76.9	93.9	78.6	81.5	76.9	78.8	73.3	56.0	50.0
8	*85.7*	80.0	80.0	64.7	64.7	75.0	78.3	83.3	78.3	88.9	88.0	83.3	66.7	80.0	81.5	63.6	57.0
24	*86.5*	*78.0*	91.7	54.5	54.5	87.0	72.7	78.3	72.7	84.6	91.7	87.0	62.9	75.9	76.9	76.2	70.0
29	*78.5*	*76.2*	*86.5*	66.7	60.6	87.0	81.4	87.0	81.8	92.3	100.0	87.0	68.6	75.9	84.6	66.7	70.0
32	*62.9*	*62.5*	*64.7*	*62.9*	100.0	50.0	51.6	50.0	51.6	68.6	66.7	62.5	86.4	63.2	62.9	46.7	41.4
33	*64.7*	*63.2*	*66.7*	*64.7*	*96.8*	50.0	45.2	50.0	51.6	68.6	66.7	62.5	86.4	63.2	62.9	46.7	48.3
36	*82.1*	*83.7*	*84.2*	*87.2*	*72.2*	*68.6*	76.2	81.8	76.2	80.0	78.3	81.8	58.8	71.4	88.0	60.0	52.6
55	*85.7*	*78.3*	*73.2*	*76.2*	*61.5*	*63.2*	*83.7*	95.2	100.0	75.0	81.8	762	60.6	74.1	75.0	52.6	55.6
56	*83.7*	*80.9*	*76.*	*79.1*	*60.0*	*56.4*	*864*	*97.9*	95.2	80.0	87.0	81.8	64.7	78.6	80.0	60.0	63.2
57	*83.7*	*85.1*	*76.2*	*79.1*	*60.0*	*61.5*	*86.4*	*97.9*	*100.0*	83.3	81.8	76.2	60.6	74.1	75.0	52.6	55.6
74	*78.0*	*75.6*	*80.0*	*68.3*	*68.4*	*64.9*	*85.7*	*80.0*	*78.3*	*78.3*	92.3	88.0	75.7	77.4	85.7	60.0	54.5
75	*71.8*	*74.4*	*84.2*	*82.1*	*77.8*	*80.0*	*90.0*	*74.4*	*81.8*	*77.3*	*95.2*	95.7	68.6	82.8	92.3	66.7	60.0
76	*76.9*	*79.1*	*84.2*	*82.1*	*83.3*	*74.3*	*85.0*	*79.1*	*81.8*	*81.8*	*90.5*	*95.0*	64.7	78.6	88.0	60.0	52.6
79	*	*	*	*	*	*	*	*	*	*	*	*	*	70.0	70.3	56.3	51.6
80	*86.5*	*82.9*	*94.4*	*81.1*	*76.5*	*72.7*	*84.2*	*78.0*	*81.0*	*81.0*	*85.0*	*89.5*	*89.5*	*	90.3	61.5	56.0
81	*73.7*	*76.2*	*81.1*	*73.7*	*74.4*	*70.6*	*87.2*	*76.2*	*74.4*	*74.4*	*92.7*	*87.2*	*87.2*	*	*86.5*	69.6	63.9
83	*74.4*	*76.6*	*71.4*	*69.8*	*60.0*	*61.5*	*81.8*	*80.9*	*79.2*	*79.2*	*78.3*	*68.2*	*72.7*	*	*76.2*	*74.4*	94.1
88	*75.0*	*72.7*	*87.2*	*75.0*	*64.9*	*66.7*	*73.2*	*72.7*	*75.6*	*71.1*	*74.4*	*73.2*	*78.0*	*	*82.1*	*70.0*	*80.0*

Table 4.1a Dice coefficients of similarity (%) between the total genome digests of *A.actinomycetemcomtians* strains cut with Sau3AI **and *HaeIII*. (Clusters of interest underlined).**

Strain No.	**8**	**24**	**29**	**32**	**33**	**36**	**55**	**56**	**57**	**74**	**75**	**76**	**79**	**80**	**81**	**83**	**88**
1	93.8	86.7	90.9	90.3	87.5	*	94.1	88.2	88.2	90.3	90.3	90.3	90.9	87.9	90.3	74.1	90.3
8		92.9	96.8	82.8	80.0	*	93.8	93.8	87.5	96.6	96.6	96.6	83.9	93.3	96.6	80.0	82.8
24			89.7	81.5	78.6	*	86.7	86.7	86.7	96.3	96.3	96.3	89.7	92.9	96.3	78.3	96.3
29				80.0	83.9	*	90.9	90.9	90.9	93.3	93.3	93.3	81.3	96.8	86.7	76.9	86.7
32					93.6	*	83.9	83.9	83.9	85.7	78.6	78.6	86.7	82.8	78.6	75.0	85.7
33						*	81.3	81.3	81.3	82.8	75.9	82.8	83.9	80.0	75.9	72.0	82.8
36							*	*	*	*	*	*	*	*	*	*	*
55								100.0	94.1	90.3	90.3	90.3	90.9	87.5	77.4	74.1	83.9
56									94.1	90.3	90.3	90.3	84.8	81.3	77.4	74.1	83.9
57										90.3	90.3	90.3	90.9	87.5	77.4	74.1	83.9
74											100.0	100.0	86.7	96.6	100.0	75.0	85.7
75												100.0	86.7	96.7	100.0	83.3	85.7
76													86.7	89.7	100.0	83.3	85.7
79														83.9	86.7	69.2	86.7
80															89.7	80.0	89.7
81																75.0	92.9
83																	83.3

Table 4.1b Dice coefficients of similarity (%) between the total genome digests of *A.actinomycetemcomtians* strains cut with *Hin*fI (Clusters of interest underlined.)

any, were common to the strains.

***Hae*III**

Several bands appeared to be common to several of the strains.

A.actinomycetemcomitans strains 55, 56, 57, 74 and 88 demonstrated bands of 40 ±1.0 kb (which may have represented unclean and unplasmid DNA).

The majority of the strains demonstrated a band of 25 ± 1.0 kb (*A.actinomycetemcomitans* strains 8, 36, 55, 56, 57, 74, 75, 76, 80, 81, 83 and 88.

Strains 32 and 36 were the only strains not to demonstrate bands at 9.7 ± 1.0 kb and 21 ± 1.0 kb. However, these strains demonstrated unique bands of 16 and 18 kb. Some of the tracks on the gel below 6.0 kb could not be digitised and therefore no comment can be made regarding commonality.

***Hin*fI**

As with *Sau*3AI the molecular weights of the bands digitised were very small - between a maximum of 5.59 kb and a minimum of 2.14 kb.

The bands which appeared common to the majority of the strains were very small e.g. 3.92 ± 0.04 and 2.52 ± 0.07.

***Sau*3AI**

With *Sau*3AI it was possible to digitise the tracks down to bands with a molecular weight of 1.81 ± 0.06. All strains examined, with the exception of 57, possessed this band.

Several other bands were common to the majority of the strains, e.g. 1.95 ± 0.05 kb and 4.69 ± 0.05 kb.

However, only small molecular weight bands were common to all strains. The largest

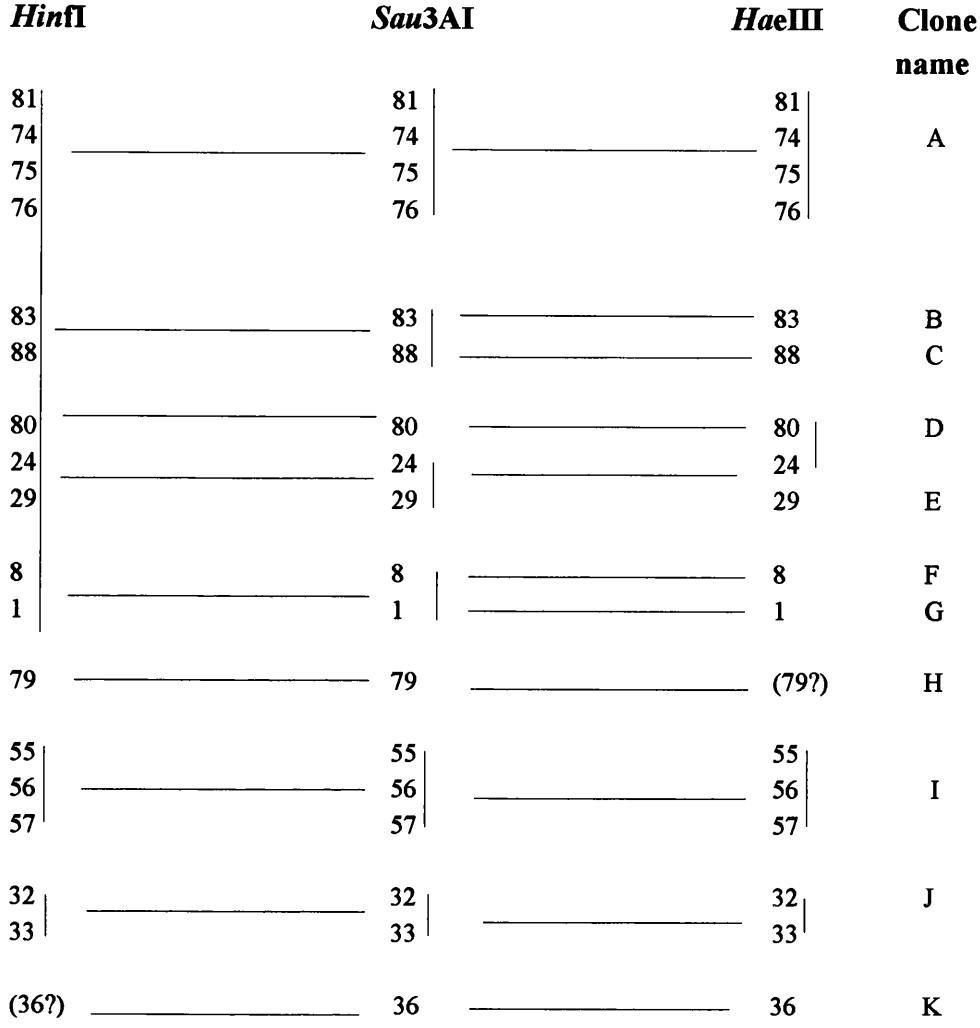

Figure 4.5 **Proposed clonal structure of *A.actinomycetemcomitans* strains: differential discrimination based upon 3 enzymes. The vertical bars represent strains which are identical to each other on the basis of restriction endonuclease fragmentation patterns with individual enzymes**

bands had a molecular weight of 6.7 kb, which were not seen in all the strains investigated.

The REFPs reflected the overall location of restriction sites within the genome and thus provided a method to estimate the genetic similarity of different strains of bacteria. Differences seen between the restriction patterns of the different strains could be due to either the presence of plasmid DNA or mutations which have resulted in a variation to the enzyme restriction sites. Spurious bands observed in REFPs from plasmid positive strains were compared to plasmid profiles (Table 3.1) and these bands discounted. Preparations of plasmid DNA would not be demonstrated to cut (see Chapter 5) and it was therefore, assumed that any plasmid DNA which was copurifed in genomic preparations had not cut. Bands with a molecular length similar to plasmid molecular lengths were found not only in plasmid positive strains, but also in other strains.

Although REFPs are relatively rapid to produce, they are not without limitations. They are dependent upon the cutting frequency of the restriction enzyme (which is related to the size of the bacterial genome) and there may be > 50 bands of varying sizes. An open window provided a greater degree of flexibility with regard to minor variations in DNA concentration and greater amenability for quantition by computer. The lower limit for numerical analysis was established empirically and standardised for each enzyme for the purpose of calculation of Dice coefficients. Further visual assessment of smaller fragments was often possible but was more subjective and therefore not included in numerical estimates.

Although windows can occur anywhere within the fingerprint, it is of most value when it occurs in the top region of the gel where the largest fragments of DNA have separated. This allowed analysis of a larger percentage of the total genome to be investigated compared to an open window at the bottom of the gel, where the smallest fragments have separated. Moreover, an open window allows visual assessment for the presence of partial digestion products and thus provides an

additional element of DNA quality control. This is particularly important when DNA is prepared from organisms that are less easily grown consistently.

4.3.4 Clonal Structure of the Glasgow Dental Hospital *A.actinomycetemcomitans* population

The bacterial population under investigation was derived from mainly patients who attended the Periodontal Department of the Glasgow Dental Hospital and School. All had periodontal disease (Table 2.1) : some were single strains from unrelated individuals, whereas others were multiple strains from the same periodontal pocket. A number of the collection were Type strains, originally held both in the USA and UK.

Type strains

Comparison of the Dice coefficients of each Type strain cut with each enzyme, did not suggest a single clonal origin for the Type strains. Tables 4.1a and 4.1b show that, for example, *A.actinomycetemcomitans* Type strain 80 cut by *Hae*III is most closely related to *A.actinomycetemcomitans* 81, (S_D = 86.5%) , whilst it shared only 76.2% similarity with *A.actinomycetemcomitans* 83. *A.actinomycetemcomitans* strains 81 and 88 were the least related of the Type Strains showing only 70% similarity.

When the Type strains were cut by *Sau*3AI, strains 80 and 81 had a Dice coefficent of 90.3%. With the same enzyme, strains 83 and 88 had a Dice coefficient of 94.1%.

When the Type strains were cut by *Hin*fI 80 compared to 81 and 88 demonstrated a Dice coefficient of 89.7%; strains 81 and 88 had a Dice coefficient of 92.9%.

These results did not suggest a single clonal origin for the Type strains of *A.actinomycetemcomitans*.

When the results of *Sau*3AI digestion are compared, there is more variation between the strains. *A.actinomycetemcomitans* 79 showed the greatest variation compared with strains 83 and 88 with similarity coefficients of 56.3 and 51.6% respectively. Once again, 80 and 81 were most similar to each other.

*Hin*fI restriction did not show the same range in variation as was seen with the other

enzymes, however, once again strains 80 and 81 were shown to be most closely related, as were strains 88 and 80. The least similar strains were 79 and 83.

Clinical isolates

Comparison of the clinical strains with each other revealed that whilst several of the strains appeared to be related to each other, major variation existed between others.

Strains 55, 56 and 57 showed very little variation between each other with each of the three enzymes used. When compared to other clinical strains they demonstrated similarities which were low - less than 50% similarity (*Hae*III, 55 compared to 33, S_D = 45.2% - Table 4a) to others which were quite high (*Hin*fI, 57 compared to 76, S_D = 90.3%). Thus, *A.actinomycetemcomitans* strains 55, 56 and 57 originally isolated from the same patient, belong to the same clone and are consistent with multiple isolates of the same strain.

Strains 74, 75 and 76 similarly showed a great deal of similarity to each other, however, strain 74 demonstrated a larger degree of variation to strains 75 and 76. Additionally, several of the other strains were shown to have Dice coefficients which were quite high, although not high enough for the strains to belong to the same clone, depending upon the enzyme used to cut the DNA. Strains 75 and 29 were shown to have a Dice coefficient of 100% when cut with *Sau*3AI, but this reduced to 82.1% when cut with *Hae*III. The Dice coefficient of similarity between 29 and 57 when cut with *Hin*fI was 93.3%, although this enzyme generally gave high results.

The degree of similarity of the remaining strains did not suggest a single clonal origin for all the strains, with the exception of strains 32 and 33. These *A.actinomycetemcomitans* strains demonstrated a similarity coefficient of 100, 96.8 and 96.6% with *Hae*III, *Sau*3AI and *Hin*fI respectively. Additionally, they demonstrated very little similarity to the other strains.

Type strains versus clinical isolates

Comparison of all the strains to each other did not suggest that the strains had arisen

from a single clone.

The Type strains isolated in America showed very little similarity to those isolated in Glasgow and to the Danish Type strain (*A.actinomycetemcomitans* strain 83). Type strains 83 and 88 demonstrated only 1 band of variation when digested with *Sau*3AI. With *Hae*III, there was a difference of 9 bands, each has a value of 2.2% - this is significant.

There would not appear to be any correlation between clonality and disease e.g. strains of *A.actinomycetemcomitans* associated with juvenile periodontitis do not all belong to the same clone.

These results demonstrated that the *A.actinomycetemcomitans* collection held at the Glasgow Dental Hospital and School comprised several clones.

Strains 55, 56 and 57 were derived from one chronic periodontal pocket from one individual. This result suggests that an individual pocket could be colonised by the one strain/ clone of *A.actinomycetemcomitans*.

Strains 74, 75 and 76 were derived from three periodontal pockets from within the one juvenile periodontal patient. This result would suggest that it is possible for one individual to be infected by more than one strain of *A.actinomycetemcomitans*. This could suggest that one clonal type is associated with juvenile periodontal disease. It is possible, however, that the individuals from whom these strains originated were colonised by more than one clonal type of *A.actinomycetemcomitans* (as is the individual from whom strains 74, 75 and 76 were derived) but that only the one clonal type was selected during the initial sampling process.

From the small subpopulation of *A.actinomycetemcomitans* investigated (18 strains from a collection of 96) it would seem that the *A.actinomycetemcomitans* population at the Glasgow Dental Hospital and School is composed of a large number of different clones.

4.4 DISCUSSION

DNA fingerprinting has been used previously in investigations of the clonal origins of *A.actinomycetemcomitans.*

The genetic structure of any given population depends upon the extent of genetic recombination within the population. It is also dependent upon the population biology of that bacterium Several different studies have investigated the genetic diversity of *A.actinomycetemcomitans*, all of which have produced different results. Each group used a different set of restriction enzymes and bacterial strains. Some groups looked at the DNA fingerprint, whereas others produced DNA probes which were then used in hybridisation studies. Poulsen *et al* (1994) suggested, on the basis of studying 97 strains, that the *A.actinomycetemcomitans* population consisted of a very large pool of genotypes, which could be divided into subpopulations - corresponding roughly to the serotype of the strain under investigation. However these authors did not properly define their interpretation of a "subpopulation". Instead of being a subpopulation, they were probably defining a clone. Evidence for this large pool of distinct genotypes was demonstrated by the results presented above. Poulsen *et al* (1994) were unable to show any association between a particular genotypic pattern and disease. This has, to limited degree, also been shown by the above results. Haubek *et al* (1995) examined 88 different strains of *A.actinomycetemcomitans* and on the basis of multilocus enzyme electrophoresis (MEE) and restriction fragment length polymorphisms (RFLPs) of the gene *lktA* (the structural leucotoxin gene) suggested that the species is composed of "subpopulations" which correspond to the serotypes of the strains. As with Poulsen *et al* (1994), Haubek *et al* (1995) were unable to show any correlation between periodontal disease and any clone.

Zambon, Sunday and Smutko (1990) used 16 restriction enzymes to digest DNA from 124 plaque strains of *A.actinomycetemcomitans* from North America, Norway and Korea. They found the strains from different continents showed little diversity by REFP analysis. It probably reflects the inappropriate choice of restriction enzymes

that failed to discriminate and this was not supported by the results which are presented here. Zambon *et al* (1990) used 2 enzymes (*Eco*RI and *Hind*III) from the panel of 16 and found 3 major restriction enzyme patterns. They related restriction pattern to the serotype of the strains which Poulsen *et al* (1994) confirmed. Zambon *et al* (1990) did not show any variation between multiple strains of *A.actinomycetemcomitans* from 1 individual - they reported 15 strains from the one individual with the same clonal type, unlike the results which were obtained for the strains 74, 75 and 76, which were not only from the same individual but from the same periodontal pocket. The variations were minor and probably represent strain divergence under the prevailing selection pressure in a chronic infection. Zambon *et al* (1990) also reported that non-human strains of *A.actinomycetemcomitans* showed a high degree of variation and diversity.

DiRienzo *et al* (1990) used probe specific DNA fingerprinting to show that individuals could be colonised by upto 2 clonal types. This technique is highly sensitive to minor genetic variations in nucleotide sequences, more so than RFLP analysis and is a precise means of characterising species and identifying individual strains of closely related bacteria.

The results reported by DiRienzo and Slots (1990) and Zambon *et al* (1990) differ, as Zambon *et al* (1990) did not find that individuals were infected with more than one strain of *A.actinomycetemcomitans*. The results reported here are similar to the results of DiRienzo and Slots (1990), although these results are based upon REFPs only.

The choice of enzyme may have resulted in the conclusion of Zambon *et al* (1990) that an individual could only be infected by one clone of *A.actinomycetemcomitans*. They also reported only distinguishing three different clonal patterns while using 2 different enzymes. This contrasts with the results presented in this thesis in which several different clones for *Sau*3AI alone were produced.

Han *et al* (1991) used three enzyme *Sal*I, *Xho*I and *Xba*I to digest 12 Type strains of

A.actinomycetemcomitans, and classified these into 10 distinct clonal types. However, the open window they examined was at the bottom of the gel where the smallest fragments of the DNA have separated - smaller than those demonstrated in the results of this investigation. These bands represent a very small fraction of the total genome available for digestion (perhaps less than 5% of the total genome).

The DNA fingerprints obtained by Zambon *et al* (1990) did show variation in an open window at the top of several of the gels, as individual bands can be seen. However the samples would have benefited from a longer period of electrophoresis to separate them out more, and so reveal much more information.

Van Steenbergen *et al* (1991) investigated three strains of *A.actinomycetemcomitans* which were differentiated from each other on the basis of the fermentation of mannitol and xylose. Isolated genomic DNA was digested with several enzymes and a double digestion of *Pst*I and *Bam*HI produced the optimum results on Reference and Type strains. However they did not show the results of restriction digest analysis of the three strains of *A.actinomycetemcomitans* which they recovered. They stated that the 11 isolates (which included both newly isolated bacteria and Reference strains) which were double digested, could be separated into 9 different "types" - but these were not defined.

From the published photographs of the results of van Steenbergen *et al* (1991) there would appear to be a lot of smeared DNA bands. The bands were also faint and difficult to differentiate.

4.5 CONCLUSIONS

It would appear that the population chosen from the collection is similar in clonal structure to that proposed by other researchers.

It is possible not only for an individual to be infected by more than one clone of *A.actinomycetemcomitans*, but also for a periodontal pocket to be colonised by two

clones of *A.actinomycetemcomitans*. This conclusion is in line with the results of other researchers who have employed hybridisation techniques.

Strains which have been isolated over a period of time, but on the same continent e.g. the American Type strains do not show much similarity, although they share a common ancestor. The Danish Type strain was shown to be different from both the American Type strains and the Glasgow strains. Additionally, there was little conservation between the strains from Glasgow and America, indicating that they have had a different clonal background.

CHAPTER 5
PLASMID RESTRICTION ENDONUCLEASE FRAGMENTATION PATTERNS OF *A.actinomycetemcomitans*

CHAPTER 5

PLASMID DNA RESTRICTION ENDONUCLEASE FRAGMENTATION PATTERNS OF *A.actinomycetemcomitans*

5.1 INTRODUCTION

Restriction Endonuclease Fragmentation Pattern (REFP) analysis of plasmid DNA has been documented by several authors in the investigation of bacterial epidemiology (Threlfall *et al*, 1985; Platt *et al*, 1986; Platt, Heraghty and Taggart, 1988; Platt and Smith, 1991). The presence of plasmids within *A.actinomycetemcomitans* has been demonstrated (Olsvik & Preus, 1989; LeBlanc *et al*, 1993; Poulsen *et al*, 1994), although there are no reports of REFP analysis of these plasmids.

Several problems were encountered throughout this investigation, most severely, the inhibition of restriction enzyme activity. The work described below is a summary of the different lines of investigation undertaken to determine the nature of the inhibition of restriction enzyme cleavage.

5.2 MATERIALS AND METHODS

5.2.1 Purification of plasmid DNA

Plasmid DNA was purified using an adaptation of the method of Kraft, Timbury and Platt (1987).

A.actinomycetemcomitans strains investigated in initial experiments, were the subset of strains listed in Table 2.2 known to be plasmid positive (Chapter 3), which were investigated in the strain diversity experiments described in Chapter 4. However, strains 24 and 29 were used exclusively in later, "problem-shooting" experiments.

Overnight cultures in TSB broth (20mls) were prepared as previously described (2.1.2). All the reagents and buffers were prepared as described in Appendices 1.3 and 1.4. The

broths were centrifuged for 10 minutes at 3000 rpm (MSE, Loughborough, England) and the pellet washed twice in 800 µl TE, pH 7.8. The final volume was divided into 4 x 200µl aliquots and to each, 400 µl alkaline SDS was added. After 5 minutes incubation on ice, 300 µl 3M sodium acetate, pH 4.8 was added, the tubes mixed and samples left on ice for a further 5 minutes. These were centrifuged for 3 minutes at 13000 rpm in a bench top microcentrifuge. The supernatant was removed to a fresh eppendorf tube, to which 500µl phenol/chloroform was added. The tubes were mixed vigorously, centrifuged as before and the upper aqueous layer removed to a fresh eppendorf tube without disruption of the interface. To this, 500 µl isopropanol (BDH) was added and the tubes left at room temperature for 60 minutes. These were centrifuged, the pellet dried and resuspended in 100 µl TE : quadruplicate tubes were pooled and 100 µl 7.5M ammonium acetate, pH 8.0, added and mixed. DNA was precipitated by the addition of 600 µl, ice cold, 100% ethanol (BDH), after which the tubes were mixed and left overnight at -20°C. After centrifugation, the pellets were dried and resuspended in 160 µl TE. To this, 18 µl RNAase was added and the tubes incubated at 37°C for 30 minutes, followed by the addition of 20 µl 2.5M sodium chloride. A second phenol/ chloroform extraction was performed as before, followed by further isopropanol and ethanol precipitation steps. The DNA was pelleted, dried then resuspended in 60 µl TE buffer and stored at +4°C until required.

The purity and concentration of the DNA suspensions were found as previously described (4.2.1).

5.2.2 Restriction enzymes

The restriction enzymes used were chosen from a panel used in initial experiments, which included *Pst* I, *Hind* III, *Sma* I, *EcoR* VI, *Ava* II, *Hinc* II and *Hae* III. After initial work, *Ava* II, *Hinc* II and *Hae* III were used exclusively.

A 20 µl aliquot of each DNA was digested with between 20 and 50 units of enzyme. To this, 5 µl of the appropriate reaction buffer was added and the total volume made upto 50µl with distilled water.

Digestion was at 37°C for 4 hours. Parallel digests of lambda DNA were carried out to quality control each enzyme and lambda DNA was additionally digested with *Pst* I enzyme, which was run in the gel as a gel calibrator.

5.2.3 Electrophoresis of plasmid DNA

Digested plasmid DNA was separated by electrophoresis in 0.8% agarose gels prepared with TBE buffer, which contained ethidium bromide. These were cast in BRL Horizon 11.14 electrophoresis tanks.

To each digest volume of 50 µl, 5 µl of gel loading buffer was added and the 55 µl volume then loaded under TBE buffer which contained ethidium bromide. The gels were run overnight at a constant current : 25 mA - typically for 18 hours. Gels were examined under ultraviolet light and photographed as before, after which they were stained in TES containing ethidium bromide, examined under ultraviolet light and photographed as described previously (3.2.3).

5.3 RESULTS AND DISCUSSION

Many of the problems which were associated with the generation of plasmid REFPs were encountered during the preparation of whole cell DNA REFPs, although these were later overcome. The problems encountered during the preparation of whole cell DNA are also discussed in this section.

The generation of a REFP is, in principle, a very straight forward and simple one.

The methods of Kraft, Timbury and Platt (1987) were used to produce both plasmid and

whole cell DNA. These have been used successfully to generate plasmid and whole cell fingerprints from *E.coli*, *Salmonella* and *Legionella* species. The method, however, was not without difficulties when applied to *A.actinomycetemcomitans*.

Problems encountered included the non-cutting of DNA, low yields of DNA and smearing of the bands.

5.3.1 Plasmid REFPs

Initial experiments were carried out on a selection of plasmid positive strains, chosen from the 18 strains of *A.actinomycetemcomitans* investigated previously in Chapter 4. However, as the full extent of the problems encountered became apparent, *A.actinomycetemcomitans* strains 24 and 29 were used exclusively. (Strain 29 had not been used previously in Chapter 4, however, it harboured 2 good sized plasmids (Chapter 3) and was used for this reason.

5.3.2 Whole cell REFPs

Initial experiments were carried out on the 18 strains of *A.actinomycetemcomitans* listed in Table 2.2. The discussion which follows applies, both to the preparation of plasmid and whole cell DNA, except where stated.

5.3.3 Low yields of DNA

This problem was an initial and intermittent one, which was solved by the addition of 1 full plate of growth by means of a dry cotton wool swab, from an 18 hour CBA plate of *A.actinomycetemcomitans*. Each broth was grown for 18 hours to ensure that the broth was in log phase when cultured.

Prior to the inoculation, CO_2 was added to the broth, by leaving in a CO_2 incubator with the top slightly loose. The choice of media was also important and TSB broth was found

to give the best yield.

5.3.4 Non-cutting of DNA

This was the greatest problem encountered. It was intermittent and could not be predicted. DNA from the same production batch would cut differently on different days with the same enzyme.

The non-cutting of the enzymes was not the result of either *dam* or *dcm* methylation as these enzymes would cut the same DNA intermittently.

The quality of the DNA was investigated spectrophotometrically. DNA was scanned over the region OD_{280} to OD_{260} and the trace was examined for contamination by protein, RNA, ethanol or phenol. DNA was rarely found to be contaminated by any of these. A further purification stage was introduced by an addition phenol/chloroform step, followed by precipitation by 95% ethanol overnight, then 70% ethanol overnight to remove any potential contaminant.

This DNA was then scanned again and if pure it was used in restriction digests. The scan also enabled the concentration of DNA to be calculated and adjusted to give a constant concentration. This, however did not help to improve the quality of the restriction digests.

5.3.5 Cell clumping

(i) Glucose

The effect of clumped cells would have reduced the surface area available for lysis with the SDS, and this would therefore have reduced the yield of DNA.

In the earliest experiments it was found that *A.actinomycetemcomitans* cells clumped together when resuspended in TGE. Clumping was seen to be strain dependent - rough strains clumped together more than smooth. Microscopic examination showed that the

cells clumped to a lesser degree when resuspended in TE as opposed to the TGE suggested by Kraft *et al* (1987). Clumping could be reduced by washing the cells in a non-glucose containing buffer e.g. TE and reduced by washing in 25mM Tris sucrose. This curing effect was reversed if cells were resuspended in TGE.

A.actinomycetemcomitans strains contain a high proportion (22.4%) of glucose in the cell wall (Brondz and Olsen, 1989) compared to other carbohydrates such as rhamnose (2.6%), galactose (5.2%) and glucosamine/ galactosamine (5.2%). It is possible that when *A.actinomycetemcomitans* cells are suspended in a solution with a high glucose content (50 mM), the cells have a tendency to become "sticky" and simply co-aggregated, forming the large clumps of cells seen. Although the effect of resuspension of *A.actinomycetemcomitans* cells in a lower concentration of glucose was not investigated, *A.actinomycetemcomitans* strains were suspended in buffers which were glucose free, with no lessening in the clumping effect.

(ii) Cations

The effect of cations upon clumping was investigated. Rough and smooth strains demonstrated different behaviour when resuspended in the same buffers.

Complete dissociation of smooth strains was observed when they were resuspended in $CaCl_2$, whilst molar tris completely dissociated rough strains. Additionally, there appeared to be an association with decreasing molarity of EDTA - the lower the molarity, the greater the clumping of rough strains seen. This was not an unexpected result, as EDTA is a chelating agent, and as such would be expected to result in clumping. The presence of cations resulted in the dissociation of smooth strains.

The clumping of *A.actinomycetemcomitans* cells has been previously investigated. Scannapieco *et al* (1987) suggested that colonies grown under anaerobic conditions were of a firmer consistency than aerobic cultures and when grown under anaerobic conditions

in a fluid medium, had a greater tendency to clump, compared with colonies grown, under aerobic conditions. Scannapieco *et al* (1987) did not suggest any reason for the clumping of the bacterial cells, however clumping could be related to the presence or absence of pseudopods and fimbriae (Section 1.2.3) and their subsequent interaction. In the presence of excess cations, chemical interactions may have occurred with, for example, the fimbrial surface layer, which resulted in cells being attracted to each other, followed by "interweaving" of the fimbriae and finally bacterial aggregation. As fimbriae do not appear to be present on the surface of smooth strains, cations would not have this effect. This may explain why chelating agents resulted in the strain specific nature of the phenomenon (Section 1.2.3).

The effect of culturing cells under aerobic conditions was not investigated but, the level of CO_2 present in broths was increased as much as possible in order to increase the yield of DNA (see DNA Yield).

Mechanical methods for the dissociation of the bacterial cells were investigated e.g. *A.actinomycetemcomitans* cells were passed through a fine bore needle. These were not used routinely as it was thought they would result in damage to the DNA.

Sonication of the cells for very brief periods of time (up to 5 seconds) was also investigated, but this method was not used, as it could not be guaranteed that the cells were only disrupted and not lysed. The latter would have probably resulted in the degradation of the DNA by certain cell contents e.g. enzymes.

Resuspension of *A.actinomycetemcomitans* in 800 µl of TE buffer rather than the 400µl suggested by Kraft *et al* (1987) reduced clumping and improved lysis by increasing the available surface area for interaction with SDS.

The effect of reducing clumping resulted in larger DNA yields, but did not have any effect upon the quality of the restriction digest.

5.3.6 DNA Yield

Preliminary experiments suggested that one cause of the lack of DNA REFPs may have been due to a low yield of DNA. One way to increase the yield of DNA was to increase the number of bacterial cells used. This was achieved by the addition of a larger initial inoculum to the growth medium (broth) and by a change in the type of broth used. Tryptic soya broth gave a greater yield of bacterial cells than BHI broth.

Additionally, to ensure an adequate supply of CO_2 for the bacterial cells, CO_2 was added to the broth prior to the addition of bacterial cells by incubation in a 5% CO_2 incubator overnight before inoculation. This increased the yield of *A.actinomycetemcomitans* cells. Also, an increase in the centrifugation time at each stage increased both the size of pellets and the resultant DNA extracted subsequently.

The combined effect of these changes from the published method of Kraft *et al* (1987), (including the resuspension in 800 µl TE) produced a visual increase in the size of the final pellet and the yield of DNA produced, determined by UV spectrophotometery. The frequency of DNA cleavage, however, remained intermittent.

5.3.7 DNA Purity

Additional washes after the phenol/ chloroform stage were performed using chloroform alone, to remove any final trace of phenol, which may have been present. This did not increase the frequency of DNA cleavage.

The purity and yield of the DNA was checked for contamination by phenols, RNA and proteins by scanning over the range 310 to 210 nm, in a UV-8 spectrophotometer, as previously described (4.2.1). No contamination of this kind was found.

The possibility of salt contamination was also investigated - DNA was washed and precipitated several times in both 95 and 100% ethanol. Again, no plasmid fingerprint was obtained.

It is known that ethanol can interfere with both the reaction buffer and the restriction enzyme, so additional care was taken to ensure that all the ethanol was removed by drying the pellet for extended periods of time, both at room temperature and in a 37°C incubator, before the addition of the TE suspension buffer. These modifications did not improve either the quality or reproducibility of the plasmid fingerprint obtained.

5.3.8 Failure of restriction endonucleases to cleave *A.actinomycetemcomitans* DNA

Enzyme inhibition in the form of *dam* and *dcm* methylation was investigated. The methylase encoded by the *dam* gene transfers a methyl group from S-adenosyl-methionine to the N^6 position of the adenine residues in the sequence GATC i.e. G_mATC. The second form of methylation is that encoded by the *dcm* gene. It methylates the internal cytosine residues in the sequences CCAGG and CCTGG at the C^5 position i.e. $C_mC(A/T)GG$. These sites are important because some or all of the sites for a restriction endonuclease may be resistant to cleavage when isolated from strains of bacteria expressing either *dam* or *dcm* methylation. If the methylation site overlaps a restriction enzyme recognition site then the DNA will not be cleaved.

The recognition sequences of the restriction enzymes (*Ava*II, *Hae*III and *Hinc*II) used in the plasmid investigations were as follows :

*Ava*II 5'----- G^G$^{A}_{T}$C C ----- 3'

3'----- C C$^{T}_{A}$G^G ----- 5'

*Hae*III 5'----- GG^CC ----- 3'

3'----- CC^GG ----- 5'

*Hinc*II 5'----- GTPy^PuAC ----- 3'

3'----- CAPu^PyTG ----- 5'

*Ava*II is blocked by overlapping *dcm* methylation, *Hae*III is not, but is blocked by GG_mCC. *Hinc*II is unaffected by methylation. Therefore, if the cause of the inhibition is *dam* methylation, then *Ava*II would not cleave the plasmid DNA, whereas *Hae*III would. This was not the case - both *Ava*II and *Hae*III were shown to cleave the plasmid DNA periodically.

There are several factors which influence enzyme activity on both plasmid and genomic DNA. These include tertiary DNA structure, base composition of the flanking sequences and position of the cleavage sites with respect to each other. The length of the piece of DNA being restricted is also important, as smaller lengths of DNA may require more enzyme. The units of enzyme used varied between 20 and 50 units, with latter experiments exclusively using 50 units of enzyme.

Methylation is important in gene regulation and expression - genes which are not transcribed may be methylated, while the absence of methyl groups in some organisms is associated with gene expression. It could therefore be possible that the intermittent nature of the enzyme restriction could be related to the growth phase of the bacteria. Care was taken that all solid cultures were grown for the same length of time before broth cultures were inoculated. However, it cannot be excluded that the periodic nature of the DNA REFPs could relate to periods of growth, when the DNA was unmethylated and in a state which would enable it to be cleaved by enzymes normally affected by methylation e.g. *Ava* II. Nevertheless, this would not explain the intermittent nature fully, as enzymes which were unaffected by methylation also showed this phenomenon.

The growth phase of the bacteria would have an effect upon the structure of the DNA. Actively dividing cells would have DNA in an open form, i.e. uncoiled, thus, enzymes would have access to restriction sites, which may have normally been hidden. Thus, as with methylation, cells which were in the stationary phase would not undergo restriction enzyme cleavage. This would explain both the intermittent nature of the cutting and also

the observation that enzymes which were not affected by methylation did not cleave the DNA.

5.3.9 Contamination

(i) Protein

The potential effect of protein contamination was minimised by the incubation of DNA preparations with proteinase K at a concentration of 10 $mgml^{-1}$, for 2 hours. The proteinase K was added at different stages e.g. before the addition of SDS (to remove any surface protein which may have interfered with the action of the restriction enzyme) and after the pellet was removed from the sodium acetate stage. The sodium acetate was incubated at 55°C on all occasions. As before, there was no improvement on the quality of profiles which were obtained. When the purified DNA was scanned in a UV spectrophotometer protein was rarely seen. Any protein contamination was removed by an additional phenol/ chloroform step, as described above

(ii) Lipopolysaccharide (LPS)

Purification of DNA with the inclusion of CetylTrimethyl Ammonium Bromide (CTAB) was carried out to exclude the co-purification of LPS, which if bound to the DNA, would have prevented enzyme restriction. This did not improve the quality of DNA which was purified and therefore LPS contamination could be excluded.

(iii) Phenol/ chloroform contamination

This potential inhibitor of enzyme activity was removed by ensuring that the interface was not disturbed when the top aqueous layer was removed. Again, the UV spectrograph traces were checked for this potential contaminant, against standards of lambda DNA, both with and without phenol/ chloroform. No contamination of the DNA due to phenol/

chloroform was found.

5.2.10 Concentration of DNA versus units of enzyme

The possibility that the concentration of DNA was too high was investigated by an increase in the number of enzyme units used in each digest, from 50 to 100.

This had no positive effect upon the reproducibility of the plasmid DNA profiles and was not continued.

5.3.11 Ethidium bromide (Plasmid only)

Ethidium bromide/ caesium chloride gradients can be used to purify large scale preparations of closed circular DNA by centrifugation (Maniatis, Fritsch & Sambrook, 1982). Ethidium bromide used in these gradients has a final concentration of 600 μgml^{-1}.

Small scale preparations of plasmid DNA from *A.actinomycetemcomitans* strains 24 and 29 were spiked with ethidium bromide at a concentration equivalent to 600 μgml^{-1}. Ethidium bromide was removed by addition of 1-butanol saturated with distilled water. The two phases were mixed vigorously, then centrifuged at 13000 rpm for 3 minutes. The lower phase was then removed to a clean eppendorf tube and the extraction repeated until all the pink colour had disappeared.

On the first occasion bacterial cell lysates were spiked by the ethidium bromide at various stages in the preparation of the plasmid DNA. When this DNA was incubated with *Hinc*II, a restriction pattern was observed, although there was only partial digestion of the DNA. However, identical results were also obtained from controls of DNA prepared from the same cells.

When further aliquots of this preparation of DNA were incubated with *Ava*II, which had been shown previously to cut *A.actinomycetemcomitans* DNA, the DNA remained uncut. Further preparation of *A.actinomycetemcomitans* plasmid DNA spiked with ethidium

bromide did not result in DNA which could be cut with either *Ava*II or *Hinc*II, which indicated that the periodic cutting/ non-cutting of the DNA could not be resolved by preparation of the DNA in an environment which contained ethidium bromide.

5.3.12 Spiked DNA Preparations

Preparations of both whole cell and plasmid DNA were spiked with lambda DNA at all stages of preparation, e.g. after addition of alkaline-SDS, sodium acetate, phenol/ chloroform etc. on both day 1 and day 2 of the preparation protocol to show that the inhibitory effect was not due to the DNA, but due instead to the presence or absence of an inhibitory element. This experiment was repeated on a number of occasions with both strains under investigation. On every occasion the lambda DNA was cut, irrespective of where it was introduced in the protocol, while the native plasmid DNA was not cut. This suggested that it was not the result of the co-purification of an agent which inhibited the enzyme, but that fault lies with the DNA. Methylation, the most obvious solution, has previously been discounted.

5.3.13 Operator error

That the lack of reproducible REFPs was due in part to operator error was also investigated. Preparations of *E.coli* and *S.enteriditis* plasmid and whole cell DNA were made using the methods of Kraft *et al* (1987). These were run in parallel to the preparations of *A.actinomycetemcomitans* plasmid and whole cell DNA and on all occasions, produced REFPs.

5.4 General Discussion

The problem of non-cutting plasmid DNA was an intermittent one, which could not be fully resolved. The problem of non-cutting whole cell DNA was overcome. However, it

proved impossible to produce a method for the preparation of plasmid DNA which could be reproduced for any length of time - even day-to-day.

The most likely explanation would appear to be some mechanism which is switched on in some preparations and off in others. The most probable mechanism which falls into this category is DNA methylation.

There are, however, problems with this conclusion - namely the intermittent nature of the DNA cleavage. Had the enzyme *Ava*II not cut the plasmid DNA at all, then this would have been an adequate explanation. However, *Ava*II, was one of the enzymes which was routinely used, for the very reason that it could be inhibited by methylated DNA, and that in preliminary experiments, it was shown to cut *A.actinomycetemcomitans* plasmid DNA. *Ava*II is, however, less sensitive to inhibition due to methylation. Generally, for the DNA to be cut by *Ava*II it would have to become unmethylated during certain periods of cell growth e.g. during transcription. Great care was taken to ensure that all DNA preparations were made with bacterial cells which were in log phase at all times. It cannot be excluded, however, that on some occasions cells may have been harvested during the stationary or lag phase and not the log phase.

The possibility of contamination by cellular components co-purified with the DNA e.g. protein was explored and excluded. Likewise, contamination of the preparations by phenol, RNA and carbohydrate.

Operator error was also examined and while it may have contributed to lack of DNA REFPs at the beginning of the experimental period it would not explain the lack of fingerprints after the long time period involved in the investigation of this problem. It was possible to produce plasmid REFPs for two bacterial species unrelated to *A.actinomycetemcomitans* i.e. *E.coli* and *S.enteriditis*. It would therefore seen unlikely to be due entirely to operator error.

Had more time been available, it may have been possible for another individual to prepare

plasmid DNA from *A.actinomycetemcomitans* isolates and to compare their results to those given above.

In the *A.actinomycetemcomitans* literature there is no reference to similar problems which have been experienced by other researchers. Problems such as possible lipopolysaccharide contamination, have been encountered by other workers, working on other bacteria, were dealt with using methods which in the past have been shown to remove this form of contamination.

All of the published literature on *A.actinomycetemcomitans* REFPs to date has been on genomic preparations. After initial problems, it was possible to obtain usable genomic profiles and therefore the problem of non-cutting was no longer an issue with respect to genomic preparations. However, there has been no published data on the REFPs of plasmid DNA, which could be the result of either similar problems such as those described above, or alternatively, the lack of plasmid positive strains which has been reported in the literature may have resulted in researchers overlooking plasmids as a means of investigating epidemiology, given their cryptic nature.

CHAPTER 6
MONOCLONAL ANTIBODY, 9D4, TO THE *Bordetella pertussis* TOXIN ADENYLATE CYCLASE AND ITS POSSIBLE CROSS REACTION WITH *A.actinomycetemcomitans* PROTEINS

CHAPTER 6

MONOCLONAL ANTIBODY, 9D4, TO THE *Bordetella pertussis* TOXIN ADENYLATE CYCLASE, AND ITS POSSIBLE CROSS REACTION WITH *A.actinomycetemcomitans* PROTEINS.

6.1 INTRODUCTION

Since Lally *et al* (1991a) demonstrated the presence of the *A.actinomycetemcomitans* leucotoxin in the outer membrane, it could be hypothesised that preparations of the OMPs from *A.actinomycetemcomitans*, separated by SDS-PAGE, would contain the leucotoxin protein. The toxin could thereafter be detected in two ways, either (i) by the presence of a protein band of the correct molecular weight and (ii) indirectly by a monoclonal antibody (MCA).

The relationship between the *A.actinomycetemcomitans* leucotoxin and adenylate cyclase toxin from *B.pertussis* has been discussed (Section 1.8). A MCA, 9D4, to adenylate cyclase was made available by Dr. Parton, Department of Microbiology, Glasgow University who in turn had received the monoclonal antibody from Professor Hewlett, Department of Medicine, University of Virginia. This was used to determine, what, if any, relationship existed between the MCA of a related toxin and outer membrane protein preparations of *A.actinomycetemcomitans*.

6.2 METHODS AND MATERIALS

6.2.1 Preparation of outer membrane proteins (OMPs)

OMPs were prepared by an adaptation of the methods of Davies (1991) and Lai *et al* (1981). All reagents were prepared as described in Appendix 1.5. Briefly, preparations of the outer membrane proteins from the 17 strains of *A.actinomycetemcomitans* under investigation were separated by SDS-PAGE, Western Blotted and the OMPs were visualised by linking the MCA to a horse radish peroxidase complex and observing a colour change.

A.actinomycetemcomitans were grown in duplicate, 400 ml volumes of TSB, as previously described (2.1.2). Cells were pelleted by centrifugation at 5000 g for 30 minutes at 4°C, then washed in 40 ml of 20 mM Tris-HCl and centrifuged as before. Cells, resuspended in 10 ml of 20 mM Tris-HCl/ 10 mM EDTA, were sonicated for 3 minutes on ice at an amplitude of 26 Hz (MSE 150 watt), with a 5 mm diameter probe, until the suspension had cleared. An aliquot of 500 µl was removed from each sonicated strain and stored at -20°C. This formed the sonicated whole cell fraction or total protein preparation and a sample from each aliquot was run as a control in initial gels to demonstrate the total protein content of the *A.actinomycetemcomitans* strains. Unbroken cells were removed by centrifugation as before and the total cell envelope fraction pelleted by centrifugation, at 83 000 g for 60 minutes at 4°C in a Beckman L8-55M ultracentrifuge. The total cell envelope pellet was resuspended in 10 ml of 0.5% (w/v) sodium N-lauroylsarcosine (Sarkosyl - Sigma) for 20 minutes at room temperature which selectively solubilised the cytoplasmic membrane (Filip *et al*, 1973). The remaining outer membrane fraction was pelleted by ultracentrifugation, as described above, resuspended in 10 ml Tris-HCl, and pelleted once more. The pellet was solubilised in 0.5 ml Tris-HCl (pH 6.8) and stored at -20°C, in 100 µl aliquots until required.

6.2.2 Adenylate cyclase

A supply of adenylate cyclase was obtained from Dr. Westrobe (Microbiology Department, Glasgow University) at a concentration of 40 μgml^{-1}. This was stored frozen until required, then thawed slowly at room temperature, when 25 µl was mixed 1:1 with double strength (2X) sample buffer which contained SDS (1%) and 2-mercaptoethanol (0.04%), bromophenol blue, glycerol and 0.05 M Tris buffer. In addition, 10 µl of the adenylate cyclase was diluted 1:10 with a mixture of equal volumes of distilled water and 2X sample buffer. Twenty microlitres of these two solutions, 20 and 2 μgml^{-1}, were as a control to detect MCA activity.

6.2.3 Monoclonal Antibody, 9D4

The monoclonal antibody 9D4 was mouse ascites fluid, which had not been purified further (Hewlett, personal communication). Hewlett also indicated that mapping by cross-reactivity with *E.coli* haemolysin indicated that the MCA reacted with the glycine-rich nanomeric repeats in the carboxyl terminal region.

This material was used at a dilution of 1:1000 in Western blots.

6.2.4 Determination of protein concentration

The protein concentrations of the sonicated fraction and the OMP preparations were estimated by the modified Lowry procedure for determination of membrane proteins (Markwell *et al*, 1987).

Briefly, 100 ml of reagent A (Appendix 1.4) were mixed with 1 ml of reagent B, to give Reagent C. From each protein sample, 50 µl was removed and mixed with 3 ml of Reagent C. This volume was then mixed with 0.3 ml of the diluted Folin-Cioacalteu reagent (Appendix 1.4) and developed at room temperature for 45 minutes, then read at OD_{660} against a blank of distilled water in a UV spectrophotometer (UVP, Cambridge).

A standard curve was constructed with bovine serum albumin (BSA - Sigma) of known concentrations and the unknown OMPs concentrations determined by interpolation. The OMPs from the seventeen strains of *A.actinomycetemcomitans* under investigation were diluted to a final protein concentration of 5 $mgml^{-1}$.

6.2.5 SDS-PAGE separation of proteins

Purified OMPs and sonicated whole cells were separated by SDS-PAGE using the SDS-discontinuous system of Laemmli (1970). Briefly, the proteins were solubilised by diluting 1:2 in double strength sample buffer, then boiled for 4 minutes and allowed to cool to room temperature.

The samples were electrophoresed in a 10% gel, with a 4% stacking gel, both of

which contained acrylamide. Twenty microlitres of each protein was loaded under running buffer. The proteins were run at a constant current of 30 mA per gel through the resolving gel. Gels were run in Bio-Rad tanks (Bio-Rad, Virginia, USA), typically for 4 hours.

When the dye front reached the lower edge of the plate/ resolving gel, electrophoresis was stopped and the gel removed from the plates. Electrophoresed proteins were stained with 0.1% Coomassie Blue (w/v) in 45% methanol/ 10% (v/v) acetic acid overnight and destained in a 30% (v/v) methanol/ 10% (v/v) acetic acid solution, until the background had cleared - typically 3 - 4 days. Protein molecular weight standards (Sigma) which ranged from 29 - 205 kDa, as below, were run at either ends of the gel and were used to prepare calibration curves from which the apparent molecular weights of the OMPs were calculated. Proteins used as standards were :

Carbonic anhydrase	29 kDa
Albumin (Egg)	45 kDa
Albumin (Bovine plasma)	66 kDa
Phosphorylase B (Rabbit muscle)	97.4 kDa
B-galactosidase	116 kDa
Myosin (rabbit muscle)	205 kDa

These were diluted 1:1 in double strength sample buffer, boiled, then stored in 60 µl aliquots at -20°C until required. Fifteen microlitres were added to each standard track and empty wells filled with sample buffer to prevent protein bands from "expanding" laterally to produce band distortions.

Gels prior to Western Blotting, were prepared as described previously (Section 6.2.6) and the purified OMPs separated, by SDS-PAGE. The gels were left unstained.

Protein gels run purely as controls - e.g. (i) sonicated whole cell/ total protein preparation and (ii) purified OMPs were photographed after they were stained and these photographs digitised as previously described (Chapter 5), with the SDS markers used as calibrators. Both the protein standard tracks were entered first

(although they were run at opposite sides of the gel) and the individual tracks entered thereafter. Individual bands were assessed visually and graded between 1 and 3 on the basis of band intensity. Faint bands scored 1 and dark bands 3. Faint, intermediate and dark bands were then entered as appropriate using one of the 3 cursor options.

6.2.6 Western blotting

After separation of the OMPs by SDS-PAGE, the proteins were electroblotted to nitrocellulose by the method of Towbin, Knobler and Buchmeier (1984), in a BioRad Trans-Blot Cell (Bio-Rad, Richmond, CA, USA). Briefly, the gels were transferred to a tray which contained blotting buffer Tris (25 mM) and glycine (192 mM) and left for 1 hour to equilibrate. Whatmann 3MM paper and nitrocellulose (Sigma) cut to the same size as the gels and the fibre pads (from the blotting apparatus) were soaked in the blotting buffer for 30 minutes to equilibrate, after which they were transferred to the gel holders. These were then assembled carefully to avoid the entrapment of air bubbles between the gels, nitrocellulose, filter paper and fibre pads.

A "sandwich" was made as described in the manufacturers instructions. Fibre pads were located on the outer edges of the sandwich (the gel holder) and filter paper placed on top of the fibre pads. The equilibrated gel was then placed on top of the filter paper and the nitrocellulose membrane was placed on top of the gel. The sandwich was completed by another piece of filter paper with the fibre pad being placed on to the nitrocellulose membrane.

The "sandwich" was located in a gel holder, which was inserted into the blotting tank with the membrane towards the anode; the gel towards the cathode. A constant voltage of 15 V was applied for 20 hours. The temperature of the tank was kept as low as possible by the insertion of a cooling coil attached to a running cold water tap.

6.2.7 Cross reactivity of OMPs with MCA 9D4

After protein transfer, the nitrocellulose membranes were removed and washed three times in PBS (ten minutes for each wash). Gels were stained with 0.1% Coomassie blue (w/v) in 45% methanol/ 10% (v/v) acetic acid overnight and destained with 30% (v/v) methanol/ 10% (v/v) acetic acid solution, to ensure that all proteins had been transferred. A portion, approximately 3 cm in width, of the nitrocellulose membrane was removed from the left edge. This corresponded to the region in the gel where the molecular weight markers had been separated. The nitrocellulose strip was stained in 0.1% amido black, then destained after 1 minute with 30% (v/v) methanol/ 10% (v/v) acetic acid solution, to (i) show that proteins had been effectively transferred to the membranes and (ii) to enable the molecular weight of any *A.actinomycetemcomitans* OMP which cross reacted with the MCA, to be estimated.

Non-specific binding of the MCA to the membrane was blocked by incubation for 1 hour, at room temperature, in 2.5% (w/v) fat-free skimmed milk in PBS with 0.15% (v/v) Tween-20 (Blocking buffer). The membranes were then washed for three 10 minute periods, in PBS with 0.15% (v/v) Tween-20 (wash buffer) and finally incubated overnight in blocking buffer, with the MCA diluted 1 in 1000.

Excess MCA was removed from the membranes by three 10 minute washes in PBS. Horseradish peroxidase labelled rabbit anti-mouse conjugate (SAPU, Law, Scotland), was then added at a dilution of 1 in 1000 in blocking buffer and incubated for 5 hours. The membranes were washed as before in PBS and developed in a substrate solution which contained 0.03% (w/v) 3,3'-diamionobenzidine (DAB - Sigma), 0.02% (w/v) $CoCl_2$ and 0.1% hydrogen peroxide. Results were visualised within 1 minute and the reaction was inhibited by the immersion of the filters in distilled water. Membranes were photographed while still damp, to enhance the bands, then stored between filter paper in the dark to prevent fading.

6.3 RESULTS

6.3.1 Total whole cell protein preparation

The sonicated whole cell preparations of the 17 strains under investigation as separated by SDS-Page are presented in Figures 6.1 (a) and (b). The protein bands demonstrated for each strain were sized and similarities and differences among strains recorded.

The total protein preparations show the variation in protein expression of the 17 strains of *A.actinomycetemcomitans* under investigation. For example, strains 1, 8, 24, 74, 75 and 76 expressed 2 proteins with approximate molecular weights of 130 and 120 kDa. Strains 55 and 56 expressed a protein of approximately 130 kDa, while the remaining strains did not express either protein.

The relative intensity of the proteins varied from strain to strain.

6.3.2 Outer membrane proteins

Purified OMPs of the 17 test strains were separated by SDS-PAGE, photographed by the photographic department at the Glasgow Dental Hospital and School and digitised as previously described (Chapter 4). The digitised images are shown in Figures 6.2 (a) and (b).

With the exception of strains 33 and 83, all appeared to lose the majority of the protein bands seen in Figures 6.1 (a) and (b) as expected.

Strains 8, 24, 32, 33, 55, 74, 75, 76, 79, 81, 83 and 88 all expressed a protein with a molecular weight within 10% of 116 kDa - the molecular weight of the *A.actinomycetemcomitans* leucotoxin.

Strains 76 - 83 appeared to have a greater number of high concentration bands i.e. digitised as triplets, or multiple comigrating proteins than the other strains

Several proteins were seen which were common to most strains. These proteins had molecular weights of approximately 29, 38, 41, 50, 65, 97 and 100 kDa.

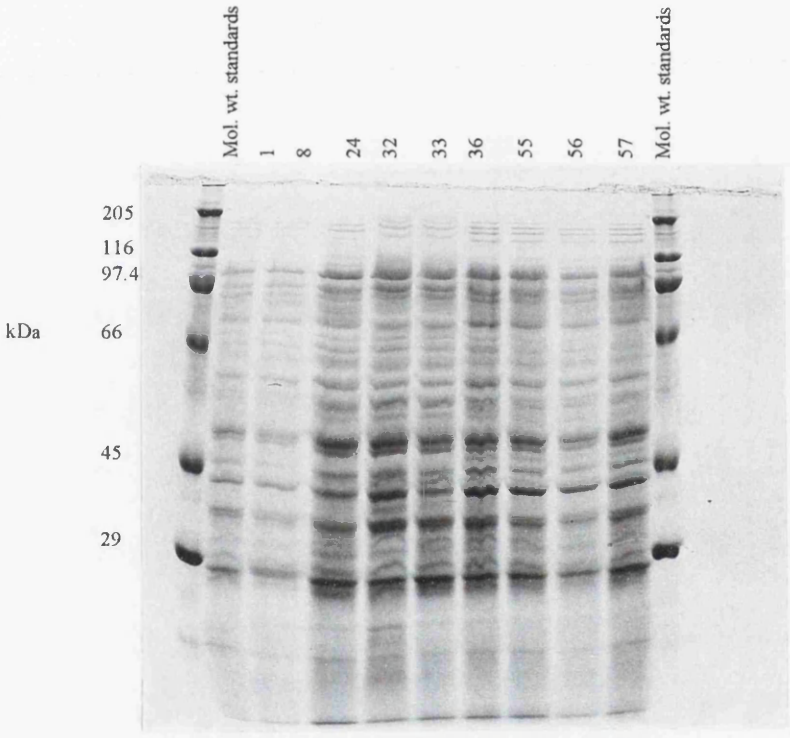

Figure 6.1a Total protein preparations of 9 strains of *A.actinomycetemcomitans*

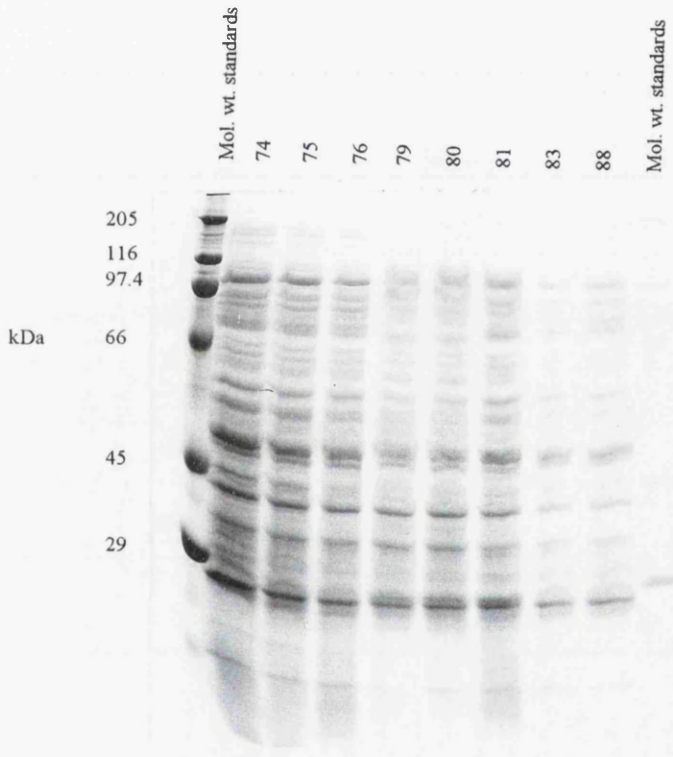

Figure 6.1b Total protein preparations of 8 strains of *A.actinomycetemcomitans*

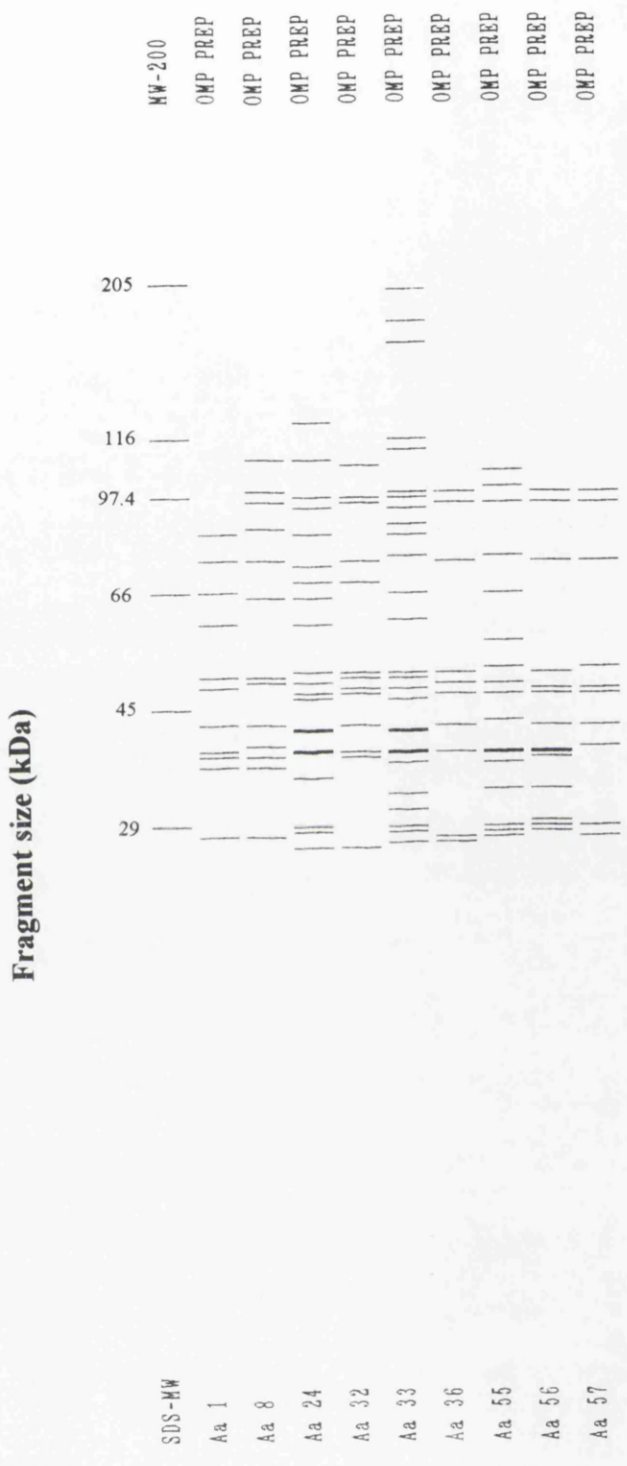

Figure 6.2a Graphical output from digitised images of outer membrane protein preparations of 9 strains of *A.actinomycetemcomitans*

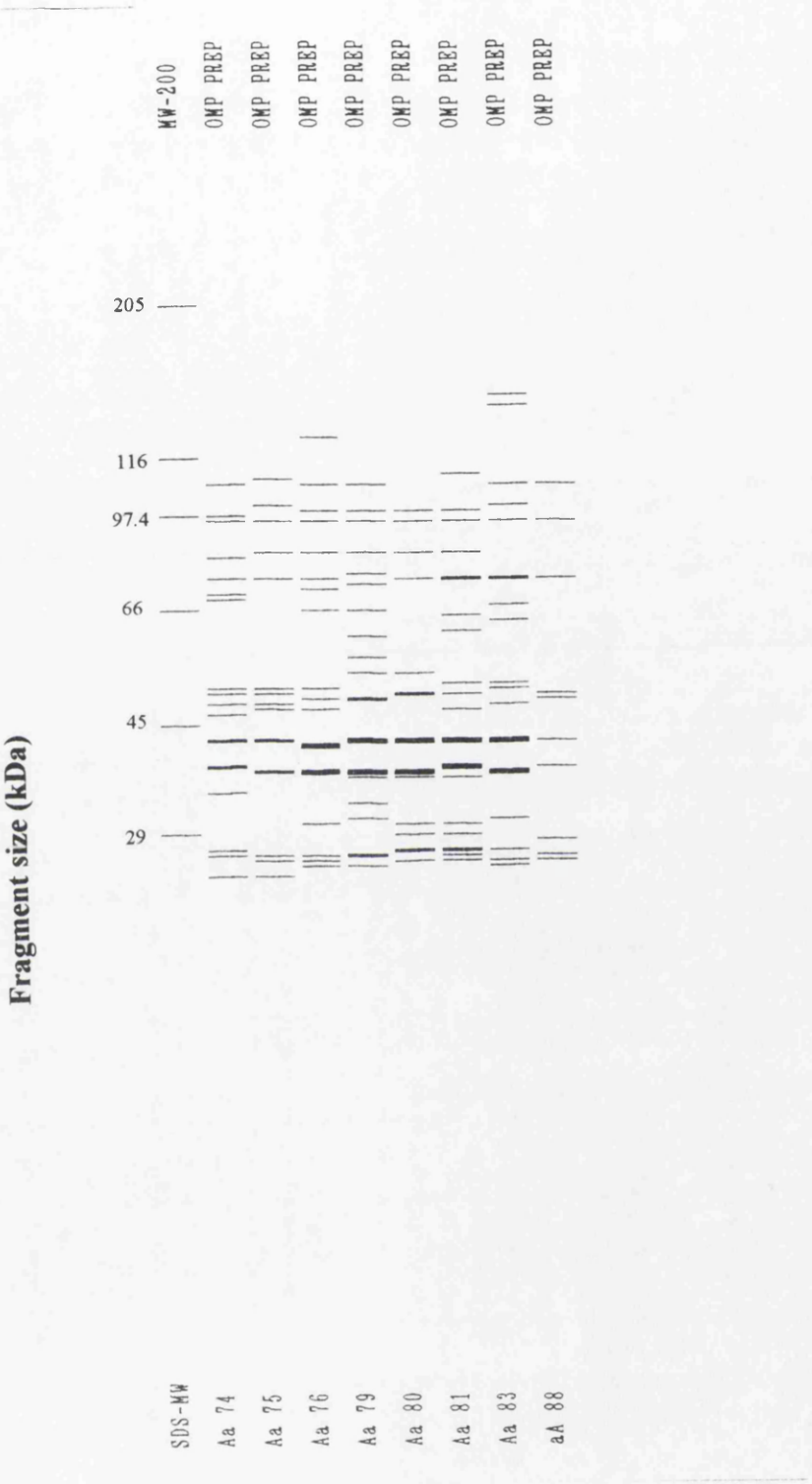

Figure 6.2b Graphical output from digitised images of outer membrane protein preparations of 8 strains of *A.actinomycetemcomitans*

By visual assessment of the OMPs, they subdivided into 11 different groups on a purely arbitory basis (Table 6.1). No one OMP profile dominated the collection of strains studied.

The large number of profiles seen was due, in part, to variation in the protein band strength e.g. the OMPs of strain 88 were very weak and although on the basis of these results it belongs to its own OMP group, this position could be altered by a higher concentration of OMPs, and the possible presence of proteins, which at present are too weak to be clearly seen.

6.3.3 Western blotting

Two SDS-PAGE gels which contained the purified OMPs from the seventeen *A.actinomycetemcomitans* strains under investigation were Western Blotted as described (6.2.4). Staining with Coomassie Blue showed that all proteins including adenylate cyclase at both concentrations, had been successfully transferred. Figures 6.3 (a) and (b) are photographs of the nitrocellulose filters after they were probed with the MCA. The molecular weight markers to the left give an approximate molecular weight of the crossreactive protein.

Reaction between the MCA and a homologous protein was seen by a distinct, sharp line. Where there had been non-specific binding of the MCA to a protein, a more diffuse pattern was seen.

All 17 *A.actinomycetemcomitans* strains showed non-specific binding of the MCA with proteins in the region 30 - 40 kDa. These corresponded to the diffuse protein bands seen in both the total protein preparation gels and the outer membrane protein preparations. There were, however, some distinct bands, which are discussed below.

Cross reactivity between the MCA and adenylate cyclase was shown to occur with both the "neat" and diluted form. The 1 in 10 dilution showed a much clearer band of approximately 200 kDa, than did the undiluted sample. There was also a reduction in the degree of non-specific binding seen.

Strain	OMP Group
1	1
8	2
24	3
75	3
76	3
32	4
33	4
36	4
56	4
57	4
80	4
55	5
74	6
79	7
81	7
88	7
83	8

Table 6.1 Proposed grouping of *A.actinomycetemcomitans* on the basis of OMP profiles

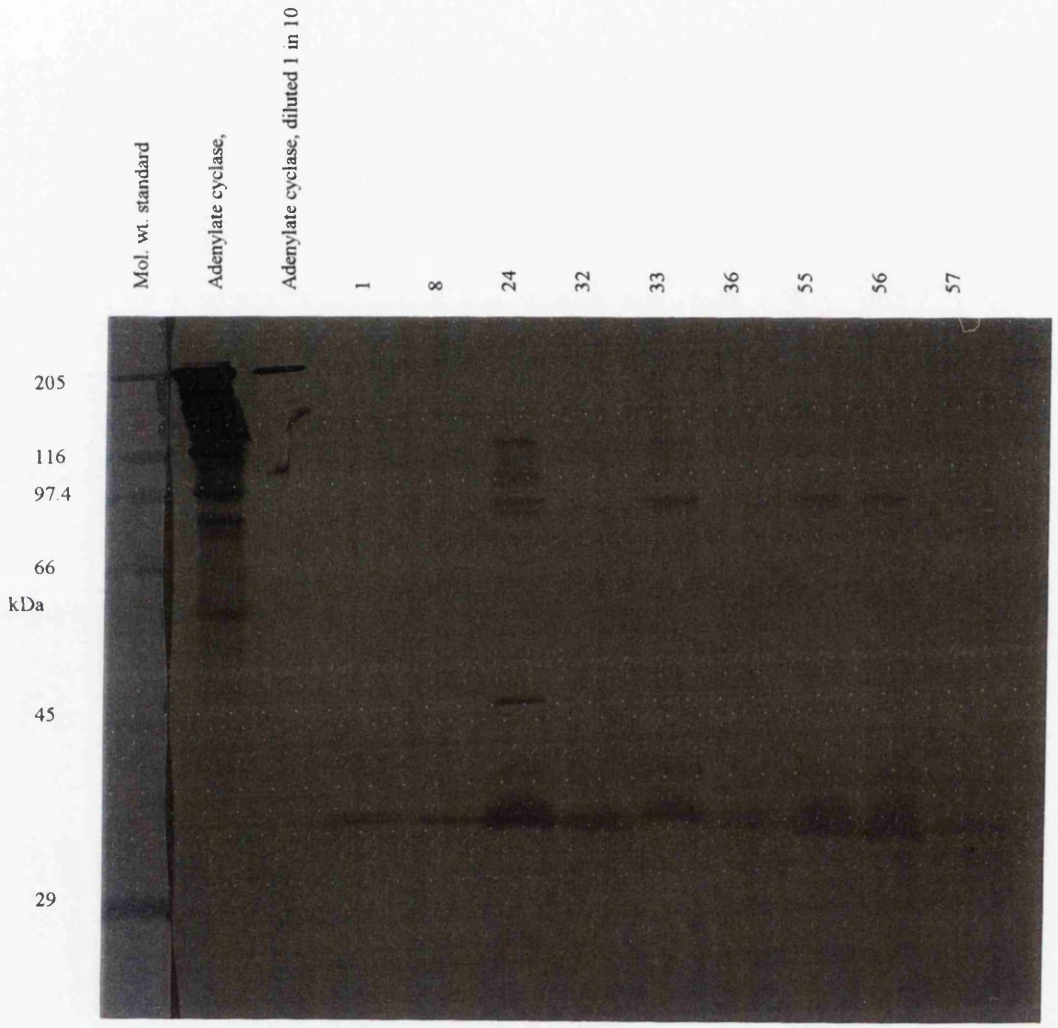

Figure 6.3a Reaction between MCA 9D4 , adenylate cyclase and the OMPs of 9 *A.actinomycetemcomitans* strains

Ac : Adenylate cyclase
Mol. wt. : molecular weight

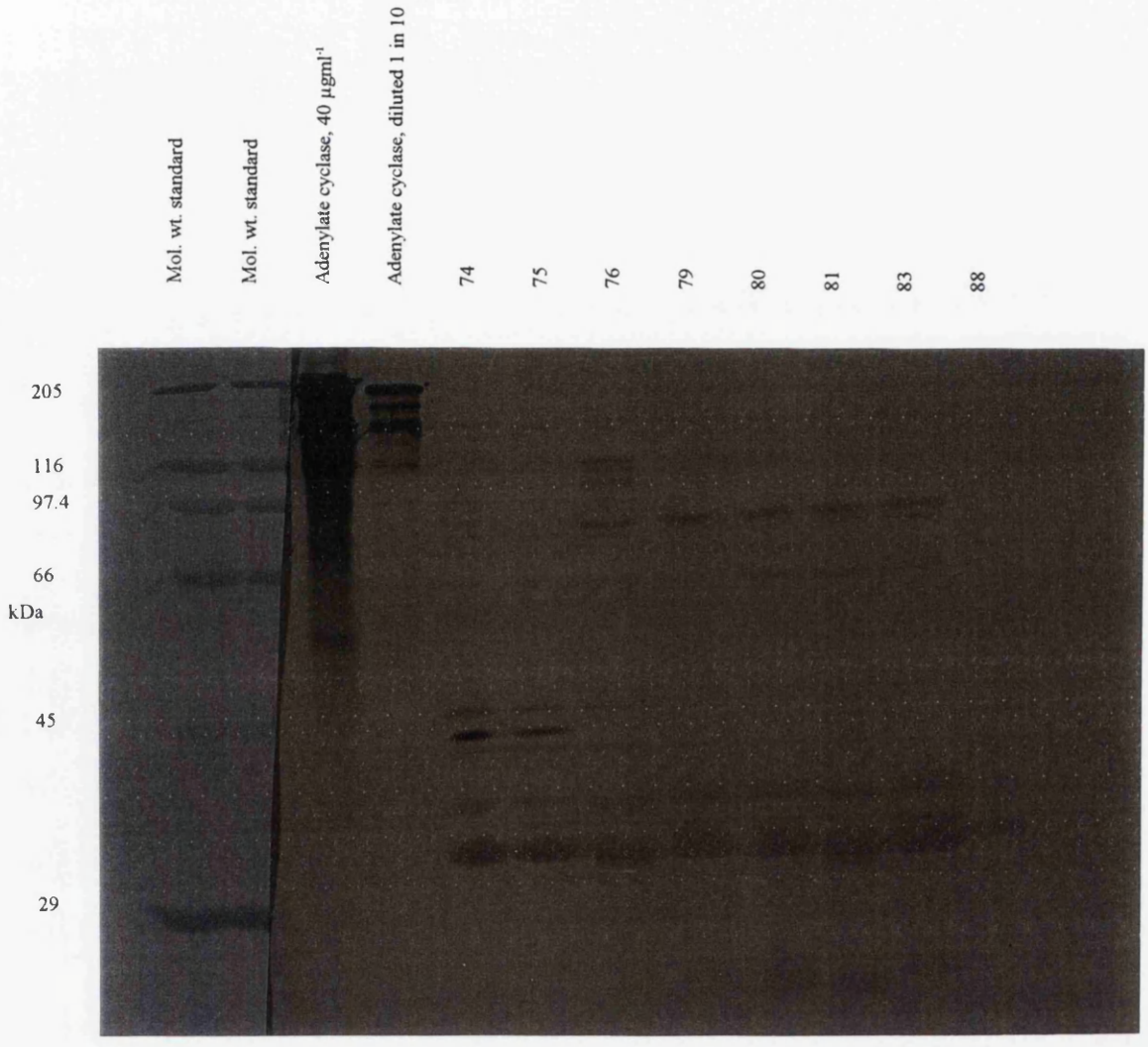

Figure 6.3b Reaction between MCA 9D4 , adenylate cyclase and the OMPs of 8 *A.actinomycetemcomitans* strains

Ac : Adenylate cyclase
Mol. wt. : molecular weight

Strains 1 and 8 did not show any distinct, sharp bands.

Strain 24 possessed a number of specific bands. These were seen at approximately 116, 97 and 95 kDa. There was also a very strong band at 45 kDa. A weaker band at 66 kDa was also seen. When the molecular weights of the 66 kDa and 45 kDa protein were added together the result was 111 kDa - which approximated to the molecular weight of the leucotoxin. Strain 76 shared many of the heavier bands with strain 24. However, it shared a similar pattern to strains 74 and 75, with its lower molecular weight bands.

Strain 33 shared 4 bands in common with strain 24 - 116, 97.4, 95 and 66 kDa. Strain 33 also appeared to have a band of approximately 45 kDa, although this was not as intense as the corresponding band of 24.

Strains 55, 56, 80, 81 and 83 were shown to have similar patterns to each other. All had bands at approximately 97 and 95 kDa and a fainter one at 66 kDa.

Strains 74 and 75 were shown to have a similar and unique pattern. Bands - which were very faint - i.e. greater than 200 and approximately 116 kDa were seen. There were also two bands of between 97.4 and 66 kDa which were very faint. These may have represented non-specific binding. Two bands were shown with a molecular weight slightly greater than and less than 45 kDa.

Strain 79 demonstrated a unique pattern. The heaviest band seen was at approximately 97 kDa. There appeared to be bands between 66 and 45 kDa, although these were faint and possibly represented non-specific binding.

Strain 88 expressed proteins of approximately 95 and 66 kDa which were very faint. It

is possible that other proteins were present, which in higher concentrations would have been recognised by the MCA.

6.4 DISCUSSION

Outer membrane proteins were prepared from 12 wild type strains of *A.actinomycetemcomitans* and 5 reference strains, separated by SDS-PAGE electrophoresis and the cross-reactivity between the OMPs and a MCA to the RTX toxin adenylate cyclase, from *B.pertussis*, investigated.

6.4.1 Whole cell preparation

The protein bands seen for each strain represented a mixture of proteins and as with any electrophoretic system, any band seen in an SDS-PAGE gel, may represent one or more proteins. Bands which appeared to be of greater intensity than others may have represented a protein more highly expressed. Equally, however, it may represent a mixture of comigrating proteins. Variation in the expression of proteins may have been the result of a genetic deletion or an effect at the expression level of the protein. Although care was taken to ensure that all strains of *A.actinomycetemcomitans* were treated equally, it cannot be excluded that variations in the total protein preparations and OMP preparations seen between strains was the result of environmental factors and not genetic variation.

Outer membrane proteins

The outer membrane of gram negative bacteria provides a variety of different functions for the bacterial cell. These include, for example, the provision of a protective environment for hydrolytic enzymes; the binding of proteins which are present in the periplasm. Outer membranes are also involved in the maintenance of the cell integrity (DiRienzo, Nakamura and Inouye, 1978) and serve as phage receptors (Datta, Arden and Henning, 1977), diffusion pores and mitogens (DiRenzo *et al*,

1978). The methods used by Wilson (1991b) were similar to those employed in this study and all strains investigated demonstrated a 29 kDa protein (Figures 6.2 (a) and (b)). Wilson (1991a), however, did not use the OMP results for taxonomic studies and therefore no protein gels were illustrated. Wilson's main interest was the presence of a heat modifiable protein of 29 kDa, and its interaction with antiserum, directed against the OmpA protein of *E.coli*.

Bolstad *et al* (1990) studied the OMPs from 10 wild strains and 4 reference strains of *A.actinomycetemcomitans*. The methodology for the isolation of the OMPs was different from that used in the current study (Bakken and Jensen, 1986, cited by Bolstad *et al*, 1990) : bacteria were freeze-dried and broken in a French pressure cell. Unbroken cells and the cell envelopes were removed by centrifugation. After several washes, cells were resuspended in a N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)/ magnesium chloride buffer.

Bolstad *et al* (1990) found four dominant protein bands of 48, 39, 34 and 28 kDa and the presence of a number of less substantial bands with an approximate molecular weight of 43, 32.5, 29, 18.5 and 16.5 kDa. They suggested that the OMPs from 10 wild strains of *A.actinomycetemcomitans* displayed a great deal of similarity in the overall patterns observed. The strains investigated in this study demonstrated proteins of approximately 29, 40, 50, 70, 97 and 100 kDa. The variation seen in the molecular weights could be due to differences in the methods used to isolate the OMPs.

The aim of Bolstad *et al* (1990) had been to use OMP profiles as a method of typing clinical strains. They demonstrated interspecies differences i.e. between *A.actinomycetemcomitans* and *H.aphrophilus* and suggested, as had others (Moore *et al* 1980; Tanner 1986), the use of SDS-PAGE techniques to distinguish between species in a mixed bacterial population.

Bolstad *et al* (1990) and Wilson (1991b) both demonstrated the presence of a heat modifiable protein of 34 and 39 kDa respectively. The heat modifiable protein referred to by both Bolstad *et al* (1991) and Wilson (1991b), could be the same protein.

Differences in the preparation of the proteins and gels may also have led to changes in mobility and apparent molecular weight. However, in the present study the effect of heat upon the electrophoretic mobility of the OMPs from the 17 strains studied was not investigated and no conclusions can be made regarding the apparent differences seen by Bolstad *et al* (1991) and Wilson (1991b).

Overall, results of the SDS-PAGE separation of the OMPs from 17 strains of *A.actinomycetemcomitans* appeared to be generally consistent with published work.

The use of proteins to type bacteria is not universally accepted, as the results can be variable, due to for example, environmental conditions. Gaston and Hunter (1986) suggested that this approach to typing bacterial strains was error prone, and is now used only in very defined circumstances.

Bolstad *et al* (1990), cultured wild strains of *A.actinomycetemcomitans* on liquid Slots medium and Reference strains in BHI then demonstrated similarities in the OMPs. This, they understood, implied that the isolated OMPs were constitutively expressed and of great use in taxonomic studies. However, had all the bacterial strains been grown under the same nutritional conditions, differences in the OMPs may have been seen and consequently the usefulness of OMPs in taxonomic studies reduced. All bacterial strains in this study were grown under the same nutritional conditions, which could explain the variation seen in the OMPs.

Variation in the OMP concentrations i.e. intensity of the protein bands, may have been as a result of operator error, in for example the calculation of the protein concentration.

There is no clear relationship between the clinical origins, plasmid status and REFP group. On the basis of OMPs, the largest group is 5, with 4 members. When the OMP profile grouping is compared to the REFP grouping, there is no clear correlation; group 5 corresponds to RFLP groups D, I and K.

One OMP result of interest is the OMP profile of strain 55. This strain types with strains 56 and 57; all three stains were isolated from the same individual. Its plasmid

profile and REFP grouping are the same, however, the OMP profiles of 56 and 57 differ from that of 55. These differences could be explained by a low concentration of proteins in strains 56 and 57.

MCA 9D4

The RTX toxins are related to each other in several ways (Welch *et al*, 1991; Strathdee & Lo, 1989; Iwase *et al*, 1990). It is therefore reasonable to expect that a MCA to the toxin from any of the RTX group members would react with purified leucotoxin or recognise the leucotoxin protein on nitrocellulose blots of separated proteins, if the epitope was to a conserved region.

Monoclonal antibodies to the leucotoxin of *A.actinomycetemcomitans* have been produced by several groups of workers (DiRienzo *et al*, 1985., Place, Scidmore and McArthur, 1988). They can be used in pathogenicity studies i.e. to neutralise the cytotoxic activity of the leucotoxin and recognise the toxin on nitrocellulose blots of separated proteins. DiRienzo *et al* (1985) described the use of MCA to the leucotoxin of *A.actinomycetemcomitans* to examine the distribution of the leucotoxin in oral strains of *A.actinomycetemcomitans*. They found that the MCA recognised 3 proteins with molecular weights which ranged from 115 to 135 kDa. No published results are available which describe the use of a MCA to one RTX toxin group member to identify toxic strains of another RTX group member.

The leucotoxin from *A.actinomycetemcomitans* has a higher percentage of amino acid sequence homology to the *E.coli* -haemolysin than to the *B.pertussis* adenylate cylase (Kraig *et al*, 1990; Hess *et al*, 1986). Therefore it might be expected that a MCA to the *E.coli* haemolysin would have greater homology to the *A.actinomycetemcomitans* leucotoxin than to adenylate cyclase of *B.pertussis*, depending upon the epitope. The MCA which was used in these experiments was one which was made available by the University of Glasgow Department of Microbiology. While it may not have been the optimal choice of MCA, it has demonstrated the principle that a MCA for one RTX

leucotoxin can recognise another RTX toxin member.

All seventeen strains of *A.actinomycetemcomitans* reacted with the MCA to some degree. With the exception of strains 1 and 8, non-specific binding of the MCA to proteins was seen at approximately 35 kDa. All seventeen strains of *A.actinomycetemcomitans* reacted with the MCA to some degree. With the exception of strains 1 and 8, non-specific binding of the MCA to proteins was seen at approximately 35 kDa.

Specific binding of the MCA with a protein of approximately 116 kDa (the molecular weight of the *A.actinomycetemcomitans* leucotoxin) was seen in 8 strains, only 1 (strain 24) of which is toxic to HL-60 cells (Chapter 7). Additional binding of the MCA was seen with proteins of 66, 95 and 97 kDa.

Strains from the same individual (54, 55 and 56) demonstrated minor differences to each other in both their total protein preparations (Figure 6.1) and purified outer membrane preparations. Some of these differences could be explained by electrophoretic variation in the SDS-PAGE gel.

Strains 74, 75 and 76 which were isolated from the same periodontal pocket, demonstrated minor differences in their total protein contents and minor differences were also seen in the outer membrane preparations, however when these proteins were probed with MCA 9D4, strains 55 and 56 reacted strongly to the MCA, whereas there was no reaction at all between the OMPs of strain 57.

Strains 74 and 75 were very similar; both demonstrated a very strong band at approximately 45 kDa. This band was not present in strain 76. Strain 76 showed a number of bands with molecular weights of approximately 116 kDa, which were not seen in either 74 or 75. Speculation that *A.actinomycetemcomitans* strain 76 could be leucotoxic to HL-60 cells were shown to be unfounded (Chapter 7).

Future work in this area could involve the use of a range of MCA and RTX toxins, to investigate homology between these. It would be interesting to examine differences in the specificity of different MCA to the RTX toxins since it is possible that one MCA

reacts more strongly with the protein toxins than another. If the purified toxin was extracted from a range of bacteria and then allowed to cross-react with a panel of MCAs at different dilutions, it would be possible to answer this question relatively simply.

This method of detecting leucotoxicity is not accurate, as only one of the 8 strains (strain 24) which reacted to the MCA at approximately 116 kDa, was toxic to HL-60 cells/ resulted in a reduction in chemiluminescence (Chapter 7). It was time consuming and requires large volumes of bacteria from which to purify the toxin. In this study no attempt was made to purify the leucotoxin from *A.actinomycetemcomitans*. Had this been done then it is likely that non-specific reactions between the MCA and the OMPs would not have occurred. As a result, any reaction between the MCA and proteins which did not have the correct molecular weight could therefore have been a reaction between the MCA and degradative products of the leucotoxin. However, had a MCA to the *A.actinomycetemcomitans* leucotoxin been available, these results may have differed, with all the toxic strains reacting to such a MCA.

These experiments demonstrated that

(i) there are variations in the total protein preparations of *A.actinomycetemcomitans* strains.

(ii) on the basis of OMPs, a number of groups can be described, which are unrelated to clinical origins, plasmid status, REFP group and leucotoxic status.

(iii) a protein with a molecular weight similar to that of the leucotoxin is present in the outer membrane of both toxic and non-toxic strain of *A.actinomycetemcomitans*

(iv) a MCA to a RTX toxin, adenylate cyclase, from *B.pertussis* would recognise the leucotoxin, despite the low (less than 50%) homology between the two toxins.

6.4.2 Future work

It would be of interest to use a panel of MCA to the RTX toxins against preparations of the OMPs and the purified toxin of *A.actinomycetemcomitans* to determine the

cross-reactivity (if any) between these MCA and the leucotoxin of *A.actinomycetemcomitans*. The reaction of purified toxin from each of the RTX members to MCAs against all the toxins would also be of interest.

CHAPTER 7
LEUCOTOXICITY OF *A.actinomycetemcomitans* TO HL-60 CELLS AND HUMAN PMNLS

CHAPTER 7

LEUCOTOXICITY OF *A.Actinomycetemcomitans* TO HL-60 CELLS AND HUMAN PMNLS

7.1 INTRODUCTION

A.actinomycetemcomitans has several virulence factors (see 1.7), the most widely studied of which is the leucotoxin (see 1.8). A number of different assays, have been developed to investigate toxin production by *A.actinomycetemcomitans*. Whole bacterial cells, sonicates of whole cells or purified leucotoxin have been used with freshly isolated human polymorphonuclear cells (Baehni *et al*, 1979., Baehni *et al*, 1981) or cultivated human tissue cell lines e.g. HL-60 (Zambon *et al*, 1983., Simpson, Berthold and Taichman, 1988). Cell death has been determined by trypan-blue exclusion (Ohta *et al*, 1987); lactate dehydrogenase release (Zambon *et al*, 1983); lysosomal elastase release (Tervahartiala *et al*, 1989); release of ^{51}Cr (Simpson *et al*, 1988) and fluorescent spectroscopy (Ohta *et al*, 1991).

Each method poses different problems, e.g. ^{51}Cr relies upon the release of a radioactive labelled element, while dye exclusion relies upon visual identification of dead/ live cells, where great care must be taken to ensure random sampling of cells.

In this thesis the leucotoxic activity of *A.actinomycetemcomitans* was investigated by two different techniques. The first used HL-60 cells, which were exposed to whole cells of *A.actinomycetemcomitans*, with or without sonication, a method previously used by Zambon *et al* (1983) and Simpson *et al* (1988). The second method measured the change in light emitted from human PMNLs exposed to *A.actinomycetemcomitans* (i.e. chemiluminescence), a method which had been used previously to investigate the toxic effect of the *B.pertussis* toxin adenylate cyclase, but which had not been previously employed to measure the leucotoxic activity of *A.actinomycetemcomitans*.

The aim of these experiments was to obtain a rapid, but accurate, method for the determination of the toxic status of strains of *A.actinomycetemcomitans* held at

Glasgow Dental Hospital and School.

7.2 MATERIALS AND METHODS

7.2.1 Toxic effect of whole cells and sonicates of *A.actinomycetemcomitans* on HL-60 cells

7.2.1.1 Preparation of *A.actinomycetemcomitans*

Duplicate, 20 ml, overnight cultures of the 17 strains of *A.actinomycetemcomitans* under investigation (Table 2.2) were prepared as previously described (2.1.2). The bacterial cells were pelleted by centrifugation at 4000 rpm for 10 minutes followed by resuspension in 10 ml ice-cold PBS, pH 7.3 (Appendix 1.5). Cells were washed in this way on 2 occasions, then the duplicate cultures (A and B) were treated as follows :

Culture A was resuspended in 5 ml ice-cold PBS and the concentration adjusted to give 1 X 10^9 cells per ml, using the formula of Lai *et al* (1983). This states that 1 X 10^9 *A.actinomycetemcomitans* per ml, has an optical density equivalent to 0.3 when read at 540 nm. The formula is set out as follows :

$$\frac{OD_{540}}{0.3} - 1 \times 5 = \text{Volume of PBS needed to adjust suspension volume to an optical density of 0.3 at 540 nm.}$$

The optical density was measured in a SP8-100 UV/ IVS spectrophotometer (Pye/ Unicam, Cambridge). The dilution of the bacterial cells in PBS was repeated until an optical density of 0.3 was achieved. From the final suspension, 3 aliquots of 1 ml were added to separate bijoux bottles labelled **A1**, **A2** and **A3**, which had been stored overnight at 4°C. These samples were stored on iced-water until required. The remaining cells were discarded.

Culture B was resuspended in 3 ml ice-cold PBS, then aliquoted into 3 x 1 ml volumes, in bijoux bottles labelled **B1**, **B2** and **B3**, which had been stored as before. All samples were stored on iced-water at 4°C until required.

These methods of preparation produced cell concentrations of approximately 8.0 - 9.0 X 10^8 (**A**) and between 9.0 X 10^9 and 1.0 X 10^{10} (**B**), which was calculated as described below. The method described by Lai *et al* (1983) thus proved to be inaccurate in the calculation of *A.actinomycetmcomtians* cell numbers and was not used in the cytotoxicity experiments.

A1/B1 : whole cell fraction.

Both bijoux bottles were shaken by hand to mix the cells and 50 µl was removed which was serially diluted in ice-cold PBS to 1.0 x 10^{-3}, 1.0 x 10^{-4} (**A** only) and 1.0 x 10^{-5}, 1.0 x 10^{-6} (**B** only). Each diluted fraction was then spiral plated (Don Whitley Sciences, Yorkshire, England) onto CBA and incubated at 37°C and 5% CO_2, in air for 48 hours. The number of colonies were counted using a manual counting grid and the colony forming units per ml calculated.

The remaining undiluted cells were left on iced water, at 4°C, until required for the HL-60 assay.

A2/B2 : Whole cells sonicated for 15 seconds

The cells were sonicated in the bijoux by introducing the probe that delivered 20% power output (Heatsystems Inc., New York, USA.) for 15 seconds. The bottles were enclosed in iced water and after sonication left in iced water at 4°C until required for the HL-60 assay. Serial dilutions and spiral plating of the sonicated fractions were made, as for fractions A1/B1.

A3/B3 : Whole cells sonicated for 5 minutes

The cells were sonicated in the bijoux over iced water for 5 minutes and serial dilutions were prepared as above. The bottles were left on iced water at 4°C until required for the HL-60 assay. Serial dilutions and spiral plating of the sonicated fractions were made, as above.

The number of viable *A.actinomycetemcomitans* cells used in each experiment was measured on three occasions and the mean value recorded. The results were subsequently expressed as the mean Log_{10} viable cell counts.

Cells were sonicated in the order 1 x 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} to ensure that the least dilute cells did not become contaminated by the more concentrated suspension. Between different strains, the sonicator probe was wiped with an "Azo wipe" to reduce the chance of cross contamination to negligible levels.

7.2.1.2 Preparation of HL-60 cells

HL-60 cells, cultured as previously described (2.3.4), were aseptically decanted into sterile plastic universals and pelleted by centrifugation at 500 rpm for 10 minutes. The cells were washed 3 times in PBS at room temperature. After the 3rd wash, the cells were resuspended in 1 ml PBS. Fifty microlitres were removed and diluted 1 in 20 in PBS with 0.1% trypan blue (Appendix 1.5). The cells were counted in a haemocytometer as described previously (2.5), to find the total number and the percentage of viable HL-60 cells present. Cells were diluted to give a final concentration of 1.0 x 10^7 cells ml^{-1}. Only cell suspensions with 95% or greater viability were used.

7.2.1.3 Determination of Toxicity

The percentage HL-60 cell death at which an *A.actinomycetemcomitans* strain was designated toxic was taken to be at an arbitrary level of 25%. Any strain of *A.actinomycetemcomitans* which resulted in HL-60 cell death of 25% or greater, irrespective of either the bacterial cell numbers or the treatment to the *A.actinomycetemcomitans*, was considered to be toxic.

7.2.1.4 Cytotoxicity assay of *A.actinomycetemcomitans*

Two hundred microlitres of each *A.actinomycetemcomitans* suspension (**A1-A3/**

B1-B3), which had been stored in ice-water, were pipetted after mixing, into a bijoux, to which 200 µl of the HL-60 cells at 1.0 x 10^7 cells ml^{-1} were added, giving a total volume of 400 µl. Two sets of controls were used; (i) a positive control, where 100% cell death of HL-60 cells was achieved by the addition of 200 µl Triton X-100 (Sigma), diluted 1 in 100 in PBS, pH 7.2, from the neat stock solution, to 200 µl of the HL-60 cells at 1.0 x 10^7 cells ml^{-1} and (ii) a negative, viability, control where 200µl of HL-60 cells were suspended in 200 µl of PBS.

The bijoux bottles were then shaken at 37°C, for 60 minutes in a waterbath (Heto, Denmark) at full power, after which they were placed on iced water at 4°C to stop any further reaction, before counting.

7.2.1.5 Counting of HL-60 cells

The contents of each bijoux were mixed thoroughly by hand, and 50 µl removed to a labelled eppendorf tube and diluted 1 in 1 with 0.1% trypan blue. Fifty microlitres of this mixture was then transferred to a microscope slide, a coverslip placed on top and the slide examined at 100X and 400X magnification using an Olympus (Japan) microscope.

Slides were examined first at 100X magnification to ensure that the concentration of cells was neither too great (i.e a confluent carpet of cells in which individual cells could not be easily distinguished or counted) nor too low (i.e less than 50 cells in each field of view). Magnification was then increased to 400X, and the central area of the coverslip examined. Fields of view which contained between 20 to 30 HL-60 cells were counted. The microscope slide was moved to the right for two fields of view, upwards for two fields of view, right for two, then down for two. This motion was repeated until 300 HL-60 cells had been counted and ensured that no HL-60 cell was counted on more than one occasion.

The number of dead cells in the PBS viability control was subtracted from the total dead cell count obtained for each test. The final result was expressed as the

"percentage dead cells".

$$\frac{\textbf{Total dead cells - PBS "background" x 100}}{300} = \textbf{Percentage HL-60 dead cells}$$

The assay was carried out in duplicate (i.e. 2 x **A1**, 2 x **A2** etc) on 3 separate occasions for each strain and these values then expressed as the "mean percentage dead cells".

Figure 7.1 shows cells stained with 0.1% trypan blue, prior to exposure of HL-60 to *A.actinomycetemcomitans*, while Figure 7.2 shows cells stained with 0.1% trypan blue, after exposure of HL-60 to the leucotoxic strain of 88.

7.2.2 Toxic effect of sonicated *A.actinomycetemcomitans* on human PMNLs measured by chemiluminescence

7.2.2.1 Isolation of human PMNLs

Twenty millilitres of venous blood were obtained from 5 healthy individuals in separate 10 ml sterile plastic bottles (Sherwood, England) containing heparin (on no occasion were blood samples from any two individuals mixed.) The sample was then mixed with 2 ml of 5% dextran (molecular weight 229 000 kDa, Sigma) in PBS (Appendix 1.5) and the red blood cells allowed to sediment for at least 1 hour. The leukocyte rich plasma layer was removed and layered carefully onto 5 ml Ficoll-hypaque (Pharmacia). The leucocytes were pelleted by centrifugation at 2500 rpm for 15 minutes, followed by one wash in 10 ml PBS, with 5 minutes centrifugation at 2500 rpm. Red blood cells were lysed by exposure of the aspirated cells to 0.2 ml distilled water for 30 seconds, followed by the addition of 10 ml PBS. PMNLs were centrifuged as described above and resuspended in PBS to give a final concentration of 1.0×10^6 cells ml^{-1}, using a haemocytometer, as previously described in section 2.5.

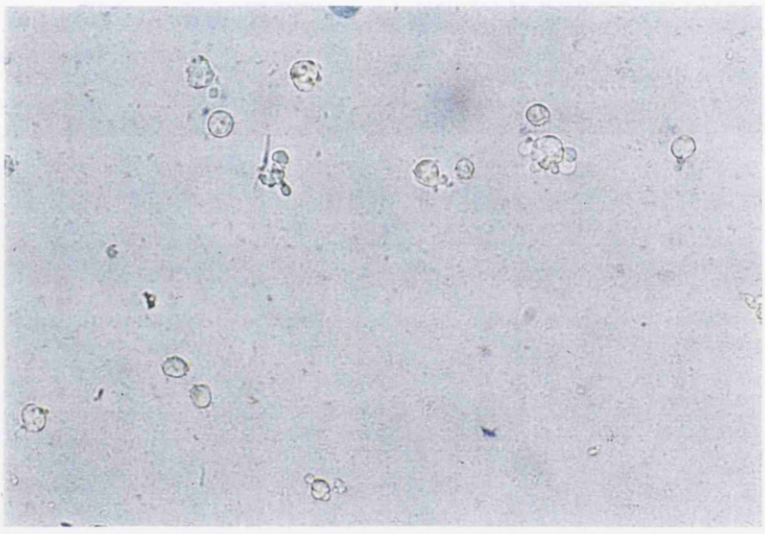

Figure 7.1 HL-60 cells stained with trypan blue, prior to exposure to *A.actinomycetemcomitans*

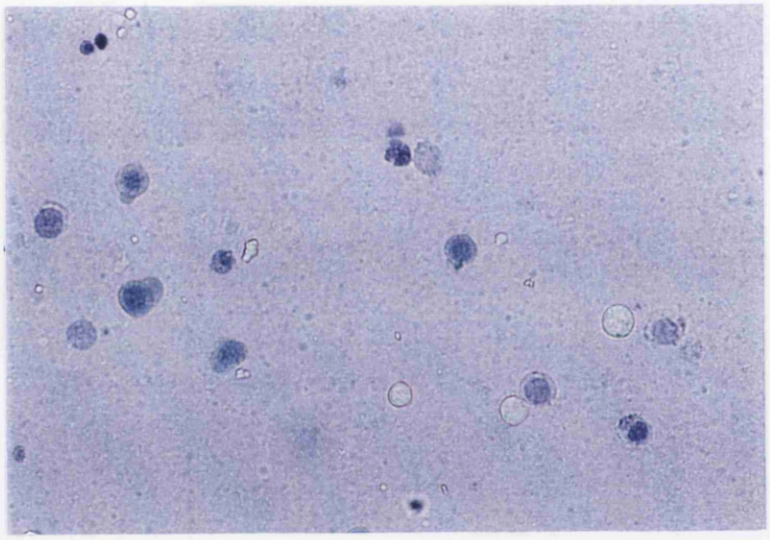

Figure 7.2 HL-60 cells stained with trypan blue, after exposure to *A.actinomycetemcomitans* strain 88 at 37°C for 60 minutes in a shaking waterbath.

7.2.2.2 Chemiluminescence Assay - Preliminary study to optimise the experimental conditions.

It became clear that the period of incubation of the PMNLs with *A.actinomycetemcomitans* prior to stimulation with phorbol-myristate-acetate (PMA) and measurement of chemiluminescence was critical - if too short a period then any reaction between the bacterial toxin and the PMNLs cell membrane would not occur; too long a period of incubation, and the natural activity of PMNLs would be reduced and little or no stimulation would be seen, after incubation with either toxic or non-toxic strains. It was also important that neither the sonicated bacterial cells nor the HL-60 cells were left for long periods of time before measuring the chemiluminescence as the activity of both would begin to decline naturally. For chemiluminescence studies, the optimum period of preincubation of PMNLs with sonicated cells of *A.actinomycetemcomitans* was first determined. Whole cells were not used as previous experience within the Department of Microbiology, Glasgow University had shown that these interfered with the test.

Chemiluminescence is light which is emitted as the result of a chemical reaction. Human PMNLs can be stimulated into producing light by the addition of PMA, which, although weak, can be enhanced by the addition of luminol. The resultant detectable levels can then be measured in a luminometer. Cells which are dead, cannot be stimulated into producing chemiluminescence. Therefore, incubation of toxic strains of *A.actinomycetemcomitans* with human PMNLs would result in a reduced level of chemiluminescence, whilst no significant change would occur if the strains were leucotoxin negative.

A preliminary study was performed with four strains of *A.actinomycetemcomitans* which were chosen on the basis of previously known leucotoxic status (7.3.1):

A.actinomycetemcomitans 1 - Non-toxic to HL-60 cells

A.actinomycetemcomitans 33 - Moderately toxic to HL-60 cells

A.actinomycetemcomitans 24 - Toxic to HL-60 cells

7.2.2.3 Preparation of A.actinomycetemcomitans sonicates

The four strains under preliminary investigation were cultured in 20 ml of TSB and the cells harvested as previously described (7.2.1). The washed bacterial pellets were resuspended in ice cold PBS, giving a concentration of 1.0 x 10^9 cells per ml. Viable cells counts were estimated as described previously (7.2.1.1).

7.2.2.4 Chemiluminescence assay

The assay was carried out in 2 ml plastic test tubes (LKB, England) with a reaction volume of 1 ml (Table 7.1). One hundred microlitres of bacterial cells, removed from a volume of 5 ml of cells sonicated for 15 seconds, were preincubated with human PMNLs (100 µl) for 0, 10, 20 and 40 minutes at 37°C prior to the addition of luminol (200 µl - Sigma) at a concentration of 1.8 μgml^{-1}, diluted in PBS from a stock solution of 1.8 $mgml^{-1}$, prepared in dimethylsulphoxide (DMSO - Appendix 1.5). Immediately prior to reading in the luminometer, PMA diluted 1 in 1000 in PBS from a stock solution, prepared in DMSO (Appendix 1.5) was added and the reaction followed during the next 20 minutes.

Two controls were run (A) a PMA control that indicated the maximum stimulation of PMNLs when they were stimulated by the addition of PMA only and (B) a blank, which consisted of PMNLs and luminol only (see Table 7.1) which supplied a baseline. Each strain was assayed in duplicate, on three separate occasions and the results expressed as the mean of these.

Samples were read in an LKB Wallac luminometer (LKB, England) and all results were measured in millivolts. The luminometer was attached to an IBM computer and a graphical output obtained through "Multiuse" - the luminometer software package supplied by LKB. The results were obtained in a graphical and numerical form. The peak millivoltage value for each strain was calculated and expressed as a percentage of

Reaction Volumes

	Test	Blank Control	PMA Control
PBS	400 µl	700 µl	500 µl
PMA	200 µl	---	200 µl
Luminol	200 µl	200 µl	200 µl
~Sonicate	100 µl	---	---
PMNLs	100 µl	100 µl	100 µl

Table 7.1 Reaction volume in chemiluminescence studies

~Sonicate : Sonicated whole cell A.actinomycetemcomitans at concentration of 1 x 10^7 cells per ml.

the maximum stimulation obtained for the PMA stimulated cells (control A). The average result for each of the four strains, for each period of preincubation was calculated from the numerical output and the results plotted onto a single graph for each time period (Figures 7.3a to 7.3d) using the spreadsheet program Quattro Pro V.1.

A strain was designated toxic to PMNLs if it resulted in greater than 70% inhibition of the maximum chemiluminescence output. This value was purely an arbitrary one, which was used routinely as a measure of toxicity in the Microbiology Department of Glasgow University. The maximum light output varied from day-to-day, depending upon the source of the PMNLs. However, as the final result was a percentage of the maximum chemiluminescence output, i.e. the area under the graph, irrespective of the peak output, comparison between the strains could easily be made.

From the results shown in Figures 7.3a to 7.3d, it could be seen that the optimum period of preincubation for sonicated *A.actinomycetemcomitans* with PMNLs was 20 minutes (Figure 7.1c). Between 10 and 20 minute preincubation, the light output reached its maximum; at 40 minutes, it had begun to decline, therefore a period of 20 minutes was chosen as the maximum period of preincubation, prior to addition of PMA.

7.2.2.5 Toxic effect of *A.actinomycetemcomitans* on human PMNLs by Chemiluminescence

Sonicates of the remaining 13 *A.actinomycetemcomitans* strains under investigation (Table 2.2) not used in the optimisation studies were prepared as previously described (7.2.2.2) and the chemiluminescence assay performed as described in 7.2.2.3 - *A.actinomycetemcomitans* sonicates were preincubated with the PMNLs for 20 minutes, since this produced maximal results.

Each strain was assayed in duplicate, on three separate occasions.

7.2.2.6 Effect of heat and dilution upon toxicity of *A.actinomycetemcomitans* to PMNLs.

The leucotoxin from *A.actinomycetemcomitans* has been reported to be heat labile (Baehni *et al*, 1979). The effect of heat upon the activity of a sonicated suspension of two *A.actinomycetemcomitans* strains was investigated, in order to help determine that the effect seen was due to a toxin and not LPS, since the LPS found in the outer membrane of bacterial cell walls are known to be toxic to mammalian cells it was important to rule out LPS as the cause of PMNL cell death. Lipopolysaccharides have also been shown to have stimulatory effect upon PMNLs, which is dose dependent (Welch, 1991) and unaffected by heat.

Two strains of *A.actinomycetemcomitans*, 76 and 88 which had been shown to react differently with the PMNLs (7.3.2), were grown, harvested and sonicated for 15 seconds as previously described (7.2.2.2). From each sonicated sample, 1 ml aliquots were removed, heated at 56°C for 30 minutes, then cooled on ice. The samples were serially diluted from 1 in 2 to 1 in 2112 and the toxicity assessed in duplicate, on two separate occasions.

7.2.2.7 Stimulatory effect of *A.actinomycetemcomitans* sonicates upon PMNLs

Many of the strains were shown to have a stimulatory effect upon the PMNLs (7.3.2) i.e. the amount of light that was produced was greater than that of the PMA control. This effect was investigated by examination of two strains, one which had been shown previously to demonstrate this effect (strain 76) and a negative strain (88). These strains were grown, harvested and sonicated for 15 seconds as before (7.2.2.2) then serially diluted from 1 in 2 to 1 in 2112 in PBS. The toxicity of each was measured in the chemiluminescence assay as described before (7.2.2.3).

7.3 RESULTS

7.3.1 Results of Cytotoxicity Assay

Seventeen *A.actinomycetemcomitans* strains were examined for their ability to kill HL-60 cells after 60 minutes incubation at 37°C.

On the basis of the criteria laid down for a strain to be designated toxic (greater than or equal to 25% HL-60 cell death, 7.2.1.3), the greatest number of strains (12 out of the 17 investigated) were shown to be non-toxic to HL-60 cells.

Tables 7.2a - 7.2g indicate the average percentage HL-60 cell death with the corresponding Log_{10} viable *A.actinomycetemcomitans* cells for all the toxic strains and a cross section of the non-toxic strains of *A.actinomycetemcomitans* studied.

Of the 17 strains which were studied, only strains 24, 79, 80, 81 and 88 were designated toxic. These results are given in Tables 7.2a to 7.2e.

Strains 32 and 74 were non-toxic and their results were typical for all non-toxic strains. These are shown in Tables 7.2f and 7.2g.

Amongst the toxic strains, the percentage HL-60 cell death decreased after 15 seconds sonication, and generally after an increase in sonication to 5 minutes cell death decreased further. There would appear to be an association between the number of viable cells for most toxic strains and HL-60 cell death e.g. strain 24; whole viable cells at a concentration of Log_{10} 8.1 resulted in the death of 32.9%, whereas counts of Log_{10} 9.7 produced kills of 44.9 (Table 7.2a). On the other hand, non-toxic strains such as 32, had little effect on HL-60 cells at both very high (Log_{10} 10.00) and low (Log_{10} 7.8) concentrations.

Sonication did not affect the overall viability of the *A.actinomycetemcomitans* strains. The variation in the results between sonicated and non-sonicated preparations could be explained by the leucotoxin being removed from the bacterial cell surface, into solution.

Sonication has previously been shown to reduce cell clumping (5.3.5). The disassociation of *A.actinomycetemcomitans* cells by sonications resulted in an increase

	Average percentage HL-60 cell death ± S.D.	Log_{10} viable bacterial cell counts ± *S.D.
A1	32.9 ± 10.2	8.1 ± 0.4
A2	18.8 ± 7.0	8.4 ± 0.6
A3	12.9 ± 4.8	8.3 ± 0.6
B1	44.9 ± 3.0	9.7 ± 0.1
B2	34.6 ± 3.2	9.8 ± 0.1
B3	21.4 ± 4.8	9.8 ± 0.2

Table 7.2a Toxicity of whole cells and sonicates of *A.actinomycetemcomitans* 24 to HL-60 cells, measured by dye exclusion

	Average percentage HL-60 cell death ± S.D.	Log_{10} viable bacterial cell counts ± *S.D.
A1	18.6 ± 5.2	8.8 ± 0.0
A2	12.3 ± 3.4	8.8 ± 0.0
A3	14.7 ± 1.4	8.8 ± 0.9
B1	43.6 ± 0.9	10.6 ± 0.1
B2	32.0 ± 1.3	10.4 ± 0.4
B3	23.9 ± 2.3	10.0 ± 0.5

Table 7.2b Toxicity of whole cells and sonicates of *A.actinomycetemcomitans* 79 to HL-60 cells, measured by dye exclusion

***S.D. : Standard Deviation**
A1 : 10^8 Whole cells
A2 : 10^8 Whole cells, sonicated for 15 seconds
A3 : 10^8 Whole cells, sonicated for 5 minutes
B1 : > 10^9 Whole cells
B2 : >10^9 Whole cells, sonicated for 15 seconds
B3 : >10^9 Whole cells, sonicated for 5 minutes

	Average percentage HL-60 cell death ± S.D.	Log_{10} viable bacterial cell counts ± *S.D
A1	17.5 ± 5.4	8.8 ± 0.4
A2	10.1 ± 6.9	8.5 ± 0.3
A3	11.0 ± 6.2	8.2 ± 0.3
B1	28.0 ± 3.6	10.0 ± 0.3
B2	12.5 ± 3.0	10.4 ± 0.0
B3	15.1 ± 1.1	10.2 ± 0.8

Table 7.2c Toxicity of whole cells and sonicates of *A.actinomycetemcomitans* 80 to HL-60 cells, measured by dye exclusion

	Average percentage HL-60 cell death ± S.D.	Log_{10} viable bacterial cell counts ± *S.D
A1	26.7 ± 5.2	8.6 ± 0.4
A2	16.1 ± 2.6	8.6 ± 0.5
A3	15.9 ± 3.0	8.4 ± 0.3
B1	30.2 ± 4.6	9.8 ± 0.3
B2	24.7 ± 1.9	10.2 ± 0.1
B3	19.1 ± 1.6	10.1 ± 0.2

Table 7.2d Toxicity of whole cells and sonicates of *A.actinomycetemcomitans* 81 to HL-60 cells, measured by dye exclusion

***S.D. : Standard Deviation**
A1 : 10^8 Whole cells
A2 : 10^8 Whole cells, sonicated for 15 seconds
A3 : 10^8 Whole cells, sonicated for 5 minutes
B1 : > 10^9 Whole cells
B2 : >10^9 Whole cells, sonicated for 15 seconds
B3 : >10^9 Whole cells, sonicated for 5 minutes

	Average percentage HL-60 cell death ± S.D.	Log_{10} viable bacterial cell counts ± *S.D
A1	31.7 ± 2.4	7.6 ± 0.6
A2	25.9 ± 9.8	9.0 ± 0.5
A3	13.5 ± 1.7	8.8 ± 0.1
B1	38.3 ± 5.0	9.9 ± 0.4
B2	36.5 ± 2.3	10.0 ± 0.4
B3	36.8 ± 6.1	9.9 ± 0.2

Table 7.2e Toxicity of whole cells and sonicates of *A.actinomycetemcomitans* 88 to HL-60 cells, measured by dye exclusion

	Average percentage HL-60 cell death ± S.D.	Log_{10} viable bacterial cell counts ± *S.D
A1	1.1 ± 0.9	7.8 ± 0.7
A2	1.0 ± 1.4	8.4 ± 0.4
A3	0.5 ± 0.3	7.8 ± 0.4
B1	2.2 ± 0.6	10.0 ± 0.2
B2	8.0 ± 5.4	9.8 ± 0.4
B3	3.2 ± 1.5	9.2 ± 0.5

Table 7.2f Toxicity of whole cells and sonicates of *A.actinomycetemcomitans* 32 to HL-60 cells, measured by dye exclusion

***S.D. : Standard Deviation**
A1 : 10^8 Whole cells
A2 : 10^8 Whole cells, sonicated for 15 seconds
A3 : 10^8 Whole cells, sonicated for 5 minutes
B1 : > 10^9 Whole cells
B2 : >10^9 Whole cells, sonicated for 15 seconds
B3 : >10^9 Whole cells, sonicated for 5 minutes

	Average percentage HL-60 cell death ± S.D.	Log_{10} viable bacterial cell counts ± *S.D
A1	0.6 ± 0.8	8.3 ± 0.3
A2	1.3 ± 1.0	8.4 ± 0.3
A3	1.4 ± 1.4	8.0 ± 0.3
B1	2.0 ± 0.9	9.4 ± 0.5
B2	2.2 ± 1.3	9.6 ± 0.3
B3	3.2 ± 2.3	9.3 ± 0.5

Table 7.2g Toxicity of whole cells and sonicates of *A.actinomycetemcomitans* 74 to HL-60 cells, measured by dye exclusion

***S.D. : Standard Deviation**
A1 : 10^9 Whole cells
A2 : 10^9 Whole cells, sonicated for 15 seconds
A3 : 10^9 Whole cells, sonicated for 5 minutes
B1 : > 10^9 Whole cells
B2 : >10^9 Whole cells, sonicated for 15 seconds
B3 : >10^9 Whole cells, sonicated for 5 minutes

in the number of viable cells, but with no increase in the leucotoxicity of the bacterial strains.

Overall, these results suggest that whole viable bacterial cells demonstrated the greatest degree of leucotoxicity in the trypan blue dye exclusion assay with HL-60 cells and the cell numbers varied depending upon the strain tested.

7.3.2 Chemiluminescence - preliminary study to optimise the experimental conditions

The results for the 4 strains of *A.actinomycetemcomitans* selected for the preliminary experiments to optimise the experimental conditions for the chemiluminescence assay are presented in Figures 7.3a - 7.3d.

Incubation of the PMNLs and whole sonicated cells for 0 minutes prior to the addition of PMA, resulted in different peaks of activity for the different strains of *A.actinomycetemcomitans* investigated (Figure 7.3a). Strains 1 and 33 (both non-toxic by dye-exclusion) produced peaks greater than the PMA stimulated control cells and this level of activity was maintained for most of the experimental period. Strain 24 (toxic by dye exclusion) resulted in a very shallow peak after 103 seconds, which was then reduced to the baseline reading of non-stimulated cells of 0 mV. Thus the toxic effect of strain 24 upon PMNLs was almost instantaneous although it failed to depress PMA activity completely. Strain 88 (toxic by dye exclusion) resulted in the complete depression of chemiluminescence: this indicated that the PMNLs did not respond to PMA stimulation and were therefore probably dead. The toxic effect of strain 88 upon PMNLs was therefore almost instantaneous.

The results for 10 minutes preincubation (Figure 7.3b) at 37°C, are very similar to those described above when no preincubation period was used. There was, however a reduction in the activity of PMNLs incubated with strains 1 and 33, whilst there was an increase in the activity of PMNLs incubated with *A.actinomycetemcomitans* strain 24. No activity was measured at all for PMNLs which were incubated with

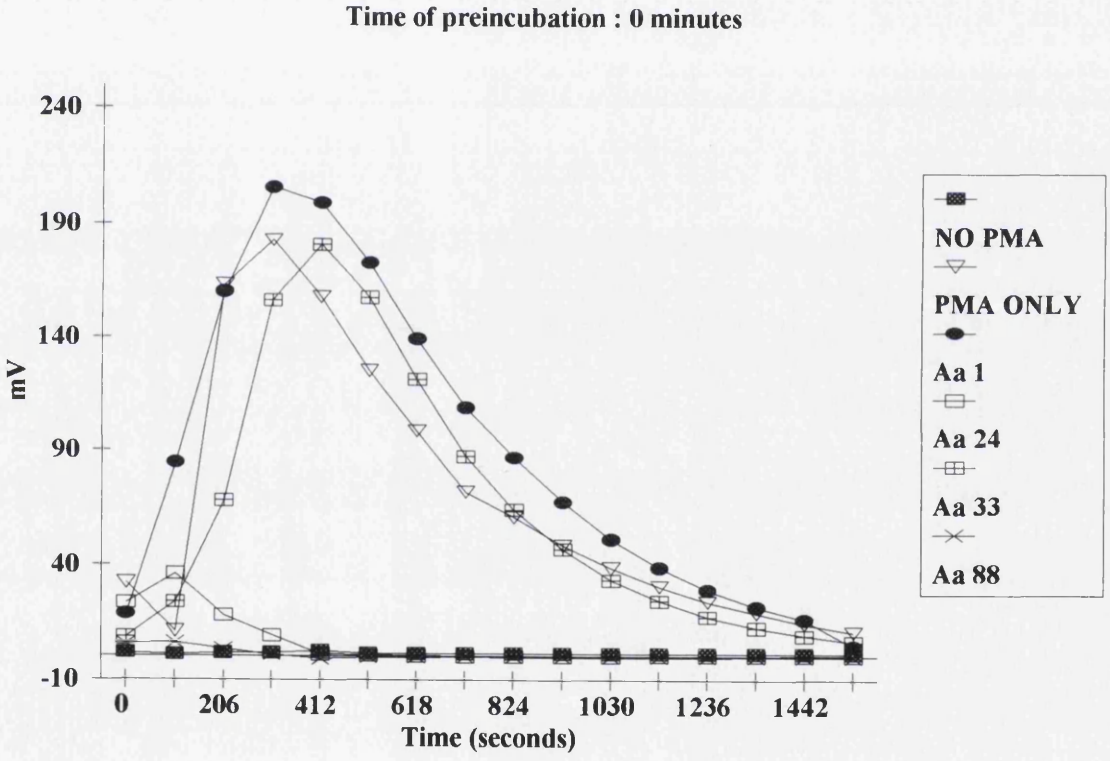

Figure 7.3a Result of chemiluminescence assay for sonicates of 4 selected strains of *A.actinomycetemcomitans*, that were preincubated for 0 minutes with PMNLs, prior to stimulation with PMA

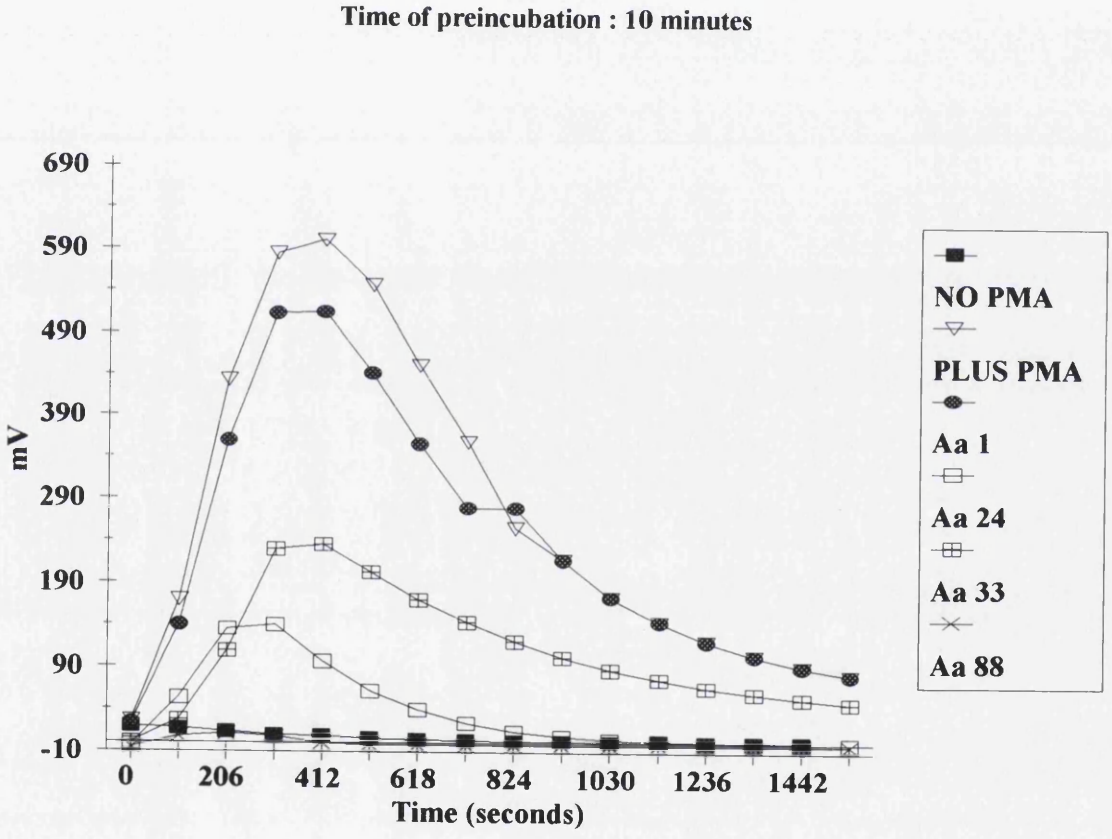

Figure 7.3b Result of chemiluminescence assay for sonicates of 4 selected strains of *A.actinomycetemcomitans*, that were preincubated for 10 minutes with PMNLs, prior to stimulation with PMA

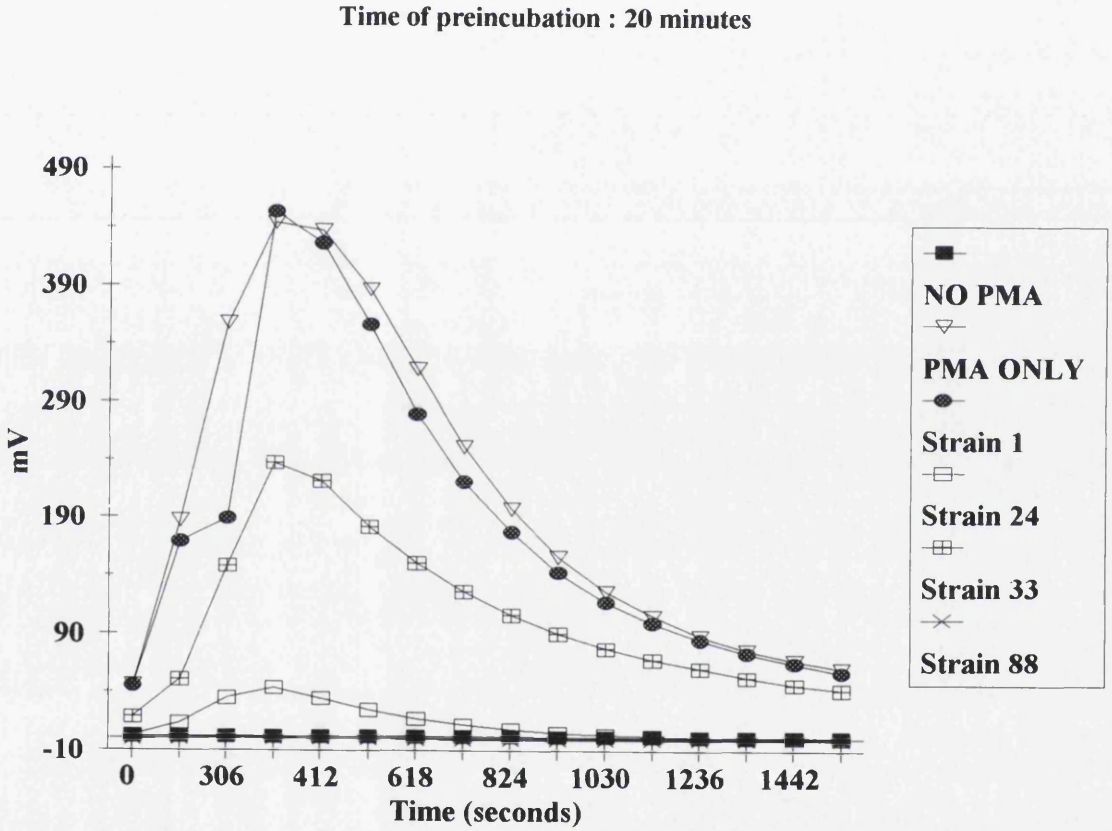

Table 7.3c Result of chemiluminescence assay for sonicates of 4 selected strains of *A.actinomycetemcomitans*, that were preincubated for 20 minutes with PMNLs, prior to stimulation with PMA

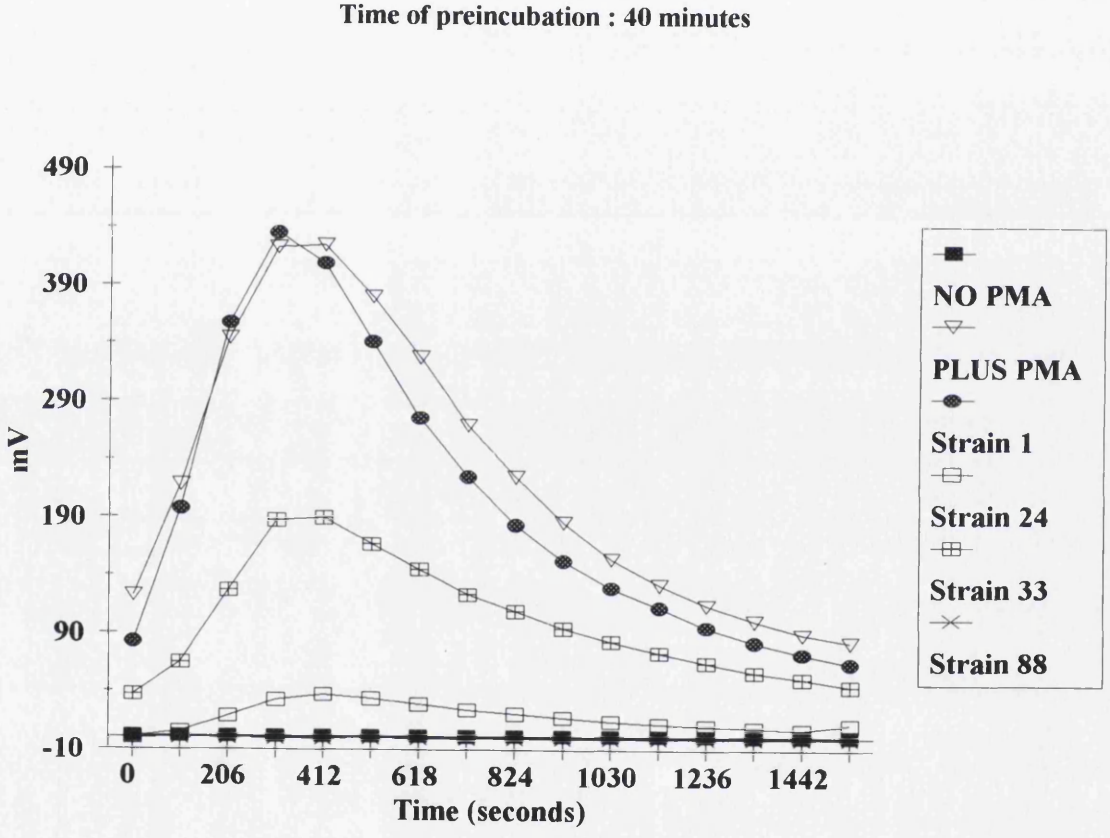

Table 7.3d Result of chemiluminescence assay for sonicates of 4 selected strains of *A.actinomycetemcomitans*, that were preincubated for 40 minutes with PMNLs, prior to stimulation with PMA

A.actinomycetemcomitans strain 88.

The results for 20 (Figure 7.3c) and 40 minutes (Figure 7.3d) preincubation did not vary greatly, but did show some differences compared with those for 0 and 10 minutes preincubation: the level of activity began to drop shown by the reduction in the maximum mV recorded after 40 minutes.

It was decided that the period of incubation of the *A.actinomycetemcomitans* strains with human PMNLs, prior to the addition of luminol and PMA would be 20 minutes as this appeared to be the point when maximum values were seen.

7.3.3 Toxic effect of *A.actinomycetemcomitans*

Figures 7.4a - 7.4d, show the variation in the effect of 14 *A.actinomycetemcomitans* strains on PMNLs, when preincubated for 20 minutes before stimulation with PMA. Several of the strains resulted in reduced PMNL activity e.g. strain 79 shown in Figure 7.4c and strains 80 and 81 in Figure 7.4d, thus indicating leucotoxic activity. Several of the strains resulted in a peak mV greater than that of the PMA peak, for example strains 8, 74 and 75 (Figure 7.4a) indeed, *A.actinomycetemcomitans* strain 74 resulted in a peak mV 1.75 times greater than the PMA control peak. The mean peak percentage values for all the strains are presented in Table 7.3.

7.3.4 Effect of heat and dilution upon toxicity of *A.actinomycetemcomitans* to PMNLs.

The effect of heat and dilution on serially diluted sonicates of two strains of *A.actinomycetemcomitans* are shown in Figures 7.5a to 7.5f. After 20 minutes incubation of the unheated sonicated whole cells of *A.actinomycetemcomitans* strain 88, no PMNL activity (Figure 7.5a) was noted. As the unheated sample was diluted, activity began to increase (Figures 7.5a and 7.5b) to levels approaching those of maximum expected from PMA stimulated cells. A maximum stimulation seen was at a serial dilution of 1 : 2112 (Figure 7.5b), when the output reached that of PMA

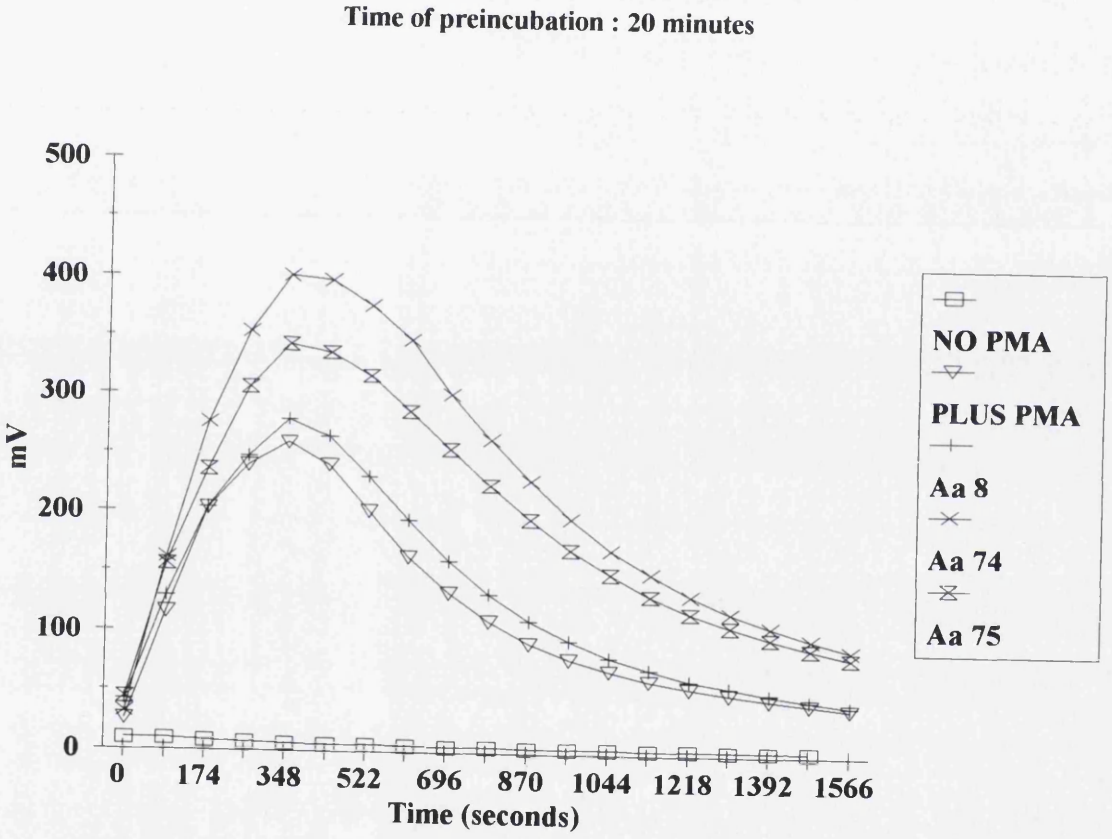

Table 7.4a Result of chemiluminescence assay for sonicates of 3 selected strains of *A.actinomycetemcomitans*, that were preincubated for 20 minutes with PMNLs, prior to stimulation with PMA

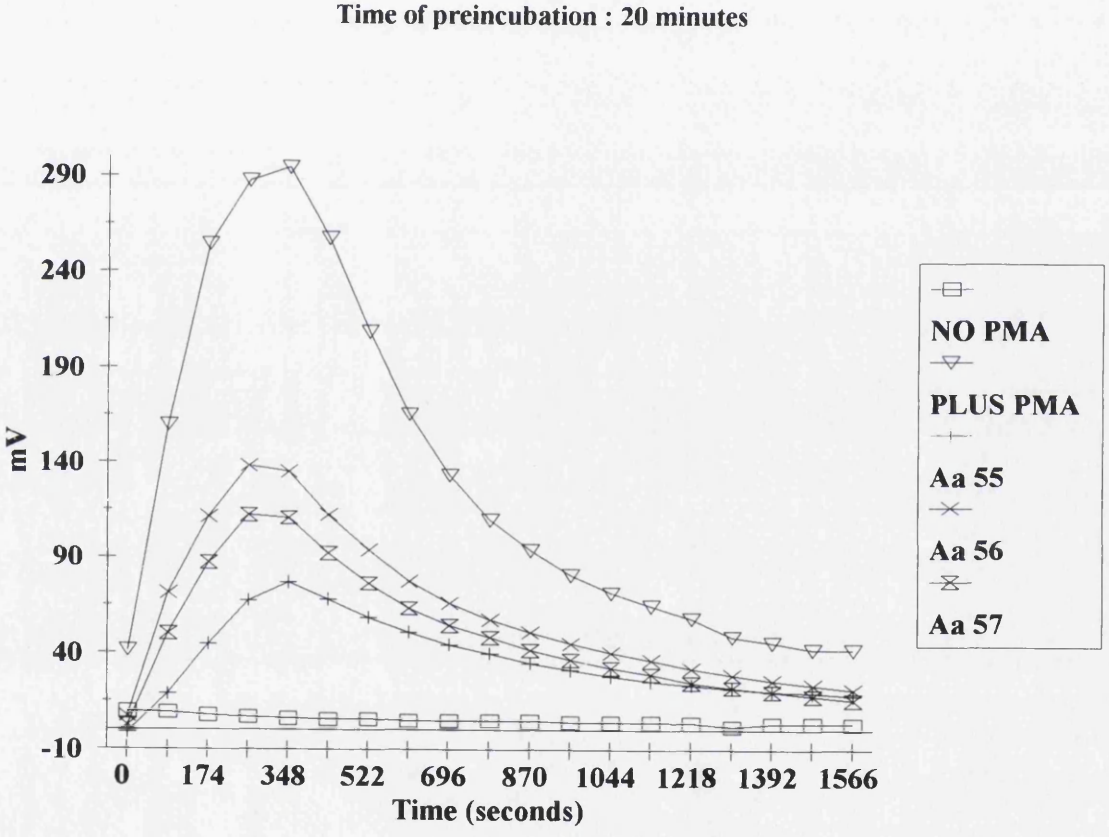

Table 7.4b Result of chemiluminescence assay for sonicates of 3 selected strains of *A.actinomycetemcomitans*, that were preincubated for 20 minutes with PMNLs, prior to stimulation with PMA

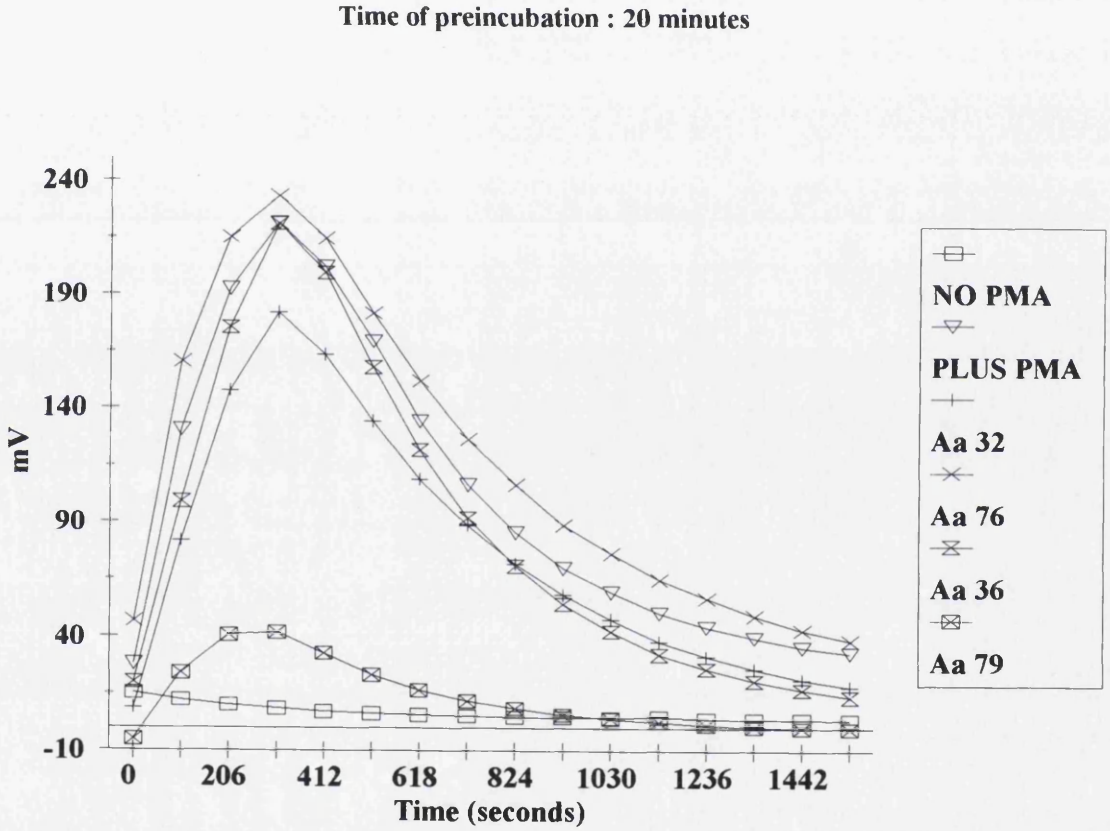

Table 7.4c Result of chemiluminescence assay for sonicates of 4 selected strains of *A.actinomycetemcomitans*, that were preincubated for 20 minutes with PMNLs, prior to stimulation with PMA

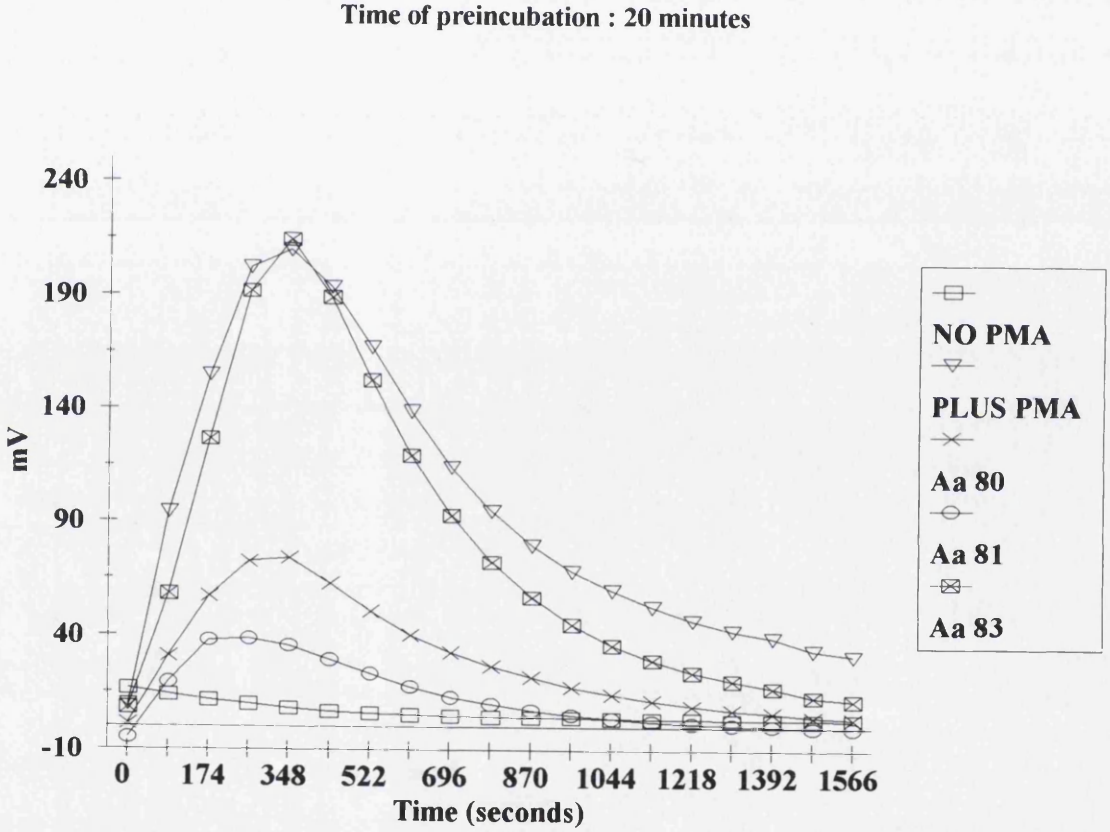

Table 7.4d Result of chemiluminescence assay for sonicates of 3 selected strains of *A.actinomycetemcomitans*, that were preincubated for 20 minutes with PMNLs, prior to stimulation with PMA

A.actinomycetemcomitans Isolate Number	Mean Percentage Chemiluminescence Produced by Isolate
1	118.0%
8	107.6%
24	10.2%
32	81.8%
33	106.9%
36	66.1%
55	95.5%
56	39.8%
57	48.7%
74	43.0%
75	138.8%
76	135.5%
79	20.5%
80	29.7%
81	15.5%
83	111.1%
88	0.2%

Table 7.3 Chemiluminescence produced by *A.actinomycetemcomitans* strains as a percentage of the light produced by stimulating PMNL cells with PMA

stimulated cells. When the sonicated sample from the same strain was heated at 56°C for 30 minutes, prior to incubation the opposite result was seen. Samples which contained the undiluted cells, produced a peak which was approximately 54% greater than that expected for PMA stimulated cells only (Figure 7.5c). Only after dilution to 1 : 32, did the stimulation reduce to a level below that of PMA stimulated cells.

When compared to *A.actinomycetemcomitans* strain 76, the unheated, undiluted sonicate sample ("neat") gave a moderately high reading, approximately 75% that of the PMA control (Figure 7.5d). However as the sample was diluted, there was an increase in the level of light output; dilutions of 1 in 2 to 1 in 8 all produced peaks one third greater than that of the PMA stimulated control (Figure 7.5d). Thereafter, as the sample was diluted, there was a decrease in chemiluminescence.

When the heated *A.actinomycetemcomitans* strain 76 was preincubated for 20 minutes prior to the addition of PMA, the undiluted sample, gave a peak approximately 75% that of the maximum PMA control. However, as this sample was diluted, peak values increased, to a maximum at a dilution of 1 in 4, approximately 2.5 times that of the PMA control (Figure 7.5e). As the sonicated sample was diluted the voltage output fell, but at a dilution of 1 in 64, the mV output began to rise again (Figures 7.5e and 7.5f).

7.3.5 Cytotoxicity Assay versus Chemiluminescence

The cytotoxicity and chemiluminescence assays identified the same strains of *A.actinomycetemcomitans* as leucotoxic, however, when the rank orders were compared, variation was seen (Table 7.4). The chemiluminescence assay used only whole cells which had been sonicated for 15 seconds, while the cytotoxicity assay used a wide variety of differently treated cells. As the rank order for the cytotoxicity assay was found as an average of all the results, this could explain the apparent discrepancies in the order of the strains.

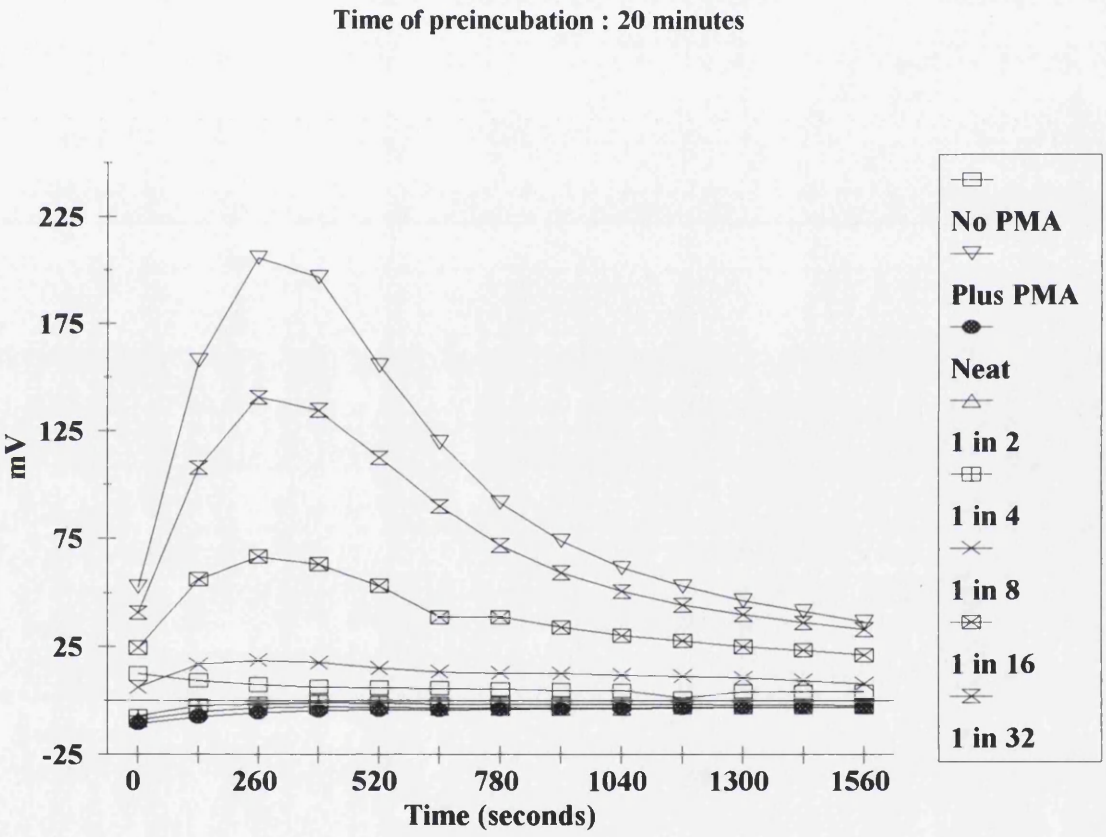

Table 7.5a Result of chemiluminescence assay for unheated sonicates of *A.actinomycetemcomitans* strain 88, diluted to 1 in32, preincubated for 20 minutes with PMNLs, prior to stimulation with PMA

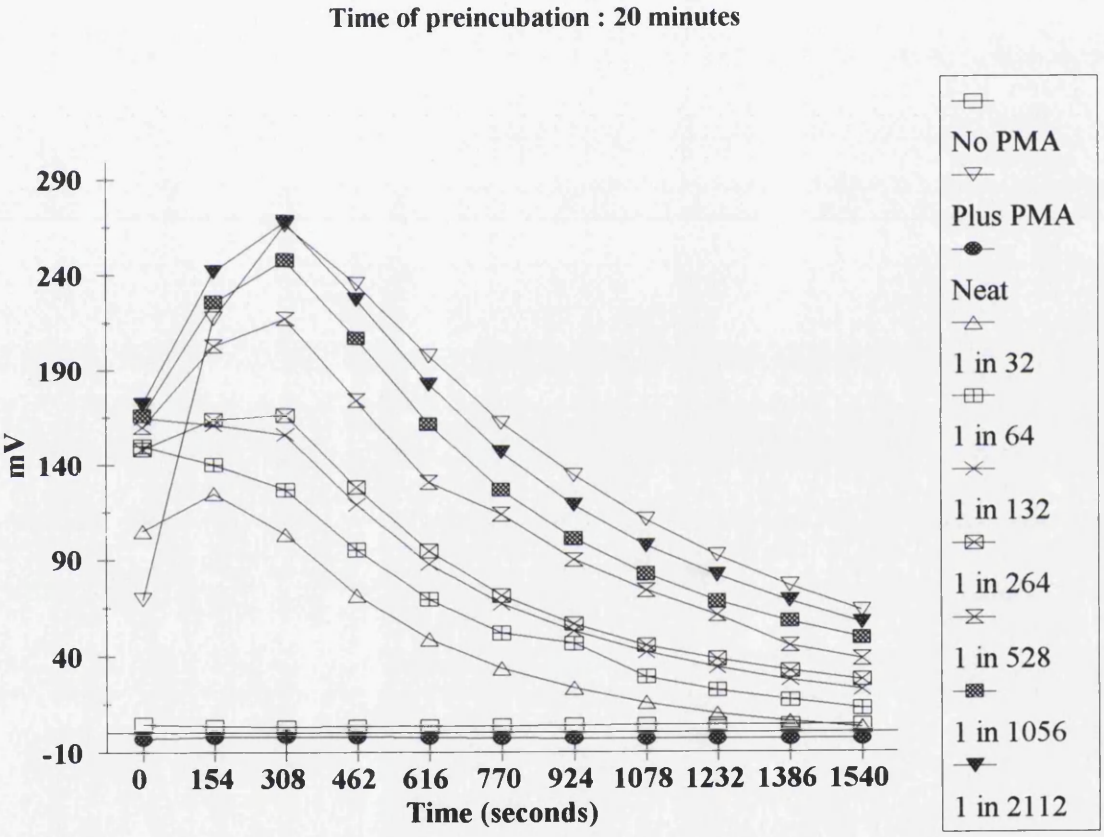

Figure 7.5b Result of chemiluminescence assay for unheated sonicates of *A.actinomycetemcomitans* 88, diluted to 1 in 2112, preincubated for 20 minutes, prior to stimulation with PMA

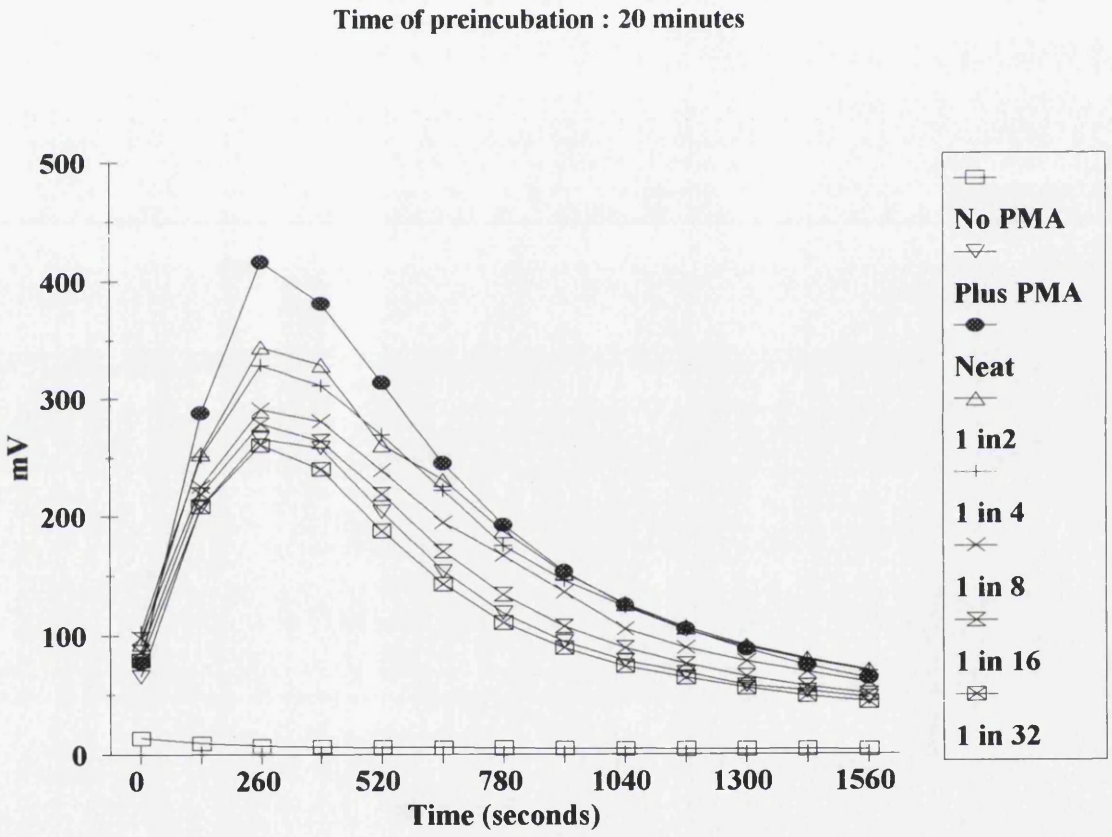

Figure 7.5c Result of chemiluminescence assay for sonicates of *A.actinomycetemcomitans* 88, heated at 56°C for 30 minutes, diluted to 1 in 32, then preincubated for 20 minutes, prior to stimulation with PMA

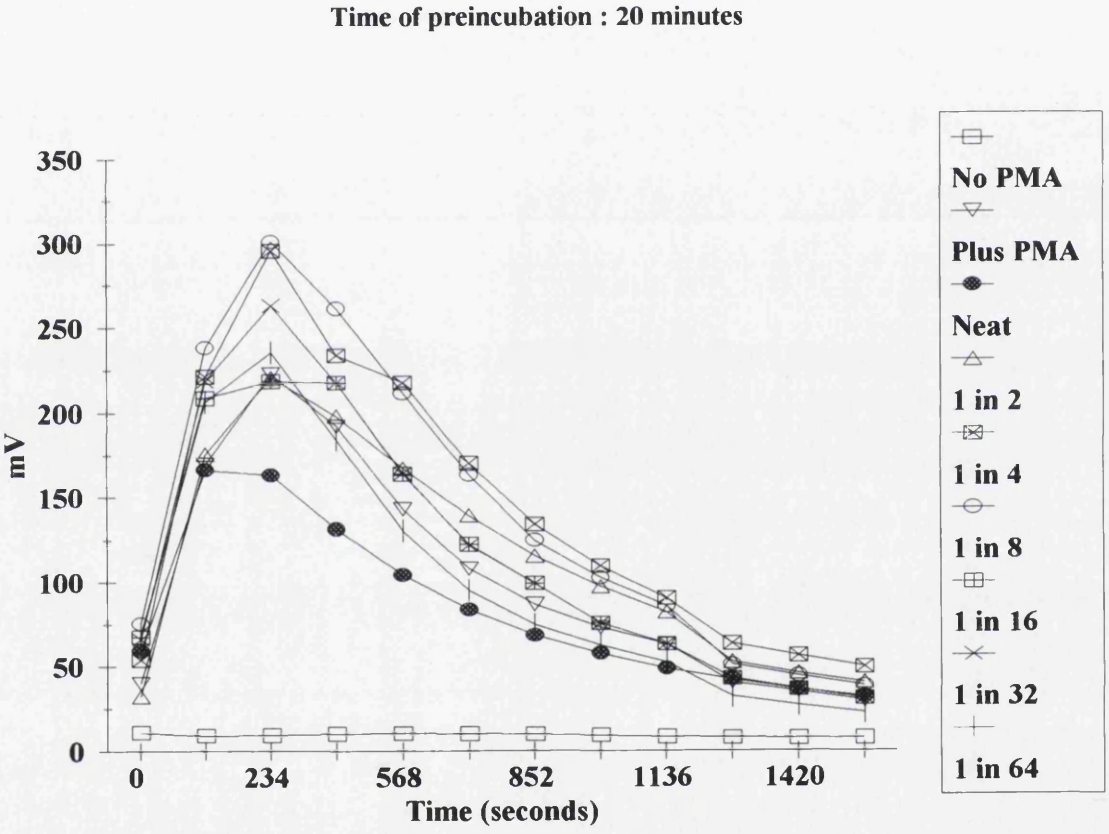

Figure 7.5d Result of chemiluminescence assay for unheated sonicates of *A.actinomycetemcomitans* 76 diluted to 1 in 64, preincubated for 20 minutes, prior to stimulation with PMA

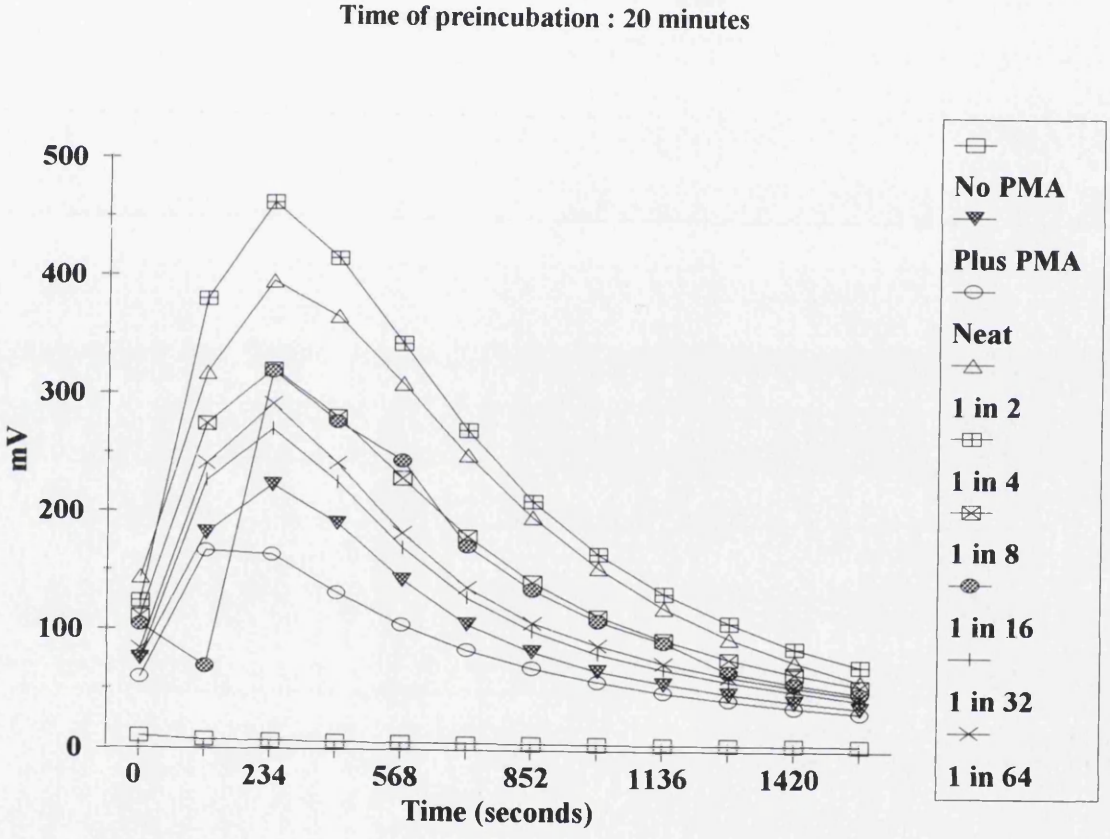

Figure 7.5e Result of chemiluminescence assay for sonicates of *A.actinomycetemcomitans* 76, heated at 55°C for 30 minutes, diluted to 1 in 64, preincubated for 20 minutes, prior to stimulation with PMA

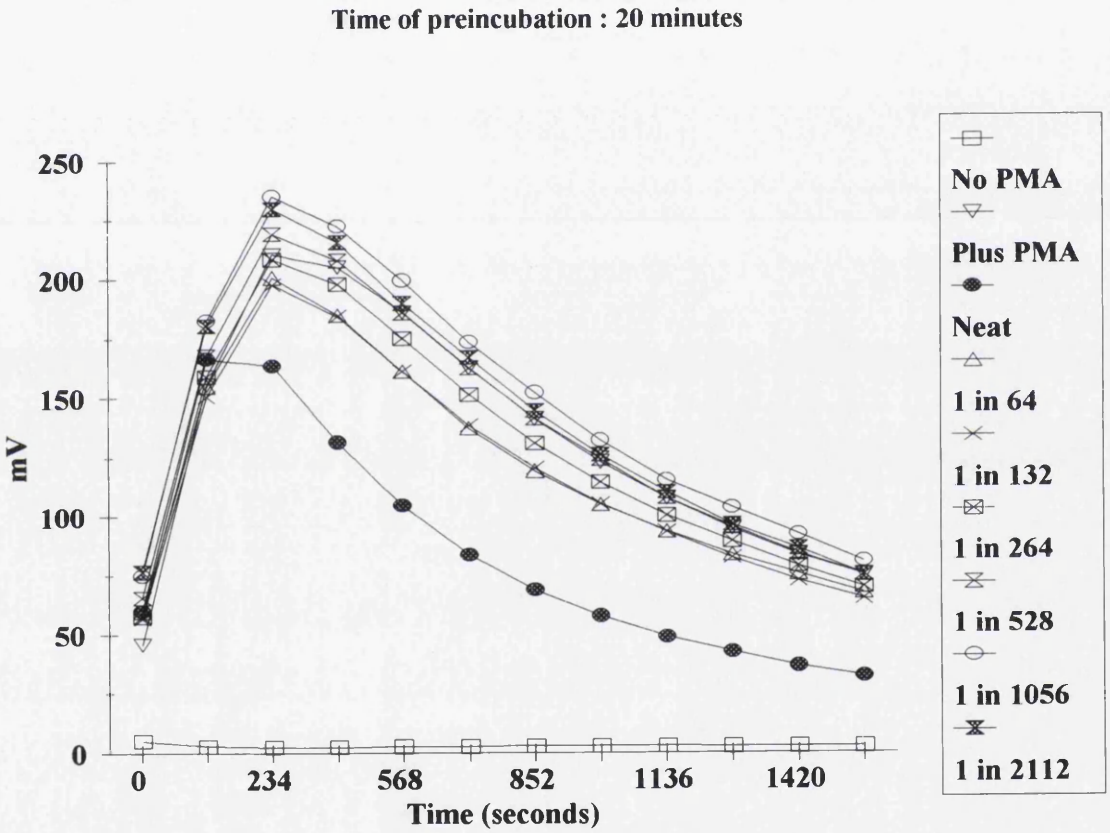

Figure 7.5f Result of chemiluminescence assay for sonicate of *A.actinomycetemcomitans* 76, heated at 55°C for 30 minutes, diluted to 1 in 2112, preincubated for 20 minutes, prior to stimulation with PMA

Rank Order of toxic A.actinomycetemcomitans strains measured by HL-60 cell death	Chemiluminescence
HL-60 cell death greater than 25%	**Chemiluminescence less than 30% that of maximum**
88	88
79	24
24	80
80	79
81	81
HL-60 cell death less than 25%	**Chemiluminescence greater than 30% that of maximum**
33	56
83	74
36	57
57	36
1	32
75	55
55	33
32	8
56	83
8	1
76	76
74	75

Table 7.4 *A.actinomycetemcomitans* strains ordered, in decreasing toxicity. The order for the two methods compared.

7.4 DISCUSSION

7.4.1 Effect of *A.actinomycetemcomitans* strains on HL-60 cells.

There are several possible different methods to measure the toxicity of *A.actinomycetemcomitans*. The majority of assays measure the effect of the toxin on susceptible viable cells e.g. LDH release, while others rely upon the purification and assay of the leucotoxin itself. The major advantages of purification of the leucotoxin, would include characterisation of the toxin which is being investigated and accurate comparisons would be possible between leucotoxins from different *A.actinomycetemcomitans* strains. However, before the leucotoxin can be purified, the toxic status of that strain must first be determined.

Previous authors (Zambon *et al*, 1983., Simpson, Berthold and Taichman, 1988) have used HL-60 cells to measure the leucotoxic effect of *A.actinomycetemcomitans*. Different methods have been employed to determine cell death e.g. trypan blue exclusion, LDH and radioactive Cr release (Zambon *et al*, 1983 and Tsai *et al*, 1979). Target cells (human PMNL and HL-60) have been exposed to whole cells of *A.actinomycetemcomitans* (Zambon *et al*, 1983); sonicated *A.actinomycetemcomitans* cells (Tsai *et al*, 1979) and purified leucotoxin (Tsai *et al*, 1984., Taichman *et al*, 1991).

In this investigation, the toxicity of *A.actinomycetemcomitans* strains for HL-60 cells, at different concentrations and after different periods of sonication was investigated and cell death measured by trypan blue exclusion, which was previously demonstrated by Ohta *et al* (1987). The HL-60 cell-line was originally derived from the peripheral blood of a patient with acute promyelocytic leukaemia (Collins, Gallo and Gallagher, 1977., Gallagher *et al*, 1979). The most commonly used cells in leucotoxicity assays were freshly isolated PMNLs (Tsai *et al*, 1979., Baehni *et al*, 1979., Baehni *et al*, 1981., Ohta *et al*, 1987). However, Zambon *et al* (1983) compared the leucotoxic nature of *A.actinomycetemcomitans* strains to both PMNLs and HL-60 cells but did not compare the two techniques. They measured the cytotoxicity of several strains of

A.actinomycetemcomitans to HL-60 by LDH release.

Strain 24 was previously tested for its toxic effect to HL-60 cells (Taher, 1990), while several of the Type strains have been previously investigated by other workers (Tsai *et al*, 1979., Baehni *et al*, 1979., Baehni *et al*, 1981., Ohta *et al*, 1987). The results of this study agree with results previously published, however, variation exists regarding the point at which a strain of *A.actinomycetemcomitans* is labelled toxic due to the different methods used to detect toxicity. The majority of authors described the measurement of LDH or ^{51}Cr release to determine toxicity. Trypan blue exclusion was often used as a secondary method, but rarely were the results of HL-60/ PMNL cell death given. When results were given cell deaths of either 20 or 25 percent were reported as the toxic threshold. In this investigation, a strain of *A.actinomycetemcomitans* was recorded as toxic to HL-60 cells, if the whole cells resulted in HL-60 cell death of greater than or equal to 25%. This value was arbitrary, but has been used by the Oral Microbiology Unit, Glasgow Dental Hospital and School to identify toxic strains. Had this value been increase to, say 30% HL-60 cell death, the the number of toxic strains would have reduced by 1 (strain 80 would no longer have been toxic). However, unless the level had been reduced to 15%, there would have been no increase in the number of toxic strains. Had the percentage cell death been raised to 50% (equivalent to LD_{50}), however, even the highly toxic strain 88 would have been designated as non-toxic.

The effect of variation in the number of *A.actinomycetemcomitans* cells was also investigated. Strains which were toxic to HL-60 cells e.g. 88 and 24, at a lower concentration of cells, were shown to have an increased killing effect when the number of *A.actinomycetemcomitans* cells increased. A similar effect was reported by Zambon *et al* (1983) with HL-60 cells, although toxicity was measured by LDH release. However, in no instance did a non-toxic strain becoming toxigenic when the concentration of bacterial product was increased.

Single Strains/ Isolates

Strains of *A.actinomycetemcomitans* isolated from unrelated individuals were all shown to be non-toxic, with the exception of *A.actinomycetemcomitans* strain designated 24. This strain demonstrated a high degree of toxicity to HL-60 cells (44.9% at the higher concentration of cells and 33% at the lower. Table 7.2a). It has been suggested by Haubek *et al* (1995) that there are very few toxic strains of *A.actinomycetemcomitans* in Northern Europe, compared to America and the results presented here would agree with this, as 24 was the only toxic wild strain out of 12 wild strains.

Multiple Strains/ Isolates

Strains of *A.actinomycetemcomitans* isolated from the same periodontal pocket, or from a different pocket but the same individual, were shown to be non-toxic to HL-60 cells, although, there was some variation in the exact levels of cell death seen between the strains. There have been no reports of multiple strains from an individual, having a different toxic status to each other.

Type Strains

Type strains of *A.actinomycetemcomitans* (coded 79, 80, 81, 83 and 88) could be grouped according to toxicity status; highly toxic (*A.actinomycetemcomitans* 79 and 88 - 43.6 and 38.3 percent respectively); moderately toxic (*A.actinomycetemcomitans* 81 - 30.2% HL-60 cell death); at the lower limits of toxic (*A.actinomycetemcomitans* 80 - 28.0% HL-60 cell death) or at the lower limits of non-toxic (*A.actinomycetemcomitans* 83 - 6.0% HL-60 cell death. Results not shown).

These results can be compared to previously published results. Strain designated 79 (ATCC 29523 - Table 2.2) has been shown to be both non-toxic (Baehni *et al*, 1981 and Zambon *et al*, 1983) and toxic (Spitznagel *et al*, 1991 and Zambon, 1985). Spitznagel *et al* (1991) suggested that this was related to the amount of mRNA

produced. They further suggested that the expression of the leucotoxin varied throughout the growth cycle and that this could explain the variable toxic nature of ATCC 29523, as reported in the literature (Baehni *et al*, 1981,. Zambon *et al*, 1983,. Zambon, 1985 and Spitznagel *et al*, 1991). The results of this study correlate with the previously published work of Zambon (1985) and Spitznagel *et al* (1991), that strain 29 is toxic.

Strains 80 and 81 (ATCC 29524 and Y4) were shown to be toxic to HL-60 cells, measured by LDH release (Baehni *et al*, 1981., Zambon *et al*, 1983., Tsai *et al*, 1984., Ohta *et al*, 1987). The results presented in this work correlate with previously published work - strains 80 and 81 were found to be toxic to HL-60 cells.

A.actinomycetemcomitans strain designated 88 (JP_2) has been shown previously to be very toxic to both PMNLs and HL-60 cells (Tsai *et al*, 1984., Iwase *et al*, 1990., Mangan *et al* 1991). In the study reported here, this strain was shown to be the most consistently toxic of all the strains investigated (Table 7.2g). Strain 24 was less toxic than either 79 or 88, but more toxic than 80 and 81.

7.4.2 Effect of *A.actinomycetemcomitans* strains on human PMNL measured by chemiluminescence

Phagocytosis is the process by which bacteria are ingested and killed by white blood cells such as PMNLs and the macrophages. This defence mechanism is important in the protection of the host against bacterial infection. Before bacteria are ingested they must first be coated with either a serum antibody or complement. This process is called opsonisation and allows white cells to recognise and phagocytose bacteria. (Technical notes, LKB)

Opsonised bacteria are ingested by PMNLs, then killed by the production of superoxide anion, hydrogen peroxide, singlet oxygen and hydroxyl radicals (Figure 7.6). This process is termed the "oxidative respiratory burst" and is one of the principal mechanisms by which PMNLs kill ingested bacteria (Technical notes, LKB).

A flash of light is associated with the release of the oxidising species and this chemiluminescence is very weak, but can be enhanced by the addition of luminol (5-amino-2, 3-hydro-1, 4-phthalazinedione) to phagocytosing cells. The luminol reacts with the oxidising species to produce a much larger and measurable amount of light (Figure 7.7) at a wavelength of 425 nm. Chemiluminescence can be produced in the absence of particulate matter by the addition of phorbol-myrisate-acetate (PMA), which is an irreversible activator of protein kinase C and results in the release of oxidising species, which causes light to be produced.

The process of phagocytosis can be monitored with a luminometer, which provides an effective technique for the study of this aspect of host-bacterial interactions. If no chemiluminescence is seen, compared with controls, it would indicate some inherent or induced deficiency in cellular metabolism. The *B.pertussis* toxin, adenylate cylase, has been shown to disrupt the cell membrane of PMNL (Friedman *et al*, 1987., Parton, 1989), which results in a reduction in chemiluminescence (Friedman *et al*, 1987). As the target cell for the *A.actinomycetemcomitans* leucotoxin is the PMNL, it is possible that chemiluminescence will provide a rapid method of identification of toxic and non-toxic strains, by measuring the amount of light which is produced when PMNLs are incubated with sonicates of *A.actinomycetemcomitans* before stimulation with PMA. A decrease in chemiluminescence produced by PMNLs exposed to bacterial products compared with cells stimulated only with PMA, would indicate that the oxidative respiratory burst pathway was disrupted in some way. Although Ashkenazi, White and Dennison (1992) have used PMA stimulation to investigate the effects of *A.actinomycetemcomitans* upon the chemotactic activity of PMNLs this method has not been used previously for the detection of the toxicity of *A.actinomycetemcomitans*.

In this study, strains of *A.actinomycetemcomitans* were considered to be toxic to PMNLs if they inhibited chemiluminescence by 70% i.e. strains with values less than 30% of the peak chemiluminescence were designated toxic. This value was purely an

arbitrary one, which was used routinely as a measure of toxicity in the Microbiology Department of Glasgow University. Had this value been altered to, say 50% (equivalent to LD_{50}), then the number of toxic strains would have increased to 8 (Table 7.3) and perhaps this should be the value that should be chosen in future.

With both the dye exclusion and chemiluminescence assays *A.actinomycetemcomitans* strains 88 (JP_2), 79 and 24 (Table 7.4) were most toxic. Strains 88 and 24 were originally obtained from patients with juvenile periodontitis. The degree of toxicity of the other 2 strains, 80 and 81, varied slightly when tested in the two assay methods.

Superoxidase generation has been used previously (Keane *et al*, 1987. Bhakdi and Martin, 1991) in the investigation of other RTX toxins. The results obtained in the present experiments suggest that it could be used equally well to study the leucotoxin of *A.actinomycetemcomitans*.

7.4.3 Effect of heating and dilution upon toxic activity of *A.actinomycetemcomitans* to PMNLs.

Welch (1991) suggested that lipopolysaccharides have the effect of priming and stimulating the neutrophils. This was also suggested by both Friedman *et al* (1987) and Hewlett *et al* (1989) who indicated that the effect was dose dependent. Baehni *et al* (1979) demonstrated the toxic effect of Y4 was heat sensitive - heating to 56°C for 50 minutes inactivated the toxin. When the reaction was carried out at 4°C, no toxic effect was observed. Interestingly, Spitznagel *et al* (1991) demonstrated that strain 79 (ATCC 29523) was partially heat resistant. When the crude lysate was heated at 65°C for 30 minutes, leucotoxic activity was detected. Heating for greater periods of time, subsequently reduced the activity to the background level. Investigation into the effect of heating and dilution, in the experiments reported here, were carried out on only strains 88 and 76 and therefore no further information is available related to the observation of Spitznagel *et al* (1991).

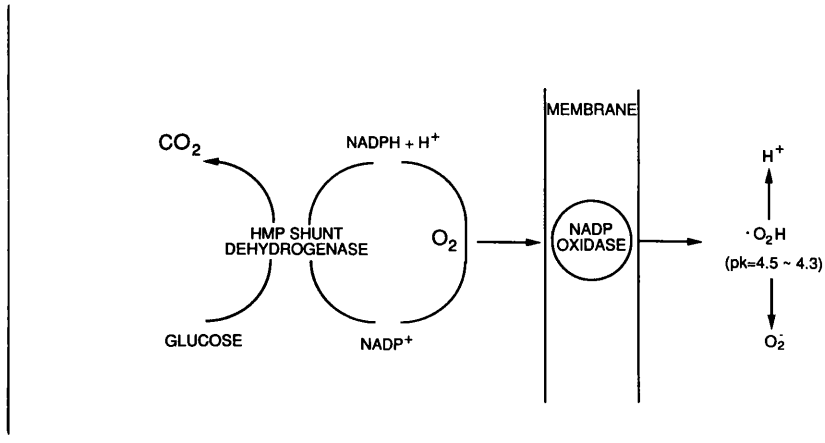

Figure 7.6 The production of bacterial oxidants on the inner surface of the phagolysosome

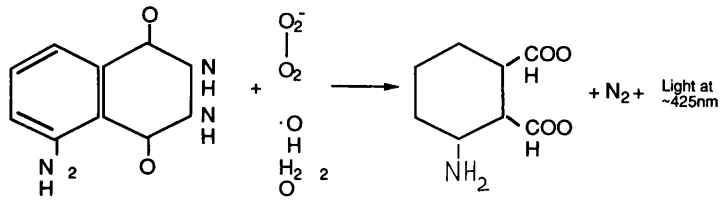

Figure 7.7 The interaction of luminol with oxidants to produce light

When strain 76 was heated and diluted, chemiluminescence increased and peaked at a dilution of 1 in 4 (Figure 7.5e). Thereafter it fell, although it never reached the level of the PMA control. This is consistent with the effect expected with a dose dependent LPS stimulation.

When a highly toxic strain 88 (JP_2) was heated at 56°C for 30 minutes no toxicity was recorded, instead a stimulatory effect was observed for the neat to 1 : 16 dilution, which indicated that the stimulation could be due to the action of LPS, which is heat resistant. Furthermore, it would suggest that the stimulatory effect observed was not due to low doses of the toxin protein, but rather a dose dependent effect by LPS.

Several of the strains e.g. 1, 8, 74, 75 and 76 appeared to have a stimulatory effect. It was suggested previously by Welch (1991) and Glasser *et al* (1991) that the stimulatory effect might be anticipated at very low doses of an RTX toxin. Keane *et al* (1987) demonstrated a 14-fold increase in superoxidase production with sublytic doses of the *E.coli* haemolysin exposed to rat renal tubular cells. This supports the view that alteration of the cell function of animal cells could occur upon exposure to sublytic or subtoxic doses of RTX toxins.

7.4.4 Effect of PMNLs

A group of 5 volunteers were used throughout these experiments to donate blood which was collected from different individuals on a daily basis. The peak chemiluminescence values for control cells varied from day-to-day which may have been the result of natural variation in the PMNLs. All donors were healthy and none were bled more frequently than once ever four days. The effect of various factors (e.g. alcohol intake the previous day/ night) on the responsiveness of the PMNLs to stimulation by PMA and/ or bacteria was not considered.

The time which elapsed between the preparation of the PMNLs and their use was also controlled. Blood was collected from donors at the Glasgow Dental Hospital and School, then transported as quickly as possible on ice to the Department of Cell

Biology, Glasgow University, where the PMNLs were prepared, before transport to the Department of Microbiology. Unfortunately no one site had all the equipment required to carry out all aspects of the chemiluminescence assay. Initial pilot experiments allowed periods of potential delay e.g. isolation of PMNLs and their transport between Oral Microbiology and the University, as well as the booking of equipment in the Departments of Cell Biology and Microbiology, to be highlighted and the appropriate action taken to ensure that once the experiments had commenced, delay did not occur at these points.

7.4.5 Effect of sonication versus whole cells

The leucotoxin of *A.actinomycetemcomitans* has been found in association with the outer membrane (Lally *et al*, 1991), therefore it would seem reasonable to assume that sonication of the whole cells for a short period of time would remove the toxin from the outer membrane of the cell and leave it free in solution. Additionally, if *A.actinomycetemcomitans* cells have clumped together, sonication would dissociate the cells, resulting in a larger surface area and thus potentially more toxin. This would not appear to be the case. Sonication for 15 seconds, in all cases, resulted in a decrease in toxicity, from the whole cell level. One possible reason for this could be that sonication for 15 seconds disrupts or destroys the toxin, however, this theory is disproved by previously published data e.g. Baehni *et al* (1981), who demonstrated toxicity to PMNLs with a sonic extract from *A.actinomycetemcomitans* strain Y4 (81).

Only sonicated cells could be used in the chemiluminescence assay, as previous experience within the University of Glasgow Microbiology Department had shown the use of whole cells to result in inaccuracies in the results. However it would appear that the sonication of whole cells for 15 seconds did not result in the designation of fewer toxic strains; the results for both experiments were comparable.

HL-60 cells are a cell-line, derived from an acute promyelocytic leukaemia patient,

whereas PMNLs are freshly harvested, immediately prior to use. If PMNLs are not as viable as the HL-60 cell line, then it is possible that PMNLs will require less toxin to be killed, than HL-60 cells. Thus, sonicated cells which would result in only the moderate killing of HL-60 cells, will kill PMNLs more easily and to a higher degree.

7.4.6 Cytotoxicity assay versus Chemiluminescence

Minor discrepancies exist between the results of the two methods i.e. the order of non-toxic strains differs however strains designated toxic by the cytotoxicity assay were also toxic by chemiluminescence, in spite of the different host target cells involved.

There are advantages and disadvantages associated with both methods : HL-60 and *A.actinomycetemcomitans* cells can be grown to order in a standardised way - whilst PMNLs are dependent on the availability of volunteers and standardisation of the cells cannot be guaranteed due to factors such as the health of the individual.

The cytotoxicity assay is observer dependent, rather tedious and relies upon the supposition that randomised selected microscopic fields will supply data that is representative of the entire sample.

The chemiluminescence assay is a faster and more accurate method - once the assay has been set up, subsequent measurements and calculations are performed by the computer and provided the assay has been set up correctly, this should eliminate operator error.

Cost is an important factor and unless access to a luminometer is possible, the capital cost of the machine may be prohibitive. Although the computer could be used for other items of equipment, the luminometer is a dedicated machine and to recoup its cost investigations involving a large number of strains would be necessary. A luminometer could also be used to investigate phagocytosis in addition to toxicity and in a busy research laboratory it may well be heavily used.

The chemicals required for the luminometer are potentially carcinogenic, which is not

the case for the cytotoxicity assay and great care must therefore be taken.

Unless care is taken, contamination of tissue culture cells may occur, but with good laboratory technique, this should not happen. The chemicals required for the luminometer are costly, although very small amounts are used. This is not the case with the cytotoxicity assay, where the chemicals are cheaper, although more are required.

Overall, cost wise, the luminometer assay is more expensive than the cytotoxicity assay, although the results with the luminometer are more accurate as they are not dependent upon subjective measurements. The chemiluminescence assay does not only give the final results, but it also allows the operator to have an insight into the reaction process itself.

7.5 CONCLUSIONS

It would appear that the collection of *A.actinomycetemcomitans* strains held at the Glasgow Dental Hospital and School is composed of a mixed population of toxic and non-toxic strains. Strains of *A.actinomycetemcomitans* which were designated toxic to HL-60 cells in a cytotoxicity assay, were also shown to be toxic to PMNLs in a chemiluminescence assay.

From the subset of 17 strains investigated, only 1 out of 12 wild strains demonstrated any toxicity to either HL-60 cells or PMNLs. In contrast however, 4 out of 5 Type strains were toxic in both the cytotoxicity and luminescence assays. It could therefore be hypothesised, that the majority of the collection is non-toxic to either HL-60 cells or PMNLs, however, in order for this hypothesis to be disproved, the remainder of the strains within the collection require to be tested for their toxic activity. If the majority of the collection is demonstrated to be non-toxic, this would be in agreement with the work of Haubek *et al* (1995) who suggested that there are very few toxic strains of *A.actinomycetemcomitans* in Northern Europe, compared to America.

Multiple strains of *A.actinomycetemcomitans* from the one individual/ individual

pocket, were all shown to be non-toxic.

Both assays studied gave reproducible and comparable results - both designated the same five strains to be toxic, although there was variation in the rank order between the methods, which could perhaps be explained by the differently treated cells.

There were advantages and disadvantages associated with each assay, however, the chemiluminescence assay gave good reproducible results, which were not dependent upon operator interpretation and if a luminometer was made available, then this should be the assay of choice.

CHAPTER 8
GENERAL DISCUSSION

CHAPTER 8

GENERAL DISCUSSION

Overall, a range of diverse experimental approaches have been used to investigate *A.actinomycetemcomitans*.

The strain diversity of the collection of *A.actinomycetemcomitans* strains was initially investigated by examination for the prevalence of plasmids (Chapter 3). Of the 96 strains held in the Glasgow Dental Hospital and School collection, 43.2% were shown to be plasmid positive (Table 3.1). The size of the plasmids ranged from 5 to 140 kb and the number of differently sized plasmids varied between 1 and 3; 73% of plasmid positive strains harboured 1 plasmid.

There are very few comparable studies in the literature; although Poulsen *et al* (1994) studied a collection of 97 strains, only 2 plasmid positive strains were found. Preus and Olsen (1989) demonstrated plasmids in 10 freshly isolated strains - however the sample population size was relatively small. Type strains, in this study, were generally found to be plasmid negative, with the exception of strain 79 (ATCC 29523), which was found to have a plasmid of approximately 5 kb.

The results presented therefore demonstrate, that in the Glasgow Dental Hospital and School collection, the occurrence of plasmids is more common than the literature would suggest. Possible reasons for the apparent lack of plasmids in previous studies include the suggestion by Poulsen *et al* (1994) that plasmids are lost through continued sub-culturing. An alternative explanation could be that *A.actinomycetemcomitans* strains from different continents reflect their different clonal origins. Preus and Olsen (1989) studied Norwegian strains of *A.actinomycetemcomitans*, whereas Poulsen *et al* (1994) investigated American strains. The study reported here demonstrated that strains isolated in the West of Scotland harboured plasmids. Although the ethnic origin of the individuals is not known, it could be hypothesised that if the patients were from the West of Scotland,

STRAIN NUMBER (Chapter 2)	CLINICAL ORIGINS (Chapter 2)	PLASMID STATUS (Chapter 3)	OMP GROUP (Chapter 6)	REFP GROUP (Chapter5)	LEUCOTOXIN STATUS (Chapter 7)	REACTION TO MCA, 9D4 (Chapter 6)
1	Cp	2 : 30, 60 kb	1	G	Negative	None
8	Cp	2 : 30, 60 kb	2	F	Negative	None
24	Jp	1 : 50	3	D	Positive	116, 97.4, 95, 66, 45 kDa
32	Cp	0	4	J	Negative	116, 97.4, 95, 66 kDa
33	Jp	0	4	J	Negative	116, 97.4, 95, 66 kDa
36	Pp	0	4	K	Negative	66 kDa
55	Cp	1 : 60	5	I	Negative	97, 95, 66 kDa
56	Cp	1 : 60	4	I	Negative	97, 95, 66 kDa
57	Cp	1 : 60	4	I	Negative	66 kDa
74	Jp	0	6	A	Negative	116, 97.4, 66, 45 kDa
75	Jp	0	3	A	Negative	116, 97.4, 66, 45 kDa
76	Jp	0	3	A	Negative	116, 97.4, 66, 45 kDa
79	Blood	1 : 5	7	H	Positive	97, 66 kDa
80	Chest aspirate	0	4	D	Positive	97, 95, 66 kDa
81	Jp	0	7	A	Positive	97, 95, 66 kDa
83	Abscess	0	8	B	Positive	97, 95, 66 kDa
88	Jp	0	7	C	Positive	95, 66 kDa

Table 8.1 Results for each investigation, compared for each strain.

Cp : Chronic periodontitis

Jp : Juvenile periodontitis

Pp : Prepubertal periodontitis

then European strains have a separate clonal origin to American strains, as suggested by other work in this study.

Investigations into the strain diversity of *A.actinomycetemcomitans* strains using RFLP techniques, have generally been conducted by American research groups, using freshly isolated clinical strains and Type strains, which have generally been isolated in America (DiRienzo & Slots, 1990., Zambon *et al*, 1990., Genco & Loos, 1991., Han *et al*, 1991.), although there have been a number of European studies (Lawon *et al*, 1990., van Steenbergen *et al*, 1991., Haubek *et al*, 1995). The results have often been contradictory: Zambon *et al* (1990) and Han *et al* (1991) linked restriction patterns to serotypes, whereas DiRienzo (1990) reported no link between restriction patterns and serotypes.

The 17 strains of *A.actinomycetemcomitans* studied could be shown to belong to one of eleven clonal types on the basis of REFPs, using three different enzymes. The large number of clonal types seen, relative to the small population size is not unusual: Han *et al* (1991) demonstrated 10 different clonal types from 12 different strains. It was not possible to relate the strain diversity of individual strains to their serotypes, as the serotypes of strains within the collection is not known.

Despite repeated attempts, the generation of a reproducible method for the production of plasmid profiles was not possible. Protocols were devised to remove potential contaminants and inhibitors: DNA was scanned in a UV-spectrophotometer, however, the exact nature of the contaminant/ inhibitor could not be detected.

The results of the study reported in this thesis are in agreement with the work of Haubek *et al* (1995), who suggested that different epidemiological situations exist in America and Europe - it would appear that *A.actinomycetemcomitans* strains isolated on different continents have a different clonal origin.

Future studies into the strain diversity of *A.actinomycetemcomitans*, should include polymerase chain reaction (PCR), a rapid and relatively straightforward method to discriminate between strains, in large scale epidemiological studies.

The characteristics of OMPs from *A.actinomycetemcomitans* have been investigated by other researchers (Bolstad *et al*, 1990., Wilson, 1991b). Bolstad *et al* (1990), showed very limited variation in the expression of the main patterns of OMPs from both wild and Type strains of *A.actinomycetemcomitans*. Additionally, Bolstad *et al* (1990) demonstrated a protein of 34 kDa, whereas Wilson demonstrated a protein of 29 kDa: both proteins were heat-modifiable.

The OMP preparations in this study, revealed little variation between the strains, which is supported by the results of Bolstad *et al* (1990). Despite this apparent lack of major variation, the OMP profiles of the 17 strains of *A.actinomycetemcomitans*, were grouped to give 8 different profiles. In this study, major proteins, common to all 17 strains, were seen with molecular weights of approximately 41 and 38 kDa. Other common proteins, had molecular weights of approximately 100, 97, 70 and 29 kDa. Several of the strains expressed a protein with a molecular weight within 10% of 116 kDa (the molecular weight of the leucotoxin), however only 3 of these strains were toxic to HL-60 cells/ inhibited chemiluminescence. These proteins could represent non-functional leucotoxin, equally, they could be unrelated to the leucotoxin protein. Bolstad *et al* (1990) reported proteins, with weights of 70, 43 kDa and 39 kDa. A protein of 34 kDa was found as well as a protein of 29 kDa. Wilson (1991) reported a heat modifiable protein with a molecular weight of 29 kDa.

Outer membrane proteins have been used to assess strain diversity (Davies, 1991a, 1991b). However their use in this context is severely limited. Fluctuations in temperature, growth media and atmospheric conditions can alter the expression of 1 or more proteins and thus alter the profile. However, if the limitations are understood and they are not used as the sole means of assigning strain diversity, then OMPs can be a useful additional means of investigation.

The OMP group to which a strain was assigned was not related to leucotoxic status, clinical origin or clonal group, on the basis of REFP analysis. From this limited study, the usefulness of OMPs, in assigning strain diversity is, for *A.actinomycetemcomitans*,

therefore restricted.

The leucotoxin of *A.actinomycetemcomitans* is known to be found in the outer membrane of the bacterial cell and is a member of a group of related toxins, RTX, to which adenylate cyclase belongs. However, there have been no reports in the literature of a MCA to one RTX toxin, reacting with the OMP proteins of a species in another genus. In this study, the MCA, 9D4, was shown to react with all the strains of *A.actinomycetemcomitans* with the exception of 1 and 8 (Table 8.1). A number of reactions between the MCA and OMPs were observed : 116 (the approximate molecular weight of the *A.actinomycetemcomitans* leucotoxin), 97, 95 and 66 kDa.

Five strains (24, 33, 74, 75 and 76) demonstrated an antigenic reaction between a protein of approximately 116 kDa and the MCA. Only 1 of these strains (24), was toxic. The reaction between the MCA with separated proteins of approximately 116 kDa, in non-toxic strains, could be due to an antigenic binding between the non-functional leucotoxin and the MCA.

In future experiments, the non-specific binding seen, could be removed by increasing the stringency of the post hybridisation-washes, however care would be needed to ensure that the washes were not so stringent as to completely remove the bound antibody. Purification of the toxin and its reaction to the MCA, would provide further confirmation. The response of the various RTX toxins to MCAs against other RTX toxins, would be of interest. Do toxins which have a greater genetic homology to each other, have a stronger reaction to each other, than to other RTX MCA, where the homology is less?

The leucotoxicity of the *A.actinomycetemcomitans* collection was investigated using a well tested method (HL-60 cells exposed to whole and sonicated whole bacterial cells, with cell death measured by trypan blue exclusion) and a novel method - chemiluminescence.

Overall, only 1 (1224/86) of the clinical strains was shown to be toxic to either HL-60 cells or freshly prepared human PMNL Ls, whereas 4 out of 5 Type strains

investigated demonstrated toxicity. It is therefore possible that the majority of the remaining clinical *A.actinomycetemcomitans* strains, untested for toxicity, could be negative. If this hypothesis is true, then this would again agree with the result of Haubek *et al* (1995), who suggested that European strains of *A.actinomycetemcomitans* were less virulent than American strains.

There did not appear to be any correlation between the leucotoxicity status of the *A.actinomycetemcomitans* strains and clinical origin of the strains (Table 8.1).

The chemiluminescence assay gave the most objective data. The results were presented as a visual and numerical output: it could be clearly seen which strains were toxic and which were not. The recording of the results was not dependent upon the operator and therefore potential error in reading HL-60 cells were eliminated.

The choice of 25% HL-60 cell death and chemiluminescence inhibition at 70% as the levels at which a strain was designated toxic, were purely arbitrary and both were set prior to the commencement of the experimental work, based upon previous studies. The number of toxic strains in the collection could have been increased or decreased merely by altering the level at which a strain was designated toxic was set. However, both methods confirmed the same 5 strains as toxic, which therefore suggests that 25% HL-60 cell death and 70% chemiluminescence inhibition were convenient points at which to designate toxicity.

Until the work of Haubek *et al* (1995), leucotoxicity was considered to be the most important virulence factor of *A.actinomycetemcomitans* and most research has focused on this area. The majority of previous studies were carried out in America and Haubek *et al* (1995) hypothesised that the northern European population of *A.actinomycetemcomitans* strains were avirulent, whereas those from America were virulent. The results presented in this thesis support this hypothesis, which brings into doubt the value of future research into the toxin since juvenile periodontal disease is found in Europe, just as in America. If the toxin produced by *A.actinomycetemcomitans* is not a significant virulence factor in the aetiology of the

juvenile periodontitis, then some factor common to all strains, irrespective of geographical and temporal isolation, must be responsible for the bacterial involvement in the disease. However, the hypothesis cannot be confirmed or rejected until a larger number of strains (ideally, the remainder of the Glasgow Dental Hospital and School collection) have been tested for their toxic effect upon either HL-60 cells or PMNLs, using one of the methods described above, preferably the chemiluminescence assay.

The results did not reveal any obvious correlation patterns. Plasmid status was not linked to disease, OMP group, REFP group (clone), leucotoxic status or reaction to MCA, 9D4.

All the Type strains, including the Danish strain (83), were leucotoxic. All belonged to different clones, although 3 belonged to the same OMP group. Only 1 of the Type (79) strains harboured plasmids.

Two clones contained both wild and Type strains. Strain 24 and 80 clustered together; both were toxic to HL-60 cells/ PMNLs. Additionally, 74, 75, 76 and 81 clustered together. Interestingly the wild strains were non-toxic. The Danish Type strain (83) did not cluster with any other strain of *A.actinomycetemcomitans*. It is therefore possible that the bacteria within these two clones share a common origin and that some event has resulted in the loss of leucotoxic activity for strains 74, 75 and 76, whereas strain 24 has retained its toxic effect. If these strains do share a common origin and there has been the loss of the leucotoxin for 3 of the European strains, then this represents an important result, as all four strains were isolated from juvenile periodontal lesions. This result, therefore confirms the work of Haubek *et al* (1995) who have suggested that the Northern European strains are less virulent than the American strains.

Two strains (32 and 33) from different clinical sources, were very similar to each other; whereas 2 strains (1 and 8) which were isolated from patients with the same disease shared a common plasmid and leucotoxic status and reaction to MCA 9D4,

but had different OMP and REFP profiles.

Strains from the same individual (55, 56 and 57; 74, 75 and 76) shared common plasmid profiles. The only variation between strains 55, 56 and 57 related to the OMP profiles - variation was seen in the OMP profile of 55, which could be an artifact of the gel. This was also the case with 74, 75 and 76 : 74 varied slightly in its OMP profile compared to 75 and 76.

In many ways the lack of any pattern in the results, linking strains together is disappointing, however, given the complex nature of the bacteria, it is not at all unexpected. Also, the number of strains investigated was very small and the study of a larger population, at least the size of the entire collection, would yield more information.

Given the problems associated with plasmid and whole genomic preparations it would perhaps be more appropriate in future work to use Polymerase Chain Reaction (PCR) based technologies. Griffen, Leys & Fuerst, 1992. Saarela *et al*, 1995), which are rapid and relatively straight-forward for use in discriminating between strains in large scale epidemiological studies.

Determination of the serotypes of the strains of *A.actinomycetemcomitans* held at the Glasgow Dental Hospital and School would be of interest, as several different authors have related the clonal type of a strain to its serotype.

If European strains of *A.actinomycetemcomitans* are less virulent than the American strains, as suggested by Haubek *et al* (1995), then future areas of research should include investigations into the other virulence factors, which appear to have been neglected in favour of the leucotoxin. These factors include collagenase activity - (Robertson *et al*, 1982. Uitto, Suomalainen and Sorsa, 1990) and adhesion (Winkler *et al*, 1988). The contribution of these factors to virulence of European strains in comparison to American strains would be of interest. For example, is the collagenase activity of European *A.actinomycetemcomitans* less, more than or equal to that of

American strains?

The results from plasmid profiles and the REFPs of whole cell DNA preparations, suggest that the population of *A.actinomycetemcomitans* strains studied is remarkably diverse and comprised a large number of distinct clones, which, on the basis of plasmid profiles and leucotoxicity status would appear to have more in common with the European than American strains of *A.actinomycetemcomitans*. However, until the strain diversity and the leucotoxic status of the entire population has been fully investigated, this hypothesis will remain unanswered.

REFERENCE LIST

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Alaluusua, S & Asikainen, S. (1988) Detection and distribution of *Actinobacillus actinomycetemcomitans* in the primary dentition. *Journal of Periodontology*, **59**, 504 -507.

Alaluusua, S, Asikainen, S. & Lai, C-H. (1991) Intrafamilial transmission of *Actinobacillus actinomycetemcomitans*. *Journal of Periodontology*, **62**, 207 - 210.

Ashkenazi, M., White, R.R. & Dennison. (1992) Neutrophil modulation by *Actinobacillus actinomycetemcomitans*. I : Chemotaxis, surface receptor expression and F-*actin* polymerisation. *Journal of Periodontal Research*, **27**, 264 - 273.

Asikainen, S., Jousimies-Somer, H., Kanervo, A. & Saxen, L. (1986) *Actinobacillus actinomycetemcomitans* and clinical periodontal status in Finnish juvenile *periodontitis patients. Journal of Periodontology*, **57**, 91 - 93.

Asikainen, S., Jousimies-Somer, H., Kanervo, A. & Summanen, P. (1987) Certain bacterial species and morphologies in localised juvenile periodontitis and in matched controls. *Journal of Periodontitis,* **58**, 228 - 230.

Asikainen, S., Alaluusua, S. & Saxen, L. (1991) Recovery of *A.actinomycetemcomitans* from teeth, tongue and saliva. *Journal of Periodontal Dieases*, **62**, 203 - 206.

Asikainen, S., Lai, C-H., Alaluusua, S. & Slots, J. (1991) Distribution of *Actinobacillus actinomycetemcomitans* serotypes in periodontal health and disease. *Oral Microbiology and Immunology*, **6**, 115 - 118.

Baardsen, R., Bakken, V., Jensen, H.B. & Hofstad, T. (1988) Outer membrane pattern of *Eubacterium plautii*, *Journal of General Microbiology*, **44**, 1561 - 1564.

Baehni, P. Tsai, C.C., McArthur, W.P., Hammond, B.F. & Taichman, N.S. (1979) Interaction of Inflammatory Cells and Oral Microorganisms. *Infection and Immunity*, **24**, 233 - 243.

Baehni, P.C., Tsai, C.C., McArthur, W.P., Hammond, B.F., Shenker, B.J. & Taichman, N.S (1981) Leukotoxic activity in different strains of the bacterium *Actinobacillus actinomycetemcomitans* isolated from juvenile periodontitis in man. *Archives in Oral Biology*, **26**, 671 - 676.

Baer, P.N. (1984) The case for periodontosis as a clinical entity. *Journal of Periodontology*, **42**, 516 - 519.

Beachey, E.H. (1981) Bacterial adherence: adhesin-receptor interactions mediating the attachent of bacteria to mucosal surfaces. *Journal of Infectious Diseases*, **143**, 325 - 345.

Berthold, P., Forti, D., Kieba, I.R., Rosenbloom, J., Taichman, N.S. & Lally, E.T. (1992) Electron immunocytochemical localisation of *Actinobacillus actinomycetemcomitans* leukotoxin. *Oral Microbiology and Immunology*, **7**, 24 - 27.

Bhakdi, S., Macjman, N., Nicaud, J. -M. & Holland, I.B. (1986) *Escherichia coli* alpha-hemolysin may damage target cell membranes by generating transmembrane pores. *Infection and Immunity*, **52**, 63 - 69.

Bhakdi, S. & Martin, E. (1991) Superoxidase generation by human neurophils induced by low doses of *Escherichia coli* hemolysin. *Infection and Immunity*, **59**, 2955 - 2962

Bimstein, E., Lustmann, J., Sela, M.N., Neriah, Z.B. & Soskolne, W.A. (1990) Periodontitis associated with Papillon-Lefevre Syndrome. *Journal of Periodontology*, **61**, 373 - 377.

Birmboim, H.C. & Doly, J. (1980) A rapid method alkaline extraction procedure for screening recombinant DNA. *Nucleic Acids Research*, 7, 1513 - 1523.

Blix, I.J.S., Preus, H.R. & Olsen, I. (1990) Invasive growth of *Actinobacillus actinomycetemcomitans* on solid medium (TSBV). *Acta Odontology Scandanavia*, **48**, 313 - 318.

Boehm, D.F., Welch, R.A. & Snyder, I.S. (1990) Calcium is required for binding of *Escherichia coli* hemolysin (HlyA) to erythrocyte membranes. *Infection and Immunity*, **58**, 1951 - 1958.

Bolstad, A.I., Kristoffersen, T., Olsen, I., Preus, H.R., Jensen, H.B, Vasstrand, E.N. & Bakken, V. (1990) Outer membrane proteins of *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* studied by SDS-PAGE and immunoblotting. *Oral Microbiology and Immunology*, **5**, 155 - 161.

Boughman, J.A., Halloran S.L., Roulston, D. *et al* (1986) An autosomal-dominant form of juvenile periodontitis: Its localisation to chromosome 4 and linkage to dentinogenesis imperfecta and Gc. *Journal of Craniofacial Genetics and Developmental Biology*, **6**, 341 - 345.

Bowden, T.J., Ochs, H.D., Altman, L.C., Price, T.H., Van Epps, D.E., Brantigan, D.L., Rosin, R.E., Perkins, W.D., Baior, B.M., Klebanott, S.J. & Wedgwood, R.J., (1982) Severe recurrent bacterial infections associated with defective adherence and chemotaxis in two patients with neutrophil deficiency in a cell-associated glycoprotein. *Journal of Pediatrics*, **101** : 932 - 940.

Bowker, C.M., Connellan, S.J. & Freeth, M.G. (1992) A case of thoracic actinobacillus infection. *Respiratory Medicine*, **86**, 53 - 54.

Brogan, J.M., Lally, E.T., Poulsen, K., Kilian, M. & Demuth, D.R. (1994) Regulation of *Actinobacillis actinomycetemcomitans* leukotoxin expression: analysis of the promoter regions of leukotoxic and minimally leukotoxic strains. *Infection and Immunity*. **62**, 501 508.

Brondz, I. & Olsen, I. (1984a) Determination of bound cellular fatty acids in *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* by gas chromatography and gas chromatography-mass spectrometry. *Journal of Chromatography*, **308**, 282-288

Brondz, I. & Olsen, I. (1984b) Differentiation between *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* based on carbohydrates in lipopolysaccharides. *Journal of Chromatography*, **310**, 216 - 272.

Brondz, I. & Olsen, I. (1989) Chemical differences in lipopolysaccharides from *Actinobacillus (Haemophilus) actinomycetemcomitans* and *Haemophilus aphrophilus*: clues to differences in periodontopathogenic potential and taxonomic distinction. *Infection and Immunity*, **57**, 3106 - 3109.

Brondz, I., Olsen, I. & Sjostrom (1990) Multivariate analysis of quantitative chemical and enzymic characterization data in classification of *Actinobacillus*, *Haemophilus* and *Pasteurella* spp. *Journal of General Microbiology*, **136**, 507 - 513.

Brown, D., Munro, D. & Platt, D.J. (1986) Recognition of cryptic plasmid pSLT by restriction fingerprinting and the study of its incidence in Scottish *Salmonella* isolates. *Journal of Hygiene*, **97**, 193 - 197.

Brown, T.A., Byres, L., Gardner, M. & Van Dyke, T.E. (1991) Subclass and molecular form of immunoglobulin A antibodies to *Actinobacillus actinomycetemcomitans* in juvenile periodontitis. *Infection and Immunity*, **59**, 1126 - 1130.

Clark, R.A., Page, R.C. & Wilde, G. (1977) Defective neutrophil chemotaxis in juvenile periodontitis. *Infection and Immunity*, **18** , 694 - 700.

Caugant, D.A., Selender, R.K. & Olsen, I. (1990) Differentiation between *Actinobacillus (Haemophilus) actinomycetemcomitans*, *Haemophilus aphrophilus* and *Haemophilus paraphrophilus* by multilocus enzyme electrophoresis. *Journal of General Microbiology*, **136**, 2135 - 2141.

Chang, Y-F., Young R. & Struck, D.K. (1989) Cloning and characterisation of a hemolysin gene from *Actinobacillus (Haemophilus) pleuropneumoniae*. *DNA*, **8**, 635 - 547.

Chang, Y-F., Young R. & Struck, D.K. (1991) The *Actinobacillus pleuropneumoniae* hemolysin determinant: unlinked *appCA* and *appBD* loci are flanked by pseudogenes. *Journal of Bacteriology*, **173**, 5151 - 5158.

Chart, H., Row, B., Threlfall, E.J. & Ward, L.R. (1989) Conversion of *Salmonella enteritidis* phage type 4 to phage type 7 involves loss of lipopolysaccharide with concomitant loss of virulence. *FEMS Microbiology Letters*, **60**, 37 - 40.

Christersson, L.A., Slots, J., Rosling, B.G. & Genco, R.J. (1985a) Microbiological and clinical effects of surgical treatment of localized juvenile periodontitis. *Journal of Clinical Periodontology*, **12**, 465 - 476.

Christersson, L.A., Slots, J., Zambon, J.J. & Genco, R.J. (1985b) Transmission and colonisation of *Actinobacillus actinomycetemcomitans* in localized juvenile periodontitis patients. *Journal of Periodontology*, **56**, 127 -131.

Christersson, L.A., Albini, B., Zambon, J.J., Wikesjo, U.M.E. & Genco, R.J. (1987a) Tissue localization of *Actinobacillus actinomycetemcomitans* I. Light, Immunofluorescence and Electron Microscopic Studies. *Journal of Periodontology*, **58**, 529 - 539.

Christersson, L.A., Wikesjo, L.M.E., Albini, B., Zambon, J.J. & Genco, R.J. (1987b) Tissue localisation of *Actinobacillus actinomycetemcomitans* in human periodontitis. II : Correlation between immunofluorescence and culture techniques. *Journal of Periodontology*, **58** , 540 - 545.

Chuba, P.J., Bock, R., Graf, G., Adam, T. & Gobel, V. (1988) Comparison of 16S rRNA sequences from the family *Pasteurellaceae* : Phlyogenetic relatedness by cluster analysis. *Journal of General Microbiology*, **134**, 1923 - 1930.

Clark, R.A., Page, R.C. & Wilde, G. (1977) Defective neutrophil chemotaxis dysfunction in human periodontitis. *Infection and Immunity*, **8**, 694 -700.

Clinkbeard, K.D., Mosier, D.A. & Confer, A.W. (1989) Transmembrane pore size and role of sell swelling in cytotoxicity casued by *Pasteurella haemolytica* leukotoxin. *Infection and Immunity*, **57**, 420 - 425.

Cogkendall A.L., Setterfield, J. & Slots, J. (1983) Deoxyribonucleic acid relatedness amongst *Actinobacillus actinomycetemcomitans*, *Haemophilus aphropilus* and other *Actinobacillus* species. *International Journal of Systematic Bacteriology*, **33**, 765 - 771.

Coia, J.E., Nor-Hussain, I. & Platt, D.J. (1988) Plasmid profile and restriction endonuclease fragmentation patterns of methacyline sensitive and methacyline resistant isolates of *Staphylococcus aureus* from hospital and the community. *Journal of Medical Microbiology*, **27**, 271 - 267.

Colebrook L. (1920) The mycelial and other microorganisms associated with human actinomycosis. *British Journal of Experimental Pathology*, **1** , 197 - 212.

Collins, S.J., Gallo, R.C. & Gallagher, R.E. (1977) Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. *Nature*, **270**, 347 - 349.

Coote, J. (1992) Structural and functional relationships among the RTX toxin determinants of Gram-negative bacteria. *FEMS Microbiology Reviews*, **88**, 137 - 162.

Crawford, A., Socransky, S.S. & Bratthall (1975) Predominant cultivable microbiota of advancd periodontitis. *Journal of Dental Research*, **54**, Special Issue. Abstract No. 209.

Crossner, C-G., Carlsson, J., Sjodin, B., Tarnvik, A., Unell, L., Venge, P. & Wranne, L. (1990) Periodontitis in the primary dentition associated with *Actinobacillus actinomycetemcomitans* infection and leukocyte dysfunction. A 3- year follow-up. *Journal of Clinical Periodontology*, **17**, 204 - 267.

Cruz, W.T., Young, R., Chang, Y.F. & Struck, D.K. (1990) Deletion anaylsis resolves cell binding and lytic domains of the *Pasteurella* leukotoxin. *Molecular Microbiology*, **4**, 1933 - 1939.

Dahlen, G & Slots, J. (1989) Experimental infections by Bacteriodes gingivalis in non-immunized and immunized rabbits. *Oral Microbiology and Immunology*, **4**, 6 - 11.

Dahlen, G., Manji, F., Baelum, V. & Fejerskov, O. (1989) Black-pigmented Bacteroides species and *Actinobacillus actinomycetemcomitans* in subgingival plaque of adult Kenyans. *Journal of Clinical Periodontology*, **16**, 305 - 310.

Datta, N. (1979) Plasmid classification: Incompatibility grouping. In : *Plasmids of Medical, Environmental and Commercial Importance*, pp 3 - 12. Eds Timmis, K.N., and Puhler, A. Elsevier/North-Holland Biomedical Press, Oxford.

Datta, D.B., Arden, B. & Henning, U. (1977) *Journal of Bacteriology*, **131**, 821 - 829.

Davies, R.L. (1991a) Outer membrane protein profiles of Yersinia ruckeri, *Journal of Veterinary Microbiology* , **26**, 125 - 140.

Davies, R.L. (1991b) Clonal analysis of *Yersinia ruckeri* based on biotypes, serotypes and outer membrane *protein-types. Journal of Fish Diseases*, **14**, 221 - 228

Davies RM, Smith RG & Porter, SR. (1985) Destructive forms of periodontal disease in adolescents and young adults. *British Dental Journal*, **158** : 429 - 436.

DeVoe, I.W. & Gilchrist, J.E. (1973) Release of endotoxin in the form of cell wall blebs during *in vitro* growth of *Neisseria meningitidis*. *Journal of Experimental Medicine*, **138** : 1156- 1167.

DeVoe, I.M., Tsai, C-C., Shenker, B.J., Taichman, N.T. & Lally, E.T. (1985) Monoclonal antibodies to *Actinobacillus actinomycetemcomitans*. *Infection and Immunity*, **47**, 31 - 36.

Dice, L. (1945) Measures of the amount of ecological association between species. *Ecology*, **26**, 297 - 302.

DiRienzo, J.M., Nakamura, K. & Inouye, M. (1978) The outer membrane proteins of Gram-negative bacteria : biosynthesis, assembly and functions. *Annual Review of Biochemistry*, **47**, 481 - 532.

DiRienzo, J.M., Tsai, C-C., Shenker, B.J., Taichman, N.S. & Lally, E.T. (1985) Monoclonal andtibodies to leukotoxin of *Actinobacillus actinomycetemcomitans*. *Infection and Immunity*, **47**, 31 - 36.

DiRienzo, J.M. & Slots, J. (1990) Genetic approach to the study of epidemiology and pathogenesis of *Actinobacillus actinomycetemcomitans* in localized *juvenile periodontitis*. *Archives of Oral Biology*, 35, suppl. 79S - 84S.

Dorward, D.W., Garon, C.F. & Judd, R.C. (1989) Export and intercellular transfer of DNA via membrane blebs of *Neisseria gonorrhoeae*. *Journal of Bacteriology*, **171**, 2499 - 2505.

Durum, S.K., Schmidt, J.A. & Oppenheim, J.J. (1985) Interleukin I: an immunological perspective. *Annual* Review of *Immunology*, **3** : 263 - 287.

Eastcott, J.W., Taubman, M.A., Smith, D.J. & Holt, S.C. (1990) *Actinobacillus actinomycetemcomitans* mitogenicity for B cells can be attributed to lipopolysaccharide. *Oral Microbiology and Immunology*, **5**, 8 - 10.

Ebersole, J.L., Taubman, M.A., Smith, D.J., Genco, R.J. & Frey, D.E. (1982) Human immune responses to oral microorganisms. I. Association of localised juvenile periodontitis (LJP) with serum antibody response to *Actinobacillus actinomycetemcomitans*. Clinical Experimental Immunology, **47**, 43 - 52.

Ebersole, J.L., Kraig, E., Bauman, G,. Spitznagel, J.k & Kolodrubetz, D. (1990) Molecular approaches to leucotoxin as a virulence component in *Actinobacillus actinomycetemcomitans*. III. DNA cloning and sequencing studies of *Actinobacillus actinomycetemcomitans* and *Bacteroides*. *Archives of Oral Biology*, **35**, 69S - 78S.

Ehrmann, I.E., Gray, M.C., Gordon, V.M., Gray, L.S. & Hewlett. E.L. (1991) Hemolytic activity of adenylate cyclase toxin from *Bordetella pertussis*. *FEMS Letters*, **273**, 79 - 83.

Eisenmann, A.A.A., Eisenmann, R., Sousa, O. & Slots, J. (1983). Microbiological study of localised juvenile periodontitis in Panama. *Journal of Periodontology*, **54,** 712 - 713.

Felmlee, R.A., Pellett, S. & Welch, R.A. (1985) Nucleotide sequence of an *Escherichia coli* chromosomal hemolysin. *Journal of Bacteriology*, **163**, 94 - 105.

Felmlee T. & Welch, R.A. (1988) Alterations of the amino acid repeats in *Escherichia coli* -haemolysin affect cytolytic activity and secretion. *Proceedings of the National Academy of Sciences USA*, **85**, 5269 - 5273.

Filip, C., Fletcher, G., Wulff, J.L. & Earhart, C.F. (1973) Solubilisation of the cytoplasmic membrane of *Escherichia coli* by the ionic deterfent sodium-laurly sarcosinate. *Journal of Bacteriology*, **115**, 717 - 723.

Forestier, C. & Welch, R.A (1991) Identification of RTX toxin target cell specificity domains by the use of hybrid genes. *Infection and Immunity*, **59**, 4212 - 4220.

Fourel, J. (1974) Periodontosis, juvenile periodontitis or Gottlied syndrome? Report of 4 cases. *Journal of Periodontology*, **45**, 234 - 237.

Frey, J. & Nicolet, J. (1988) Purification and partial characterisation of a hemolysin produced by *Actinobacillus pleuropneumoniae* typr strain 4074. *FEMS Microbiology Letters*, **55**, 41 - 46.

Friedman, R.L., Fiederlein, R.L., Glasser, L. & Galgiani, J.N. (1987) *Bordetella pertussis* adenylate cyclase: Effect of affinity-purified adenylate cyclase on human polymorphonuclear leukocyte function. *Infection and Immunity*, **55**, 135 - 140.

Gallagher, R., Collins, S., Trujillo, J., McCredie, K., Ahearn, M., Tsai, S., Metzgar, R., Aulakh, G., Ting, R., Ruscetti, F. & Gallo, R. (1979) Characterisation of the continuous, differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia. *Blood*, **54**, 713 - 733.

Gaston, P.R., & Hunter, M.A. (1988) Numerical index of the discriminatory ability of typing systems : an application of Simpson's index of diversity. *Journal of Microbiology*, **26**, 2465 - 2466.

Genco, R.J. (1987) Tissue localisation of *Actinobacillus actinomycetemcomitans* in human periodontitis. II : Correlation between immunofluorescence and culture techniques. *Journal of Periodontology*, **58**, 540 - 545.

Genco R.J. & Loos, B.G. (1991) The use of genomic DNA fingerprinting in studies of the epidemiology of bacteria in periodontitis. *Journal of Clinical Periodontology*, **18** : 396 - 405.

Gillett, R. & Johnson, N.W. (1982) Bacterial invasion of the periodontium in a case of juvenile periodontitis. *Journal of Clinical Periodontology*, **9**, 93 - 100.

Glasser, P., Sakamoto, H,. Bellalou, J., Ullmann, A. & Danchin, A. (1988) Secretion of cyclolysin, the calmodulin sensitive adenylate cyclase-haemolysin bifunctional protein of *Bordetella pertussis*. *EMBO Journal*, **7**, 3997 - 4004.

Griffen, A.L., Leys, E.J. & Fuerst, P.A. (1992) Strain identification of *Actinobacillus actinomycetemcomitans* using the polymerase chain reaction. *Oral Microbiology and Immunology*, **7**, 240 - 243.

Gunsolley, J.C., Tew, J.G, Gooss, C.M., Burmeister, J.A. & Schenkein HA (1988) Effects of race and periodontal status on antibody reactive with *Actinobacillus actinomycetemcomitans* strain Y4 *Journal of Periodontal Research*, **23**, 303 - 307.

Gyri, D., Nicolet, J., Frey, J., Cross, M., Koronakis, V. & Hughes, C. (1990) Isolation of the *Actinobacillus pleuropneumoniae* haemolysin gene and the activation and secretion of the prohaemolysin by the HlyC, HlyB and HLyD proteins of *Escherichia coli*. *Molecular Microbiology*, **4**, 123 - 128.

Hammond, B.F. & Steven, R.H. (1982) *Capnocytophaga* and *Actinobacillus actinomycetemcomitans*: occurrence and pathogenic potential in juvenile periodontitis, p 46 - 61. In : R. J. Genco and S. E. Mergenhagen (ed.) Host-parasite interaction in periodontal diseases, American Society for Microbiology, Washington, D.C.

Hammond, B.F., Stevens, R.H., Bonner, P. & Lillard, S.E. (1984) Toxicity of *Actinobacillus actinomycetemcomitans* (Aa) extracts for crevicular bacteria. *Journal of Dental Research*, **63** (abst.)

Hammond, B.F., Lillard, S.E. & Stevens, R.H., (1987) A bacteriocin of *Actinobacillus actinomycetemcomitans*. *Infection and Immunity*, **55**, 686 - 691.

Han, N., Hoover, C.I., Winkler, J.R., Ng, C. Y. & Armitage, G.C. (1991) Identification of Genomic Clonal types of *Actinobacillus actinomycetemcomitans* by restriction endonuclease analysis. *Journal of Clinical Microbiology*, **29**, 1574 - 1578.

Hardie, K.R., Issartel, J.-P., Koronakis, E., Hughes,C. & Koronakis, V. (1991) *In vitro* activation of *Escherichia coli* prohaemolysin to the mature membrane-targeted toxin requires HlyC and a low molecular weight cytosolic poplypeptide. *Molecular Microbiology*, **5**, 1669 - 1679.

Hart, T.C., Maeazita, M.L., Schenkein, H.A. & Diehl, S.R. (1992) Re-interpretation of the evidence for X-linked dominant inheritance of juvenile periodontitis. *Journal of Periodontology*, **63**, 169 - 173.

Hart, T.C., Marazita, M.L., Gunsolley, J.A., Schenkein, H.A. & Diehl, S.R. (1991) No female preponderance in juvenile periodontitis after correction for ascertainment bias. *Journal of Periodontology*, **62**, 745 - 750.

Haubek, D., Poulen, K., Asikainen, S. & Killian, M. (1995) Evidence for Absence in Northern Europe of Especially Virulent Clonal Types of *Actinobacillus actinomycetemcomtians*, **33**, 395 - 401.

Heinrich, S. & Pulverer, G. (1954) Zur Aetologir und Mikrobiologie der Actinoykose. II. Definition und praktische Diagnostik des Actinobacil *Bacillus actinomycetemcomitans*. *Zentralbl Bakteriol [A]*, **174**, 123 - 135.

Hess, J., Wels, W., Voel, M. & Goebel, W. (1986) Nueclotide sequence of a plasmid-encoded hemolysin determinant and its comparison with a corresponding chromosomal hemolysin sequence. *FEMS Microbiological Letters*, **34**, 1 - 11.

Hewlett, E.L., Gordon, V.M., MCCaffery, J.D., Sutherland, W.M. & Gray, M.C. (1989) Adenylate cyclase toxin from *Bordetella pertussis*. *The Journal of Biological Chemistry*, **264**, 19379 - 19384

Highlander, S.K., Engler, M.J. & Weinstock, G.M. (1990) Secretion and expression of the *Pasteurella haemolytica* leukotoxin. *Journal of Bacteriology*, **172**, 2343 - 2350.

Hill, H.R., Gerrard, J.M., Hogan, N.A. & Quie, P.G. (1974) Hyperactivity of neutrophil leukotactic responses during bacterial infection. *Journal of Clinical Investigation*, **53**, 996 - 1002.

Hillman, J.D. & Socransky, S.S. (1982) Bacterial interference in the oral ecology of *Actinobacillus actinomycetemcomitans* and its relationship to human periodontosis. *Archives of Oral Biology*, **27**, 75 -77.

Hillman, J.D., Socransky, S.S. & Shivers, M. (1985) The relationship between strepopcoccal species and periodontopathic bacteria in human dental plaque. *Archives in Oral Biology*, **30**, 791 - 795.

Hillman, J.D., Shivers. M. (1988) Interaction between wild-type, mutant and revertant forms of the bacterium *Streptococcus sanguis* and the bacterium *Actinobacillus actinomycetemcomitans in vitro* and in the gnotobiotic rat. *Archives in Oral Biology*, **33**, 395 - 401.

Hiromasa, Y., Taubman, M.A., Olson, C.L., Ebersole, J.L. & Smith, D.J. (1987) Periodontal bone loss and immune characteristics after adaptive transfer of Actinobacillus-sensitive T cells to rats. *Journal of Periodontal Research*, **22**, 499 - 505.

Hirst, T.R. & Welch, R.A. (1988) Mechanisms of secretion of extracellular proteins by Gram-negative bacteria. *Trends in Biochemical Science*, **13**, 265 - 269.

Holland, I.B., Blight, M.A. & Kenny, B. (1990) The mechanism of secretion of hemolysin and other poplypeptides from Gram-negative bacteria. *Journal of Bioenergenics and Biomembranes*, **22**, 473 - 491

Holm, A., Kalfas, S. & Holm, S.E. (1989) *In vivo* chemotaxis evoked by *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*. *Oral Microbiology and Immunology*, **4**, 30 - 34.

Holmberg, S.D., Wachsmuth, I.K., Hickman-Brenner, F.W. & Cohen, M.L. (1984) Comparison of plasmid profile analysis, phage typing and antimicrobial susceptibility testing in characterizing *Salmonella typhimurium* isolates from outbreaks. *Journal of Clinical Microbiology*, **19**, 100 - 104.

Holt, S.C., Tanner, A.C.R. & Socransky S.S. (1980) Morphology and ultrastructure of oral strains of *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*. *Infection and Immunity*, **30** : 588 - 600.

Horton, J.E., Oppenheim, J.J. & Mergenhagen, S.E. (1974) The role for cell-mediated immunity in the pathogenesis of periodontal disease. *Journal of Periodontology*, **45** : 351 - 360.

Hovi, M., Sukupolvi, S., Edwards M.F., & Rhen, M. (1988) Plasmid-associated virulence of *Salmonella enteritidis*. *Microbial Pathogenesis*, **4**, 385 - 391.

Hugoson, A. (1970) Ginigival inflammation and female sex hormones. *Journal of Periodontal Research*, (Suppl.5)

Iino, Y & Hopps, P.M. (1984) The bone-resorbing activities in tissue culture of lipopolysaccharides from the bacteria *Actinobacillus actinomycetemcomitans*, *Bacteriodes gingivalis* and *Capnocytophaga achracea* isolated from human mouths. *Archives in Oral Biology*, **29**, 59 - 63.

Inouye, T., Ohta, H., Kokeguchi, S., Fukui, K. & Kato, K. (1990) Colonial variation and fimbriation of *Actinobacillus actinomycetemcomitans*. *FEMS Microbiology Letters*. **69** : 13 - 18.

Issartel, J.-P., Koronakis, V & Hughes, C. (1991) Activation of *Escherichia coli* prohaemolysin to the mature toxin by acyl carrier protein-dependent fatty acylation. *Nature*, **351**, 759 - 761.

Iwase, M., Lally, E.T., Berthold, P., Korchak, H.M. & Norton, N.S. (1990) Effects of cations and osmotic protectants on cytolytic activity of *Actinobacillus actinomycetemcomitans* leucotoxin. *Infection and Immunity*, **58**, 1782 - 1788

Jantzen, E. Berdal, B.P. & Omland, T. (1980) Cellular fatty acid composition of *Haemophilus* species, Pasturella multocida, *Actinobacillus actinomycetemcomitans* and *Haemophilus vaginalis (Corynebacterium vaginale) Acta pathology Microbiology Scandanavia. Section B*. **88**, 89 - 93.

Johnson, N.W., Grittiths, G.S., Wilton, J.M.A., Maiden, M.F.J., Curtis, M.A., Gillet, I.R., Wilson, D.T. & Sterne, J.A.C. (1988) Detection of high-risk groups and individuals for periodontal diseases. Evidence for the existence of high-risk groups and individuals and approaches to their detection. *Journal of Clinical Periodontology*, **15**, 276 - 282.

Jones, D. & Kreig, N.R. (1984) Bacterial classification V. Serology and chemotaxonomy. In: Kreig N.R. & Holt, J.G. eds. Bergey's manual of systematic bacteriology. Vol I. Baltimore: Williams & Wilkins, 1984 : 15 - 18.

Kagermeier, A.S., London, J. (1985) *Actinobacillus actinomycetemcomitans* strains Y4 and N27 adhere to hydroxyapatite by distinctive mechanisms. *Infection and Immunity*, **47**, 645 - 658.

Kahn, M.E., Barany, F. & Smith, H.O. (1983) Transformasomes: specialized membranous structures that protece DNA during *Haemophilus* transformation. *Proceedings of the National Academy of Sciences USA*, **80**, 6927 - 6931.

Kamin, S., Harvey, W., Wilson, M. & Scutt, A. (1986) Inhibition of fibroblast proliferation and collagen synthesis by capsular material from *Actinobacillus actinomycetemcomitans*. *Journal of Medical Microbiology*, **22**, 245 - 249.

Kamin, S., Wilson, M. & Harvey, W. (1988) The role bacterial surface components in destruction. *Dental Journal of Malaysia*, **10**, 21 - 25.

Kaplan, H.A., Weber, D.J., Oddone, E.Z. & Perfect, (1989) Infection due to *Actinobacillus actinomycetemcomitans*: 15 cases and review. *Reviews of Infectious Diseases*, **2**, 46 - 63.

Keane, W.F., Welch, R., Gekker, G. & Peterson, P.K. (1987) Mechanism of *Escherichia coli* alpha-hemolysin-induced injury to isolated renal tubular cells. *American Journal of Pathology*, **126**, 350 - 357.

Kiley, P. & Holt, S.C. (1980) Characterisation of the lipopolysaccharide from *Actinobacillus actinomycetemcomitans* Y4 and N27. *Infection and Immunity*, **30**, 862 - 873.

Kilian, M., Frederiksen, W. & Biberstein, E.L. (1981) Preface *In*: *Haemophilus, Pasteurella and Actinobacillus*. Eds. Kilian, M., Frederiksen, W. & Biberstein, E.L. Academic Press, London.

Kilian, M. & Reinholdt, J. (1986) Interference with IgA defence mechanisms by extracellular bacterial enzymes. p 173 - 208. In : Medical Microbiology Volume 5. Eds Eason, C.S.F. & Jeljaszewicz, J. Academic Press, London.

Kinane, D.F., Cullen, C.F., Johnson, F.A. & Evans, C.W. (1988a) Neutrophil chemotactic behaviour in patients with early-onset forms of periodontitis (I). Leading front analysis in boyden chambers. *Journal of Clinical Periodontology*, **16**, 242 -246.

Kinane, D.F., Cullen, C.F., Johnson, F.A. & Evans, C.W. (1988b) Neutrophil chemotactic behaviour in patients with early-onset forms of periodontitis (II). Assessment using the under agarose technique. *Journal of Clinical Periodontology*, **16**, 247 - 251.

King, E.O. & Tatum, H.W. (1962) *Actinobacillus Actinomycetemcomitans* and *Haemophilus aphrophilus*, *Journal of Infectious Diseases*, **111**, 85 - 94.

Kolodrubetz, D., Dailey, T., Ebersole, J. & King, E. (1989) Cloning and expression of the leukotoxin gene from *Actinobacillus actinomycetemcomitans*. nfection and Immunity, **57**, 1465 - 1469.

Kornman, K.S. & Robertson, P.B. (1985) Clinical and microbiological evaluation of therapy for juvenile periodontitis. *Journal of Periodontology*. **56** : 443 - 446.

Kraft, C.A., Timbury, M.C. & Platt, D.J. (1987) Restriction enzyme fingerprinting of trimethoprim resistance plasmids. *Epiemiology and Infection*, **98**, 241 - 252.

Kraig, E., Dailey, T. & Kolodrubetz, D. (1990) Nucleotide sequence of the leukotoxin gene from *Actinobacillus actinomycetemcomitans*: Homology to the alpha-haemolysin/leukotoxin gene family. *Infection and Immunity*. **58** : 920 - 929.

Laemmli, U.K. (1970) Cleavage if the structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)*, **227**, 680 - 685.

Lai, C-H., Listgarten, M.A & Hammond, B.J. (1981) Comparative ultrastructure of leukotoxic and non-leukotoxic strains of *Actinobacillus actinomycetemcomitans*. *Journal of Periodontal Research*. **16** : 379 - 389.

Lally, E.T., Kieba, I.R., Demuth, D.R., Rosenbloom, J., Golub, E.E., Taichman, N.S. & Gibson, C.W. (1989) Identification and expression of the *Actinobacillus actinomycetemcomitans* leukotoxin gen. *Biochemical and Biophysical Research Communication*, **159**, 256 - 262.

Lally, E.T., Kieba, I.R., Taichman, N.S., Rosenbloom, J., Gibson, C.W., Demuth, D.R., Harrison, G. & Golub, E.E. (1991a) *Actinobacillus actinomycetemcomitans* leukotoxin is a calcium-binding protein. *Journal of Periodontal Research*, **26**, 268 - 271.

Lally, E.T., Golub, E.E., Kieba, I.R., Taichman, N.S., Decker, S., Berthold, P., Gibson, C.W., Demuth, D.R. & Rosenbloom, J. (1991b) Structure and function of the B and D genes of the *Actinobacillus actinomycetemcomitans* leukotoxin complex. *Microbial Pathogenesis*, **11**, 111 - 121.

Lalonde, G., McDonald, T.V., Gardner, P. & O'Hanley, P.D. (1989) Identification of a hemolysin from *Actinobacillus pleuropneumoniae* and characterisation of its channel properties in planar phospholipid bilayers. *Journal of Biological Chemistry*, **264,** 13559 -13564.

Lawson, D.A., Haas, R., Meyle, J. & Meyer, T.F. (1990) Molecular analysis of periodontal pathogens. *Archives in Oral Biology*, **35** Supplement, 101S - 105S.

Lavine, W.S., Maderazo, E.G., Stolman, J, Ward, P.A., Cogan, R.B., Greenblatt, I. & Robertson, P.B. (1979) Impaired neutrophil chemotaxis in patients with juvenile and rapidly progressing periodontitis. *Journal of Periodontal Research*. **14**, 10 - 19.

LeBlanc, D.J., Lee, L.N., Abu-Al-Jaibat, A.R., Sreenivasan, P.K. Fives-Taylor, P.M. (1993) Identification of plasmids in *Actinobacillus actinomycetemcomitans* and construction of intergeneric shuttle plasmids. *Oral Microbiology and Immunology*. **8**, 94 - 99.

Lennon, M.A. & Davies, R.M. (1974) Prevalence and distribution of alveolar bone loss in a population of 15-year-old school children. *Journal of Clinical Periodontology*, **1**, 175 - 182.

Lenski, R.E. (1993) Assessing the genetic structure of microbial populations. *Proceedings of the National Academy of Sciences, USA*, **90**, 4334 - 4336.

Levin, B.R. (1981) Periodic selection, infectious gene exchange and the genetic structure of *E.coli* populations. *Genetics*, **99**, 1 - 23.

Lewin, B. (1985) The replicon: Unit of replication. In : *Genes II.* pp 513 - 531. (Ed. Lewin, B.) John Wiley and Sons, Chichester.

Liljenberg, B & Lindhe, J. (1980) Juvenile periodontitis. Some microbiological, histopathological and clinical *characteristics. Journal of Clinical Periodontology*, 7, 48 - 61.

Ludwig, A., Jarchau, T., Benz, R. & Goeble, W. (1988). The repeat domain of

Escherichia coli haemolysin (HlyA) is responsible for its Ca^{2+}-dependent binding to erythrocytes. *Molecular General Genetics*, **214**, 553 -561.

McArthur, W.P., Tsai, C-C., Baehni. P.C., Genco, R.J. & Taichman, N.S. (1981) Leukotoxic effects of *Actinobacillus actinomycetemcomitans.* Modulation by serum components. *Journal of Periodontal Research.* **16**, 159 - 170.

McArthur, W.P., Stroup, S. & Wilson, L. (1986) Detection and serotyping of *Actinobacillus actinomycetemcomitans* isolates on nitrocellulose paper blots with monoclonal antibodies. *Journal of Clinical Periodontology*, **13** , 684 -

McGee, Z.A., Street, C.H., Chappell, C.L., Cousar, E.S., Morris, F. & Horn RG. (1979) Pili of *Neisseria meningitidis* : Effects of media on maintenance of pilation, characterisation of pili and colonial morphology. *Infection and Immunity.* **24** : 194 - 201.

Macmann, N., Nicaud, J.-M., Gray, L. & Holland, I.B. (1986) Secretion of haemolysin by *Escherechia coli. Current Topics in Microbiology and Immunology*, **125**, 159 - 181.

Maiden, M.F.J., Carman, R.J., Curtis, M.A., Gillett, R.J., Griffiths, G.S., Sterne, J.A.C., Wilton, J.M.A. & Johnson, N.W. (1990) Detection of high-risk groups and individuals for periodontal disease: laboratory markers based on the microbiological analysis of subgingival plaque. *Journal of Clinical Periodontology.* **17** : 1 - 13.

Mandell, R.F. & Socransky, S.S. (1981) A selective medium for *Actinobacillus actinomycetemcomitans* and the incidence of the organism in juvenile periodontitis. *Journal of Periodontology,* **52**, 593 - 597.

Mangan, D.F., Taichman, N.S,. Lally, E.T. & Wahl, S. (1991) Lethal effects of *Actinobacillus actinomycetemcomitans* leukotoxin on human T lymphocytes. *Infection and Immunity*, **59**, 3267 - 3272.

Maniatis, T., Fritsch, E.F. & Sambrook, J. (1982) Molecular Cloning. A Laboratory Manual. Published Cold Spring Harbor Laboratory. Various chapters.

Manson, J.D. & Lehner, T. (1974) Clinical features of Juvenile periodontitis (periodontosis). *Journal of Periodontology,* **17**, 204 - 267.

Markwell, M.A.K., Haas, S.M., Bieber, L.L. & Tolbert, N.E. (1987) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Analytical Biochemistry*, **87**, 206

Martin, B.F., Derby, B.M., Budzilovich, G.N. & Ransohoff, J. (1967) Brain abscesses due to *Actinobacillus actinomycetemcomitans. Neurology*, **17**, 833 - 837.

Mashimo, P.A., Yamamoto, Y., Slots, J., Park, B.H. & Genco, R.J. (1983) The periodontal microflora of juvenile diabetics. *Journal of Periodontology.* **54** : 420 - 430.

Melnick, M., Shields, E.D. & Bixter, D. (1975) Periodontosis : a phenotypic and genetic analysis. *Oral Surgery*, **40**, 115 - 118.

Meyer, D.H., Sreenvasan, P.K. & Fives-Taylor, P.M. (1991) Evidence for invasion of a human oral cell line by *Actinobacillus actinomycetemcomitans. Infection and Immunity,* **59**, 2719 - 2726.

Meyers, B.R., Buttone, E., Hirschman, S.Z., Schneierson, S.S. & Gershengorn, K. (1971) Infection due to *Actinobacillus actinomycetemcomitans. American Journal of Clinical Pathology*, **56**, 206 - 211.

Miller, R.D. & Hartl, D.L. (1986) Biotyping confirms a nearly clonal population structure in *Escherichia coli. Evolution (Lawrence, Kans.)*, **40**, 1 - 12.

Miyasaki, K.T., Wilson M.E., Brunetti, A.J. & Genco, R.J. (1986) Oxidative and nonoxidative killing of *Actinobacillus actinomycetemcomitans* by human *neutrophils. Infection and Immunity*, **53**, 154 - 160.

Miyaski, K.T., Bodeau, A.L. & Fleming, T.F. (1991) Differential killings of *Actinobacillus actinomycetemcomitans* and *Capnocytophaga* spp by human neutrophil granule components. *Infection and Immunity*. **59**, 3760 - 3767.

Miyasaki, K.T. & Bodeau, A.L. (1991) *In vitro* killing of *Actinobacillus actinomycetemcomitans* and *Capnocytophaga* spp. by human neutrophil cathepsin G and Elastase. *Infection and Immunity*, **59**, 3015 - 3020.

Moore, W.E.C., Holdman, L.V. & Cato, E.P.(1980) Polyacrylamide slab gel electrophoresis of soluble proteins for studies of bacterial flora. *Applied Environmental Microbiology,* **39**, 900 - 907.

Moore, W.E.C., Holdeman, L.V., Smibert, R.M., Hash, D.E., Burmeister, J.A. & Ranney, R.R. (1982) Bacteriology of severe periodontitis in young adult humans. *Infection and Immunity*. **38** : 1137 - 1148.

Moore,W.E.C., Hildeman, L.V., Cato, E.P., Smibert, R.M., Burmeister, J.A. & Ranney, R.R. (1983) Bacteriology of moderate (chronic) periodontitis in mature adult humans. *Infection and Immunity*, **42**, 510 - 515.

Moore, W.E.C., Holdeman, L.V., Cato, E.P., Burmeister, J.A., Palcanis, K.G. & Ranney, R.R. (1985) Comparative bacteriology of juvenile periodontitis. *Infection and Immunity*, **48**, 507 - 519.

Moore, W.E.C (1987) Microbiology of periodontal disease. *Journal of Periodontal Research*, **22**, 336 - 341.

Muller, H-P, Muller, R.F. & Lange, D.E. (1989) *Actinobacillus actinomycetemcomtians* contamination of toothbrushes from patients harbouring the organism. *Journal of Clinical Periodontology*, **16**, 388 - 390.

Muller, H-P, Muller, R.F. & Lange, D.E. (1990) Morphological compositions of subgingival microbiota *in Actinobacillus actinomycetemcomitans-associated* periodontitis. *Journal of Clinical Periodontology*. **17** : 549 - 556.

Naiming, H., Xiaorong, X., Liangsheng, Z., Xinquan, Ri., Juzhi, Z., Yuehua,T., Meiru, Y. & Zhuoran, X. (1991) Bacteriological study of juvenile periodontitis in China. *Journal of Periodontal Research*, **26**, 409 - 414.

Nishihara, T., Maki, E., Ishihara, Y., Kato, H., Noguchi, T. & Koga, T. (1989) Inhibitory effects of rabbit antisera on mitogenic activity of *Actinobacillus actinomycetemcomitans* lipopolysaccharide. *Journal of Periodontal Research*. **24**, 165 - 170.

Ngan, P.W.H., Tsai, C-C. & Sweeney, E. (1985) Advanced periodontitis in the primary dentition : case report. *Pediatric Dentistry*, 7, 255 - 258.

Nicaud, J. -M., Mackman, N., Gray, L. & Holland, I.B. (1985) Characterisation of the HlyC and mechanism of activation and secretion of haemolysin from *E.coli* 2001. *FEMS Letters*, **187**, 339 - 344.

Okuda, K., Naito, Y., Ohta, K., Fukumoto, Y., Kimura, Y., Ishikawa, I., Kinoshita, S. & Takazoe, I. (1984) Bacteriological study of periodontal lesions in two sisters with juvenile periodontitis and their mother. *Infection and Immunity*, **45**, 118 - 121.

Ohta, H., Kato, K., Kokeguchi, S., Hara, H., Fukui, K. & Murayama, Y. (1991) Nuclease-sensitive binding of an *Actinobacillus actinomycetemcomitans* leukotoxin to the bacterial cell surface. *Infection and Immunity*, **59**, 4599 - 4605.

Olsvik, B. & Preus H.R. (1989) Plasmid in *Actinobacillus actinomycetemcomitans* strains isolated from periodontal lesions of patients with rapidly destructive periodontitis. *Oral Microbiology and Immunology*, **4**, 219 - 221

Oropeza-Wekerle, R.L., Muller, E., Kern, P., Meyermann, R. & Goebel, W. (1989) Synthesis, inactivation and localisation of extracellular and intracellur *Escherichia coli* hemolysins. *Journal of Bacteriology*, **171**, 2783 - 2788.

Orskov, F. & Orskov, I. (1983) Summary of a workshop on the clonal concept in epidemiology, Taxonomy, and evolution of the Enterobacteriaceae and other bacteria. *The Journal of Infectious Diseases*, **148** (2), 346 - 357.

Owen, R.J. (1989) Chromosomal DNA fingerprinting - a new method of species and strain identification applicable to microbial pathogens. *Journal of Medical Microbiology*, **30**, 89 -99.

Page, M.I. & King, E.O. (1966) Infection due to *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*. *The New England Journal Of Medicine*, **275**, 181 - 188.

Page, R.C. & Schoreder, H.F. (1973) Biochemical aspects of the connective tissue alterations in inflammatory gingival and periodontal disease. *International Dental Journal*. **23**, 455 - 469.

Page, R.C. (1983) Prepubertal periodontitis. I. Definition of a clinical disease entity. *Journal of Periodontology*, **54**, 257 -

Page, R.C., Altman, L.C., Ebersole, J.L., Vendesteen, G.E., Dahlberg, W.W., Williams, B.L. & Osterberg, S.K. (1983) Rapidly progressive periodontitis. A distinct clinical condition. *Journal of Periodontology*, **54**, 197 - 209.

Page, R.C., Vandesteen, G.E., Ebersole, J.L., Williams, B.L., Dixon, I.J. & Altman, L.C. (1985). Clinical and laboratory studies of a family with a prevalence of juvenile periodontitis. *Journal of Periodontology*, **56**, 602 - 610.

Page, R.C. (1986) Current understanding of the aetiology and progression of periodontal disease. *International Dental Journal*, **36**, 153 - 161.

Parton, R. (1989) *Bordetella pertussis* toxins and the human host. *Current Options in Infectious Diseases*, **2**, 788 - 795.

Pellet, S., Boehm, D.F., Snyder, I.S., Rowe, G. & Welch, R.A. (1990) Characterisation of monoclonal antibodies against the *Escherichia coli* hemolysin. *Infection and Immunity*, **58**, 822 - 827.

Pennel, B.M. & Kealgle, J.G. (1977) Predisposing factors in the etiology of chronic inflammatory periodontitis. *Journal of Periodontology*, **48**, 517

Pettit, M.D.A., Van Steenbergen, T.J.M., Scholte, L.M.H., Van der Velden, U. & De Graff., J. (1993). Epidemiology and transmission of *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* among children and their family members. A report of 4 surveys. *Journal of Clinical Periodontology*, **20**, 641 - 650.

Place, D.A., Scidmore, N.C. & McArthur, W.P. (1988) Monoclonal antibodies to *Actinobacillus actinomycetemcomitans*. *Infection and Immunity*, **56**, 1394 - 1398.

Plikaytis, B.D., Carlone, G.M., Edmonds, P. & Mayer, L.W. (1986) Robust estimation of standard curves from protein molecular weight and linear-duplex DNA base-pair number after gel electrophoresis. *Analytical Biochemistry*, **152**, 346 - 364.

Platt, D.J., Chesham, J.S., Brown, D.J., Kraft, C.A. & Taggart, J. (1986) Restriction enzyme fingerprinting of enterobacterial plasmids: a simple strategy with wide application. *Journal of Hygiene*, **97**, 205 - 210.

Platt, D.J., Heraghty, K.A. & Taggart J. (1988) Molecular divergence of the serotype-specific plasmid (pSLT) among strains of *Salmonella typhimurium* of human and veterinary origin and comparison of pSLT with the serotype specific plasmids of *S.enteritidis* and *S.dublin*. *Journal of Medical Microbiology*, **27**, 277 - 284.

Platt, D.J. & Sullivan, L. (1990) Molecular epidemiology: molmatch and beyond. *International Labmate*, **16**, 11 - 13.

Platt, D.J. & Smith, I. (1991) Gentamicin-resistant *Salmonella typhimurium* phage type 204c: molecular studies and strain diversity in a putative bovine outbreak. *Epidemiology and Infection*, **107**, 213 - 223.

Platt D.J, Browning, L & Candlish, D. (1996) Molecular anaylsis of *Salmonella enteredius* serotype Dublin : building bridges between population genetics and epidemiological studies. *Electrophoresis* : *In Press*

Potts, T.V. & Berry, E.M. (1983) Deoxyribonucleic acid to deoxyribonucleic acid hybridisation analysis of *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*. *International Journal of Systematic Bacteriology*, **33**. 765 - 771.

Potts, T.V., Zambon, J.J. & Genco, R.J. (1985) Reassignment of *Actinobacillus actinomycetemcomitans* to the genus *Haemophilus* as *Haemophilus actinomycetemcomitans* comb. nov. *Journal of General Microbiology*, **35**, 337 -341.

Potts, T.V., Mitra, T., O'Keefe, T., Zambon, J.J. & Genco, R.J. (1986). Relationships among isolates of oral haemophili as determined by DNA-DNA hybridisation. *Archives in Microbiology*, **145**, 136 - 141.

Poulsen, K,. Theilade, E., Lally, E.T., Demuth D.R. & Kilian, M. (1994) Population structure of *Actinobacillus actinomycetemcomitans* : a framework for studies of disease-associated properties. *Microbiology*, **140**, 2049 - 2060.

Preus, H. & Gjermo, P. (1987) Clinical management of prepubertal periodontitis in 2 siblings with Papillon-Lefevre Syndrome. *Journal of Clinical Periodontology*, **14**, 156 - 160.

Preus, H.R., Olsen, I. & Narmork, E. (1987) Association between bacteriophage-infected *Actinobacillus actinomycetemcomitans* and rapid periodontal destruction. *Journal of Clinical Periodontology*, **14** : 245 - 247.

Preus, H.R., Narmork, E. & Olsen, I. (1988) Fimbriation of *Actinobacillus actinomycetemcomitans*. *Oral Microbiology and Immunology*, **3** : 93 - 94.

Preus, H.R. & Olsen, I. (1989) Possible transmission of *A.actinomycetemcomtians* from a dog to a child with rapidly destructive periodontitis. *Journal of Periodontal Research*, **23**, 68 - 71.

Pugsley, A.P. & Schwartz, M. (1985) Export and secretion of proteins by bacteria. *FEMS Microbiological Reviews*, **32**, 3 - 38.

Rabie, G., Lally, E.T. & Shenker, B.J. (1988) Immunosuppressive properties of *Actinobacillus actinomycetemcomitans* leukotoxin. *Infection and Immunity*, **56**, 122 - 127.

Rifkind, B.R. & Heijl, L. (1979) The occurrence of mononuclear cells at sites of osteoclastic bone resorption in experimental periodontitis. *Journal of Periodontitis*. **50**, 633 - 642.

Robertson, P.B. & Simpson, J. (1976) Collagenase : current concepts and relevance to periodontal disease. *Journal of Periodontology*. **47**, 29 - 33.

Robertson, P.B., Lantz., M., P.T,. Marucha, P.T., Kornman, K.S., Trummel, C.L. & Holt, S.C. (1982) Collagenolytic activity associated with *Bacteroides* species and *Actinobacillus actinomycetemcomitans*. *Journal of Periodontal Research*, **17**, 275 - 283.

Rodenburg, J.P., Van Winkelhoff, A.J., Goene, R.J., Abbas, F. & De Graaff, J. (1990) Occurrence of Bacteroides gingivalis, *Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* in severe periodontitis in relation to age and treatment history. *Journal of Clinical Periodontology*, **17**, 392 - 399.

Rosan, B., Slots, J., Lamont, R.J., Listgarten, M.A. & Nelson, G.M. (1988) *Actinobacillus actinomycetemcomitans* fimbriae. *Oral Microbiology and Immunology*, **3** : 58 - 63.

Saarla, M., Asikainen, S., Alaluusua, S., Pyhala, L., Lai, C-H. & Jousimies-Somer, H. (1992) Frequency and stability of mono- or poly-infection by *Actinbacillus actinomycetemcomitans* serotypes a, b, c, d or e. *Oral Microbiology and Immunology*, **7**, 277 - 279.

Saarela, M., von, Troil-Linden, B., Torkko, H., Stuki, A-M., Alaluusua, S., Jousimies-Somer, H & Asikainen S. (1993). Transmission of oral bacterial species between spouses. *Oral Microbiology and Immunology*, **8**, 349 - 354.

Saarela, M., Asikainen, S., Chen, C., Alaluusua, S & Slots, J. (1995) Comparison of Arbitraily Primed Polymerase Chain Reaction and Ribotyping for Subtyping *Actinobacillus actinomycetemcomitans. Anaerobe*, **1**, 97 - 102.

Saglie, F.R., Carranza, F.A. Jr. & Newman, M.G. Cheng, L. & Lewin, K.J. (1982) Identification of tissue invading bacteria in human periodontal disease. *Journal of Periodontal Research*, **17**, 452 - 455.

Saglie, F.R., Carranza, F.A. Jr. & Newman, M.G. (1985). The presence of bacteria in the oral epithelium in periodontal disease. I : Scanning and transmission electron microscopic study. *Journal of Periodontology*. **56**, 10 -19.

Saglie, F.R., Smith, C.T., Newman, M.G., Carranza, F.A. Jr., Pertuiset, J.H., Cheng, L., Auil, E. & Neisengard, R.J (1986) The presence of bacteria in the oral epithelium in periodontal disease. II: Immunohistochemical identification of bacteria. Journal of Periodontology, **57**, 492 - 500.

Saglie, F.R., Simon, K., Merrill, J. & Koeffler, H.P. (1990) Lipopolysaccharides from *Actinobacillus actinomycetemcomitans* stimulates macrophages to produce interleukin-1 and tumor necrosis factor mRNA and protein. *Oral Microbiology and Immunology*, **5**, 256 - 262.

Saunders, JR. (1986) Genetic basis of phase and antigenic variation in bacteria. *In "Antigenic Variation in Infectious Diseases"* (Eds. Birkbeck, TH and Penn, CW) pp 57-76. Society for General Microbiology/IRL Press, Oxford.

Saxby, M. (1984) Prevalence of juvenile periodontitis in a British school population. *Community Dental Oral Epidemiology*, **12**, 183 - 185.

Saxby, M.S. (1987) Juvenile periodontitis : An epidemiological study in the West Midlands of the United Kingdom. *Journal of Clinical Periodontology*, **14**, 594 - 598.

Saxen, L. (1980a) Juvenile periodontitis. *Journal of Clinical Periodontology*, **7**, 1 - 19.

Saxen, L. (1980b) Heredity of juvenile periodontitis. *Journal of Clinical Periodontology*, **7**, 276 - 288.

Scannapieco, F.A., Miller, S.J., Reynolds, H.S., Zambon, J.J. & Levine, M.J. (1987) Effect of anaerobiosis on the ultrastructure and surface proteins of *Actinobacillus actinomycetemcomitans* (*Haemophilus actinomycetemcomitans*). *Infection and Immunity*, **55** : 2320 -2323.

Schack, S.H., Smith, P.W., Penn, R.G. & Rapoport J.M. (1984). Endocarditis caused by *Actinobacillus actinomycetemcomitans*. *Journal of Clinical Microbiology*, **20**, 579 - 581.

Schulger (1990) In : Schulger, Yuodelis, Page & Johnson : *Periodontal Diseases. 2nd Edition.* Published by Lea & Febiger. London.

Selender, R.K. & Levin, B.R. (1980) Genetic diversity and structure in *Escherichia coli* populations. *Science*, **210**, 545 - 547.

Shenker, B.J,. Tsai, C.C. & Taichman, N.S. (1982a) Immune suppression induced by *Actinobacillus actinomycetemcomitans*. I. Effect on human peripheral blood lymphocyte responses to mitogens and antigens. *Journal of Immunology*, **128**, 148 - 154.

Shenker, B.J,. Tsai, C.C. & Taichman, N.S. (1982b). Suppression of lymphocyte responses by *Actinobacillus actinomycetemcomitans*. *Journal of Periodontal Research*. **17**, 462 - 465.

Shenker, B.J., Kusher, M.E. & Tsai, C.C. (1992) Inhibition of fibroblast proliferation by *Actinobacillus actinomycetemcomitans*. *Infection and Immunity*, **38**, 985 - 992.

Shurin S.B., Socransky, S.S., Sweeney, E. & Stossel, T.P. (1979) A neutrophil disorder induced by *Capnocytophaga*, a dental microrganism. *The New England Journal of Medicine,* **301**, 849 - 854.

Simpson, D.L., Berthold, P. & Taichman, N.S. (1988) Killing of human myelomonocytic leukemia and lymphocytic cell lines by *Actinobacillus actinomycetemcomitans* leukotoxin. *Infection and Immunity*, **56**, 1162 - 1166.

Slots, J., Reynolds, H.S. & Genco, R.J. (1980) *Actinobacillus actinomycetemcomitans* in human periodontal disease : a cross-sectional microbiological investigation. *Infection and Immunity*, **29**, 1013 - 1020.

Slots, J., Zambon, J.J., Rosling, B.G., Reynolds, A.S., Christersson, L.A. & Genco, R.J. (1982) *Actinobacillus actinomycetemcomitans* in human periodontal disease; Association, serology, leukotoxicity and treatment. *Journal of Periodontal Research*, **17**, 447 - 448.

Slots, J., Rosling, B.G. & Genco, R.J. (1983) Suppression of penicillin-resistant oral *Actinobacillus actinomycetemcomitans* with tetracycline: considerations in endocarditis prophylaxis. Journal *of Periodontology*, **54**, 193 - 196.

Slots, J. & Genco, R.J. (1984) Blackpigmented Bacteroides species, *Capnocytophaga* species and Actinobacillus actinomycetemcomitans in human periodontal disease : virulence factors in colonisation, survival and tissue destruction. Journal of Dental Research, **63**, 412 - 421.

Slots, J. (1986) Bacterial specificity in adult periodontitis. A summary of recent work. *Journal of Clinical Periodontology*, **13**, 912 - 917.

Slots, J. & Listgarten, M.A. (1988) *Bacteroides gingivalis*, *Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* in human periodontal disease. *Journal of Clinical Periodontitis*, **15**, 85 - 93.

Slots, J., Feik, D. & Rams, T.E. (1990) *Actinobacillus actinomycetemcomitans* and *Bacteroides intermedius* in human periodontitis. Age, relationship and mutual association. *Journal of Clinical periodontitis*, **17**, 659 - 662.

Slots, J. & Rams, T.E. (1990) Antibiotics in periodontal therapy : advantages and disadvantages. *Journal of Clinical Periodontology*, **17**, 479 - 493.

Smith, G.L.F., Sansone, C. & Socransky, S.S. (1989) Comparison of two methods for the small-scale extraction of DNA from subgingival microorganisms. *Oral Microbiology and Immunology*, **4**, 135 - 140.

Sneath, P.H.A. & Johnson, R. (1973) Numerical taxonomy of *Haemophils* and related bacteria. *International Journal of Systematic Bacteriology*, **23** 405 - 418.

Sneath, P.H.A. & Stevens, M. (1985) Numerical taxonomic study of *Actinobacillus*, *Pasturella* and *Yersinia*. Journal of General Microbiology, **131**, 2711 - 2738.

Sofaer, J.A. (1990) Genetic approaches in the study of periodontal diseases. *Journal of Clinical Periodontology*, **17**, 401 - 408.

Sonis, A.L. (1979) Periodontosis of the primary dentition : a case report. *Pediatric Dentistry*, **2**, 53 - 55.

Spektor, M.D., Vandesteen, G.E. & Page, R.C. (1984) Clinical studies of one family manifesting rapidly progressive, juvenile and prepubertal periodontitis. *Journal of Periodontology*, **56**. 93 - 101.

Spitznagel Jr, J., Kraig, E. & Kolodrubetz, D. (1991) Regulation of leukotoxin in leukotoxic and non-leukotoxic strains of *Actinobacillus actinomycetemcomitans. Infection and Immunity*, **59**, 1394 - 1401.

Stevens, R.H., Gatewood, C. & Hammond. B.F. (1983) Cytotoxicity of the bacterium *Actinobacillus actinomycetemcomitans* extracts in human gingival fibroblasts. *Archives of Oral Biology*, **28**, 981 - 987.

Stevens, R.H., Lillard, S.E. & Hammond, B.F. (1987) Purification and biochemical properties of a bacteriocin from *Actinobacillus actinomycetemcomitans*. *Infection and Immunity*, **55**, 92 - 697.

Strathdee, C.A. & Lo, R.Y.C. (1989) Cloning, nucleotide sequence and characterisation of genes encoding the secretion function of the *Pasteurella haemolytical* leukotoxin determinant. *Journal of Bacteriology*, **171**, 916 - 928

Suomalainen, K., Sorsa, T., Saxen, L., Vankonen, M. & Uitto, V-J. (1991) Collagenase activity in gingival crevicular fluid of patients with juvenil periodontitis. *Oral Microbiology and Immunology*, **6**, 24 - 29

Sutcliffe, P (1972) A longitudinal study of gingivitis and puberty. *Journal of Periodontal Research*, 7, 52- 58.

Sveen, K. & Skang, N. (1980) Bone resorption stimulate by lipopolysaccharides from *Bacteriodies*, *Fusobacterium* and *Veillonella* and by the lipid A and the polysaccharide part of the *Fusobacterium* lipopolysaccharide. *Scandanavian Journal of Dental Research*, **88**, 535 - 542.

Swanson, J. (1982) Colony opacity and protein II compositions of gonococci. *Infection and Immunity*, **37**, 359 - 368.

Taher, I.A.A. (1989) *Actinobacillus actinomycetemcomitans* and periodontal disease (Laboratory and clinical studies). PhD Thesis, University of Glasgow, Scotland.

Taher, I.A.A., MacFarlane, T.W. (1991a) Hydrophobicity of *Actinobacillus actinomycetemcomitans*. *Microbial Ecology in Health and Disease*, **4**, 101 - 104.

Taher, I.A.A., MacFarlane, T.W. (1991b) Adherence of *Actinobacillus actinomycetemcomitans* to buccal epithelial cells *in vitro*. *Biomedical Letters*, **46**, 113 - 120.

Taichman, N.S., Dean, R.T. & Sanderson, C.J. (1980) Biochemical and morphological characterisation of the killing of of human monocytes by a leukotoxin derived from *Actinobacillus actinomycetemcomitans*. *Infection and Immunity*, **28**, 258 - 268.

Taichman, N.S., Shenker, B.J., Tsai, C-C., Glickman, L.T., Baehni, P.C., Stevens, R. & Hammond, B.F. (1984) Cytopathic effects of *Actinobacillus actinomycetemcomitans* on blood leukocytes. *Journal of Periodontal Research*, **19**, 133 - 145.

Taichman, N.S., Simpson, D.L., Sakurada, S., Cranfield, M., DiRienzo, J. & Slots, J. (1987) Comparative studies on the biology of *Actinobacillus actinomycetemcomitans* leukotoxin in primates. *Oral Microbiology and Immunology*, **2**, 97 - 104.

Taichman, N.S, Iwas, M., Korchak, H., Berthold, P. & Lally, E.T. (1991) Membranolytic activity of *Actinobacillus actinomycetemcomitans* leukotoxin. *Journal of Periodontal Research*, **26**, 258 - 260.

Tanner, A.C.R., Haffer, C., Brathall, G.T., Visconti, R.A. & Socransky, S.S. (1979) A study of bacteria associated with advancing periodontitis in man. *Journal of Clinical Periodontitis*, **6**, 278 - 303.

Tervahartiala, B., Uitto, V-J., Kari, K. & Laakso, T. (1989) Outer membrane vesicles and leukotoxic activity of *Actinobacillus actinomycetemcomitans*, from subjects with different periodontal status. *Journal of Dental Research*, **97**, 33 - 42.

Therlfall, E.J., Rowe, B, Ferguson, J.L., Ward, L.R. (1983) Gentamicin resistant *Salmonella typhimurium*. *Veterinary Record*, **113**, 627.

Timmis, K.N. (1979) Mechanisms of plasmid incompatibility. In: *Plasmids of Medical and Commercial Importance*. pp 13 - 22. Eds Timmis, K.N. and Puhler, A. Elsevier/North-Holland Biomedical Press. Oxford.

Topley, W.W.C. & Wilson, G.S. (1955) *Principles of Bacteriology and Immunology. I.* Revised by GS Wilson and AA Miles. Published by Edward Arnold & Co. London.

Tsai, C-C., McArthur, W.P., Baehni, P.C., Hammond, B.F. & Taichman, N.S. (1979) Extraction and partial characterization of a leukotoxin from a plaque-derived gram-negatice microorganism. *Infection and Immunity*, **25**, 427 - 439.

Tsai, C-C., Shenker, B.J., DiRienzo, J.M., Malamud, D. & Taichman, N.S. (1984) Extraction and isolation of a leukotoxin from *Actinobacillus actinomycetemcomitans* with polymyxin B. *Infection and Immunity*, **43**, 700 - 705.

Tsai, C-C. & Taichman, N.S. (1986) Dynamics of infection by leucotoxic strains of *Actinobacillus actinomycetemcomitans* in juvenile periodontitis. *Journal of Clinical Periodontology*, **13**, 330 - 331.

Uitto, V-J., Suomalainen, K. & Sorsa, T. (1990) Salivary collagenase. Origin, characteristics and relationship to periodontal health. *Journal of Periodontal Research*, **25**, 135 - 142.

Uphoff, T.S. & Welch, R.A. (1990) Nucleotide sequencing of the *Proteus mirabilis* calicium-independent hemolysin genes (*hpmA* and *hpmB*) reveals sequence similarity with the *Serratia marcescens* hemolysin genes (*shla* and *shlb*). *Journal of Bacteriology*, **172**, 1206 - 1216.

Vandepitte, J., De Geest, H. & Jousten, P. (1977) Subacute bacterial endocarditis due to *Actinobacillus actinomycetemcomitans*. *Journal of Clinical Pathology*, **30**, 824 - 846.

Vandesteen, G.E., Williams, B.L., Ebersole, J.L., Altman, L.C. & Page, R.C. (1984) Clinical, microbiological and immunological studies of a family with a high prevalence of early-onset periodontitis. *Journal of Periodontology*, **55**, 159 - 169.

Van der Velden, U., Van Winkelhoff, A.J., Abbas, F. & De Graaff, J. (1986). The habitat of periodontopathic micro-organisms. *Journal of Clinical Periodontology*, **13**, 243 - 248.

Van Dyke, T.E., Horoszewicz, H.V., Cianciola, L.J. & Genco, R.J. (1980) Neutrophil chemotaxis dysfunction in human periodontitis. *Infection and Immunity*, **27**, 124 - 132.

Van Dyke, T.E., Taubman, M.A., Ebersole, J.L., Haffajee, A.D., Socransky, S.S., Smith, D.J. & Genco, R.J. (1984) The Papillon-Lefevre Syndrome : Neutrophil dysfunction with severe periodontal disease. *Clinical Immunology and Immunopathology*, **31**, 419 - 429.

Van Dyke, T.E., Bartholomew, E., Genco., R.J., Slots, J. & Levine, M.J. (1982a) Inhibition of neutrophil chemotaxis by soluble bacterial products. *Journal of Periodontology*, **53**, 502 - 508.

Van Dyke, T.E., Horoszweicz, H.U. & Genco, R.J. (1982b) The polymorphonuclear leukocyte (PMNL) locomotor defect in Juvenile periodontitis. *Journal of Periodontology*, **53**, 682 - 687.

Van Dyke, T.E., Levine, M.J., Al-Hashimi, I. & Genco, R.J. (1982c). Periodontal diseases and impaired neutrophil function. *Journal of Periodontal Research*, **17**, 492 - 494.

Van Dyke T.E., Warbington, M., Gardner, M. & Offenbacher, S. (1990) Neutrophil surface protein markers as indicators of defective chemotaxis in localised juvenile periodontitis. *Journal of Periodontology*, **61**, 180 - 184.

van Steenbergen, T.J.M., Van der Velden, U., Abbas, F. & de Graff, J. (1991) Microflora and bacterial DNA restriction enzyme analysis in young adults with periodontitis. *Journal of Periodontology*, **62**, 235 - 241.

Waldrop, T.C., Anderson, D.C., Hallman, W.W., Schmalsteig, F.C. & Jacobs, R.C. (1987) Periodontal manifestations of the heritable Mac-1, LFA-1 deficiency syndrome. *Journal of Periodontology*, **58**, 400 - 416.

Weiss, S.J., Lampert, M.B. & Test, S.T. (1983) Long live oxidants generated by human neutrophils : characterization and bioactivity. *Science*, **222**, 625 - 627.

Welch, R.A. & Pellet, S. (1988) Transcriptional organisation of the *Escherichia coli* hemolysin genes. *Journal of Bacteriology*, **170**, 1622 - 1630.

Welch, R.A. (1991) MicroReview. Pore-forming cytolysins of Gram-negative bacteria. *Molecular Microbiology*. **5**, 521 - 528.

Wilson, M., Kamin, S. & Harvey, W. (1985) Bone resorbing activity of purified capsular material from *Actinobacillus actinomycetemcomitans*. *Journal of Periodontal Research*, **20**, 484 - 491.

Wilson, M.E. (1991a) IgG antibody response of localized juvenile periodontitis patients to the 29 Kilodalton outer membrane protein of *Actinobacillus actinomycetemcomtians*. *Journal of Periodontology*, **62**, 211 - 218.

Wilson, M.E. (1991b) The heat-modifiable outer membrane protein of *Actinobacillus actinomycetemcomitans*: relationship to OmpA proteins. *Infection and Immunity*, **59**, 2505 - 2507.

Winkler, J.R., Matarese, V., Hoover, C.I., Kramer, R.H. & Murray, P.A. (1988) An *in vitro* model to study bacterial invasion of periodontal tissues. *Journal of Periodontology*, **59**, 40 - 45.

Wilton, J.M.A., Griffiths, G.S., Curtis, M.A., Maiden, M.F.J., Gillett, I.R., Wilson, D.T., Sterne, J.A.C. & Johnson, N.W. (1988) Detection of high-risk groups and individuals for periodontal disease. *Journal of Clinical Periodontology*. **15**, 339 - 346.

Whittam, T.S. (1995) Genetic poopulation structure and pathogenicity in enteric bacteria. pp 217 - 245 *In* :Fifty-second symposium of the Society for General Microbiology, held at the University of Leicester, January 1995. S. Baumberg & J. P. W.Young, E.M.H. Wellington & J.R.Saunders (ed.) The Society for General Microbiology, The Press Syndicate of the University of Cambridge, England.

Wolff, L.F., Liljemark, W.F., Bloomquist, C.G., Philstrom,B.L., Schaffer, E.M. & Bandt, C.L. (1985) The distribution of *Actinobacillus actinomycetemcomitans* in human plaque. *Journal of Periodontal Research*. **20**, 237 - 250.

Woodward, M.J., McLaren, I. & Wray, C. (1989) Distribution of virulence plasmids within *Salmonellae*. *Journal of General Microbiology*. **135**, 305 - 511.

Yoshie, H., Taubman, M.A., Ebersole, J.L., Olsen, C.L., Smith, D.J. & Pappo, J. (1985) Activation of rat B lymphocytes by *Actinobacillus actinomycetemcomitans*, **47**, 264 - 270.

Yoshie, H., Taubman, M.A., Olson, C.L., Ebersole, J.L. & Smith, D.J. (1987) Periodontal bone loss and immune characteristice after adoptive transfer of *Actinobacillus*-sensitized T cells to rats. *Journal of Periodontal Research*, **22**, 499 - 505,

Zambon, J.J., Deluca, C., Slots, J. & Genco, R.J. (1983) Studies of leukotoxin from *Actinobacillus actinomycetemcomitans* using the promyelocytic HL-60 cell line. *Infection and Immunity*, **40**, 205 - 212.

Zambon, J.J., Slots, J. & Genco, R.J. (1983) Serology of oral *Actinomycetemcomitans actinomycetemcomitans* and serotype distribution in human periodontal disease. Infection and Immunity, **41**, 19 - 27.

Zambon, J.J., Christersson, L.A. & Slots, J. (1983b) *Actinobacillus actinomycetemcomitans* in human periodontal disease. Prevalence in patient groups and distribution of biotypes and serotypes within families. *Journal of Periodontology*, **54**, 707 - 711.

Zambon, J.J. (1985) *Actinobacillus actinomycetemcomitans* in human periodontal disease. *Journal of Clinical Periodontology*, **12** : 1- 20.

Zambon, J.J., Clinemoto, T,. De Nardin, E., Nakazawa, F., Christersson, L.A. & Genco R.J. (1988) *Actinobacillus actinomycetemcomitans* in the pathogenesis of human periodontal. *Advances in Dental Research*, **2**, 269 - 274.

Zambon, J.J., Sunday, G.J. & Smutko, J.S. (1990) Molecular genetic analysis of *Actinobacillus actinomycetemcomitans* epidemiology. *Journal of Periodontology*, **61**, 75 - 80.

Zinnemann, K. (1981) History of HPA. p 1 - 10. *In*: Haemophilus, Pasteurella and Actinobacillus. Eds. Kilian, M., Frederiksen, W. & Biberstein, E.L. Academic Press, London.

APPENDICES

APPENDIX 1.1
General Methods (Chapter 2)

Columbia Blood Agar (CBA)	**per litre**
Columbia Blood Agar base	44.0 g
Defibrinated horse blood (7.5%)	75.0 ml
Vitamin K/ Haemin (1.0%)	10.0 ml
Distilled water	1.0 l

pH 7.3

The CBA powder was dissolved by boiling and frequent mixing. It was then autoclaved at 121°C for 15 minutes. After cooling to blood heat, the horse blood was added with the Vitamin K/ Haemin solutions and mixed by swirling to avoid bubbles and the plates then carefully poured.

Tryptic Soy Broth	**per litre**
TSB powder	30.0 g
distilled water	1.0 l

pH 7.3 ± 0.2

The powder was dissolved in the distilled water and aliquoted into 100 or 400 ml volumes prior to being autoclaved at 121°C for 14 minutes. It was stored at room temperature until require.

Constituents of TSB	**g/ litre**
Peptone 140	17.0
Peptone 110	3.0
NaCl	5.0
K_2HPO_4	2.5
Dextrose	2.5

Constituents of RPMI 1640 MEDIUM	**mg/ litre**
Calcium nitrate	100.0
Potassium chloride	400.0
Magnesium sulphate	100.0
Sodium chloride	6000.0
Sodium hydrogen chloride	2000.0
Sodium phosphate (anhydrous)	800.0
D-Glucose	2000.0
Glutathione (reduced)	1.0
Phenol Red	5.0
L-Arginine HCl	200.0
L-Asparagine	50.0
L-Aspartic Acid	20.0

Constituents RPMI 1640 Medium continued../

L-Cystine	50.0
L-Glutamic Acid	20.0
L-Glutamine	300.0
Glycine	10.0
L-Histidine (free base)	15.0
L-Hydroproline	20.0
L-Isoleucine (allo free)	50.0
L-Leucine (methonine free)	50.0
L-Lysine.HCl	400.0
L-Methionone	15.0
L-Phenylalanine	15.0
L-Proline (hydroxy L-proline free)	20.0
L-Serine	30.0
L-Threonine (allo free)	20.0
L-Trytophan	5.0
L-Tyrosine	20.0
L-Valine	20.0
Biotin	0.2
D-Ca Pantothenate	0.3
Choline Chloride	3.0
Folic acid	1.0
i-Insitol	35.0
Nicotinamide	1.0
Para-animobenzoic acid	1.0
Pyridoxal HCl	1.0
Riboflavin	0.2
Thiamine HCl	1.0
Vitamin B12	0.005

Foetal Calf Serum (FCS)

This was heated to 56°C for 60 minutes then aliquoted into 10 and 20 ml volumes for storage at -20°C, until required.

RPMI 1640/ FCS	**ml/ 100 ml**
RPMI 1640	90.0
FCS	10.0

The FCS was defrosted then, both the RPMI 1640 and the FCS were allowed to reach room temperature before mixing. From the mixed solution, 5 ml was removed and the pelleted cells resuspended in this volume, then returned to the original 100 ml. Cells were incubated at 37°C as previously described.

APPENDIX 1.2
Prevalence of Plasmids (Chapter 3)

Tris Borate buffer (TBE)	**g/ 5 litres**
Tris - 89 mM	53.9
Boric acid - 89 mM	27.5
EDTA (disodium) - 1.2 mM	2.3

pH 8.2

The dry chemicals were weighed out, mixed and stored at room temperature until required. This mixture was then added to 1 litre of distilled water and dissolved by stirring and heat. After making up to 5 litres, the buffer was stored at room temperature.

10% Sodium dodecyl sulphate (SDS)

SDS	10 g
TBE	up to 100 ml

CAUTION : SDS is an irritant to both the skin and respiratory system.

Disposable gloves and mask were worn to avoid contact with the powder.

The SDS was dissolved in 50 ml TBE, then made up to 100 ml. The solution was stored at room temperature.

Gel loading buffer	**per 100 ml**
Sucrose	23 g
Sodium acetate	60 mg
SDS	100 mg
Bromophenol blue	50 mg
Distilled water	up to 100 ml

The dry chemicals were dissolved in distilled water and stored at room temperature.

Agarose gels - 0.7%

Agarose type II	0.9 g
TBE	125.0 ml

The agarose was melted in 125 ml TBE for each vertical gel and allowed to cool slightly before casting.

Ethidium bromide - stock solution

Ethidium bromide - 0.495 mM	0.39 g
Distilled water	200.0 ml

CAUTION : ETHIDIUM BROMIDE is a suspected carcinogen

Disposable gloves and mask were worn to avoid contact with the powder. Disposable gloves were worn to avoid contact with the solution.

The ethidium bromide was made up in 200 ml distilled water and left at 37°C overnight to aid solution. It was then stored at +4°C in the dark until required.

Tris/ EDTA/ Salt (TES) Stock solution : 10 X concentrate

	per 10 litres
Tris (50 mM)	0.6 g
Sodium chloride (50 mM)	29.2 g
EDTA (disodium)	18.6 g
Distilled water	upto 1 l
Hydrochloric acid - concentrated	
pH 8.0	

The dry chemicals for 10 litres were dissolved in 900 ml distilled water. The pH was adjusted with the concentrated hydrochloric acid and made up to 1 litre. This stock solution was stored at +4°C, then diluted 1 in 10 with distilled water to give 1X working solution as required.

Ethidium bromide - staining of gels

0.5 ml of the stock ethidium bromide solution was diluted in 300 ml 1 X TES and stored at room temperature.

APPENDIX 1.3

Strain Diversity of *A.actinomycetemcomitans* (Chapters 4 and 5)

Tris EDTA (TE)	**g/ 500 ml**
Tris 10 mM	0.6 g
EDTA 1 mM	0.186 g
Glacial acetic acid	
pH 7.4	

The dry chemicals were dissolved in distilled water and the pH adjusted to 7.4 with 1 - 2 ml glacial acetic acid.

TE10	**per 500 ml**
Tris 10 mM	0.60 g
EDTA 10 mM	1.86 g
Distilled water	up to 500 ml
Glacial acetic acid	
pH 7.8	

The Tris and EDTA were dissolved in 400 ml distilled water then the pH adjusted to 7.8 with the glacial acetic acid. After autoclaving at 121°C for 15 minutes, the buffer was stored at room temperature.

TE50	**per 500 ml**
Tris 50 mM	3.25 g
EDTA 50 mM	9.3 g
Distilled water	up to 500 ml
Glacial acetic acid	
pH 7.8	

The Tris and EDTA were dissolved in 400 ml distilled water then the pH adjusted to 7.8 with the glacial acetic acid. After autoclaving at 121°C for 15 minutes, the buffer was stored at room temperature.

2M Sodium hydroxide (with care)	**per 100 ml**
Sodium hydroxide	8.0 g
Distilled water	up to 100 ml

The sodium hydroxide was dissolved in the distilled water, then autoclaved at 121°C for 15 minutes before storing at room temperature.

10% SDS	**per 100 ml**
SDS	10.0 g
Distilled water	up to 100 ml

The SDS was dissolved in 50 ml distilled water then the volume made up to 100 ml. The solution was stored at room temperature.

20% SDS	**per 100 ml**
SDS	20.0 g
Distilled water	up to 100 ml

The SDS was dissolved in 50 ml distilled water then the volume made up to 100 ml. The solution was stored at room temperature.

Alkaline SDS	**per 10 ml**
10% SDS	1.0 ml
2M Sodium hydroxide	1.0 ml
Distilled water	8.0 ml

This solution was prepared fresh immediately prior to use. Any unused portions were discarded.

Lysozyme - TE	
lysozyme	25 mg
TE	5 ml

The lysozyme powder was dissolved in the TE buffer immediately prior to use.

3M Sodium acetate	**per 500 ml**
Sodium acetate	123.0 g
Distilled water	500.0 ml
Glacial acetic acid	
pH 4.8	

The sodium acetate was dissolved in 100 ml of distilled water and the pH adjusted to 4.8 with the glacial acetic acid. After the volume was adjusted to 500 ml, the solution was autoclaved at 121°C for 15 minutes then stored at room temperature.

Proteinase K (10 $mgml^{-1}$)	
Proteinase K	100 mg
Distilled water	10 ml

The proteinase K was dissolved in the distilled water, then stored in 500 µl volumes at -20°C until required. It was defrosted immediately prior to use and any unused Proteinase K from the defrosted volume was discarded.

Tris EDTA glucose (TGE)	**per 500 ml**
Tris - 25 mM	1.5 g
EDTA - 10 mM	1.85 g
Glucose - 50 mM	4.5 g
Hydrochloric acid	
pH 7.4	

The tris and EDTA were dissolved in 100 ml of distilled water and the pH adjusted with the concentrated hydrochloric acid. The volume was then made up to 500 ml and autoclaved at 121°C for 15 minutes, after which 5 - 10 ml was aseptically removed. The glucose was dissolved in this volume and then added to the the autoclaved solution by filtering through a 0.2 µm filter.

Phenol/ Chloroform	**per 250 ml**
Phenol	250 g
Chloroform	250 ml
TGE	50 ml

CAUTION : PHENOL IS HIGHLY TOXIC

Disposable gloves and mask were worn to avoid contact with the phenol powder. Disposable gloves were worn to avoid contact with the solution.

The phenol was dissolved in the chloroform by leaving overnight at room temperature. TGE was added and mixed well. After the aqueous layer had separated, the solution was stored in the dark at +4°C.

7.5 M Ammonium acetate	**per 100 ml**
Ammonium acetate	57.8 g
Distilled water	up to 100 ml
Glacial acetic acid	
pH 8.0	

The ammonium acetate was dissolved in 40 ml of distilled water and the pH adjusted with glacial acetic acid. The volume was then made up to 100 ml and the solution stored at room temperature.

RNAase (1 mgml^{-1})	
RNAase	3 mg
Distilled water	3 ml

The RNAase was dissolved in the distilled water then heated at 100°C in a boiling water bath for 10 minutes. It was stored at +4°C.

RNAase (10 mgml^{-1})

RNAase	30 mg
Distilled water	3 ml

The RNAase was dissolved in the distilled water then heated at 100°C in a boiling water bath for 10 minutes. It was stored at +4°C.

2.5M Sodium chloride

Sodium chloride	14.6 g
Distilled water	up to 100.0 ml

The sodium chloride was dissolved in 50.0 ml of distilled water then made up to 100 ml. After autoclaving at 121°C for 15 minutes it was stored at room temperature.

TBE plus Ethidium bromide

0.5 ml Ethidium bromide stock was diluted in 3 litres of TBE and stored at room temperature.

Agarose gels - 0.8% (plasmid)

Agarose Type II	0.8 g
TBE plus ethidium bromide	100 ml

The agarose was melted in TBE containing ethidium bromide and allowed to cool slightly before casting. Once set, the tank was flooded with TBE containing ethidium bromide and the comb removed.

Agarose gels - 0.6% (genomic)

Agarose Type II	1.8 g
TBE	300 ml

The agarose was melted in TBE and allowed to cool slightly before casting. Once set, the tank was flooded with TBE and the comb removed.

APPENDIX 1.4

Monoclonal Antibody, 9D4, and its Possible Cross Reaction with *A.actinomycetemcomitans* Proteins (Chapter 6)

20 mM Tris HCl	**per 250 ml**
Tris HCl	0.8 g
Distilled water	up to 250 ml
Hydrochloric acid - concentrated	
pH 7.2	

The Tris was dissolved in 150 ml distilled water then the pH adjusted to pH 7.2 with the concentrated hydrochloric acid. The buffer was made up to 250 ml with distilled water, autoclaved at 121°C for 15 minutes and stored at room temperature.

10 mM EDTA/ 20 mM Tris	**per 100 ml**
Tris HCl	0.32 g
EDTA	0.37 g
Hydrochloric acid - concentrated	
pH 7.2	

The Tris was dissolved in 150 ml distilled water, then the pH adjusted to pH 7.2 with the concentrated hydrochloric acid. The buffer was then made up to 250 ml with distilled water, autoclaved at 121°C for 15 minutes and stored at room temperature.

0.5 % Sarkosyl	**per 50 ml**
Sodium N-lauroyl sarcosinate	0.25 g
Distilled water	up to 50 ml

The sarkosyl was dissolved in 30 ml distilled water then the volume made up to 50 ml. This was stored at room temperature.

Protein determination : modified Lowry procedure

Reagent A	**per 100 ml**
Sodium carbonate (2%)	2.0 g
Sodium hydroxide (0.4%)	0.4 g
Sodium tartrate (0.16%)	0.16 g
SDS (1%)	1.0 g
Distilled water	up to 100 ml

The reagents were dissolved in 70 ml of distilled water and the volume made up to 100 ml. This was stored at room temperature.

Reagent B	**per 100 ml**
Copper sulphate ($\cdot 5H_2O$)	4.0 g
Distilled water	up to 100 ml

The copper sulphate was dissolved in the distilled water and the volume made up to 100 ml. It was stored at room temperature.

Folin-Ciocalteu
Diluted 1 in 1 with distilled water as required, immediately prior to use.

Acrylamide : Bis (30% : 2.67%)	**per 100 ml**
Acrylamide	29.2 g
N'N" - Bis methene acrylamide	0.8 g
Distilled water	up to 100 ml

CAUTION : ACRYLAMIDE IS A NEUROTOXIN
Disposable gloves and mask were worn to avoid contact with the powder. Disposable gloves were worn to avoid contact with the solution.
The acrylamide and bisacrylamide were dissolved in 50 ml of distilled water then the volume made up to 100 ml. After filtering, the solution was stored at +4°C in a dark bottle for a maximum of 1 month.

1.5M Tris-HCl	**per 100 ml**
Tris - HCl	18.15 g
Distilled water	up to 100 ml
Hydrochloric acid - concentrated	
pH 8.8	

The Tris-HCl was dissolved in 50 ml distilled water and the pH adjusted with the concentrated hydrochloric acid before the volume was made up to 100 ml. The buffer was stored at +4°C.

0.5M Tris-HCl	**per 50 ml**
Tris - HCl	3.0 g
Distilled water	
Hydrochloric acid - concentrated	
pH 6.8	

The Tris-HCl was dissolved in 30 ml distilled water and the pH adjusted with the concentrated hydrochloric acid before the volume was made up to 100 ml. The buffer was stored at +4°C.

10% SDS	**per 50 ml**
SDS	5.0 g
Distilled water	

The SDS was dissolved in 30 ml distilled water the volume made up to 50 ml. It was stored at room temperature until required.

N,N.N',N' - tetra methyl-ethlene diamine (TEMED)
Undiluted stock was used.

10% Ammonium persulphate

Ammonium persulphate	50.0 µg
Distilled water	0.5 ml

This was prepared freshly as required.

Running buffer - 2X Stock solution	**per litre**
Tris base	6.0 g
Glycine	28.8 g
SDS	2.0 g
Distilled water	
pH 8.3	

The reagents were dissolved in 1 litre of water and the pH checked, although in practice it did not need to be adjusted. It was stored at +4°C until required, then diluted 1 in 1 before use, with distilled water.

Sample Buffer	**2X Stock solution**
Distilled water	4.0 ml
Tris (0.5M)	1.0 ml
Glycerol	0.8 ml
SDS (10%)	1.6 ml
2-mercaptoethanol	0.4 ml
bromophenol blue (0.05%)	0.2 ml

The buffer was prepared and stored at +4°C until required. It was diluted 1 in 1 with the protein sample prior to boiling.

Separating gel - 10% GEL	**for 2 x 1.5 mm gels**
Tris - 1.5M	20.0 ml*
SDS - 10%	0.8 ml**
Acrylamide : bis	26.6 ml*
ammonium persulphate	0.4 ml**
TEMED	40 µl**
distilled water	32.2 ml*

Chemicals marked * were mixed and degassed for 10 minutes, prior to the addition of the chemicals marked **. The mixture was mixed carefully to avoid bubbles before being poured.

Stacking gel - 4% gel	**for 2 x 1.5 mm gels**
Tris - 0.5M	5.0 ml*
SDS - 10%	0.2 ml**
Acrylamide : bis	2.6 ml*
Ammonium persulphate	0.1 ml**
TEMED	20 µl**
Distilled water	12.2 ml*

Chemicals marked * were mixed and degassed for 10 minutes, prior to the addition of the chemicals marked **. The mixture was mixed carefully to avoid bubbles before being poured.

Coomassie Blue staining solution	**per 5 litres**
Methanol	2.5 l
Glacial Acetic acid	0.5 l
Coomassie Brilliant Blue R	0.05 g
Distilled water	2.0 l

The Coomassie Brilliant Blue R was dissolved in the methanol, before the addition of the acetic acid and distilled water. This solution was stored at room temperature in a dark bottle.

Destaining solution	**per 2 litres**
Methanol	600 ml
Acetic acid	200 ml
Distilled water	1200 ml

The three liquids were mixed together and stored at room temperature in a dark bottle.

Blotting buffer	**per 4 litres**
Tris	12.12g
Glycine	57.60g
Distilled water	up to 4 litres

pH 8.3

The tris and glycine were dissolved in 3 litres of distilled water and the pH checked, although in practice it did not need to be altered. The volume was then made up to 4 litres and the buffer was stored at +4°C in a dark bottle until required.

Phosphate buffer saline (PBS)	**10 X solution per litre**
Sodium chloride	87.66g
Sodium hydrogen phosphate (Anhydrous)	10.22g
Disodium dihydrogen phosphate ($\bullet 2H_2O$)	4.37g
Distilled water	up to 1 litre

pH 7.2

The sodium hydrogen phosphate and disodium dihydrogen phosphate were dissolved by heat and stirring in 200 ml of distilled water. The sodium chloride was then added and the volume made up to 1 litre after the pH had been checked. The buffer was then sterilised by autoclaving at 121°C for 15 minutes.

Blocking buffer	**per litre**
PBS	up to 1 litre
Skimmed milk powder	25g
Tween-20	1.5 ml

The milk powder was dissolved in the PBS by heating and stirring, then the Tween-20 added. The solution was mixed carefully to ensure the Tween-20 dissolved fully and allowed to reach room temperature before use.

Washing buffer	**per litre**
PBS	up to 1 litre
Tween-20	1.5 ml

The Tween-20 was allowed to dissolve in the PBS by heating and stirring. It was then cooled to room temperature before use. This buffer could be stored overnight at +4°C, but before use it was allowed to reach room temperature.

1 % Cobalt chloride solution	**per 100 ml**
Cobalt chloride	1.0 g
Distilled water	up to 100 ml

The cobalt chloride was dissolved in 50 ml distilled water and the volume made up to 100 ml. It was stored at room temperature.

0.1 % Imido black solution	**per 100 ml**
Imido black	0.1 g
Distilled water	up to 100 ml

The imido black was dissolved in 50 ml distilled water and the volume made up to 100 ml. It was stored at room temperature.

Diaminobenzidine (DAB) substrate solution

3,3'-diaminobenzidine (DAB)	0.03 g
cobalt chloride - 1%	2.0 ml
PBS	98.0 ml
Hydrogen peroxide - 30%	0.1 ml

CAUTION : DAB is a suspected carcinogen.

Disposable gloves and mask were worn to avoid contact with the powder. Disposable gloves were worn to avoid contact with the solution.

The cobalt chloride and DAB were dissolved in the PBS. This was left at room temperature until the hydrogen peroxide was added immediately prior to use.

Appendix 1.5
Leucotoxic Activity (Chapter 7)

Phosphate buffered saline (PBS)

Phosphate buffered saline tablets, pH 7.3, were used. Each tablet was dissolved in 100 ml distilled water, autoclaved at 121°C for 15 minutes and stored at room temperature.

Trypan Blue

Trypan blue	0.1 g
PBS	100 ml

The trypan blue powder was dissolved in the PBS, then filtered through Whatmann 3MM paper. It was stored at room temperature.

5% Dextran

Dextran	5 g
PBS	100 ml

The dextran was dissolved in the PBS then stored at +4°C until required. Before use it was allowed to reach room temperature.

Phorbol-Myristate-Acetate (PMA) Stock solution

PMA	2 mg
PBS	1 ml

CAUTION : PMA IS HIGHLY TOXIC AND A SUSPECTED CARCINOGEN

Disposable gloves and mask were worn to avoid contact with the powder. Disposable gloves were worn to avoid contact with the solution.

The PMA was dissolved in the PBS, then stored at -20°C until required. Before use it was brought to room temperature and diluted by the addition of 50 µl of the stock solution to 10 ml PBS.

Luminol Stock solution

Luminol	1.77 mg
Dimethyl sulphoxide	1.0 ml

The luminol was dissolved in the PBS, then stored at -20°C until required. Before use it was brought to room temperature and diluted by the addition of 10 µl of the stock solution to 10 ml PBS to give a working concentration of 10^{-4} M.

SOURCE OF REAGENTS

Unless otherwise stated, all reagents and chemicals were obtained from

Sigma : Chemical Company, Poole, Dorset
BDH : Poole, Dorset

RPMI 1640 : Life Technologies, Paisley
CBA : Life Technologies, Paisley
Vitamins : Life Technologies, Paisley
FCS : Globepharm, Esher, Surrey
API strips : BioMerieux, Basingstoke
Protect beads : Lab M, Bury

Restriction Endonucleases : New England Biolabs, Bishop's Stortford

Skimmed milk powder: Boots The Chemist