Detection and Identification of oral *Treponema* species using molecular methods

A thesis submitted to the University of Manchester for the degree of PhD in the Faculty of Science and Engineering

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List of Tables

List of Figures

Abstract

The aim of this study was to apply two molecular methods, Random Amplification of Polymorphic DNA by the polymerase chain reaction (RAPD-PCR) and partial 16S rDNA sequencing, to characterise five oral treponemal isolates, previously purified from the subgingival plaque of five patients.

RAPD-PCR utilises a single primer, of arbitrary sequence, in a PCR of low stringency, to amplify potentially polymorphic regions of the genome. Five ATCC strains of *Treponema* including *T. socranskii*, *T. pectinovorum*, *T. vincentii* and two serovar strains of *T. denticola* were also characterised by this method, using four different primers. The different isolates gave distinct patterns of amplified fragments following agarose gel electrophoresis. The presence or absence of major bands was recorded in a binary matrix and used to calculate Dice and simple matching coefficients in a pair-wise manner between isolates. Phenograms were drawn based on average linkage cluster analysis. Three of the patient isolates clustered with *T. denticola* strains. The remaining isolates were distinct from each other and from the other ATCC species.

Partial 16S rDNA sequences were determined for 3 of the patient isolates and for *T. socranskii*, *T. denticola* and *T. vincentii*. These sequences were aligned, a distance matrix with the Kimura correction was constructed and a phenogram was produced as above. With this analysis, two of the isolates were shown to be virtually identical to each other and to cluster very closely with *T. socranskii*. These isolates were distinct in the RAPD analysis. The two *T. denticola* strains and the remaining patient isolate had virtually identical partial 16S rDNA sequences, corroborating the RAPD clustering for these.

From these data, three of the patient isolates were identified as *T. denticola* and two were identified as *T. socranskii*.

Two regions of variability were located in the sequenced region of the 16S rRNA gene that had potential as DNA probe and PCR primer sequences to detect *T. socranskii* and *T. denticola* species. The probes were tested for specificity and sensitivity using dot-blot hybridisation and the primers tested by PCR, in order to assess the validity of using these probes to elucidate the microbial content of subgingival plaque.

Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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The electron micrographs of *Treponema* species on page 90 were taken by Alan Curry and Helen Cotterill from the Public Health Laboratory, Manchester.

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A final thank you to my parents, for encouraging my study, and for their financial and moral support.

Dedication

This thesis is dedicated to Frankie: my partner and my rock.

Abbreviations

AFLP	Amplified fragment length polymorphism
ANUG	Acute necrotising ulcerative gingivitis
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CAL	Clinical attachment level
D	Dice similarity coefficient
dATP	Deoxyadenosinetriphosphate
dCTP	Deoxycytidinetriphospahte
den1	*Treponema denticola* ATCC 35405
den2	*Treponema denticola* ATCC 33520
den3	*Treponema denticola* ATCC 33521
den4	*Treponema denticola* ATCC 35404
dGTP	Deoxyguanosinetriphosphate
DI	Discriminatory index
DIG	Digoxigenin
dNTPs	Deoxynucleotidetriphosphates
DTT	Dithiothreitol
dTTP	Deoxythymidinetriphosphate
DMSO	Dimethylsulphoxide
EDTA	Ethylenediaminetetra-acetic acid disodium salt
EMBL	European Molecular Biology Laboratory
ERIC	Enterobacterial repetitive intergenic consensus
GI	Gingival index
IPTG	*iso*propyl β-D- thiogalactopyranoside
J-C	Jukes-Cantor correction
K	Kimura correction
LB agar	Luria-Bertani medium
Li	*Leptospira icterohaemorrhagiae*
LJP	Localised juvenile periodontitis
LS-PCR	Low-stringency polymerase chain reaction
ML	Maximum likelihood
MLEE	Multilocus enzyme electrophoresis

MPC	Magnetic particle concentrator
NOS	New Oral Spirochaete medium
NJ	Neighbor-joining
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PD	Pocket or probing depth
pect	*Treponema pectinovorum* ATCC 33768
PFGE	Pulsed-field gel electrophoresis
PMNL	Polymorphonuclear leucocyte
PROS	Pathogen-related oral spirochaete
Pi	*Prevotella intermedia*
Pwo	DNA polymerase from *Pyrococcus woesei*
RAPD	Random amplification of polymorphic DNA
REA	Restriction endonuclease analysis
REP	Repetitive extragenic palindromic
RFLP	Restriction fragment length polymorphism
SDS	Sodium dodecyl sulphate
SM	Simple matching similarity coefficient
soc1	*Treponema socranskii* subsp. *buccale* ATCC 35534
soc2	*Treponema socranskii* subsp. *paredis* ATCC 35535
soc3	*Treponema socranskii* subsp. *socranskii* ATCC 35536
SSC	Sodium chloride, sodium citrate buffer
STE	Sucrose, Tris-Cl, EDTA buffer
Taq	DNA polymerase from *Thermus aquaticus*
TBE	Tris-Cl, boric acid, EDTA buffer
TE	Tris-Cl, EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
T_m	Melting point of a duplex
TNE	Tris-Cl, NaCl, EDTA buffer
TPE	Tris phosphate, EDTA buffer
Tris-Cl	Trishydroxymethylaminomethane chloride
UPGMA	Unweighted Pair Group Method using Arithmetic mean
vin	*Treponema* vincentii ATCC 35580

1.0 Introduction

1.1 Taxonomy of *Treponema*

Spirochaetes are helically-shaped, motile bacteria that exist either in association with animal and human hosts or can be free-living (Canale-Parola, 1984). According to Holt (1978), the first description of these bacteria was by van Leeuwenhoek in 1674, although Barantón & Old (1995) accord this honour to Ehrenberg in 1833.

The order Spirochaetales contains two families (Spirochaetaceae and Leptospiraceae) as outlined in Figure 1.1. The latter family consists of aerobic bacteria with hooked ends that utilise long chain fatty acids or long chain fatty alcohols as energy sources. The family Spirochaetaceae consists of anaerobic, facultatively anaerobic or microaerophilic bacteria which utilise carbohydrate and/or amino acids as energy sources. The distinctively-shaped bacteria are divided into different genera on the basis of morphology, environment and, more recently, genetics (Canale-Parola, 1984; Stanton *et al.*, 1991). The genus *Treponema* contains obligate anaerobic and microaerophilic bacteria that are indigenous to the mouth, intestinal tract and genital areas of humans and animals. Some species are pathogenic whereas the pathogenic potential of other species have yet to be determined. The best characterised of the oral species are *T. denticola* (Chan *et al.*, 1993), *T. socranskii* (Smibert *et al.*, 1984), *T. pectinovorum* (Smibert & Burmeister, 1983) and *T. vincentii* (Smibert, 1984), with *T. maltophilum* (Wyss *et al.*, 1996), *T. medium* (Umemoto *et al.*, 1997) and *T. amylovorum* (Wyss *et al.*, 1997) recently being isolated and characterised. Tables 1.1 and 1.2 outline distinguishing characteristics of the species in this genus.

1.1.1 Morphology and motility of *Treponema*

The characteristic helical shape of treponemal bacteria is formed from the so-called 'protoplasmic cylinder', consisting of the cytoplasmic and nuclear regions surrounded by the membrane cell-wall complex (Holt, 1978). Axial filaments (also known as periplasmic or axial fibrils) are found wound around this structure, one end anchored near the pole and the other end free. The number of such filaments differs between species, ranging from one to over one hundred (Canale-Parola, 1978), although fewer numbers are attributed to the described species of *Treponema* (Table 1.1 and 1.2).

The same number of filaments are usually inserted at each end of the cell, with the free ends overlapping in the middle, giving rise to the commonly cited 1-2-1 or 2-4-2 arrangements. The number of filaments for each cell can also be quoted simply as the number inserted into one pole. The axial fibril number is not a constant, with a temporary modification in the number and arrangement of filaments occurring during cell division (Listgarten & Socransky, 1964).

The outermost structure of the cell is the outer cell envelope, which completely surrounds the protoplasmic cylinder and the axial filaments. Despite the endocellular nature of the filaments, it is believed that they have a role to play in the motility of the bacteria (Canale-Parola, 1978). The chemical and structural components of the filaments are very similar to bacterial flagella (Bharier & Allis, 1974), but their mode of action differs. External bacterial flagella can propel the cell by acting against the environment (Berg, 1975), which is not possible for the internal axial fibrils of *Treponema*. Various theories have been suggested to explain the movement of *Treponema* in liquid media, by modelling the effect of the rotation of the filaments on the protoplasmic cylinder (Berg, 1976; Canale-Parolá, 1978), however, the experimental evidence in support of such theories is not conclusive. *Treponema* bacteria show an increase in motility when the viscosity of the medium is increased (Klitorinos *et al.*, 1993), an observation that can explain the invasive capabilities of pathogenic species of *Treponema*.

1.1.2 In *vitro* growth of oral *Treponema*

The growth of oral *Treponema* species *in vitro* is hampered by their low oxygen tolerance (Loesche, 1969) and complex nutritional requirements (Wyss, 1992). The American Type Culture Collection (Gherna *et al.*, 1992) recommend various media to cultivate *Treponema* species in the laboratory, although there is no one medium that will satisfy the growth requirements of all the different species. Oral treponemes require either serum or rumen fluid (which can be replaced with a mixture of fatty acids: Caldwell & Bryant, 1966) and thus a general purpose medium, containing both fatty acids and rabbit serum, is often used for the primary isolation of treponemes from plaque samples (Wardle, 1997). Individual requirements of the different oral species are outlined in Table 1.1, and the constituents of various media are detailed in Table 1.3.

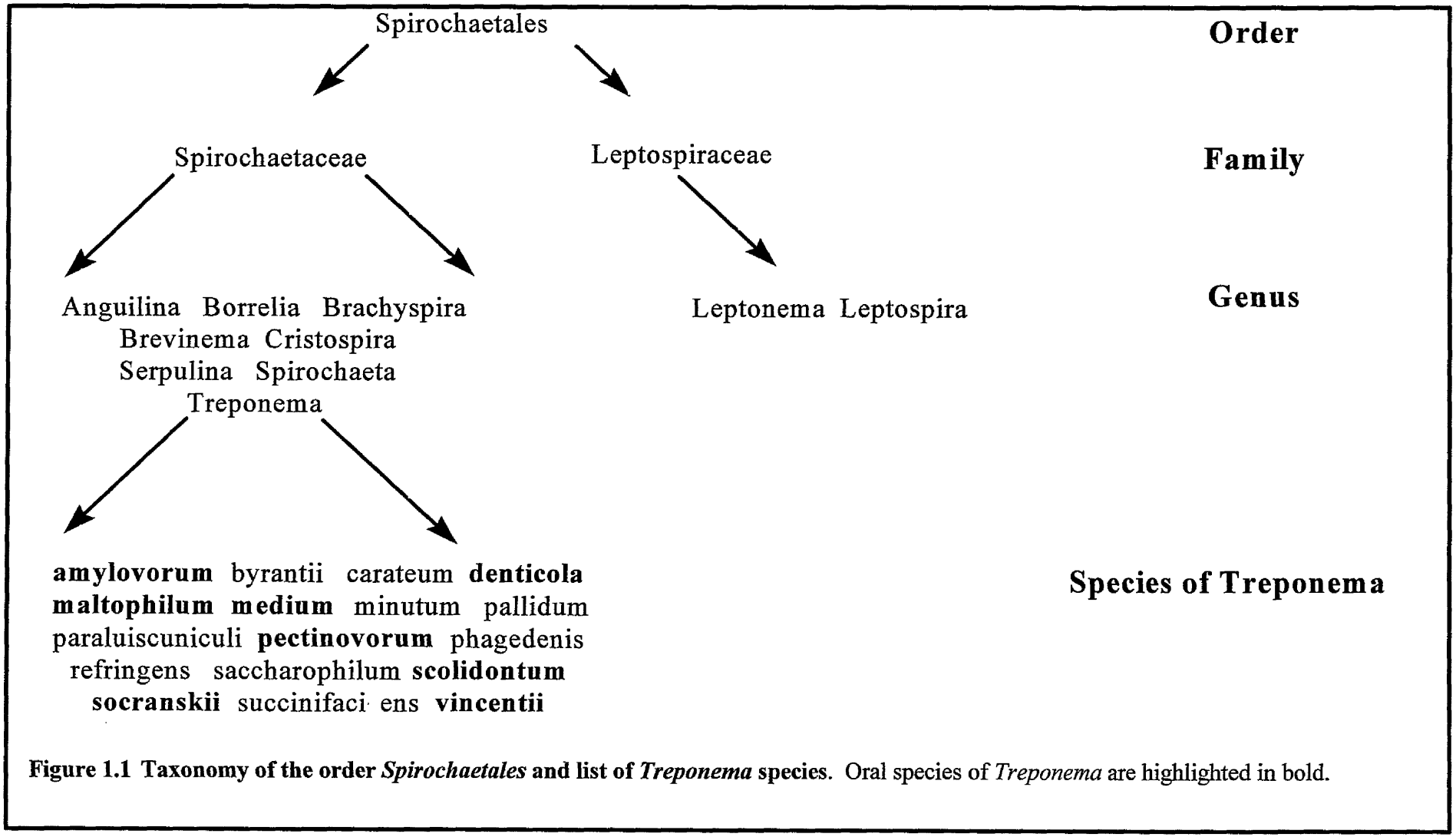

Figure 1.1 Taxonomy of the order *Spirochaetales* and list of *Treponema* species. Oral species of *Treponema* are highlighted in bold.

	.denticola[a]	vincentii[a]	socranskii[b]	pectinovorum[c]	scolidontum[a]	medium[d]	amylovorum[e]	maltophilium[f]
Host	human oral cavity	human oral cavity	human oral cavity	human oral cavity	human oral cavity	human oral cavity	human oral cavity	human oral cavity
Disease[g]	Pd, H	Pd	Pd, H	Pd	Pd	Pd	Pd	Pd
% GC content	37 - 38	44	50 - 52	39	ND	51	ND	ND
Cell length[h]	6 - 16	5 - 16	6 - 15	7 - 15	6 - 16	5 - 16	7	5
Diameter[h]	0.15 - 0.2	0.2 - 0.25	0.16 - 0.18	0.28 - 0.3	0.15 - 0.2	0.2 - 0.3	0.25	0.2
Wavelength[h]	0.9	1.3	ND[m]	ND	ND	ND	1.2	0.7
Amplitude[h]	0.15	0.2 - 0.3	ND	ND	ND	ND	0.3	0.3
Ends of cells	Blunt	Blunt	Tapered	Pointed	Pointed	Blunt	ND	ND
No. of fibrils	2 or 3	4 to 6	1	2	ND	5 to 7	3	1
Carbohydrates[i]	no	no	yes	no	no	yes	yes	yes
Amino acids[i]	yes	yes	no	no	yes	yes	yes	yes
Serum[j]	yes	yes	no	no	yes	yes	yes	no
Fatty acids[j]	no	no	yes	yes	no	yes	yes	yes
Glucose[j]	no	no	no	no	no	yes	yes	no
Cocarboxylase[j]	yes	yes	no	no	yes	yes	no	no
Pectin[j]	no	no	no	yes	no	no	no	no
Growth medium[k]	1357	1357	modified M10	1367	1357	MTYGVS	OMIZ-pat	OMIZ-pat
End products[l]	A, l, s, f	A, B, l, s, f	A, L, S, f	A, F, p, l	a, f, s, p, b	A, B, v	ND	ND

Table 1.1 Characteristics of oral treponemes.

Information in this table was compiled from: **a:** Smibert (1984); **b:** Smibert *et al.*, (1984); **c:** Smibert & Burmeister, (1983); **d:** Umemoto *et al.*, (1997); **e:** Wyss *et al.*, (1997); **f:** Wyss *et al.*, (1996).

g: Pd = periodontal diseases; H = isolated from healthy patients

h: all measurements in μm. **i:** ability to ferment carbohydrate or amino acids. **j:** required for growth. **k:** 1357 (NOS spirochaete medium) and 1367 (modified OTI medium) from Ghema *et al.*, (1992); Modified M10 from Koseki *et al.*, (1996); MTYGVS from Salvador *et al.*, (1987); OMIZ-pat from Wyss (1992) and Wyss *et al.*, (1996).

l: acid fermentation end products A = acetic; B = *n*-butyric; F = formic; L = lactic; P = propionic; S = succinic; V = valeric. Capital letters denote major product, lowercase denotes minor or trace amounts. **m:** ND no data.

	pallidum	carateum	para-luiscuniculi	minutum	phagedenis	succini-faciens	saccharo-philum	bryantii	refringens
Host	humans/ monkeys	humans	rabbits	human genital area	chimpanzee genital area	swine colon	bovine rumen	bovine rumen	human genital area
Disease[a]	VS, NVS, Yaws	pinta	VS	normal flora	normal flora	normal flora	normal flora	normal flora	normal flora
% GC content	52 - 54	37 - 38	37 - 38	37	38 - 39	36	54	36	39 - 43
Cell length[b]	6 - 16	6 - 16	6 - 16	9 - 12	6 - 12	4 - 8	12 - 20	3 - 8	5 - 16
Diameter[b]	0.15 - 0.2	0.15 - 0.2	0.15 - 0.2	0.15 - 0.2	0.2 - 0.25	0.3	0.6 - 07	0.3	0.2 - 0.25
Wavelength[b]	0.9	0.9	0.9	1.3	1.4 - 1.6	ND	ND	ND	1.8
Amplitude[b]	0.15	0.15	0.15	0.2	0.2 - 0.3	ND	ND	ND	0.2 - 0.3
Ends of cells	Pointed	Pointed	Pointed	Tapered	Blunt	ND	ND	ND	Tapered
No. of fibrils	2 or 3	2 or 3	2 or 3	2 or 3	3 to 8	2	16	1	2 to 4
Carbohydrates[c]	ND[g]	ND	ND	no	yes	yes	yes	yes	no
Amino acids[c]	ND	ND	ND	yes	yes	no	no	no	yes
Serum[d]	ND	ND	ND	yes	yes	no	no	no	yes
Fatty acids[d]	ND	ND	ND	no	yes	no	yes	yes	no
Glucose[d]	ND	ND	ND	no	no	yes	yes	yes	no
Cocarboxylase[d]	ND	ND	ND	no	no	yes	no	no	no
Pectin[d]	ND	ND	ND	no	no	no	yes	no	no
Growth medium[e]	X	X	X	1828	1828	1367	MTYGVS	MTYGVS	1828
End products[f]	ND	ND	ND	A, s, l, p, b	A, B, p, f, l	A, L, F, S	F, A	A, F, S	A, s, l, p, b

Table 1.2 Characteristics of non-oral treponemes

Information in this table was compiled from Smibert (1984) and Paster & Canale-Parola, (1985). **a:** VS venereal syphilis; NVS non-venereal syphilis. *T. pallidum* subsp. *pallidum* is the causative agent of VS; *T. pallidum* subsp. *pertenue* is the causative agent of yaws and *T. pallidum* subsp. *endimium* is the causative agent of NVS. **b:** all measurements in μm. **c:** ability to ferment carbohydrates or amino acids. **d:** required for growth. **e:** X not cultured on artificial medium; 1828 (peptone - yeast medium) and 1367 (modified OTI medium) from Gherna *et al.*, (1992); MTYGVS from Salvador *et al.*, (1987). **f:** acid fermentation end products: A = acetic; B = *n*-butyric; F = formic; L = lactic; P = propionic; S = succinic; V = valeric. Capital letters denote major product, lowercase denotes minor or trace amounts. **g:** ND no data.

Medium	Constituents	Reference
1357 (NOS)	Brain Heart Infusion broth, trypticase, yeast extract, sodium thioglycollate, L-cysteine HCl, L-asparagine, glucose, cocarboxylase, volatile fatty acids, sodium bicarbonate, rabbit serum	Gherna *et al.*, (1992)
1367 (modified OTI)	Polypeptone, Heart Infusion broth, yeast extract, pectin, glucose, starch, sucrose, maltose, sodium pyruvate, ribose, K_2HPO_4, $MgSO_4$, L-cysteine HCl, rumen fluid, rabbit serum, cocarboxylase,	Gherna *et al.*, (1992)
1828 (peptone - yeast)	Peptone, trypticase, yeast extract, resazurin, $CaCl_2$, $MgSO_4$, K_2HPO_4, KH_2PO_4, $NaHCO_3$, NaCl, haemin, vitamin K1, L-cysteine HCl, cocarboxylase, rabbit serum	Gherna *et al.*, (1992)
modified M10	Polypeptone, yeast extract, glucose, L-cysteine HCl, K_2HPO_4, KH_2,PO_4, NaCl, $CaCl_2$, $MgSO_4$, $(NH_4)_2HPO_4$, haemin, L-ascorbic acid, sodium bicarbonate, volatile fatty acids	Koseki *et al.*, (1996)
OMIZ-pat	A very enriched medium containing over 100 ingredients	Wyss (1992); Wyss *et al.*, (1996)
MTYGVS	Tryptone, Heart Infusion broth, yeast extract, pectin, gelatin, $(NH_4)_2SO4$, $MgSO_4$, K_2HPO_4, NaCl, glucose, L-cysteine HCl, cocarboxylase, sodium pyruvate, volatile fatty acids, rabbit serum	Salvador *et al.*, (1987)

Table 1.3 Growth media for treponemes

1.2 Periodontal disease

The expression 'periodontal disease' covers a number of distinct clinical conditions that affect the periodontium (the area surrounding the teeth). These conditions range from transient gingivitis, which effects nearly all of the population at some time, to the more serious refractory periodontitis, that can result in tooth loss. The diseases are chronic bacterial infections (Section 1.2.3) and are exacerbated by host and other factors (Section 1.2.4).

1.2.1 Gingival disease

The soft gum tissue surrounding the teeth is known as the gingiva. The healthy gingiva appear pale pink, firm and usually have well-defined scalloped margins. Histologically, healthy tissue is characterised by absence of inflammatory infiltrate, although it is common to see a minimal inflammatory infiltrate in gingiva that appear clinically healthy (Nisengard *et al.*, 1988). The gingival margin (the area where the teeth and gums meet) is not a sterile environment, harbouring a microbial flora whose composition is not static (Section 1.2.3). Gingivitis (inflammation of the gingiva) is very common and occurs as a response to the bacteria that accumulate around the gingival margin. The gingiva appear swollen, redden and can bleed on probing. The condition is both transient and reversible if the bacteria are removed, and can be prevented by good oral hygiene practices. The disease can progress to periodontitis in the continued presence of bacteria (Section 1.2.2).

Acute necrotising ulcerative gingivitis (ANUG) is an inflammatory destructive gingival condition. In typical acute form, it is characterised by painful, malodorous, necrotic ulceration of gingival tissue.

1.2.2 Periodontitis

Periodontitis is an inflammatory disease characterised by destruction of the periodontal ligaments, 'pocket' formation and loss of alveolar bone support. Pockets are formed by an increase in separation of the soft tissues from the tooth, caused by breakdown of connective tissue and epithelium by host inflammatory responses to the presence of bacteria. In advanced stages of periodontitis, tooth loss can occur and is the major cause of loss of teeth in adults (Douglas *et al.*, 1983). It is rare for periodontitis to occur without previous gingivitis, although gingivitis does not always

lead to periodontitis. Active disease is associated with increased proportions of putative pathogenic bacteria (Section 1.2.3), changes in host responses and possibly bacterial invasion within the tissues. Several types of periodontitis have been described that can be differentiated both clinically and microbiologically (Nisengard *et al.*, 1988; Tanner & Stillman, 1993). The classification tends to reflect the patient's age at onset and also the extent of the disease. Adult periodontitis ranges from 'slight', 'moderate', 'advanced', 'early-onset' (also known as 'rapidly progressing') to 'refractory'. In the latter state, the disease does not respond to treatment, leading to severe tooth loss. It is assumed that this lack of response to therapy is due to host or bacterial factors that are not yet understood. Early-onset periodontitis is a rare condition characterised by the young age of onset (from postpubertal to age 35). The diseases are not continually progressive conditions, but undergo quiescent periods of low activity followed by bursts of active periodontal destruction (Socransky *et al.*, 1984).

Localised juvenile periodontitis (LJP) is an aggressive periodontal condition found in younger patients (12 - 20 years old). The disease is characterised by alveolar bone loss localised to certain teeth (often first permanent molars and central incisors) and the absence of gingival inflammation and supragingival plaque (Section 1.2.3). LJP has a genetic component, being more common in females (Nisengard *et al.*, 1988).

Prepubertal periodontitis (or 'periodontitis associated with leukocyte adhesion deficiency'; Dibart, 1997) is a rare condition that occurs during or immediately after the eruption of the primary teeth. The disease can present as a localised or generalised condition, with the appearance of 'fiery red' gingivae and the rapid destruction of alveolar bone, causing the early loss of primary teeth. As with LJP, there is a genetic element to the condition, with affected patients demonstrating abnormal polymorphonuclear leu cocyte (PMNL) adhesion function (Page *et al.*, 1987).

There is difficulty in standardising the concepts of 'healthy' and 'diseased' sites in periodontal disease. In latter years, several clinical parameters have been described to define sites as diseased (Genco, 1996). These parameters include clinical attachment level (CAL; measured from the cemento-enamel junction to the base of the periodontal pocket), probing or pocket depth (PD; measured from the gingival

margin to the base of a periodontal pocket) and the gingival index (GI; a score reflecting the condition of the gingiva, including a measurement of the extent of bleeding). Measurement of alveolar bone loss using radiography is also an important indicator of disease severity, but as this is harder to measure, is not often quoted for routine studies. Bone loss is correlated with CAL and so this parameter is generally recorded. Advanced periodontitis is diagnosed if the PD is greater than, or equal to, 6 mm and CAL is greater than or equal to 3 mm (Riviere *et al.*, 1996), although these cut-off levels can be arbitrary. Machtei *et al.* (1992) suggest a diagnosis of advanced periodontitis if CAL is greater than or equal to 6 mm in two or more teeth, and PD greater than or equal to 5 mm in at least one site. Healthy sites are recorded if the GI is less than or equal to 1, and if PD is less than or equal to 3 mm; gingivitis is diagnosed when the GI is greater than or equal to 2, and PD are less than 3 mm (Riviere *et al.*, 1996).

1.2.3 Oral Microbiology

Dental plaque may be defined as bacterial aggregation on the teeth or other solid oral structures (Dawes *et al.*, 1963). Plaque is generally described as being supragingival (*i.e.* deposited on the clinical crowns of teeth), or subgingival (*i.e.* located in the gingival crevice or the periodontal pocket). The latter occurs when a deposit of the former establishes itself in the gingival crevice. Acquisition of pathogenic bacteria and a general increase in the number of bacteria are responsible for gingivitis (Löe *et al.*, 1965). Bacteria initiate an inflammatory response in the gingiva, resulting in oedema, swelling and an increase in gingival fluid. Bacteria can utilise nutrients in this fluid and can thus proliferate. The outcome of the initial bacterial infection is dependent on the oral hygiene practices of the patient, host responses and other factors (Section 1.2.4). The subgingival environment favours anaerobic growth, causing a shift in the microbial population, resulting in the differing compositions of sub- and supragingival plaque.

Periodontal diseases are polyclonal infections, and so microbiological examination of diseases sites can be difficult: sites of disease are not constantly active resulting in the change in range and number of bacteria over time; areas of interest can easily be

contaminated with bacteria from neighbouring lesions, and healthy sites can harbour bacteria in a carrier state to complicate the overall situation.

Methods to characterise and enumerate the subgingival flora include microscopy & culture (Section1.3.1.i), ELISA (Section 1.3.1.iv), DNA probes (Section 1.3.2.iv), PCR (Section 1.3.2.xi.d) and fluorescent *in situ* hybridisation (FISH; Section1.3.2.x). All methods have their advantages and disadvantages and have all been used extensively in clinical studies (Slots, 1986; Dahlén, 1993; Söder *et al.*, 1993; Wolff *et al.*, 1994; Ashimoto *et al.* 1996; Clerehugh *et al.*, 1997; Moter *et al.*, 1998).

1.2.3.i Normal oral flora

At birth, the mouth is probably a sterile environment, or at most contains very few organisms (Sutter, 1984). During the first twenty-four hours, there is a rapid increase in bacterial numbers, including the appearance of the anaerobic bacteria *Veillonella* and *Lactobacillus* species (McCarthy *et al.*, 1965), although in this study, many bacteria may have been lost due to the anaerobic technique employed.

With the eruption of teeth, the flora increases and changes composition. In periodontally healthy individuals, Gram-positive cocci are the major types of bacteria recovered from the gingival crevice and compose almost two thirds of the total flora (Nisengard *et al.*, 1988). Gram-negative rods and spirochaetes can also be present but in much lower numbers. In the study by Socransky *et al.* (1977), the developing flora in the supragingival plaque from the surface of upper first molars was analysed using culture. Samples were taken from the subjects, all of whom had no initial gingivitis, at various time points (from 5 min to 16 days). Anaerobic bacteria (*Peptostreptococcus, Veillonella* and *Actinomyces*) were detected sporadically on different days. As subgingival plaque developed, the number and types of anaerobic bacteria increased, making up to 25 - 68 % of healthy sites. These species included *Bacteroides, Fusobacterium, Peptostreptococcus, Veillonella, Actinomyces, Clostridium* and *Lactobacillus*. Other studies have shown a similar range of bacteria as well as *Treponema* species, *Porphyromonas gingivalis, Prevotella intermedia, Campylobacter rectus* (Dahlén *et al.*, 1992) and *Eikenella corrodens* (Riviere *et al.*, 1996). Other studies have failed to find these potential periodontopathogens in healthy patients (Frisken *et al.*, 1990), even by the sensitive PCR method (Conrads *et*

al., 1996), detecting instead higher levels of *Prevotella nigrescens* and *Capnocytophaga* species.

1.2.3.ii Microbiology of periodontal disease

Over 300 species of bacteria have been isolated and identified from the gingival crevice (Melvin *et al.*, 1994; Wolff *et al.*, 1994), although only a few have been implicated as the primary aetiological agents of periodontitis. Important species are generally anaerobic organisms and have been identified as *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Prevotella nigrescens, Bacteroides forsythus, Campylobacter rectus, Eikenella corrodens, Fusobacterium nucleatum, Peptostreptococcus micros, Selenomonas* spp., *Eubacterium* spp. and various *Treponema* spp. including *denticola, socranskii, pectinovorum* and *vincentii* (Dzink *et al.*, 1988; Loesche *et al.*, 1985; van Winkelhoff & de Graaff, 1991; Dahlén, 1993; Söder *et al.*, 1993; Wolff *et al.*, 1994; van Steenbergen *et al.*, 1996; Papapanou *et al.*, 1997). *Veillonella & Actinomyces* spp. and *Streptococcus oralis* are associated with less active disease, being harboured in sites that respond well to treatment (Socransky *et al.*, 1988b).

Treponema species have been implicated in ANUG (Loesche *et al.*, 1982), as have *Prevotella intermedia* and *Selenomonas & Fusobacterium* species (Moore *et al.*, 1982; Slots & Listgarten, 1988; Papapanou *et al.*, 1993).

Actinobacillus actinomycetemcomitans is found in 85 % of lesions from LJP patients (Zambon, 1985). This species is also associated with Papillon-LeFèvre syndrome (Section 1.2.4), although elevated levels of *Eikenella corrodens, Porphyromonas gingivalis, Prevotella intermedia* and *Fusobacterium nucleatum* have also been recovered from these patients (Clerehugh *et al.*, 1996).

Different clinical symptoms, such as deep pockets and gingival bleeding, have been correlated with the presence of particular species of bacteria, although the studies are not always in agreement. Okuda (1994) record that elevated levels of *Porphyromonas gingivalis, Bacteroides forsythus* and *Treponema denticola* are correlated with the level of bleeding in a patient, whereas Dahlén (1993) note that *Porphyromonas gingivalis* is correlated with pocket depth, and not GI. Renvert *et al.* (1990) found that *Actinobacillus actinomycetemcomitans* was correlated with bleeding, but not deep pockets nor CAL.

The interaction of different bacteria is probably more important than presence of an organism alone in causing disease, with bacterial synergy and antagonism demonstrated for several species. *Treponema denticola* and *Porphyromonas gingivalis* were always found in pockets together (Simonson *et al.*, 1992) and in the study by Socransky *et al.* (1988a), the successful establishment of *Porphyromonas gingivalis* was favoured by the presence of *Actinomyces* species but hampered by the presence of *Streptococcus oralis*. The growth stimulation of *Treponema denticola* by *Prevotella intermedia, Veillonella parvula, Fusobacterium nucleatum* and *Eubacterium nodatum* has been demonstrated by ter Steeg & van der Hoeven (1990). Coaggreggation of bacteria probably enhances metabolic communication among strains aiding the proliferation of both organisms, and has been demonstrated between *Treponema denticola* and *Porphyromonas gingivalis* (Grenier, 1992 a&b), *Treponema* and *Fusobacterium* species (Kolenbrander *et al.*, 1995) and between the commensal organism *Streptococcus crista* and *Bacteroides forsythus* (Yao *et al.*, 1996). The presence of certain bacteria can inhibit the growth of others. *Streptococcus mutans* inhibits *T. denticola; Staphylococcus aureus* inhibits *T. denticola, Porphyromonas gingivalis* and *Prevotella intermedia* (Grenier, 1996); *Actinobacillus actinomycetemcomitans* is inhibited by the presence of *Streptococcus sanguis*, probably by the production of hydrogen peroxide (Hillman *et al.*, 1985).
Microbiological investigation of lesions from refractory patients reveal that the prevalence of *Actinobacillus actinomycetemcomitans, T. denticola, Porphyromonas gingivalis* and *Bacteroides forsythus* is the same in patients who show full recovery and those who are deemed refractory (Colombo *et al.*, 1998). *Prevotella nigrescens* was significantly more prevalent in the successfully treated patients, who also harboured a larger proportion of *Streptococcus constellatus*.

1.2.3.iii *Treponema* species in periodontal disease

Treponemes are not found in infants without erupted teeth (Fiehn, 1989a), but can be found in increased numbers in the gingival crevice of children and adults. In the healthy mouth, the number of treponemes is low and are found on the tonsils and the dorsum of the tongue as well as the gingival crevice. Listgarten & Helldén. (1978) report that *Treponema* species make up to 50 % of the microflora from the pockets of periodontitis patients, but only 2 % of bacteria from healthy sites. The bacteria can

be visualised by phase-contrast or darkfield microscopy (Listgarten & Helldén, 1978) and by electron microscopy (Heylings, 1967). There is a positive correlation between number of treponemes and severity of periodontal disease (Simonson *et al.*, 1988; Riviere *et al.*, 1995), with successful treatment of a site resulting in the reduction in numbers (Loesche *et al.*, 1982). In a study by Macphee & Muir (1986), high numbers of treponemes were found in 8 % of relatively healthy sites sampled, whereas no treponemes at all were found in 18 % of diseased sites. This indicates that there may be species of *Treponema* that are not involved with the disease process, but may be commensal bacteria. Similar figures are quoted by Riviere *et al.* (1995) with 4 - 10 % of healthy sites harbouring *Treponema* species (*denticola, socranskii* and Pathogen-related oral spirochaete - PROS), 20 - 40 % of periodontitis sites and 6 - 20 % of gingivitis sites. PROS (Riviere *et al.*, 1991) do not represent a single species but are probably a heterogeneous group of treponemes of which *T. vincentii* is the only cultivable species (Choi *et al.*, 1996).

There are few studies investigating the species distribution of treponemes. Smibert *et al.* (1984) claim that *T. socranskii* is the treponeme most frequently isolated from diseased sites, whereas Chan *et al.* (1993) make the same claim for *T. denticola.* Riviere *et al.* (1992) claim that PROS are more likely to be present in severe periodontitis than *T. denticola*, and that it is rare to find *denticola* and *socranskii* in the same pocket (Riviere *et al.*, 1995). The presence of many uncultivable treponemes from subgingival plaque has been demonstrated using molecular techniques by Choi *et al.* (1994) and Moter *et al.* (1998), suggesting the most frequently isolated species may be a reflection of the organism's ability to survive *in vitro*, rather than as an indicator of the organism's clinical importance.

1.2.3.iii.a Putative role for *Treponema*

Possible virulence factors have been suggested for *Treponema* but their actual role in the disease process has not been definitively established (Loesche, 1988; Fenno & McBride, 1998). *Treponema* have the ability to adhere to epithelial cells under aerobic and anaerobic conditions (Olsen, 1984; Reijntjens *et al.*, 1986), resulting in morphological damage and detachment of epithelial cells after 24 hours. Endotoxin release, comprising of lipopolysaccharide, may be less potent than that from other bacteria (Merganhagen, 1961), but can still be toxic to host cells or can activate host

immune responses. Immune complexes can then promote tissue damage and exacerbate the disease progression (Chung *et al.*, 1983; Schenkein & Berry, 1991; Lavelle, 1992). The major outer sheath 53 KDa protein (Msp) from *T. denticola* (Egli *et al.*, 1993) has been shown to induce pores in epithelial cells (Mathers *et al.*, 1996). The exact role of these ion channels has not been established for *Treponema,* although for other bacteria these pores in host cell play a part in bacterial nutrient uptake (Jap & Walian, 1990), host cell destruction (Jonas *et al.*, 1994) and movement of bacterial products into host cells (Eriksen *et al.*, 1994).

Enzymes released by *Treponema* can degrade or disrupt surrounding tissues. Phospholipase C production has been demonstrated in oral *Treponema* species and may play a role in hydrolysing the membrane phospholipid of epithelial cells (Siboo *et al.*, 1989). Trypsin, phosphatase and proteolytic enzymes released from *Treponema* may also be involved with host damage (Fiehn, 1989a; Rosen *et al.*, 1995). The metabolic products from *Treponema* can also be toxic to host cells (Mäkinen *et al.*, 1986). These products include ammonia, indole and hydrogen sulphide.

The function of host cells can be disrupted as a result of colonisation by *Treponema* species. *Treponema* species have been shown to inhibit superoxide production in phagocytes (Sela *et al.*, 1988), resulting in the suppression of phagocytosis. The suppression of fibroblast proliferation, leading to the loss of collagen in supportive tissues, has also been documented (Boehringer *et al.*, 1984).

The invasion of epithelial and underlying connective tissue by treponemes in ANUG has been shown using electron microscopy (Listgarten, 1965). The invasive capabilities of *Treponema* species known as PROS have been demonstrated using the penetration of mouse abdominal wall as an *in vitro* model (Riviere *et al.*, 1991). *Treponema* that show this invasive capability share antigens with the human pathogen *Treponema pallidum* subsp. *pallidum*, an organism that has the ability to disseminate through tissue.

The evidence indicates that *Treponema* have the capabilities to cause periodontal diseases, although the pathogenic potential of all the individual species is not clear.

1.2.4 Risk factors for periodontal disease

A significant percentage of the general population is susceptible to periodontal diseases, especially in the absence of oral hygiene measures, but a larger proportion is not susceptible to the severe forms (Genco, 1996). Several phenomena have been postulated as potential risk factors, although the exact mechanisms are not fully understood. An increase in the incidence and severity of periodontal disease is found in older populations when compared to younger patients (Abdellatif & Burt, 1987), however, this is probably due to cumulative gingival tissue destruction over a lifetime, rather than any other age-specific factor (Genco, 1996).

Severe disease is reported more often in male patients than female (Grossi *et al.*, 1995), although the difference is not seen when post-menopausal women are compared with men of a comparable age. If the women receive hormone-replacement therapy, the level of gingival disease is again decreased, suggesting that oestrogen may have a protective effect (Norderyd *et al.*, 1993). During pregnancy, an increase in gingivitis often occurs due to a heightened response to plaque bacteria at periods of imbalance between progesterone and oestrogen levels. This hyper-response can also occur during puberty, menstruation and when using oral contraceptives, again suggesting a role for oestrogen in the prevention of disease. Severe forms of periodontal disease are linked to genetic conditions such as Papillon-LeFèvre, Chediak-Higashi and Down's syndromes (Sofaer, 1990; Dibart, 1997). These conditions are associated with PMNL deficiencies which may explain the increase in disease severity. Diabetes mellitus (both insulin-dependent and insulin-independent) is also linked with destructive periodontal diseases (Oliver & Tervonen, 1994). Mechanisms by which diabetes may contribute to the progression of periodontal disease include thickening of blood vessels in gingival tissues, resulting in inhibition of phagocyte migration (Mowat & Baum, 1971), and alteration of collagen metabolism by gingival and periodontal ligament fibroblasts (Schneir *et al.*, 1981).

Smoking has long been associated with the progression of periodontal diseases (Pindborg, 1947; Solomon *et al.*, 1968). Cigarette smoke, or metabolites of smoke, adversely affect phagocyte function (MacFarlane *et al.*, 1992) and may encourage a different microflora to thrive in the gingival crevice (Zambon *et al.*, 1996). Anxiety and stress can enhance the path ogenesis of periodontal diseases (particularly

ANUG). During periods of stress, host tissue resistance may be altered by mechanisms acting though the nervous system and endocrine glands, so that salivary flow and gingival circulation become affected (Shannon *et al.*, 1969; Manhold *et al.*, 1971). Cortico-steroids associated with stress may provide a nutrient advantage to help establish specific bacteria in subgingival plaque (Loesche *et al.*, 1982).

1.2.5 Treatment of periodontal disease

The control of plaque formation aids in the prevention and treatment of periodontal disease, and thus the primary strategy to encourage health is the promotion of good oral hygiene practices. Mechanical removal of plaque by brushing and flossing can be performed by the patient, or scaling and root planing can be achieved by the dentist. Löe *et al.* (1965) demonstrated that ceasing to brush the teeth for a few weeks resulted in a build-up of supragingival plaque and the development of gingivitis. A resumption of normal oral hygiene measures removed the build-up of plaque and resulted in the restoration of gingival health. In most cases this will be adequate, but in more aggressive cases of periodontal disease, antimicrobial therapy may be appropriate. Tetracycline, clindamycin or metronidazole are useful for conditions such as LJP but are not recommended for long-term plaque control, due to the side-effects of taking medication and the potential problem of encouraging drug resistance. In the most severe cases, periodontal surgery to remove pockets may be necessary, although this is becoming increasingly rare.

1.3 Detection and Identification of bacteria

Identification of bacteria has been defined as 'both the act and the result of determining whether an unknown organism belongs to a previously defined group' (Goodfellow & O'Donnell, 1993). This process of 'diagnostic microbiology' involves determining the relevant characters of the unknown strain and matching these characteristics with those of known organisms, in order to assign names to the unknown strains. Organisms are assigned to a succession of categories of different seniority (domain, kingdom, division, class, order, family, genus, species), of which the species is regarded as the basic rank (Wayne *et al.*, 1987). The arrangement of organisms into such categories is known as 'classification', with the term 'taxonomy' describing the theory of classification and identification.

There is no universally accepted definition of a species in microbiology, despite much discussion on the subject (Wayne *et al.*, 1987; Fox *et al.*, 1992; Goodfellow & O'Donnell, 1993). However, the traditional view is that members of a given 'species' have a combination of characters peculiar to it, and can be distinguished from other organisms on the basis of these characters. Subspecies designations are used for genetically close organisms that diverge in other attributes.

Methods to detect and identify bacteria to both the species and subspecies level can be divided into phenotypic (Section 1.3.1) or genotypic procedures (Section 1.3.2). Phenotypic techniques detect distinguishing characteristics expressed by the organism, such as physiological and biochemical attributes, whereas genotypic techniques detect differences in the nucleic acid content of the organism. Genotypic procedures have the advantage over phenotypic ones as they are not generally influenced by the environmental conditions of the bacteria (except for the loss and gain of extra-chromosomal elements such as plasmids; Section 1.3.2.v).

The differentiation of strains at the subspecies level is known as 'typing'. The purpose of typing systems is often to trace the origin and spread of an organism causing an outbreak of infection (the 'epidemiology' of the infection).

Many typing methods involve 'fingerprinting', whereby a method produces a unique pattern for a given isolate. The fingerprints can be compared and used to differentiate strains and species, and thus such techniques have use as identification methods as well as for epidemiological studies.

For all identification methods, the most important characteristics are the typeability, reproducibility and the discriminatory power of the method (Maslow & Mulligan, 1996; Power, 1996). Typeability refers to the capacity of the method to obtain a definite result for all samples tested. Reproducibility is the capacity to produce the same results, on separate occasions, with the same samples, as well as the attribute's stability over time. The discriminatory power is described as the probability that an identification method will distinguish two strains chosen at random from a population of unrelated strains (Hunter & Gaston, 1988), and can be calculated using Simpson's index of diversity (Simpson, 1949). A discriminatory index (DI) of over 0.9 is recommended as a threshold for declaring a technique an adequate identification method, which means that if two strains are sampled randomly from a population, on over 90 % of occasions, they will fall into different sets. Other considerations to assess the potential utility of characterisation methods are: availability of the method (specialist equipment may be needed), speed, ease of interpretation and cost.

1.3.1 Phenotypic methods

Phenotypic methods characterise the products of gene expression in order to differentiate strains (Tenover *et al.*, 1997). Biochemical profiles and cell characteristics are examples of phenotypic properties that have been used in such a way. These properties are not always stable, as they rely on gene expression, which can be susceptible to changes in growth conditions and growth phase.

1.3.1.i Culture and microscopy

The growth of bacteria *in vitro* can reveal important differences between species. Colony characteristics such as morphology, colour and pattern of growth are first indications that two strains may be distinct. The Gram-stain, developed by Christian Gram in 1884, is an important method to show the presence or absence of the outer membrane in bacteria (Barrar & Feltham, 1993), and has long been used for in initial identification. Cellular characteristics such as size, shape, presence of flagella and motility are relatively easy to determine using light or electron-microscopy, and provide a basis for classifying bacteria, as are physiological characteristics such as

nutritional requirements, growth temperature and growth conditions. Tables 1.1 and 1.2 outline defining characteristics of *Treponema* species.

Microscopy has been used to elucidate the various components of subgingival plaque (Listgarten, 1986; Slots, 1986; Lembariti *et al.*, 1995). Different cell types can be quoted as a percentage of the total cell population, although this method does not allow for speciation.

Bacteria can be cultured from the gingival sulcus by selectively removing subgingival plaque, either with a curette or a paper-point (Dahlén, 1993) and transporting the subgingival plaque to the laboratory in a transport medium that does not encourage bacterial growth, but in which the bacteria can survive (Syed & Loesche, 1972). After vortexing the plaque, in order to disperse any 'clumps', the sample is serially diluted and plated out on differing media, and in various environments, for growth. The bacteria can be identified by biochemical tests (Section 1.3.1.ii) and the cell numbers determined, and quoted as a percentage of total plaque bacteria. The proportions of various periodontal bacteria at diseased sites have been determined, including *Treponema* species, *Porphyromonas gingivalis, Prevotella intermedia, Prevotella nigrescens, Actinobacillus actinomycetemcomitans, Bacteroides forsythus, Fusobacterium nucleatum, Eikenella corrodens* and *Campylobacter rectus* (van Steenbergen *et al.*, 1996; Papapanou *et al.*, 1997).

The disadvantage of relying on culture alone is that not all bacteria are able to be cultured *in vitro* due to fastidious nutritional requirements or the need for specific environmental conditions, resulting in a bias in calculated distributions. This is particularly true for oral *Treponema* species, which are often omitted from studies, despite making up a large proportion of the bacteria at diseased sites (Listgarten & Helldén, 1978).

1.3.1.ii Biochemical properties

Biochemical properties have long been used to identify and speciate bacteria (Krieg & Holt, 1984; Barrar & Feltham, 1993; Busse *et al.*, 1996). The individual tests can be performed separately or commercial kits can be used to test for a whole panel of biochemical phenomena, such as presence of indole and catalase or production of hydrogen sulphide and hydrogen peroxide. Suspensions of the test bacteria are added

to dried substrates in the test strip, which may require the addition of other reagents. Each reaction is scored as positive or negative according to the colour change seen, to produce a coded number. The kits provide a database of identifying codes of known bacteria to compare the unknown isolate, and hopefully provide an identification. Examples of commercial kits are API (bioMèrieux SA, France), RapID (Mercia Diagnostics, Guildford, UK), BBL Crystal (Becton Dickinson Microbiological Systems, Sparks, MD, USA) and the Biolog systems (Biolog, Inc., Hayward, CA, USA). Each system has a different kit for a range of bacteria (*e.g.* for Gram-negative anaerobic bacteria or for enteric organisms) and so other phenotypic tests may have to be performed initially.

The kits are relatively easy to use and can provide an answer within a few hours. However, the reproducibility of the kits can vary with inoculum size, incubation time and incubation temperature (Busse *et al.*, 1996). In addition, the kits are only useful to identify known species, as isolates previously uncharacterised may provide substrate utilisation results similar to those of known strains, to provide misleading results. Bacteria derived from environmental sources can also be misidentified as these strains may have atypical biochemical properties (Busse *et al.*, 1996).

The API-ZYM system has been used to identify species of *Treponema denticola* and *vincentii* (Laughon *et al.*, 1982), and the RapID-ANA system has been shown to differentiate *T. denticola*, *T. vincentii* and *T. socranskii* (Syed *et al.*, 1988) although neither are recognised kits for the detection of oral *Treponema*.

1.3.1.iii Chemotaxonomy

The chemical constituents of bacterial cells can be used to classify and differentiate bacteria. Examples are quinones (Collins & Jones, 1981), lipopolysaccharide (Chart, 1995), diaminoacids (Schleifer & Kandler, 1972) and fatty acids (Drucker, 1995).

Fatty acids are mainly located in cell membranes as components of phospholipids and lipopolysaccharides. Structural differences are found within these molecules consisting of length variation, presence of saturated and unsaturated acids or occurrence of branched fatty acids. The relative amounts and types of fatty acids can be analysed using spectrometry (Drucker, 1995) or chromatography (Brondz & Olsen, 1986), and can be distinct to a particular bacteria genus or species (Heller *et*

al., 1987; Aluyi *et al.*, 1992; Busse *et al.*, 1996). The fatty acid profiles produced can be compared to reference strains in order to speciate and type the organisms. Oral *Treponema* species have been differentiated on the basis of fatty acid composition (Brondz *et al.*, 1991) as have species of *Candida* (Abdi & Drucker, 1996).

Multi-locus enzyme electrophoresis (MLEE) differentiates organisms by analysing the mobilities of numerous metabolic enzymes in starch gels (Boerlin & Piffaretti, 1995). The migration of the protein is a function of its molecular mass, electrical charge and conformation, which in turn are dependent on the amino acid sequences of the proteins. The location of each enzyme is detected by exposing the gel to a substrate that changes colour with that specific enzyme. Differences in mobilities reflect variations in the genetic loci and can be characteristic for a given isolate. The mobilities of proteins are also affected by the pH and composition of the buffer/gel system, and thus different buffers need to be tested in order to establish the most discriminatory system. The method has been used to differentiate strains of oral *Treponema* species (Dahle *et al.*, 1995), intestinal spirochaetes from the genera *Serpulina* (Stanton *et al.*, 1996) and *Prevotella* (Pearce *et al.*, 1996), as well as the subspecies discrimination of strains of *E. coli* (Selander & Levin, 1980).

Polyacrylamide gel electrophoresis (PAGE) of proteins extracted from bacterial samples can reveal important relationships between different isolates. The method of sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970), is a method in which anionic SDS binds to the proteins of the cell in proportion to the size of the proteins. As SDS is a detergent , the proteins become rods of negative charge with equal charge densities. The subsequent migration in the polyacrylamide is thus inversely proportional to the size of the proteins, although the running conditions of the gels and isolation of the proteins needs to be carefully standardised for reproducible results. The patterns of proteins can be compared for two strains, with differing strains producing different patterns. The technique is widely used in bacterial systematics (Kersters & DeLey, 1975; Moore *et al.*, 1980; Fotos *et al.*, 1984), and has been used to differentiate oral *Treponema* species (Tall & Nauman, 1986). The method is often used as a first stage in Western blotting (Section 1.3.1.iv).

The total cell constituents (including nucleic acids) can be analysed using the technique of pyrolysis mass spectrometry (PyMS). The organic matter is heated rapidly in a vacuum, to vaporise the sample. The vapour is ionised as it passes across a beam of low energy electrons and analysed quantitatively by mass spectrometry, to reveal a bacterial fingerprint which can identify and type the organism (Freeman *et al.*, 1995). *Streptococcus pyogenes* and *Staphylococcus aureus* have been typed using this method (Magee *et al.*, 1989; Gould *et al.*, 1991).

Additional methods that utilise the whole cell properties of bacteria include antibiotic resistance profiling (Hammonds, 1995) and bacteriophage typing (Pitt & Gaston, 1995). The latter technique takes advantage of the natural ability of particular viruses (known as bacteriophages) to infect bacteria. The susceptibility to infection by certain 'phages varies between strains of a species, and thus the technique can be highly discriminatory. *Staphylococcus aureus* and *Salmonella typhi* have been typed using this method (Williams & Rippon, 1952; Anderson & Williams, 1956), although problems with reproducibility and the stability of the technique over time are major limitations.

Antibiotic resistance profiling involves determining the relative sensitivities of strains of bacteria to a range of antibiotics, to obtain a pattern of sensitivities known as an 'antibiogram'. The technique can have poor discrimination due to unrelated isolates acquiring the same plasmid during an outbreak, and the resistance profiles can change over time due to the loss or gain of plasmids and other mobile genetic elements.

1.3.1.iv Immunological methods

Serotyping, whereby antibodies selectively recognise antigenic determinants on cell surfaces, has been used to identify bacteria for over ninety years (Bowden, 1993), with various methods existing to detect the antibody-antigen interaction.

The simplest methods involve agglutination directly visualised on a microscope slide. Antisera are mixed with cell cultures and 'clumping' is seen when the antisera and antigens are matched. This method can be used as an initial screening technique, or by using antibodies raised to distinct antigenic determinants, can be made more

specific and can be used to differentiate between strains (Delmee & Avesani, 1990; Putnins & Bowden, 1993). The subspecies of *T. socranskii* (subsp. *buccale, socranskii* and *paredis*) were defined in this way (Smibert *et al.*, 1984).

Precipitin tests utilise the ability of antibodies to precipitate soluble antigens. The method is usually carried out in agar gels, where the cellular extract and antibodies are allowed to diffuse towards each other from adjacent wells. A precipitate is seen at the point where the proportions of reactants are optimal, and thus the number and presence of antigens in different extracts can be determined and compared. *Bacteroides* species were compared and differentiated using this method (Okuda *et al.*, 1986).

In an extension of this technique, the bacterial extract can be subjected to electrophoresis in one direction, followed by electrophoresis at right angles into gel which contains antibody (crossed immunoelectrophoresis). Different antigens in the extract will produce arcs of precipitation within the gel, yielding distinct patterns for different bacteria. Isolates of *Porphyromonas gingivalis* have been differentiated using this method (Parent *et al.*, 1986), although as the technique can be difficult to interpret, it is not often used for routine identification.

Immunoblotting (or 'Western blotting'; Renart *et al.*, 1979) provides a method of identifying bacteria and demonstrating antigenic relatedness between different organisms. Following PAGE, cellular materials can be transferred to nylon or nitro-cellulose membranes. The membrane is then probed with a specific antibody, and then subsequently incubated with an enzyme-labelled secondary antibody, which recognises the first antibody. The substrate for the enzyme is added, producing a colour-change reaction, so that coloured bands representing the antigens recognised by the primary antibody can be seen. The technique allows more differentiation than SDS-PAGE on its own. Different bacteria can have different patterns of proteins on the membrane, enabling the method to be used for the identification and epidemiological study of bacteria. *Clostridium difficile* (Poxton *et al.*, 1984), *Campylobacter (Helicobacter) pylori* (Burnie *et al.*, 1988), *Staphylococcus aureus* (Thompson-Carter & Pennington, 1989), black-pigmented oral bacteria (Bowden & Nolette, 1990), oral isolates of *Actinomyces* species (Putnins & Bowden, 1993) and

Treponema species (Tall & Nauman, 1994; Sela *et al.*, 1997) have been characterised in this way.

The technique of ELISA (enzyme-linked immunosorbent assay; Engvall & Perlmann, 1972; van Weemen & Schuurs, 1972) relies on the addition of two antibodies to detect specific antigens on bacterial cell surfaces, in the same manner as immunoblotting. The primary antibody is coated to the wells of microtitre plates, followed by the addition of the bacterial sample, the secondary antibody and finally the enzyme substrate. The subsequent colour-change for a positive reaction can be measured by eye or by using a spectrophotometer, and the results for different bacteria can be compared. Commercial kits are available to detect food bacterial pathogens such as *Salmonella* and *Listeria* (Robison, 1995). *Treponema* species have been detected in periodontal pockets using this method (Wilson *et al.*, 1993), as have *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Prevotella intermedia* (Clerehugh *et al.*, 1997).

Organisms can be directly detected in clinical samples by tagging bacteria with antibodies that have been fluorescently-labelled. Fluorochromes such as rhodamine or fluoroscein can be visualised by epifluorescent microscopy. Species of *Actinomyces* and strains of *Prevotella intermedia* have been detected by this method (Firtel & Fillery, 1988).

1.3.2 Genotypic methods

Differences in the genomic content of asexually reproducing organisms occur by various mechanisms. DNA recombination (Sigal & Alberts, 1972), mobile elements such as transposons (Kleckner, 1981) and misincorporation of bases during DNA replication due to DNA polymerase error (Loeb & Kunkel, 1982), can all effect the nucleic acid content of the organism. These genetic differences can be detected by genotypic methods, which for fastidious, anaerobic or slow-growing organisms can be of more value than phenotypic techniques.

1.3.2.i % G+C content

The duplex nature of DNA and hydrogen bonding between the bases guanine (G) and cytosine (C), and between adenine (A) and thymine (T) ensures that there are equivalent amounts of G+C and A+T present in bacterial cells. The mol % content of G+C can be calculated for a species of bacteria (Austin & Priest, 1986), which generally ranges from 25 - 75 %. Beyond these limits, the skewed genetic code would exhibit scant coding regions to produce few sensible proteins (Austin & Priest, 1986). Bacteria that are genetically and biologically unrelated can have very similar % G+C values and little can be inferred in cases such as these. However, if two organisms have widely different mol % G+C compositions, they can have few sequences in common, and will therefore be distantly related. The % G+C value is thus a negative criterion on which to distinguish two organisms, with differences in base composition reflecting differences in sequences, and hence dissimilar organisms. The % G+C values for *Treponema* species varies from 36 - 54 % (Section 1.1, Table 1.1).

1.3.2.ii DNA homology

Single-stranded DNA molecules can form duplexes by the formation of hydrogen bonds between the amino and carboxyl groups of their complementary bases. The pairing is typically between guanine-cytosine and adenine-thymine, although other non-canonical pairing is thermodynamically permissible (Watson *et al.*, 1987). The duplex molecules can be separated into single strands by chemical or physical means, and under the appropriate conditions can reassociate to form the original duplex. The usefulness of this DNA reassociation as a means to identify complementary

sequences has been recognised for nearly forty years (Marmur & Lane, 1960) and has often been quoted as the 'gold-standard' to determine the genetic relatedness of organisms (Stackebrandt & Goebel, 1994). Estimation of similarity between two organisms is achieved by denaturing the DNA from one organism to single strands and allowing reassociation in the presence of a second denatured molecule. If the base sequences of both DNA molecules are homologous, a duplex will be formed. If the two sequences share few common nucleotides, there will be negligible duplex formation. An important consideration in DNA homology studies is the concept of the 'melting point' (T_m) of the duplex. The T_m is defined as the temperature corresponding to the mid-point in the transition from double helix to random coil, and is dependent on various factors such as length and base composition of the duplex, and the ionic strength of the solution. The T_m is decreased by 1 - 2.2 % for each percent mis-pairing in the duplex (Bautz & Bautz, 1964), and thus similarity values can be calculated for pairs of organisms. Other methods are available to calculate the complementarity of duplex molecules (Austin & Priest, 1986): single-stranded DNA is labelled radioactively and allowed to reassociate with other single-stranded DNA. S1 nuclease hydrolyses single-stranded DNA, leaving intact double-stranded DNA to be precipitated, and the radioactivity counted. A second method follows the shift in absorbance of UV light at 260 nm. Double-stranded DNA absorbs less light than single-stranded DNA, and thus the relative rate of reassociation between two DNA molecules can be estimated.

If two organisms show high DNA homology, this does not necessarily imply high sequence identity, although the converse is true, whereby very similar sequences will have a high DNA homology. As with % G+C content, low DNA homology is indicative of low sequence similarity, and thus suggests two organisms are not related.

Disadvantages of DNA homology estimations are that the techniques are laborious, slow and there is a need for careful standardisation to give reproducible results.

1.3.2.iii 16S rRNA gene sequencing

The ribosomal structures present in all cells are an integral part of the protein translation system, and thus the genes encoding these structures are present in all organisms. The major structural rRNA of the ribosomal small subunit in bacteria has

an average sedimentation coefficient of 16 Svedberg units and can be sequenced directly using reverse transcriptase, or the DNA sequence of the gene can be obtained (Lane *et al.*, 1985). The 16S rRNA gene consists of approximately 1500 bases and is highly conserved amongst all organisms (Amann *et al.*, 1995). The gene is not uniformly conserved, with several hypervariable regions being interspersed with the highly conserved domains. These variable regions are useful to distinguish unrelated organisms and for the design of specific oligonucleotide probes (Section 1.3.2.iv.c).
For many organisms, the complete nucleotide sequence of the 16S rRNA gene has been determined, and the sequences have been deposited in databases such as the EMBL DNA database. These can be accessed via remote computer, to enable any user to compare 16S rDNA sequences of any organisms.
16S rDNA sequence analysis is less discriminatory than DNA-DNA homology studies, but it is a lot quicker and easier to perform and the results are often enough to distinguish related organisms. If two organisms have 100 % identical 16S rDNA sequences, then the strains will almost certainly have a high genomic DNA similarity and are thus the same species. If two organisms show little similarity in the 16S rRNA gene, then the organisms are distinct species and show low genomic DNA homology. However, if the two sequences differ in only a few nucleotides, the variation in genomic DNA homology can range from as little as 23 % to almost 100 % (Fox *et al.*, 1992). The concept of an 'rRNA species complex' has been coined by Fox *et al.* (1992) to describe such species. The similarity of 16S rDNA sequences in isolation is therefore not a guarantee of species identity and other factors such as morphological or phenotypic similarities need to be examined.

The conserved and ubiquitous nature of the 16S rRNA gene, with its interspersed hypervariable regions, makes the gene an ideal candidate to study the evolution of organisms (Ludwig & Schleiffer, 1994).
The differing methods of phylogenetic analysis are discussed in Section 1.3.3.

1.3.2.iv. DNA Probes

DNA probes exploit the ability of DNA from heterogeneous sources to form duplexes. Probes are single-stranded DNA elements and can hybridise with complementary sequences in a target sample, in order to detect that sequence.

Several different types of probe have been described which have differing specificities and sensitivities: whole genomic, cloned genomic and oligonucleotide probes (Sections 1.3.2.iv.a,b,c). Probes can either be used to detect organisms directly (Section 1.3.2.ix) or can be used for comparative typing in epidemiological studies (1.3.2.vii).

A successful hybridisation between the probe and the target sequence is indicated by a signal generated by reporter molecules incorporated into the DNA probe. The signal can be generated by one of two methods: either the label is attached directly to the probe via a covalent bond, or a reporter group can be attached to the probe which is detected with a labelled protein (Matthews & Kricka, 1988).

The use of radioisotopes (such as the incorporation of ^{32}P in the phosphate backbone of the DNA) for directly labelling probes has traditionally been the method of choice (Stahl & Amann, 1991). However, problems with the safety and disposal of radioisotopes, as well as the expense and inconvenience of the short shelf-life of labelled probes (the half-life of ^{32}P is 14.3 days) has led to the development of safer, more stable, non-radioactive probes such as biotin or digoxigenin (DIG).

Cloned genomic probes can have DIG incorporated during PCR (polymerase chain reaction, Section 1.3.2.xi), whereas oligonucleotide probes can be specifically labelled by the addition of DIG to the 3' or 5' end of the molecule. The labelled probe is detected by the hybridisation of an anti-DIG antibody-alkaline phosphatase conjugate, followed by the addition of a chemiluminescent substrate. The chemiluminescent signal is detected on X-ray film for a permanent record.

1.3.2.iv.a Whole genomic DNA probes

Whole genomic DNA probes are the easiest and cheapest probes to construct, and are useful to detect organisms whose genetics are not well described. The probes are constructed from chromosomal DNA extracted from bacterial cells and then labelled. The probe can then be used in a variety of techniques such as restriction fragment length polymorphism analysis (Section 1.3.2.vii) or in dot-blot hybridisations (Section 1.3.2.ix). Disadvantages in using this type of probe are the lack of specificity exhibited, and that the DNA extraction to produce the probe can be of variable quality and quantity causing difficulties with standardising the labelling procedure.

1.3.2.iv.b Cloned genomic DNA probes

These types of probes are much smaller than whole genomic probes but can be as sensitive and show more specificity (French *et al.*, 1986). The probes can be generated randomly from a restriction digest of chromosomal DNA (Section 1.3.2.vi) or can be specific gene sequences. The probes are generally constructed by ligating the fragments into plasmid vectors, which are then transformed into competent *E. coli* cells. Plasmid DNA is extracted from the transformants and the insert DNA fragments isolated, labelled and used as DNA probes as described in Section 1.3.2.iv.a. The specificity of the randomly-generated probes needs to be tested very carefully as the random fragments could be conserved elements. The reaction kinetics of these probes allow shorter hybridisation times than whole chromosomal probes.

1.3.2.iv.c Oligonucleotide DNA probes

Much shorter DNA probes (12 - 50 bp) can be easily and cheaply manufactured that allow even shorter hybridisation times than cloned genomic probes (2 - 3 h versus 12 h). The shorter probes can be reproducibly labelled, the labelled probes are stable in storage and have greatly increased specificity. The sensitivity of the probe may be less than that of larger probes, but this can be increased by directing the probes towards the 16S rRNA gene (Section 1.3.2.iii), and targeting cellular rRNA as well as DNA. The presence of multiple rRNA copies of the gene sequence within cells can amplify the signal of the probe. However, this can make the estimation of the number of target organisms difficult, as the amount of rRNA can vary 1000-fold from cell to cell (Savitt *et al.*, 1990).

General bacterial probes can be directed towards the conserved regions of the 16S rRNA gene, whereas species-specific probes can be directed towards the variable regions. Putative probe sequences (usually between 15 - 30 bp) can be compared with *all* sequences, not just 16S rDNA, recorded in the public domain EMBL DNA database, using computer software such as 'FASTA' (Wisconsin Package, Version 8.1-UNIX, Genetics Computer Group, Madison, Wisconsin, USA). In this way, any potential probe sequences that are coincidentally identical to other DNA sequences can be discounted from further use.

The % G+C content will affect the annealing temperature (T_m) of the probe, where T_m is the temperature at which half the probe is annealed to the target sequence, and can be estimated from the following equation:

(Equation 1.1) $T_m = [2°C \times (\text{no. of A+T residues}) + 4°C \times (\text{no. of G+C residues})]$

Probes with a high % G+C content should be chosen if at all possible in order to increase the annealing temperature. The higher the annealing temperature, the less likely non-specific or non-canonical base-pairing is to occur, and thus the specificity of the hybridisation is increased.

These measures ensure that the manufactured probe (in theory at least) will be as specific and sensitive as possible.

1.3.2.v Plasmid analysis

Plasmids are self-replicating, extrachromosomal DNA molecules that can be extracted from bacteria and separated by electrophoresis in agarose gels. The negatively-charged DNA moves through the gel matrix towards the anode, with shorter, lighter fragments moving more quickly through the gel than longer, heavier DNA. The gel is stained with ethidium bromide, to visualise the DNA under UV light, and can be photographed. The plasmid profile is determined by the number and size of plasmids present, to yield a distinguishing pattern for a given bacterial species. However, not all species contain plasmids, and those that do can lose or gain plasmids over time, to give poorly reproducible profiles (Maslow & Mulligan, 1996).

Plasmids have been found in *Treponema denticola* strains (Ivic *et al.*, 1991) that are homologous to plasmids found in Gram-positive bacteria (MacDougall *et al.*, 1992). No plasmids have been isolated from other species, and strains of *T. denticola* that do not have plasmids have also been identified (Reedy *et al.*, 1994; Caudry *et al.*, 1995). These plasmid-free strains have no obvious morphological, antigenic or genetic differences to plasmid-carrying strains, thus plasmid-profiling is probably not a useful technique to identify *Treponema* species.

1.3.2.vi Restriction Endonuclease analysis (REA)

Restriction endonuclease enzymes are found naturally in many bacteria and function to protect the bacteria from invading foreign DNA (Smith & Wilcox, 1970; Roberts, 1983). Each enzyme cleaves at a particular point in a DNA sequence (usually a four or six nucleotide sequence) to create either two blunt-ended DNA fragments or two fragments with complementary overhangs. The frequency with which the restriction enzymes will cleave genomic DNA depends on the frequency of the signature cutting sites.

REA utilises restriction endonuclease enzymes to cleave extracted genomic DNA, either in combination or singularly, to produce few to many fragments of different sizes. The cleaved DNA is then subjected to electrophoresis in agarose gels. There will be differences between two isolates in the positions at which the restriction enzymes will cut the DNA, reflected in the differing sizes and lengths of DNA fragments in the gel. This is known as a 'DNA fingerprint', and forms the basis of many molecular identification methods.

REA has been used to characterise isolates of *Porphyromonas gingivalis* obtained from patients with periodontitis and root-canal infections (Loos *et al.*, 1990). The study found that 33 different isolates of *Porphyromonas gingivalis* produced 29 different fingerprint patterns. The patterns consisted of many closely running bands that were difficult to interpret, other than to comment that two isolates were visually distinct. Other groups have used REA to characterise oral bacterial isolates including *Porphyromonas gingivalis* & *Actinobacillus actinomycetemcomitans* (Petit *et al.*, 1993a), *Actinomyces* species (Barsotti *et al.*, 1993) and *Porphyromonas endodontalis* (Petit *et al.*, 1993b). In addition, REA has been used to distinguish isolates of *Serpulina (Treponema) hyodysenteriae* (ter Huurne *et al.*, 1992). In all these studies, the band profiles were very reproducible, but the complicated patterns made the interpretation of the results difficult. Choice of restriction enzyme obviously can affect the fingerprint patterns, with regard to the number and lengths of fragments. Forbes *et al.* (1991) developed a rigorous selection method for the choice of restriction enzymes, based on a statistical analysis of cutting frequency and the genome in question; however, the fingerprint patterns are still too complicated for easy analysis. As an additional problem, the presence of plasmids in various strains of bacteria, that can easily be lost with repeated subculture, can complicate the

chromosomal fingerprint patterns. The use of REA as a characterisation method is thus limited, with the discriminatory power often too great to identify at the species level. The method is perhaps a better epidemiological tool to differentiate individual strains of the same species. Refinements to the method have been developed which have led to simplified fingerprints for analysis. These methods include Restriction Fragment Length Polymorphism analysis (RFLP; Section 1.3.2.vii), Pulsed-Field Gel Electrophoresis (PFGE; Section 1.3.2.viii) and Polymerase Chain Reaction (PCR) methods (Section 1.3.2.xi).

1.3.2.vii Restriction Fragment Length Polymorphism analysis (RFLP)

RFLP analysis is an extension of REA. The genomic DNA is digested by restriction enzymes and separated by electrophoresis as previously described. The fragments are then transferred and immobilised onto a membrane, using the method of Southern blotting (Southern, 1975) as modified for nylon membranes (Stahl & Amann, 1991). The membrane is then incubated with a labelled DNA probe (Section 1.3.2.iv), in order for the probe to hybridise to any complementary sequences in the DNA fragments on the membrane. The bound probes will highlight the differences ('polymorphisms') in the DNA fragment patterns, and these patterns can be compared. The band patterns seen in RFLP analysis are much simpler than those seen after REA, and thus have more utility as an identification and typing method.

The probes can be cloned genomic probes such as toxin or flagellar protein genes (Pappenheimer & Murphy, 1983; ter Huurne *et al.*, 1992), or can be generated randomly from chromosomal digests of DNA. This latter type of probe has been used to distinguish oral isolates of *Treponema* (DiRienzo *et al.*, 1991) and *Actinobacillus actinomycetemcomitans* (DiRienzo *et al.*, 1990).

The use of 16S rDNA sequences (Section 1.3.2.iii) from *E. coli* as universal probes is known as ribotyping (Grimont & Grimont, 1986) and has been used successfully for *Treponema* isolates to differentiate strains of *Treponema denticola* and *T. socranskii* (Fiehn *et al.*, 1995).

The disadvantages of this technique are the requirement for relatively large quantities of DNA (in the order of micrograms), compared to the smaller amounts needed for PCR methods (Section 1.3.2.xi), and the method is more time-consuming than REA and PCR methods due to the additional Southern blotting and hybridisation steps.

1.3.2.viii Pulsed-Field Gel Electrophoresis (PFGE)

PFGE (Smith & Cantor, 1987) allows the separation of very large DNA fragments in an agarose gel (from 10 kb to more than 1.5 Mb), in contrast to standard electrophoresis which can only separate fragments less than approximately 20 kb. The technique applies an electric field alternately in two directions. The DNA must change conformation and reorient itself before it can move in the direction of the second current. The time required for this reorientation is proportional to the molecular weight of the DNA, and thus larger molecules will take longer to move through the gel. The technique has been used to differentiate strains of methicillin-resistant *Staphylococcus aureus* (Bannerman *et al.*, 1995), *Listeria monocytogenes* (Destro *et al.*, 1996) and *Clostridium difficile* (Chachaty *et al.*, 1994). The technique offers good discrimination and is reproducible, but is time-consuming, technically demanding and relatively expensive to perform, requiring specialist equipment.

1.3.2.ix Dot-blot hybridisation

This technique is less complex than those methods requiring digestion and electrophoresis of DNA; instead the probe is able to hybridise with the target sample directly. This is not a fingerprint method of detection as the labelled probe will produce a positive or negative result. The samples to be detected can either be extracted chromosomal DNA, whole cells or clinical specimens, which are immobilised on nylon membranes by irradiating with UV light for 1 min or by baking at high temperature (120°C) for 1 h (Tenover, 1988). Cell samples and clinical specimens either need to be lysed to release the DNA or need to be treated in order to make the cells permeable to the probe (Tenover, 1988).

The labelled probe is incubated with the membrane either overnight (if a longer cloned DNA probe is used) or for 2 - 6 h (oligonucleotide probe). After hybridisation, any unbound probe is washed off, and by a series of stringent washes, any non-specifically bound probe can be removed. The presence of bound labelled probe is detected depending on the type of label used (Section 1.3.2.iv).

This method has been successfully used to detect many different oral micro-organisms with various types of DNA probe. Whole chromosomal probes (Section

1.3.2.iv.a) have been developed to detect species of *Bacteroides* (Roberts *et al.*, 1987; Smith *et al.*, 1989; Tay *et al.*, 1992) and *Actinobacillus actinomycetemcomitans* (Smith *et al.*, 1989; Savitt *et al.*, 1990). All studies could specifically detect in the range of 10^3 - 10^4 organisms, although only the study by Roberts *et al.* did not report slight problems with cross-reactivity to other species. Socransky *et al.* (1994) describe a method whereby plaque samples are subjected to DNA hybridisation with numerous whole genomic probes, to species such as *Treponema denticola*, *Fusobacterium nucleatum* and *Prevotella nigrescens,* in addition to those species already mentioned. This 'Checkerboard' method of dot-blot hybridisation allows for the quick processing of large numbers of plaque samples, with a detection limit of 10^3 bacteria.

Randomly-cloned gene probes (Section 1.3.2.iv.b) to the intestinal spirochaete *Serpulina (Treponema) hyodysenteriae* (Sotiropoulos *et al.*, 1993) were able to detect approximately 10^3 - 10^4 bacteria, although this type of probe proved to be less sensitive when detecting *Treponema socranskii* and *Treponema denticola* (DiRienzo *et al.*, 1991), being able to detect 2×10^5 organisms. Wong *et al.* (1996) compared the use of randomly-cloned probes with whole genomic probes to detect species of *Porphyromonas gingivalis* and *Bacteroides forsythus* in a dot-blot hybridisation. The genomic probes were potentially more sensitive, but the randomly-cloned probes were much more specific, exhibiting less cross-reactivity to other organisms.

Oligonucleotide probes are the most specific type of probe and offer several advantages over other probes (Section 1.3.2.iv.c). Many oligonucleotide probes, corresponding to variable regions of the 16S rRNA gene have been developed to detect oral bacteria, including *Actinobacillus actinomycetemcomitans*, *Bacteroides* species, *Fusobacterium nucleatum, Porphyromonas gingivalis, Prevotella intermedia* and *Prevotella nigrescens* (Chuba *et al.*, 1988; Dix *et al.*, 1990; Conrads & Brauner, 1993; Conrads *et al.*, 1997). The level of detection has been quoted from 10^3 - 10^6 organisms with excellent specificity.

1.3.2.x Fluorescent *in situ* hybridisation (FISH)

FISH can be used to visualise specific bacterial cells by microscopy (Amann & Ludwig, 1994). In this technique, cultures or plaque samples are fixed to a microscope slide in such a way that the cell structures are maintained, but are permeable to oligonucleotide (Stahl & Amann, 1993). Probes are labelled with fluorochromes such as fluoroscein or rhodamine (Amann & Ludwig, 1994) and hybridised to the fixed cells in a sealed moisture chamber, at the required temperature. The probes can migrate through the cell walls into the cell and can hybridise with the DNA, or if directed towards ribosomal sequences, can additionally bind to rRNA. The slides can be viewed using an epifluorescent microscope, which enables the fluorochromes to be seen. Differently-coloured probes can be used to visualise several bacteria on one slide. For slow-growing organisms, the amount of rRNA in a cell may be quite low (Choi *et al.*, 1994), leading to problems with sensitivity. However, this technique has been used to visualise different species of *Treponema* from biopsy material taken from digital dermatitis lesions of cattle (Choi *et al.*, 1997), and has recently been successful in detecting both cultivable and uncultivable species of *Treponema* in plaque samples from patients with early-onset periodontitis (Moter *et al.*, 1998).

1.3.2.xi Polymerase Chain Reaction (PCR) -based methods

PCR is a means to synthesise ('amplify') millions of copies of a specific DNA segment ('DNA template'), using DNA polymerase enzymes and is now an established laboratory procedure (Mullis & Faloona, 1987). The DNA segment to be amplified is mixed with a DNA polymerase, dNTPs, and two oligonucleotide primers which are complementary to the sequences on either side of the template DNA. The DNA polymerase widely used today is a commercially available, heat-stable enzyme, purified from the thermophilic bacterium *Thermus aquaticus* (*Taq* polymerase), which is capable of withstanding repeated cycles of high temperature needed in PCR. A typical PCR reaction requires approximately 30 - 35 cycles of amplification, where each cycle consists of a heat denaturation step, in which double-stranded DNA is melted into single strands; an annealing step, in which the primers bind to the complementary strands in the template; and an extension step, in which DNA synthesis proceeds from the primers along each strand of the template by the action

of the *Taq* polymerase. After each amplification cycle, the newly synthesised DNA acts as template in subsequent cycles, resulting in the selective amplification of the target sequence. After 30 such cycles, a single copy of the template DNA can be amplified into millions of copies.

Each step in the PCR is usually held for between 30 s - 1 min. The denaturation step is at a temperature between 93 - 95°C and the extension step is at 72°C (the temperature at which the *Taq* polymerase efficiently synthesises DNA). The temperature of the annealing step is dependent on the primer sequences and is an important consideration for optimising the specificity of the reaction. The temperature is usually between 30 - 65°C and is calculated from Equation 1.1 (Section 1.3.2.iv.c) in the same way as annealing temperatures for oligonucleotide probes are calculated.

PCR has been used to create DNA fingerprints to identify species (Sections 1.3.2.xi.a, b & c). and to detect organisms directly in clinical samples (Section 1.3.2.xi.d).

1.3.2.xi.a PCR-RFLP

The innovation of PCR in the laboratory has improved existing DNA fingerprinting techniques such as REA (Section 1.3.2.vi) and RFLP/ribotyping (Section 1.3.2.vii). These methods involved restriction endonuclease digests of chromosomal DNA, leading to a DNA fingerprint of many bands. In order to decrease the number of bands, PCR can be used to amplify known sequences from the genome of the target organism, subjecting the amplified fragment to a restriction digest to create a fingerprint and then comparing the fingerprints from different strains. Various sequences can be the targets for PCR-RFLP, *e.g.* flagellin gene sequences (Nachamkin *et al.*, 1993) and genes encoding surface antigens (Regenery *et al.*, 1991).

There are several advantages over conventional RFLP: smaller quantities of genomic DNA are required (nanograms rather than micrograms) and less complicated fingerprints are produced, which may remove the requirement for a Southern blot followed by hybridisation with a labelled probe. As a result, the technique is significantly faster to perform than RFLP.

Polymorphisms in the intergenic spacer regions in ribosomal operons can be detected by PCR, a method that has been called PCR-ribotyping (Kostman *et al.*, 1992; Jordens & Leaves, 1997). The method can be modified if polymorphisms are not apparent, by performing a restriction digest of the PCR products. The method is very reproducible and easy to perform, but is not applicable for all organisms. Jordens & Leaves (1997) found that the method was not discriminatory enough for isolates of *Haemophilus*, although standard ribotyping was adequate.

1.3.2.xi.b Random amplification of polymorphic DNA by polymerase chain reaction (RAPD-PCR)

The technique of RAPD-PCR or AP-PCR (arbitrarily primed PCR) was described by two research groups in 1990 (Welsh & McClelland, 1990; Williams *et al.*, 1990). Various other names have been coined to describe the technique, including DAF (DNA amplification fingerprinting; Bassam *et al.*, 1992) and AFLP (amplified fragment length polymorphism; Power, 1996; but see also Section 1.3.2.xi.c.), although the fundamentals of the method are the same in all studies. The technique differs from standard PCR in that only a single oligonucleotide primer of arbitrary sequence is used to amplify template DNA, instead of two specific primers. Prior knowledge of the template DNA sequence is therefore not required. The annealing temperature in the PCR is lower than in standard PCR to allow the primer to anneal to sites in the DNA template that are not exactly complementary ('mismatch' annealing). After several rounds of amplification, fragments of varying sizes are generated and are separated by electrophoresis in agarose gels as with other PCR methods. The resultant fragment band patterns form a fingerprint which can be unique to the original genome. Several groups have used this technique to differentiate organisms as diverse as mammals (Welsh *et al.*, 1991), plants (Chalmers *et al.*, 1992; Castiglione *et al.*, 1993), bacteria (Ménard et al 1992; Ralph *et al.*, 1993), fungi (Bidochka *et al.*, 1994; Anderson *et al.*, 1996) and parasites (Bandi *et al.*, 1995; Gomes *et al.*, 1995). The technique is highly discriminatory, being able to differentiate strains other techniques cannot (Wachira *et al.*, 1995; Hilton *et al.*,1997) and is gaining prominence as a method to investigate epidemiology (Giesendorf *et al.*, 1994). RAPD has been used to identify and characterise intestinal spirochaetes (Dugourd *et al.*, 1996) but not, as yet, oral *Treponema*.

The criteria of typeability and reproducibility can be generally met with RAPD by choosing suitable primers and taking care with the preparation of the PCR. The discriminatory index (DI) can be used to compare molecular methods to select the most discriminatory system. RAPD has been compared with many other traditional and molecular techniques in order to determine its potential use as a means to characterise bacteria. Hilton *et al.* (1997) calculated a DI of 0.999 from the RAPD data from isolates of *Campylobacter*. Bandi *et al.* (1995) compared RAPD with allozyme analysis of 8 taxa of *Trichinella*. There were only minor differences in clustering but these differences were not supported statistically. Clemons *et al.* (1997) compared RAPD with REA & PFGE of isolates of *Candida albicans*. The respective DIs calculated for each method were 0.978, 0.954 and 0.715, demonstrating that RAPD is more discriminatory than the two other methods. However, the PFGE data were easier to interpret, being analysed visually rather than with computer software. REA was also compared with RAPD for isolates of *Leptospira* (Corney *et al.*, 1993). RAPD was useful in typing these isolates down to the level of serovar and subtype and the patterns were judged to be easier to interpret than REA. Chachaty *et al.* (1994) compared the techniques of RAPD, PFGE and ribotyping of isolates of *Clostridium difficile*. The three methods were in good agreement with each other. DIs were 0.986 (RAPD), 0.984 (PFGE) and 0.931 (ribotyping). PFGE was judged to be the most time-consuming assay but yielded patterns that were easier to analyse than RAPD. Brown & Levett, (1997) compared RAPD with PCR-REA and with Low-Stringency (LS)-PCR (Section 1.3.2.xi.c) with isolates of *Leptospira*. The DIs calculated were 0.77 (PCR-REA), 0.986 (RAPD) and 0.956 (LS-PCR). All methods showed good agreement with each other in typing the isolates. Multilocus enzyme electrophoresis (MLEE) (Wang *et al.*, 1993) was concluded to be less sensitive than RAPD in distinguishing isolates of *E. coli*.

Several methods of interpreting RAPD patterns have been used. Corney *et al.*, (1997) compared the patterns by eye and placed type strains from 45 serovars of *Leptospira* species in 8 'RAPD similarity groups'. Mondon (1995) tested several primers in order to find one that would amplify a single discriminatory band. The band patterns were not very similar, but the presence of one particular band enabled

different strains to be distinguished. Other groups have found the band patterns too complex for direct analysis and have opted for a band-matching method where reproducible bands are scored as present or absent between pairs of isolates. Demeke *et al.*, (1992) scored for intensity of bands in addition to presence/absence.

Many different similarity coefficients have been calculated and the choice as to which one to use is subjective, with many being demonstrated to be useful in bacterial taxonomy (Austin & Colwell, 1977). The choice is often confined to those which can be calculated easily with given computer software. After creating similarity coefficients, the data can be subjected to cluster analysis (Virk *et al.*, 1995) or Principal Co-ordinate Ordination (PCO) (Heun *et al.*, 1994). The binary matrix can also be used directly for parsimony analysis (Bayman & Cotty, 1993; Daud Khaled *et al.*, 1997). Ritchie *et al.*, (1997) calculated similarity coefficients followed by cluster analysis as well as parsimony analysis and found the clustering virtually the same by both methods. Häne *et al.*, (1993) suggests that an alternative statistical analysis (Pearson product-moment correlation coefficient, PPCC) is better suited to interpreting DNA band patterns than the band matching method. X-ray films of the lane profiles are scanned using a laser densitometer. Special computer software allows comparisons of lane profiles using the contours of the densitometric curves. Other groups have used this type of analysis to describe similarities between strains (van Belkum, 1994; van Rossum *et al.*, 1995).

The RAPD profiles can be very sensitive to changes in reaction conditions. The number of PCR cycles (30 - 45 cycles) does not seem to have any affect on the profiles (Yu & Pauls, 1992; Power, 1996), although at less than 30, some bands are absent. Yu & Pauls, (1992) optimised the cycling profile for 10 bp primers and found that a denaturing time of 5 sec at 94°C, rather than the more usual 30 or 60 s, resulted in the best band profiles, with regard to the brightness and quantity of bands. This is probably due to the limited life-span of *Taq* polymerase at high temperatures. The annealing time is correlated with % G+C content of the primer. Generally an annealing temperature of 36°C for 30 s results in the best band profiles for all primers. If the % G+C content is 70 - 80 %, then the annealing time can be reduced to 5 s with no loss of band clarity. However, if the % G+C content is 50 - 60 %, an annealing time of 5 s is too short and results in loss of bands. The extension time is

proportional to the maximum length of band fragment generated, with extension times of 1 min resulting in fragments of up to 3 kb.

The choice of annealing temperature does not alter the band patterns to a great extent. If too stringent (high) an annealing temperature (60°C) is selected, then no product is seen (Welsh & McClelland, 1990). Cycling at lower temperatures for a couple of cycles, followed by 40 cycles of standard PCR, results in the characteristic band profile. The pattern of bands changes only slightly if the annealing temperature is raised (30 - 50°C). Williams *et al.* (1993), concluded that raising the annealing temperature can affect the relative intensity of some amplified bands. It is sufficient then, to perform RAPD at a given (low) annealing temperature, and to stick with this same temperature at all times.

In order to increase the number of bands, multiplex RAPD-PCR has been performed, where more than one arbitrary primer is added to the PCR reaction (Daud Khaled *et al.*, 1997).

RAPD has been described as a quick, simple, relatively inexpensive technique, requiring only small quantities of DNA that (in theory) can be applied to all organisms with a high discrimination (Power, 1996). The technique compares well with other genotypic identification methods; however, there are major drawbacks to the technique, including the need to standardise all parameters and reagents to ensure maximum reproducibility both between and within laboratories (Penner *et al.*, 1993; Louden *et al.*, 1995; Schweder *et al.*, 1995; Gao *et al.*,1996).

1.3.2.xi.c Other PCR fingerprinting methods

RAPD patterns can be made more reproducible and easier to interpret by adapting the standard method. After separating fragments in agarose gels, individual fragments can be excised, cloned and sequenced. New primers are produced, based on the old RAPD primer and the first few bases of the excised fragment. This new primer can then be used for a more 'specific-random' PCR to produce a sharper and more reproducible pattern. This technique has been used to differentiate strains of *Aspergillus fumigatus* and has been called sequence-specific DNA primer (SSDP) analysis (Mondon *et al.*, 1997).

In an alternative technique, the RAPD profile can be used to design specific PCR primers or probes. Fragments can be excised, gel-purified and labelled prior to being used as a probe in RFLP analysis or in a dot-blot hybridisation. The excised fragment can also be cloned and sequenced and then used to develop PCR primers. Some groups have been successful in producing primers in this manner to detect species of the archaeal bacteria *Haloferax mediterranei* (Martínez-Murcia & Rodríguez-Valera, 1994), or to detect the oral bacteria *Prevotella intermedia* and *Prevotella nigrescens* (Guillot & Mouton, 1997)

Low-Stringency (LS)-PCR combines the use of specific PCR with the low stringency conditions of RAPD , thus allowing the production of patterns for epidemiological studies and a gene-specific amplified band. This technique has also been called COGEDET (combined gene detection and epidemiological typing) and has been used with *Escherichia coli, Clostridium difficile* and *Staphylococcus aureus* (Saulnier *et al.*, 1997) and *Leptospira* species (Damas de Caballero *et al.*, 1994; Brown & Levett, 1997). The technique is reproducible, highly discriminatory and by careful selection of primers, allows typing of almost all organisms.

The presence of repetitive extragenic palindromic (REP) elements and enterobacterial repetitive intergenic consensus (ERIC) sequences distributed throughout the genome has been shown for many Gram-negative bacterial species (Versalovic *et al.*, 1991). The function of these sequences is uncertain, although various roles including mRNA stability and transcriptional termination have been proposed (Gilson *et al.*, 1986). PCR primers can be designed to amplify from these sequences, producing a characteristic fingerprint for a given bacterial species. *Campylobacter jejuni* has been characterised in this way (Giesendorf *et al.*, 1994) as have *E. coli* and *Pseudomonas aeruginosa* (Woods *et al.*, 1993) and species of *Listeria* (Jeršek *et al.*, 1996),

As with other PCR fingerprinting techniques, REP/ERIC PCR is highly discriminatory if the primers are chosen carefully, but may not be applicable for all bacteria.

The technique of amplified fragment length polymorphism (AFLP) is based on the selective PCR amplification of restriction digest fragments from a total digest of genomic DNA (Vos *et al.*, 1995). The method has been called AFLP due to the similarity to RFLP, although this name has also been coined as an alternative to RAPD-? . Genomic DNA is digested with two restriction enzymes followed by the ligation of specific oligonucleotide adaptor sequences to each fragment. A subset of the fragments are selectively amplified by PCR, by choosing primers that recognise part of the adaptor sequence and extend into the restriction fragment by one or more bases. The amplified fragments can be separated in polyacrylamide gels, and if the primers have been tagged with a fluorescent label, can be visualised using automated sequencer software. This technique was originally used to characterise plant genomes, but has also been used to fingerprint *Salmonella* (Aarts *et al.*, 1998), *Xanthomonas,* strains and *Aeromonas* strains (Janssen *et al.*, 1996), and to investigate the epidemiology of a *Streptococcus pyogenes* outbreak (Desai *et al.*, 1998). The technique has the advantage of high specificity and reproducibility, and can be used to characterise any genome.

1.3.2.xi.d Specific detection using PCR

Bacteria in clinical samples can be detected by PCR using primers directed to species-specific sequences. The specific product can be visualised following agarose or polyacrylamide gel electrophoresis, and sized by comparison with molecular weight markers. In order to use PCR primers in a clinical setting (*e.g.* to detect specific organisms directly in plaque samples), the reaction conditions of the PCR, and thus the specificity of the primers, must be optimised. Two important parameters which affect the PCR are magnesium concentration and annealing temperature. The latter condition is dependent on the sequence and length of the primers. If the number of G+C residues is increased and the length increased, then the T_m of the primer is raised. Usually, an annealing temperature a few degrees lower than the T_m is adequate for the PCR, but this can be raised if non-specific products are formed.

Magnesium ions are an essential component of the PCR, and the concentration can be crucial. High concentrations of magnesium stabilise double-stranded DNA and prevent denaturation into single-stranded templates (Kidd & Ruano, 1995). In

addition, high levels of magnesium can stabilise primer-template mismatches, resulting in an increase in non-specific bands, as well as an overall decrease in yield. However, magnesium ions are required by the DNA polymerase as a co-factor and thus if the concentration is too low, the extension of product is inhibited, resulting in low yield (Kidd & Ruano, 1995). Thus a balance has to be found between reducing non-specificity and maximising the desired specific product, which can be achieved by performing the PCR with a range of magnesium ion concentrations.

The PCR can be performed either with extracted DNA from cultures or directly from the clinical samples themselves. Ashimoto *et al.* (1996) designed species-specific primers from variable regions in the 16S rRNA gene for eight putative periodontal pathogens (*Treponema denticola, Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Bacteroides forsythus, Campylobacter rectus, Eikenella corrodens, Prevotella intermedia* and *Prevotella nigrescens*) and used these to detect the organisms directly in plaque samples from patients. The group reported no cross-reactivity for the primers, even with closely related organisms. Watanabe & Frommel (1996) also designed primers to detect oral species, but the primers chosen were to genes encoding specific proteins: *Porphyromonas gingivalis* (fimbrial gene); *Actinobacillus actinomycetemcomitans* (leukotoxin gene) and *Treponema denticola* (gene encoding a surface antigen).

Several improvements have been described that either increase the sensitivity or the speed of the standard PCR method:

Multiplex PCR has been developed, where several specific primers are added to one PCR reaction, in order to detect more than one species, thus removing the need to set up individual PCR reactions for each organism. Tran & Rudney (1996) and Wahlfors *et al.* (1995) have shown this can be done for *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*.

The method of PCR followed by Southern blotting has been developed for the specific detection of bacteria species including *Treponema pallidum* (Burstain *et al.*, 1991) and *Bacteroides forsythus* (Guillot & Mouton, 1996). The Southern blot is a sensitive method of detecting DNA (Southern, 1975), and thus blank lanes can be confirmed as actual absence of product and not due to lack of visualisation under UV light. The probe will also confirm that the PCR product is that particular sequence.

An additional method to increase the sensitivity of the PCR is to combine PCR with immunological and DNA probe technologies. Chandad *et al.* (1997) described a method to detect *Bacteroides forsythus* in plaque samples consisting of three steps: separation of *Bacteroides forsythus* from mixed plaque samples, using magnetic beads coated with antibodies against surface antigens on the bacteria; PCR amplification, using specific primers, of DNA released by boiling the captured bacteria; detection by dot-blot hybridisation of the amplified products using a labelled DNA probe. The use of immunomagnetic beads, to separate the target bacteria from a complex mixture, concentrated the bacteria and provided a purer specimen of DNA for the PCR. PCR is known to be inhibited by compounds present in crude clinical samples (Wintzingerode *et al.*, 1997) and thus using this refined technique increased the relative sensitivity of the reaction. The assay was highly sensitive and could detect as few as 10 cells per ml in pure cultures, and 100 bacteria per ml in mixed suspensions. The technique has also been used for *Shigella* species (Islam & Lindberg, 1992).

Increased sensitivity can also be achieved by targeting the 16S rRNA of a cell in a reverse PCR reaction using reverse transcriptase. Centurion-Lara *et al.* (1997) detected *Treponema pallidum* in the sera of infected patients using this technique, and concluded that the sensitivity was higher than when a DNA-based detection technique was used.

PCR is an excellent technique to detect bacteria, potentially down to single organisms, but has disadvantages when compared to other methods. No distinction is made between viable and dead cells, as both will be amplified, and PCR cannot easily quantify the number of bacteria present, other than to quote a threshold figure.

False positives and false negatives can occur in PCR to mask the true detection levels of the technique. False positives can occur if contaminating DNA or carry-over from previous PCR reactions is mistakenly amplified, although good laboratory practice can reduce such occurrences (Kwok & Higuchi, 1989; Vaneechoutte & van Eldere, 1997). False negatives can occur if inhibiting compounds are present in the clinical sample (Wintzingerode *et al.*, 1997) or if the sensitivity of the primers is too low, but optimal primer design and DNA purification can, again, reduce this problem (Vaneechoutte & van Eldere, 1997).

1.3.3 Molecular phylogenetics

Phylogenetic studies aim to describe the evolutionary relationships among organisms, enabling the establishment of a logical and rigorous basis for taxonomic classifications. Bacteria do not display the varied morphologies and behaviour patterns that form the basis of classification for higher organisms such as plants and animals. As a result, taxonomy for micro-organisms has largely been based on numerical systems, whereby organisms that share the same metabolic traits are grouped together, although these groupings do not necessarily reflect the evolutionary history of the organisms (Schmidt & Relman, 1994). The use of molecular sequence data, as opposed to biochemical or morphological data, to infer phylogenetic relationships is recommended by Zuckerkandl & Pauling (1965). There are several reasons why DNA and protein sequence data are suitable for phylogenetic analysis: the evolution of such molecules is much more regular than physiological or morphological traits; sequence data are generally easier to establish in a reproducible and consistent manner; sequence data is more abundant, allowing for more character states to be compared.

Ribosomal RNA has been used extensively to estimate phylogenetic relationships (Olsen, 1988), with the 16S rRNA molecule particularly attractive for such studies as discussed in Section 1.3.2.iii. Relationships based on individual molecules will reflect the evolution of that molecule, rather than the whole organism. However, phylogenies based on 16S rRNA genes are in good agreement with phylogenies derived from other macromolecules, such as elongation factors (Bachleitner *et al.*, 1989) and subunits of ATPase (Iwabe *et al.*, 1989), suggesting that the evolution of 16S rRNA genes reflect the evolution of the whole organism.

Various methods exist to establish the phylogeny of related organisms using 16S rDNA sequences (Section 1.3.3.i,ii,iii) and various computer packages are available for the calculations. The three most common packages are PHYLIP - Phylogeny Inference Package, (written by Felsenstein and available free from evolution.genetics.washington.edu); PAUP - Phylogenetic Analysis Using Parsimony, (Swofford, 1993) and MEGA - Molecular Evolutionary Genetic Analysis, (Kumar, 1993).

All methods rely on the comparison of sequence data that have been correctly aligned so that only comparable positions are considered. Computer software is available to produce an alignment of sequences. The results needs to be checked manually, however, as the programs favour inserting gaps to increase the potential similarity of the sequences, and thus computer alignments may not reflect an alignment of truly homologous positions (Schmidt & Relman, 1994). As all 16S rRNA molecules have the same core secondary structure (Gutell, 1993), it is possible to align regions that have little primary structure by comparing their common secondary arrangements. However, Lane *et al.* (1985) recommend omitting such regions, in order to avoid introducing error into the subsequent phylogenetic analysis.

1.3.3.i Distance matrix methods

There are two steps in this type of analysis: a) the creation of distance values by comparing sequences over all positions and b) cluster analysis or additive tree construction of the pair-wise distances.

Simple comparison between sequences will not be a true reflection of the evolutionary distances between two sequences. Multiple changes can occur at the same position, with later changes masking the record of earlier mutation events. The distance values can be corrected for this 'superimposition' of events with various probability theory algorithms. Three such 'corrections' are available in the PHYLIP package of programs - Jukes-Cantor (J-C), Kimura (K) and Maximum likelihood (ML). All corrections have the same general assumptions:

a) all base positions are equally likely to change in a given sequence

b) substitutions at a given base position are independent

c) the base composition over time does not change, and any substitutions maintain the base composition equilibrium.

Gaps are not counted and are ignored.

In addition the K correction assumes:

a) transition substitutions (purine-purine or pyrimidine-pyrimidine) occur more often than transversion substitutions (purine-pyrimidine or *vice-versa*). The default setting is for a ratio of 2:1 transition : transversion.

Both J-C and K assume that all four nucleotides occur with equal frequency.
The ML correction is the most rigorous and differs from the K correction by allowing for different frequencies of the four nucleotides.
In effect the J-C correction is a special case of the K correction (when transition substitutions occur at the same rate as transversions). The K correction is in turn a special case of the ML correction (when the numbers of each base in the sequences are equal). The computing time for the ML correction is significantly longer than for the other corrections, especially when bootstrap analysis is performed (Section 1.3.3.iv).

The distance data can be represented graphically by many methods. Two common methods are UPGMA (Unweighted Pair Group Method using Arithmetic mean) and NJ (Neighbor-joining) clustering.
For both cluster methods, similarities are represented in a table as distances between all pairs of sequences (taxa). For UPGMA, the tree is constructed by linking the most similar pair of taxa, followed by successively linking more distant taxa. When two or more taxa are linked, they lose their individual identities and are subsequently referred to as a single cluster. When the next taxon is to be joined to one such cluster, the new distance is the arithmetic mean of all the taxa contributing to the cluster. NJ is an algorithm for inferring an additive tree and the method clusters the sequences in a pair-wise manner as for UPGMA. However, instead of selecting the next pair to cluster by looking for the smallest distance in the table, this method seeks to form pairs that minimise the sum of the branch lengths for the whole tree. The new cluster replaces the two or more pair-wise distances from which it was created in the table.

1.3.3.ii Maximum likelihood method

The maximum likelihood method chooses the tree topology that maximises the probability that the observed sequence data would have occurred given a model of evolution. The model has the same assumptions as for the distance matrix correction algorithms as well as the following:
a) Each site undergoes substitution at an expected rate (each with a probability of occurrence) specified by the program

b) Substitutions are either transitions, transversions or no change depending on the frequency of the bases present.

1.3.3.iii Parsimony

The parsimony model assumes that sequences were derived from their ancestors by acquiring the minimum number of changes. The most parsimonious trees are the topologies with the minimum overall length (*i.e.* the minimal number of changes). Several algorithms are available to search for all possible trees and to evaluate and rank the results.

The method of phylogenetic analysis used is largely due to personal preference, with drawbacks existing to all methods (Li, 1997; Swofford *et al.*, 1996). Evaluating the different methods is not easy as the 'right' answer describing evolutionary relationships is usually not known. Empirical studies have been used to try and answer the question 'which is the best method?' by using data from organisms where the evolutionary history *is* known. Atchley & Fitch (1991) used inbred strains of mice and Hillis *et al.* (1994) manipulated bacteriophage T7 in the laboratory to generate molecular data to test the predictive phylogenetic methods. However, the limited data available from these experiments was not enough to draw any conclusion as to the relative performances of the different methods. Computer simulation studies have been used to understand the limitations of a particular phylogenetic method (Felsenstein, 1988; Saitou & Nei, 1987; Li, 1997). The general conclusions of such studies are that UPGMA and Parsimony perform less well than Neighbor-joining, which in turn is less robust than Maximum Likelihood, although Li (1997) warns that more research needs to be done for a full evaluation of all methods.

1.3.3.iv Bootstrap analysis

The various methods to infer phylogenetic relationships usually produce different trees, even with the same data. In order to decide which is the 'best' tree, it is recommended to calculate some level of significance for the overall topology (Schmidt & Relman, 1994) and the method most frequently used is bootstrap analysis. Bootstrap analysis involves creating a new data set by sampling random characters from the original data. The new data set has the same number of

characters as the original, with some characters being sampled more than once and some not at all. The re-sampling and creation of new data sets is repeated usually 100 times and the statistical analysis is then repeated with all the new data sets. The branches that are most frequently found in the trees are used to draw a consensus tree, with values at each node indicating the number of times that position was found in all the data sets. This gives a percentage confidence limit for the nodes of the tree. The higher the percentage, the more confidence there is in that node, with values upwards of 50 % being deemed statistically significant (Zharkikh & Li, 1992).

1.3.4 Detection and identification of oral *Treponema* species

Oral *Treponema* species can be visualised by phase-contrast microscopy for enumeration in plaque samples, but this does not allow for speciation. Phenotypic tests are traditionally the way in which oral *Treponema* species have been characterised. Morphology of the cells, biochemical tests, acid end-products and nutritional requirements are determined, although many of these qualities do not distinguish between species (Section 1.1, Table 1.1), so that several different attributes of the cells need to be examined. *Treponema* species are difficult to culture, and can be difficult to maintain in the laboratory (Fiehn *et al.*, 1995; Wardle, 1997) and thus identification methods that do not rely on culture are desired.

Genotypic methods have been used to identify and characterise *Treponema* species isolated from the periodontal pocket. The % G+C content of cells and DNA-DNA homology studies are used to classify bacteria, but are not enough to identify species, nor are they routine laboratory tests. Plasmid-carrying strains of *T. denticola* have been isolated, but plasmids have not been demonstrated in other oral treponemes, and so the utility of this method is limited. Ribotyping has shown reproducible differences between *T. denticola* and *T. socranskii*, but the method has not been developed to include all oral species.

Sequencing 16S rDNA, PCR detection, DNA probe techniques and, more recently, FISH, have all been used to identify treponemes found in plaque, without the prior isolation of individual species of bacteria. This has led to the discovery of many distinct, previously uncharacterised treponemes, that have not been detected by other identification methods relying on culture.

Examples of studies using different techniques are listed in Table 1. 4.

Phenotypic Methods	References	Genotypic Methods	References
Phase-contrast/ darkfield microscopy	Listgarten & Socransky, 1964; Listgarten & Hellden, 1978	**% G+C content**	Smibert & Burmeister, 1983; Smibert, 1984; Cheng *et al.*, 1985
Electron microscopy	Cheng & Chan, 1983; Smibert, 1984; Chan *et al.*, 1993; Wyss *et al.*, 1996; Umemoto *et al.*, 1997; Wyss *et al.*, 1997	**DNA homology**	Smibert *et al.*, 1984; Cheng *et al.*, 1985; Chan *et al.*, 1993; Olsen *et al.*, 1995; Umemoto *et al.*, 1997
Nutritional requirements	Cheng & Chan, 1983; Smibert & Burmeister, 1983; Smibert, 1984; Wyss *et al.*, 1992; Koseki *et al.*, 1996; Wyss *et al.*, 1996; Wyss *et al.*, 1997	**Plasmid analysis**	Reedy *et al.*, 1994; Caudry *et al.*, 1995
Fatty acid analysis	Brondz *et al.*, 1991; Dahle *et al.*, 1996	**16S rDNA sequencing**	Choi *et al.*, 1994; Wyss *et al.*, 1996; Umemoto *et al.*, 1997; Wyss *et al.*, 1997
Fermentation end products	Cheng & Chan, 1983; Smibert & Burmeister, 1983; Smibert *et al.*, 1984; Chan *et al.*, 1993	**DNA probe**	DiRienzo *et al.*, 1991; Loesche, 1992b; Koseki *et al.*, 1995; Moter *et al.*, 1998
Biochemical tests	Laughon *et al.*, 1982; Cheng & Chan, 1983; Smibert & Burmeister, 1983; Smibert, 1984; Smibert *et al.*, 1984; Syed *et al.*, 1988; Seida *et al.*, 1992; Koseki *et al.*, 1995; Dahle *et al.*, 1996; Wyss *et al.*, 1996; Umemoto *et al.*, 1997; Wyss *et al.*, 1997	**Ribotyping**	Fiehn *et al.*, 1995
SDS PAGE	Tall & Nauman, 1986; Umemoto *et al.*, 1989; Koseki *et al.*, 1995; Dahle *et al.*, 1996; Wyss *et al.*, 1996; Umemoto *et al.*, 1997; Wyss *et al.*, 1997	**PCR detection**	Ashimoto *et al.*, 1996; Watanabe & Frommel., 1996
MLEE	Dahle *et al.*, 1995	**FISH**	Moter *et al.*, 1998
Agglutination	Cheng & Chan, 1983; Smibert *et al.*, 1984; Tall & Nauman, 1986; Umemoto *et al.*, 1989; Chan *et al.*, 1993		
Immunoassay	Cheng & Chan., 1983; Umemoto *et al.*, 1989; Simonson *et al.*, 1992; Chan *et al.*, 1993; Riviere *et al.*, 1995; Dahle *et al.*, 1996; Wyss *et al.*, 1997		

Table 1.4 Detection and Identification of oral *Treponema* species

1.4 Aims

Treponema species have been extensively studied using phenotypic methods of detection and identification (Table 1.4), however, studies using genotypic methods are not so well developed. In order to study fully the pathogenic potential of these bacteria, quicker and more rigorous techniques are desired to speciate treponemes.

Five uncharacterised isolates of *Treponema* had previously been isolated from the subgingival plaque of several patients. The aims of this study were as follows:

1) to examine the validity of using RAPD-PCR to identify *Treponema* species of known ATCC species

2) to use the molecular techniques of RAPD-PCR and partial 16S rDNA sequencing to identify the patient isolates of unknown species

3) to examine the phylogeny of oral treponemes using partial 16S rDNA sequence analysis

4) to develop molecular probes to detect and identify oral treponemes, including the species *T. denticola* and *T. socranskii*.

2.0 Materials

2.1 General

The majority of materials were manufactured by BDH, Lutterworth, Leicestershire, UK, or Sigma Chemical Company, Poole, Dorset, UK and were of analytical grade. Exceptions are as follows:

Material	Manufacturer
Agar no. 1	Oxoid Ltd., Basingstoke, Hampshire, UK
Agarose	Appligene, Chester-le-Street, Co. Durham, UK
Anti-DIG antibody	Boehringer Mannheim, Lewes, E. Sussex, UK
[α- ^{35}S] dATP	Dupont NEN™ (UK) Ltd., Hertfordshire, UK
Blocking Reagent	Boehringer Mannheim
Bluo-Gal	Gibco BRL, Life Technologies™, Paisley, UK
Brain Heart Infusion Broth	Lab M, Wash Lane, Bury, Lancashire, UK
Buffer IV	Advanced Biotechnologies, Leatherhead, Surrey, UK
CDP-*Star*™	Boehringer Mannheim
DIG Easy Hyb	Boehringer Mannheim
dNTPs	Pharmacia Biotech Ltd., Milton Keynes, Bucks, UK
Dynabeads®	Dynal, Wirral, UK
Easigel	Scotlab, Coatbridge, UK
Epicurian Coli®cells	Stratagene Ltd., Cambridge, UK
Filter paper (3MM)	Whatman International Ltd., Maidstone, Kent, UK
High molecular wt. markers	Gibco BRL
Lambda DNA (uncut)	Gibco BRL
Magnesium chloride soln.	Advanced Biotechnologies
Nitrocellulose filter	Whatman International Ltd.
NuSieve®3:1 agarose	Flowgen Instruments Ltd., Sittingbourne, Kent, UK
Nylon membrane	Boehringer Mannheim
pGEX-2T	Promega Corporation, Southampton, UK
Phenol, pH 8, Tris buffered	Fisons, Loughborough, Leicestershire, UK

ϕX174 DNA *Hae*III digest	Advanced Biotechnologies
Polaroid 667 film	Polaroid (UK) Ltd., St Albans, Hertfordshire, UK
Polynucleotide kinase	Boehringer Mannheim
Polypeptone	Oxoid Ltd
Proteinase K	Boehringer Mannheim
Pwo polymerase	Boehringer Mannheim
Taq enzyme	Advanced Biotechnologies
Tryptone	Oxoid
X-Ray Film (Blue)	X-ograph Ltd., Malmesbury, Wiltshire,UK
Yeast extract	Oxoid

2.2 Molecular Biology Kits

For kit contents and manufacturers' protocols see Appendix 7.3

TA Cloning®Kit version 2.3 (Invitrogen, Leek, The Netherlands)

Sequenase™ version 2.0 T7 DNA Polymerase Sequencing Kit (USB™, Amersham Life Sciences, Amersham, UK)

Wizard™ PCR Preps DNA Purification System (Promega Corporation, Southampton, UK)

Rapid DNA Ligation Kit (Boehringer Mannheim, Lewes, E. Sussex, UK)

DIG Oligonucleotide 3' -End Labeling Kit (Boehringer Mannheim, Lewes, E. Sussex, UK)

2.3 Buffers and Solutions

2.3.1 DNA extraction

STE (pH 8.0)	25 % (w/v) sucrose
	50 mM Tris-Cl
	50 mM EDTA disodium salt
TE (pH 8.0)	10 mM Tris-Cl
	1 mM EDTA disodium salt

2.3.2 Agarose gel electrophoresis

6 x Glycerol loading buffer	0.1 % (w/v) bromophenol blue
	0.1 % (w/v) xylene cyanol
	30 % glycerol
	5 mM EDTA disodium salt pH 8.0
	10 mM Tris-Cl pH 7.0
10 x TPE (pH 8.0)	0.9 M Trizma® base
	1.3 % (w/v) H_3PO_4
	20 mM EDTA disodium salt

2.3.3 PCR

10 x Buffer IV	200 mM $(NH_4)_2SO_4$
	750 mM Tris-Cl
	0.1 % w/v Tween
10 x *Pwo* polymerase buffer	100 mM Tris-Cl pH 8.85
	250 mM KCl
	50 mM $(NH_4)_2SO_4$
	20 mM $MgSO_4$

2.3.4 Sequencing

LB broth/(agar)	1 % tryptone 0.5 % yeast extract 1 % NaCl (0.7 % Oxoid Agar no. 1)
Easigel	6 % (w/v) acrylamide in 1 x TBE 0.3 % bisacrylamide 7 M urea
Sequencing fixative solution	10 % glacial acetic acid 10 % methanol
5 x TBE	0.5 M Trizma® base 0.5 M *ortho*-boric acid 10 mM EDTA disodium salt pH 8.0
TNE	10 mM Tris-Cl pH 7.5 100 mM NaCl 10 mM EDTA disodium salt pH 8.0

2.3.5 Dot-blot Hybridisation

Blocking solution	1 % (w/v) Blocking Reagent in maleic acid buffer
Detection buffer (pH 9.5)	100 mM Tris-Cl pH 7.5 100 mM NaCl
Maleic acid buffer (pH 7.5)	100 mM maleic acid 150 mM NaCl
20 x SSC	3 M NaCl 0.3 M sodium citrate pH 7.0

Standard hybridisation buffer	5 x SSC
	0.1 % (w/v) N-laurolysarcosine
	0.02 % (w/v) SDS
	1 % (w/v) Blocking solution
Stripping solution	0.2 M NaOH
	0.1 % (w/v) SDS
Washing buffer	0.3 % (w/v) Tween® 20 in maleic acid buffer
2 x Washing solution	2 x SSC
	0.1 % (w/v) SDS
1 x Washing solution	1 x SSC
	0.1 % (w/v) SDS
0.1 x Washing solution	0.1 x SSC
	0.1 % (w/v) SDS

2.4 PCR Primers and oligonucleotide probes

*L10 5'-GTAGAGCTCGCGGCACTTG-3'

*US 5'-GTAAAACGACGGCCAGT-3'

*RSP 5'-GGAAACAGCTATGACCATGA-3'

*970-11 5'-GTAAGGCCG-3'

*TPU1 5'-AGAGTTTGATC(A/C)TGGCTCAG-3'

*RTU3 5'-G(A/T)ATTACCGC(G/T)GCTG-3'

*TREP 5'-GACTTGCATGCTTAA(G/A)AC-3'

+806R 5'-*biotin*GGACTACCAGGGTATCTAAT-3'

◊SOC2 5'-CCGCTCTCGGCATTGCTGCCTGCC-3'

◊S2P 5'-GGCAGGCAGCAATGCCGAGAGCGG-3'

◊DEN2 5'-TTACCTTTATGTAAATGAGCAC-3'

* manufactured by University of Manchester oligonucleotide synthesis service

+ manufactured by Gibco BRL, Paisley, UK. 806R was 5' biotinylated

◊ manufactured by Perkin-Elmer, Applied Biosystems, Warrington, Cheshire, UK

L10 and US: Corney *et al.*, 1993

RSP: Welsh *et al.*, 1992

970-11: Ménard *et al.*, 1992

TPU1, RTU3 & TREP: Choi *et al.*, 1994

806R: Wilson *et al.*, 1990

2.5 Bacterial cultures

2.5.1 Strains

Strain	Source information	Abbreviation
Treponema socranskii subsp. *buccale*	ATCC 35534	soc1
Treponema socranskii subsp. *paredis*	ATCC 35535	soc2
Treponema socranskii subsp. *socranskii*	ATCC 35536	soc3
Treponema pectinovorum	ATCC 33768	pect
Treponema vincentii	ATCC 35580	vin
Treponema denticola	ATCC 35405	den1
Treponema denticola	ATCC 33520	den2
Treponema denticola	ATCC 33521	den3
Treponema denticola	ATCC 35404	den4
Leptospira icterohaemorrhagiae	donated by H. Wardle *	Li
Prevotella intermedia	donated by L. Maddocks +	Pi

* Helen Wardle, formerly of Hope Hospital, Salford, UK

+ Louise Maddocks, Turner Dental School, Manchester, UK

2.5.2 Clinical isolates

All clinical isolates were cultured by Helen Wardle, formerly of Hope Hospital, Salford, UK, from the subgingival plaque of five patients attending the Periodontology clinic at the Turner Dental Hospital, Manchester, UK (Appendix 7.2). The isolates are as follows:

Patient isolate	Source	Diagnosis
3	44 year old	Early-onset periodontitis
54	age unknown	Unknown diagnosis
63	55 year old	Adult periodontitis
64	60 year old	Adult periodontitis
68	28 year old	ANUG

3.0 Methods

3.1 Growth and maintenance of Culture Collection strains

The strains *Treponema socranskii* subsp. *buccale* ATCC 35534 (soc1), *Treponema pectinovorum* ATCC 33768 (pect), *Treponema vincentii* ATCC 35580 (vin), *Treponema denticola* ATCC 35405 (den1), and *Treponema denticola* ATCC 33520 (den2) were all obtained from ATCC and initially cultured by Helen Wardle, formerly of Hope Hospital, Salford, UK. I obtained *Treponema socranskii* subsp. *paredis* ATCC 35535 (soc2), *Treponema socranskii* subsp. *socranskii* ATCC 35536 (soc3), *Treponema denticola* 33521 (den3) and *Treponema denticola* 35404 (den4) directly from ATCC. The bacteria were resuscitated from either frozen (soc2 & soc3) or freeze-dried stocks (den3 & den4).

Den1, den2, den3, den4 and vin were cultured in ATCC culture medium 1357 (New Oral Spirochete broth) supplemented with rifampicin (Appendix 7.1.1). Initially, pect was cultured in ATCC broth culture medium 1367 supplemented with rumen fluid and rifampicin (Gherna *et al.*, 1992). The rumen fluid was replaced by fatty acids and haemin on subculturing (Appendix 7.1.2). Soc1 was cultured in this modified 1367 medium as well. Soc2 and soc3 were cultured in modified M10 broth medium (Appendix 7.1.3). All strains were grown at 37°C in an anaerobic environment consisting of 80 % CO_2, 10 % N_2 and 10 % H_2 (Compact M Anaerobic Workstation, Don Whitley Ltd., Shipley, West Yorkshire, UK). Purity was confirmed by phase-contrast microscopy at x 40 magnification and Gram-stain (Barrar & Feltham, 1993) at x 100, before being subcultured, on a monthly basis, into fresh broth.

3.1.1 Maintenance of clinical isolates

Clinical isolates were cultured by Helen Wardle, formerly of Hope Hospital, Salford, UK (Appendix 7.2). Clinical isolates 3, 54 & 68 were cultured in ATCC broth medium 1357 (Appendix 7.1.1). Isolates 63 and 64 were cultured in 1367 ATCC broth medium supplemented with fatty acids and haemin (Appendix 7.1.2). All samples could grow in either medium but optimal growth was achieved as described.

3.2 Nucleic acid extraction

5 ml of 3 week old bacterial suspension was centrifuged (10 min, 3000 x *g*) and resuspended in 0.5 ml STE. Lysozyme (20 µl of 50 mg ml^{-1}) was added followed by incubation at 37°C for 30 min. Proteinase K (7.5 µl of 20 mg ml^{-1}) and SDS (60 µl of 10 % w/v) were added before incubating at 55°C for 1 hour. The suspension was vortexed with an equal volume of phenol/chloroform/*iso*-amyl alcohol (25:24:1 v:v:v) and centrifuged. An equal volume of *iso*-propanol and NaCl (24 µl of 5 M) were added to the supernatant fluid. After centrifugation, the pellet was resuspended in water and ammonium acetate (100 µl of 7.5 M) added. The suspension was left at 0°C for 1 hour. An equivalent volume of ethanol was added to the supernatant and after centrifugation, the pellet was washed with 70 % ethanol. The final pellet was resuspended in 50 µl TE, overnight at 4°C.

3.2.1 Agarose gel electrophoresis

To quantify the amount of DNA extracted from broth cultures, 5 µl of DNA was mixed with 6 x glycerol loading buffer and subjected to electrophoresis in 0.8 % agarose gels (5 V cm^{-1}) and 1 x TPE buffer. Known quantities of high molecular weight DNA markers or uncut lambda DNA were also mixed with glycerol loading buffer and run in the gel as comparisons. DNA was visualised under ultraviolet light after ethidium bromide staining (1 µg ml^{-1} in 1 x TPE) with a model STX 20.M transilluminator with wavelength of 254 nm (UVItec Ltd., Cambridge, UK) and photographed on Polaroid 667 film. The amount of extracted DNA in each sample was compared to the known amounts of DNA. DNA was diluted in water to give approximately equivalent concentrations in each sample.

3.2.1.i Visualisation of PCR products and restriction digest fragments

DNA fragments to be separated were mixed with 6 x glycerol loading buffer and subjected to electrophoresis in 1.8 % agarose gels (5 V cm^{-1}) and 1 x TPE buffer. A *Pst*1 digest of lambda DNA, or *Hae*III digest of ϕX174 were loaded as molecular weight markers. DNA fragments were visualised and photographed as described in Section 3.2.1.

3.3 RAPD-PCR

Approximately 50 ng of bacterial DNA was subjected to PCR, in a total volume of 50 µl, containing 2.5 units of *Taq* DNA polymerase in a Crocodile II™ thermalcycler (Appligene, Chester-le-Street, Co. Durham, UK). The reaction mix contained 1 x buffer IV, 3 mM $MgCl_2$, 0.2 mM of each dNTP (dATP, dCTP, dGTP, dTTP) and 1 µM primer. Primers used were L10, US, RSP and 970-11. The mixture was overlaid with mineral oil and denatured for 3 min at 95°C, prior to a thermal cycling profile (for primers L10, US and RSP) of 1 min at 94°C, 1 min at 40°C and 1 min at 72°C for 5 cycles, then 1 min at 94°C, 1 min at 55°C and 1 min at 72°C for 25 cycles. Primer 970-11 had the profile: 1 min at 94°C, 1 min at 32°C and 1 min at 72°C for 35 cycles. PCR products (10 µl) were electrophoresed and visualised as described in Section 3.2.1.i. Each primer amplification was repeated to check for reproducibility of bands. To avoid exogenous DNA contamination and carry-over of PCR product into new PCR, the guidelines laid down by Kwok & Higuchi (1989) were adhered to in this study. The area where template DNA was manipulated and PCR reagents were aliquotted were physically separated. Stock solutions were divided into small aliquots and a negative control, with no added DNA was always included. In addition, aerosol-resistant disposable pipette tips (Rainin Instrument Co., Inc., Woburn, Massachusetts, USA) specially prepared for PCR were used.

3.3.1 Pattern analysis

Gel photographs were scanned using an OmniMedia scanner (OmniMedia Technology Inc., Santa Clara, California, USA) and major bands sized using Whole Band Analyzer software (Millipore UK Ltd., Watford, Hertfordshire, UK) on a Sun Sparc workstation (Sun Microsystems Inc., Palo Alto, California, USA). The gels were visually scored for presence (= 1) or absence (= 0) of major bands in each track and the results expressed in a binary matrix.

3.3.2 Statistical analysis

The binary matrix was used to create simple matching (SM) and Dice (D) coefficients for each isolate in a pair-wise fashion (Austin & Colwell, 1977). SM coefficients give equal weight to positive and negative matches (Equation 3.1). D

coefficients disregard negative matches (Equation 3.2). Bands of the same size were scored as common bands, with the relative brightness or thickness of bands not considered.

Equation 3.1 $SM = \frac{N_1 + N_0}{N_t}$ **Equation 3.2** $D = \frac{2 \times N_1}{N_x}$

N_1 = number of bands in common between two isolates

N_0 = number of absent bands in common between two isolates

N_t = total number of bands scored for all isolates

N_x = total number of bands scored for two isolates

Phenograms were drawn based on average linkage cluster analysis of the results calculated using SPSS for Windows software.

3.4 Partial 16S rDNA sequencing

Sequence data for ATCC strains soc1, soc2, soc3, vin, & den1 and the clinical isolates 63, 64 & 68 were obtained by one or more of the following methods:

3.4.1 Method A: Cloning of PCR fragments using the TA Cloning® Kit

Partial 16S rDNA sequences were amplified by PCR in a Crocodile II™ thermal cycler using primers TPU1 and RTU3, to create a fragment of approximately 500 bp (Choi *et al.*, 1994). The primers correspond to positions 8 - 27 and 536 - 516 respectively in the 16S rRNA gene in *Escherichia coli*. The PCR contained approximately 50 ng of DNA, 1 x Buffer IV, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.3 µM of each primer and 1.25 units of *Taq* polymerase, in a final volume of 50 µl, overlaid with an equal volume of mineral oil. The reaction mix and mineral oil were UV irradiated for 4 min on a model STX 20.M transilluminator with wavelength of 254 nm prior to addition of template DNA. The mixture was incubated for 3 min at 94°C before the cycling profile of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C for 31 cycles. The last cycle had a 5 minute extension step. PCR product was confirmed by agarose gel electrophoresis as outlined in Section 3.2.1.i. Reagent

controls were always set up with each PCR, containing all the components of the PCR except template DNA and were confirmed blank before continuing with the cloning protocol. PCR product was cleaned by adding four times the volume of chloroform/*iso*-amyl alcohol (24:1 v:v). After vortexing, NaCl (20 µl of 5 M) and an equal volume of ethanol were added to the aqueous phase. The mixture was left on ice for 10 min and then centrifuged for 20 min at 12 000 x *g*. The pellet was resuspended in 100 µl of TE.

PCR product (150 ng) was ligated into pCR™II vector (50 ng) and transformed into TA Cloning® One Shot™ competent cells supplied in the TA Cloning® Kit version 2.3 as per the manufacturer's instructions (Appendix 7.3.1). Transformed (white) colonies were picked and subcultured into 2 x LB broth and left overnight at 37°C. The plasmid was purified using a phenol/chloroform protocol (Sambrook *et al.*, 1989) and the insert size confirmed by agarose gel electrophoresis after an *Eco*RI digest (Section 3.4.1.i). Insert sequence data from at least two clones were obtained for each DNA sample as described in Section 3.4.1.i.

3.4.1.i Restriction digest

DNA to be digested was incubated for 1 h at 37°C with 10 units of restriction enzyme (*Bam*HI or *Eco*RI) and 1 x restriction buffer supplied with the enzyme. The digest was then subjected to electrophoresis for 1 h and visualised as described in Section 3.2.1.i.

3.4.1.ii Manual sequencing

Sequence data were obtained using the Sequenase™ version 2.0 T7 DNA Polymerase Sequencing Kit (Appendix 7.3.2), the supplied kit universal sequencing primers or primers TPU1 & RTU3 and [α- ^{35}S] dATP. The sequencing reactions were set up several times for both forward and backward strands. Gels were prepared using Easigel, 0.7 % (w/v) ammonium persulphate and 0.05 % (v/v) TEMED in a model V3F Sequencing Apparatus (Anachem Ltd., Luton Bedfordshire, UK). Sequence reactions (3 µl) were heated to over 85°C for 5 min before being loaded and the gels were run for 1.5 h or 3.5 h. The gels were then soaked in sequencing fixative

solution for 30 min before being dried, under vacuum, onto 3 MM filter paper at 80°C using a model 583 gel drier (Bio-rad, Hemel Hempstead, Hertfordshire, UK). The dried gels were exposed to Blue X-ray film for periods ranging from 12 h to 5 days. Films were developed automatically using a Compact X4 Imaging System (X-ograph Ltd., Malmesbury, Wiltshire, UK) and the sequences read by eye. A consensus sequence was compiled from all the different runs for each isolate. The forward and backward strands were compared with each other, with reference made to the original X-ray films in the event of any discrepancies, before the final data was entered into my directory on the computer at Daresbury, Warrington, Cheshire, UK. This was done using the Seqed program, which is part of the Genetics Computer Group (GCG) software package (GCG, Madison, Wisconsin, USA).

3.4.2 Method B: Cloning of PCR fragments using pGEX-2T

PCR was performed as described in Section 3.4.1 using 2.5 units of *Pwo* polymerase with the supplied buffer instead of *Taq* polymerase and buffer IV. The total PCR product was cleaned using a Wizard™ PCR Preps DNA Purification System according to the manufacturer's instructions (Appendix 7.3.3) and eluted in 50 µl of water. The 5' ends of the fragment were phosphorylated (Section 3.4.2.i) and then the phosphorylated product (150 ng) was ligated into the vector pGEX-2T using the Rapid DNA Ligation Kit, as per the manufacturer's instructions (Appendix 7.3.4). Epicurian Coli®cells were transformed by the same method as One Shot™ cells (Appendix 7.3.1) and plated out on LB agar containing ampicillin (225 µl of 5 mg ml $^{-1}$), Bluo-Gal (25 µl of 40 mg ml $^{-1}$) and IPTG (40 µl of 0.1 M). Transformants were subcultured and plasmids recovered as described in Section 3.4.1. Plasmids containing the correct insert were verified using a *Bam*H1 and *Eco*R1 restriction digest (Section 3.4.1.i). Sequence data for isolate 68 were obtained as described in Section 3.4.1.ii.

3.4.2.i Phosphorylation of 5' ends of PCR product using polynucleotide kinase

PCR product (0.5 µg) was incubated with polynucleotide kinase (10 units), 1 x supplied buffer and 1 µM ATP at 37°C for 30 min in a final volume of 20 µl. The mixture was then heated to 68°C for 10 min, in order to inactivate the enzyme. The DNA was purified by the addition of 2 volumes of phenol/chloroform/*iso*-amyl alcohol (25:24:1 v:v:v) and

centrifuged for 10 min at 12 000 x *g*. Four times the volume of ethanol and 2.4 µl of 5M NaCl were added to the aqueous phase. The mixture was left on ice for 10 min and centrifuged for 10 min at 12 000 x *g*. The pellet was washed with 70 % ethanol and resuspended in 10 µl water.

3.4.3 Method C: Direct sequencing using Dynabeads® M-280 Streptavidin (Dynal, Wirral, UK)

PCR was performed as described in Section 3.4.1 with a biotinylated primer 806R (Wilson *et al.*, 1990) instead of RTU3. Dynabeads® M-280 Streptavidin (50 µl) were aliquotted into a clean micro-centrifuge tube for each reaction. The beads were washed with an equal volume of TNE. The beads were placed next to a Magnetic Particle Concentrator ('MPC', Dynal, Wirral, UK) and the supernatant fluid discarded. The MPC was removed and the wash repeated. PCR product (50 µl) and TNE (50 µl) were added to the beads. The mixture was left at room temperature and tapped occasionally over a 5 min period. The mixture was placed next to the MPC and the supernatant fluid discarded. NaOH (100 µl of 150 mM) was added to the beads and the mixture left for 5 min at room temperature. After placing the tube next to the MPC, the supernatant fluid was removed and placed in a clean micro-centrifuge tube. This supernatant fluid contained one strand of the DNA template and was processed as follows: sodium acetate pH 5.2 (10 µl of 3M) and 250 µl ethanol were added and the solution left at - 80°C for 15 min. After centrifuging for 10 min at 12 000 x *g*, the pellet was washed twice with 70 % ethanol. The dried pellet was resuspended in 7 µl of water and stored at - 20°C.

The other strand of DNA, immobilised on the Dynabeads®, was washed once with TNE and then twice with water - the supernatant fluids being discarded each time. The beads were resuspended in 14 µl of water and stored at - 20°C.

Seven microlitre of each strand were used as template in the subsequent sequencing reactions, as described in Section 3.4.1.ii.

The initial PCR was set up twice for soc1 and vin and both amplifications were subsequently sequenced for each isolate. For den1, clinical isolate 63 and 64, the PCR was performed once and the fragment sequenced.

3.4.4 Method D: ABI Prism™ Dye Terminator Cycle Sequencing

PCR was performed as described in Section 3.4.1. PCR product was cleaned using a Wizard™ PCR Preps DNA Purification System (Appendix 7.3.3) and eluted in 50 µl of water. Approximately 90 ng of purified product, 3.2 pmol of primer RTU3 or TPU1 and 8 µl of Terminator Ready Reaction Mix (containing A-Dye Terminator, C-Dye Terminator, G-Dye Terminator, T-Dye Terminator, dITP, dATP, dCTP, dTTP, Tris-Cl pH 9.0, $MgCl_2$, thermal stable pyrophosphatase, AmpliTaq® DNA Polymerase, FS) were made up in a final volume of 20 µl, and were cycle sequenced in a Perkin-Elmer GeneAmp PCR System 2400 thermal cycler. The cycling profile was 10 s at 96°C, 5 s at 50°C, 4 min at 60°C for 25 cycles. Sodium acetate pH 5.2 (3M) and 50 µl of ethanol were added to the total product. The solution was left at 0°C for 10 min and then centrifuged for 25 min at 12 000 x *g*. The pellet was washed twice with 70 % ethanol and left to air-dry. Sequencing reactions were analysed in an ABI Prism 377 DNA Sequencer (Perkin-Elmer Corporation). Soc1, soc2, soc3, vin, den1 and isolate 68 were sequenced by this method. The automated data were analysed using DNA Sequencing Analysis software (Perkin-Elmer Corporation). Forward and backward strands were compared using the command GAP (Wisconsin Package, Version 8.1-UNIX, Genetics Computer Group, Madison, Wisconsin, USA) and any discrepancies checked by reference to the electropherogram from the original sequencing run.

Sequence data derived from different methods were compared to arrive at a final consensus sequence for each isolate. These sequences were then entered into my directory on the computer at Daresbury, Warrington, Cheshire, UK. This was done using the Seqed program, which is part of the Genetics Computer Group (GCG) software package (GCG, Madison, Wisconsin, USA).

3.4.5 Statistical analysis of sequencing data to compare with RAPD results

Sequence data were aligned with sequences obtained from the EMBL DNA database using the program PILEUP (Wisconsin Package, Version 8.1-UNIX, Genetics Computer Group, Madison, Wisconsin, USA). Only unambiguously aligned sequences were used for distance calculations. The program LINEUP (Wisconsin

Package) was used to delete regions of uncertainty. A distance matrix with the Kimura correction was calculated using the EDNADIST program (modified version of PHYLIP version 3.572c's DNADIST by Joseph Felsenstein), and a phenogram drawn based on average linkage cluster analysis using SPSS for Windows software.

3.4.6 Phylogenetic analysis of *Treponema*

All distinct *Treponema* species in the EMBL DNA database available to date (December, 1997) were aligned with the sequence data as described in Section 3.4.5. Sequences were available (accession number in brackets) for: *T. succinifaciens* ATCC 33096^T (M57738), *T. bryantii* ATCC 33254^T (M57737), *T. saccharophilum* ATCC 43261^T (M71238), *T. phagedenis* strain K5 (M57739), *T. pallidum* Nichols strain (M34266), *T. medium* G7201^T (D85437), *T. amylovorum* isolate HA2P (Y09959), *T. maltophilum* ATCC 51939^T (X87140), *T. pectinovorum* ATCC 33768^T (M71237) and *T. denticola* ATCC 33520 (M71236). Pair-wise distances with the Kimura correction were calculated from the aligned *Treponema* sequences using the program EDNADIST. Only unambiguously aligned sequences were compared, with the program LINEUP (Wisconsin Package) used to remove ambiguously aligned bases, as indicated in Appendix 7.4.2. Phylogenies were estimated using the program ENEIGHBOR (modified version of PHYLIP version 3.572c's NEIGHBOR by Joseph Felsenstein) from the distance matrix data using the NJ method of clustering.

Bootstrap analysis was performed on the data as follows:

The aligned sequence data were converted to PHYLIP format using the program READSEQ (Wisconsin Package). A multiple data set (100 repetitions) was formed using the program ESEQBOOT (Wisconsin Package). The output of this program was used as the input for EDNADIST, with the additional 'analyse multiple data sets' option, and left to run overnight. The consensus tree was provided by the programs ENEIGHBOR and ECONSENSE (Wisconsin Package), with the confidence levels for the nodes of the tree recorded.

3.5 Design of oligonucleotide probes and PCR primers

The aligned *Treponema* sequences were scrutinised by eye for regions of species variation. Two such regions were identified. These were positions 95 to 61 (SOC2) and positions 194 to 171 (DEN2). The program FASTA (Wisconsin Package,

Version 8.1-UNIX, Genetics Computer Group, Madison, Wisconsin ,USA) was used to check for the absence of these potential probes in all DNA sequences registered in the EMBL DNA database. The program STEMLOOP confirmed the absence of significant levels of secondary structure in the oligonucleotide sequences. Two oligonucleotide sequences were manufactured by Perkin-Elmer: SOC2 (24- mer) and DEN2 (22- mer). The sequence S2P was manufactured to be used as a PCR primer and corresponds to the reverse and complement of SOC2. The TREP probe (18-mer) was designed to detect spirochaetal species by Choi *et al.* (1994).

3.5.1 Digoxigenin oligonucleotide 3' -end labelling

The probes SOC2 and DEN2 were concentrated by ethanol precipitation to a final concentration of 10 mM (Sambrook *et al.*, 1989). SOC2, DEN2 and TREP probes (100 pmoles) were labelled with digoxigenin (DIG) using a DIG Oligonucleotide 3' - End Labeling Kit as per the manufacturer's instructions (Appendix 7.3.5).

3.5.1.i Quantification of labelled probe

Labelled probe was serially diluted in water from an initial dilution of 1:50 to a final dilution of 1:31 250. Labelled control DNA, supplied with the kit, was also serially diluted from 50 fmol μl^{-1} to 0.08 fmol μl^{-1}. Each dilution (1 µl) was dotted onto a positively-charged nylon membrane. The samples were fixed to the membrane by UV cross-linkage using a model STX 20.M UV transilluminator, 75 s for each side of the membrane. The DIG - labelled DNA was then detected using CDP -*Star* ™ .

3.5.1.ii Detection of DIG - labelled DNA with CDP - *Star*™

Membranes were rinsed in washing buffer for 2 min and then agitated in 1 % blocking solution for 30 min. Anti - digoxigenin- antibody conjugated to alkaline phosphatase was diluted to 37.5 mU ml^{-1} in 1 x blocking solution. Membranes were incubated with the antibody solution for 30 min. After rinsing twice in washing buffer, the membranes were equilibrated in detection buffer for 2 min. Membranes were incubated with CDP - *Star*™ (250 µM final concentration) in sealed bags for 5 min, after which they were removed, the excess liquid drained, and then sealed in fresh bags.

The membranes were exposed to Blue X -ray film for 3 min (immobilised probe) or between 20 - 60 min (dot - blot hybridisation). Films were developed using a Compact X4 Imaging System.

3.6 Dot-blot Hybridisation

DNA extracted from *Treponema* species, *Prevotella intermedia* (Pi) and *Leptospira icterohaemorrhagiae* (Li) were quantified as described in Section 3.2.1 and then diluted to concentrations of 100, 25 & 5 ng μl^{-1}. Each dilution was denatured by heating to 95°C for 10 min and then immediately placed on ice. The denatured, diluted DNA samples (1 µl) were dotted onto a positively-charged nylon membrane. The samples were fixed by UV irradiation as described in Section 3.5.1.i. Membranes were pre-hybridised in either standard hybridisation buffer (for probes DEN2 and TREP) or DIG Easy Hyb (SOC2) for 2 h, at 37°C, in roller tubes in a Hybaid Shake 'n' Stack hybridisation oven (Hybaid Ltd., Teddington, Middlesex, UK). The pre-hybridisation solution was discarded and replaced with DIG-labelled probe (5 pmol) in 6 ml of the corresponding hybridisation buffer. The probes were left to hybridise for 3 h at 37°C. After this time the probe solution was decanted and stored at - 20°C to be used in subsequent hybridisation reactions. The membranes were removed from the roller tubes and placed in clean, large petri-dishes and washed twice with 2 x washing solution at room temperature. Each membrane was then washed twice, for 15 min, in roller tubes with more stringent conditions as described in Section 4.6.1.

The membranes were then processed for detection as described in Section 3.5.1.ii.

3.6.1 Stripping probe from membranes

Membranes to be stripped were rinsed in distilled water. The membranes were incubated with stripping solution for 2 x 20 min at 37°C in roller tubes in a Hybaid Shake 'n' Stack hybridisation oven. The membranes were rinsed in 2 x SSC and stored in sealed bags at 4°C prior to re-probing.

3.7 Specific PCR detection

The oligonucleotide pairs DEN2/TPU1 and S2P/RTU3 were tested as specific PCR primers with soc2, soc3, pect, vin, den1, den2, den3, den4, Li, Pi, and patient isolates 3, 54, 63, 64 & 68. The PCR contained varying amounts of either extracted DNA (0.2 pg - 100 ng) or whole cells (5 - 2 x 10^6 cells; Sections 3.7.1 & 3.7.2), 1 x buffer IV, 1.5 mM $MgCl_2$ (primer S2P) or 3 mM $MgCl_2$ (primer DEN2), 0.2 mM of each dNTP, 0.3 μM of each primer and 1.25 units of *Taq* polymerase, in a final volume of 50 μl. The reaction mix and the mineral oil were UV irradiated at 254 nm for 4 min prior to the addition of template DNA. The mixture was incubated for 3 min at 94°C before the cycling profile of 1 min at 94°C, 1 min at 57°C (for primer DEN2) or 1 min at 62°C (for primer S2P) and 1 min at 72°C for 30 cycles. The last cycle had a 5 minute extension step. PCR product was confirmed by agarose gel electrophoresis as described in Section 3.2.1.i. As the expected fragment was under 250 bp for primers DEN2/TPU1, a 3 % gel was used with NuSieve® 3:1 agarose. PCR was also performed with all the samples with the primer pair TPU1/RTU3 as described in Section 3.4.1.

3.7.1 Cell counts

Three week old cultures of den2 and soc2 were centrifuged for 1 min at 12 000 x *g*. The pellets of cells were resuspended in water and diluted 10 -fold. An aliquot of each was placed in an Improved Neubauer haemocytometer (Weber Scientific International Ltd., Teddington, Middlesex, UK) and left to settle for a few minutes. Twenty-five small squares were counted for each sample. This was repeated with freshly spun-down aliqouts on four occasions. The average number of cells per square was calculated (Y). The average number of cells ml^{-1} was calculated according to the following manufacturer's equation:

Equation 3.3 Cells ml^{-1} = Y x 10 x 400 x 1000 x dilution factor

3.7.2 Preparation of cells for PCR

After samples were taken for cell counts, the cultures were stored at 4°C before use in the PCR. An aliquot was spun down for 3 min (12 000 x *g*) and the pellet of cells

resuspended in the same volume of water. Samples were serially diluted 10, 10^2, 10^3, 10^4 -fold in water and boiled for 10 min. Aliquots of the boiled cell samples were used in the PCR as template.

4.0 Results

4.1 Growth and maintenance of Culture Collection strains

All strains of *T. denticola* grew well in NOS broth (medium 1357) supplemented with rifampicin (Appendix 7.1.1), producing turbid growth adequate for DNA extraction after 7 days, and surviving continuous subculture into fresh broth on a monthly basis. The pH of the medium was between 7.0 and 7.5 and did not appreciably affect the growth of the bacteria. Purity was confirmed by phase-contrast microscopy at x 40 magnification and Gram-stain at x 100. These methods proved the existence of only spirochaetal-shaped bacteria in the culture. The potential problem of cross-contamination with a different treponeme cannot be ignored, however, great care was taken when subculturing stocks to use sterile, individually wrapped, plastic pastettes (Alpha Laboratories Ltd., Eastleigh, Hampshire, UK) for each strain. Screw-capped bijoux bottles were used as culture vessels, which further reduced the likelihood of cross-contamination.

The growth of *T. socranskii* strains (soc1, soc2 & soc3), vin and pect was difficult. A very sparse growth of vin was achieved in modified NOS broth which only survived for a few rounds of subculture.

Initially, pect and soc1 were grown in ATCC 1367 medium, and then in a modified medium supplemented with fatty acids and haemin instead of the rumen fluid (Appendix 7.1.2). After several rounds of subculture, soc1 could not be recovered, even from frozen stocks. Soc2 and soc3 were resuscitated in 'modified M10 medium' (Appendix 7.1.3). Soc2 and soc3 grew well in this medium for the first few rounds of subculture. In subsequent rounds, the growth became sparse and viable organisms could not be recovered.

4.1.1 Maintenance of clinical isolates

The clinical isolates were all purified and donated by Helen Wardle, formerly of Hope Hospital, Salford, UK (Appendix 7.2). All five isolates were initially recovered in ATCC culture medium 1357 (Appendix 7.1.1) but were subsequently subcultured in other media - 1367 medium containing rumen fluid and a modified 1367 medium containing fatty acids (Appendix 7.1.2). Patient isolates 3, 54 and 68

all grew well in 1357 medium and were consequently always subcultured into this medium, on a monthly basis. Patient isolates 63 and 64 (Figure 4.1) grew sparsely in 1357 medium, but produced a denser growth on subculturing into modified 1367 medium. These patient isolates were thus subsequently subcultured in this medium. After several rounds of subculture, it was not possible to recover patient isolate 64, although enough DNA had been extracted for the ensuing experiments.

All the patient isolates were examined microscopically to confirm purity in the same manner as the ATCC strains (Section 4.1).

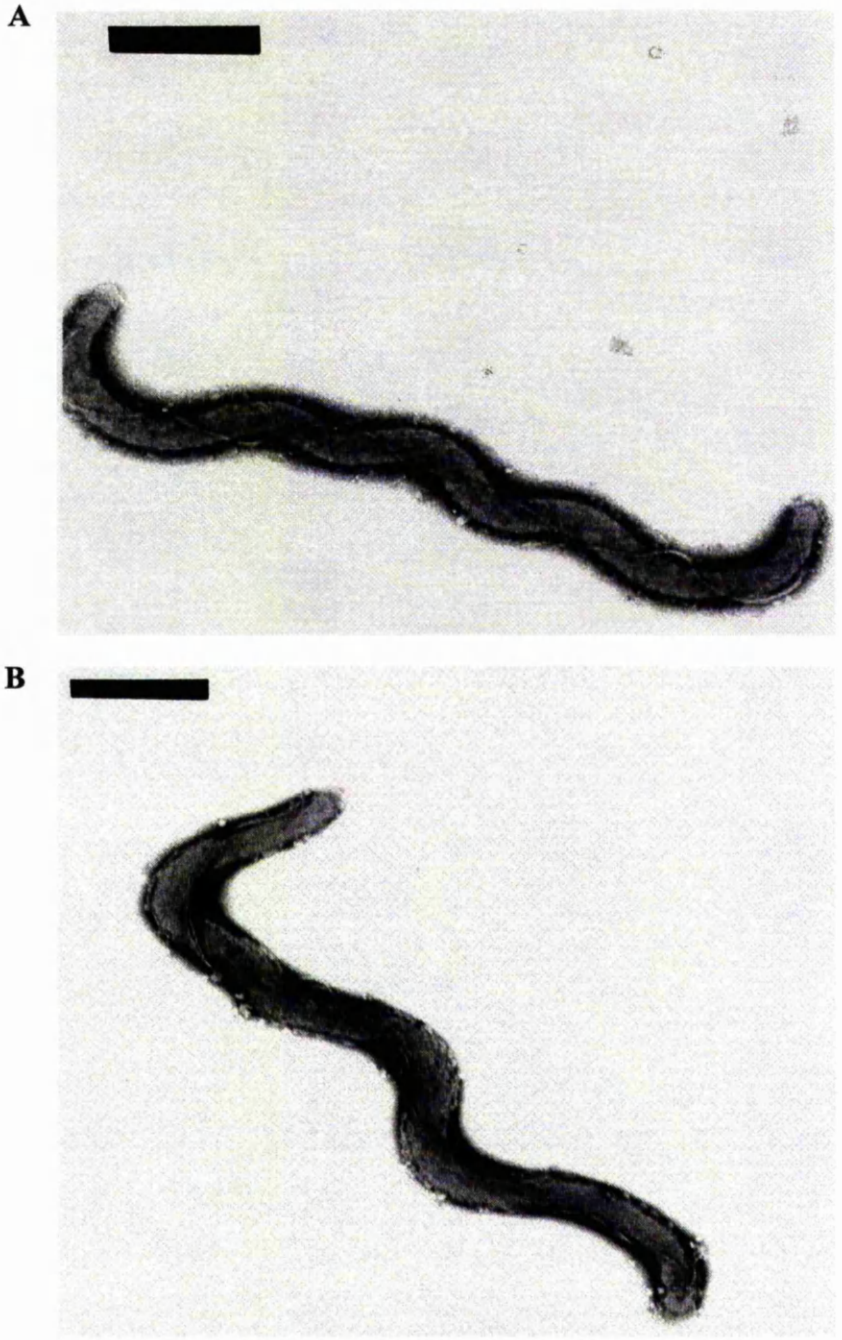

Figure 4.1 Electron micrographs of negatively-stained *Treponema*, isolated from subgingival plaque from two patients with adult periodontitis. (A) Patient isolate 64; (B) Patient isolate 63. Samples were fixed with 5% glutaraldehyde and stained with 3 % phosphotungstic acid, prior to being examined using a Phillips 420 transmission electron microscope at 80 kV, and photographed on Kodak film. The scale bar represents 1 μm. *Pictures courtesy of A. Curry and H. Cotterill, PHL, Manchester, UK*

4.2 DNA extraction and quantification

All four *T. denticola* strains, soc2, soc3, vin and all patient isolates yielded large quantities of DNA following a DNA extraction protocol (Section 3.2). Estimation of concentrations varied from 50 to over 200 ng μl^{-1} following agarose gel electrophoresis (Section 3.2.1). DNA from soc1 was recovered from only two batches of broth, at low concentration (under 20 ng μl^{-1}). This was enough DNA for the RAPD and sequencing experiments, but not enough for the dot-blot hybridisation or specific PCR detection experiments. DNA extraction from pect was successful on only a few occasions and did not generally yield large quantities (under 100 ng μl^{-1}). However, this was enough DNA for all subsequent experiments.

4.3 RAPD-PCR

ATCC strains (soc1, pect, vin, den1 & den2) and all five clinical isolates (3, 54, 63, 64, & 68) were characterised by RAPD-PCR with four different primers. Distinct RAPD patterns were produced for all the strains tested with each of the primers (Figures 4.2 - 4.5). Negative control reactions were always performed for each PCR, containing all the reagents except added DNA. Occasionally, faint bands were evident in the negative control and on these occasions all the reactions for that run were discarded. Fresh aliquots of all reagents were used in the subsequent repeat experiments. Only reactions that had a blank negative control were considered for analysis.

To ensure reproducibility, DNA was extracted from at least two broth cultures of the same isolate and both subjected to RAPD-PCR on separate occasions. Occasionally the PCR failed completely (no bands visible) or partially (only low molecular weight bands visible). This problem was rectified by using either fresh aliquots of *Taq* polymerase or fresh dilutions of template DNA. Faint bands were sometimes found in one RAPD pattern and not in another, but generally the band patterns for each isolate were reproducible.

After this initial study, the newly acquired ATCC strains of soc2, soc3, den3 and den4 were also subjected to RAPD-PCR using the four primers, with the intention of adding this data to the analysis. Very few bands were present on the gels (data not shown), and the few bands that were present were not reproducible, even when the

PCR was repeated with fresh *Taq* and new primers. Several months later, it was found that the manufacturer, without communicating this fact to its customers, had changed the formulation of the *Taq* polymerase, making the concentration one third of the original and this was the probable reason for the failure of the PCR. For this reason, the statistical analysis did not include data from these strains.

4.3.1 Optimisation of band patterns

The optimal magnesium and primer concentrations were determined by performing RAPD-PCR with den1 and a range of concentrations of magnesium (0.5 - 5.0 mM) and primer (0.5 - 5.0 μM). The optimum concentrations were 3 mM (magnesium) and 1 μM (primer) for all four primers. Lower concentrations of either, resulted in fainter or no bands at all, rather than less bands and a simpler pattern (data not shown).

The amount of template DNA (0.5 - 100 ng) added to the reaction did not affect the number or size of bands in the RAPD patterns (data not shown).

4.3.2 Pattern analysis

As the band profiles for each isolate were very different, it was not possible to quantify the similarity between pairs of isolates visually. The gel photographs were thus scanned into, and analysed on, a Sun Sparc workstation using Whole Band Analyzer software. This software allows sizing of bands by reference to the molecular weight markers, taking into account the slant of the gel. The Polaroid photographs of each RAPD pattern were also enlarged to allow analysis by eye. To confirm standardised measurement, the bands were scored on one day and then re-scored on a separate day, with all bands scored, as far as possible, subject to the following criteria:

a) only bands clearly reproducible in both RAPD-PCR reactions were scored.

b) only bands that could be easily sized using the Whole Band Analyzer software were included. In effect this meant that bands under 400 bp and greater than 2800 bp were not scored.

c) bands running very close together to produce a 'fuzzy' appearance on the gel were not scored.

The presence (= 1) and absence (= 0) of bands were recorded in binary matrices (Tables 4.1 - 4.4), so that data from all four primers could be compiled together. In total, 191 bands were scored.

Two of the primers, (L10 and US) had been used successfully as RAPD-PCR primers on isolates of *Leptospira* (Corney *et al.*, 1993). Primer RSP had been used to differentiate isolates of *Borrelia burgdorferi* (Welsh *et al.*, 1992) and primer 970-11 had been used to distinguish isolates of *Porphyromonas gingivalis* (Ménard *et al.*, 1992).

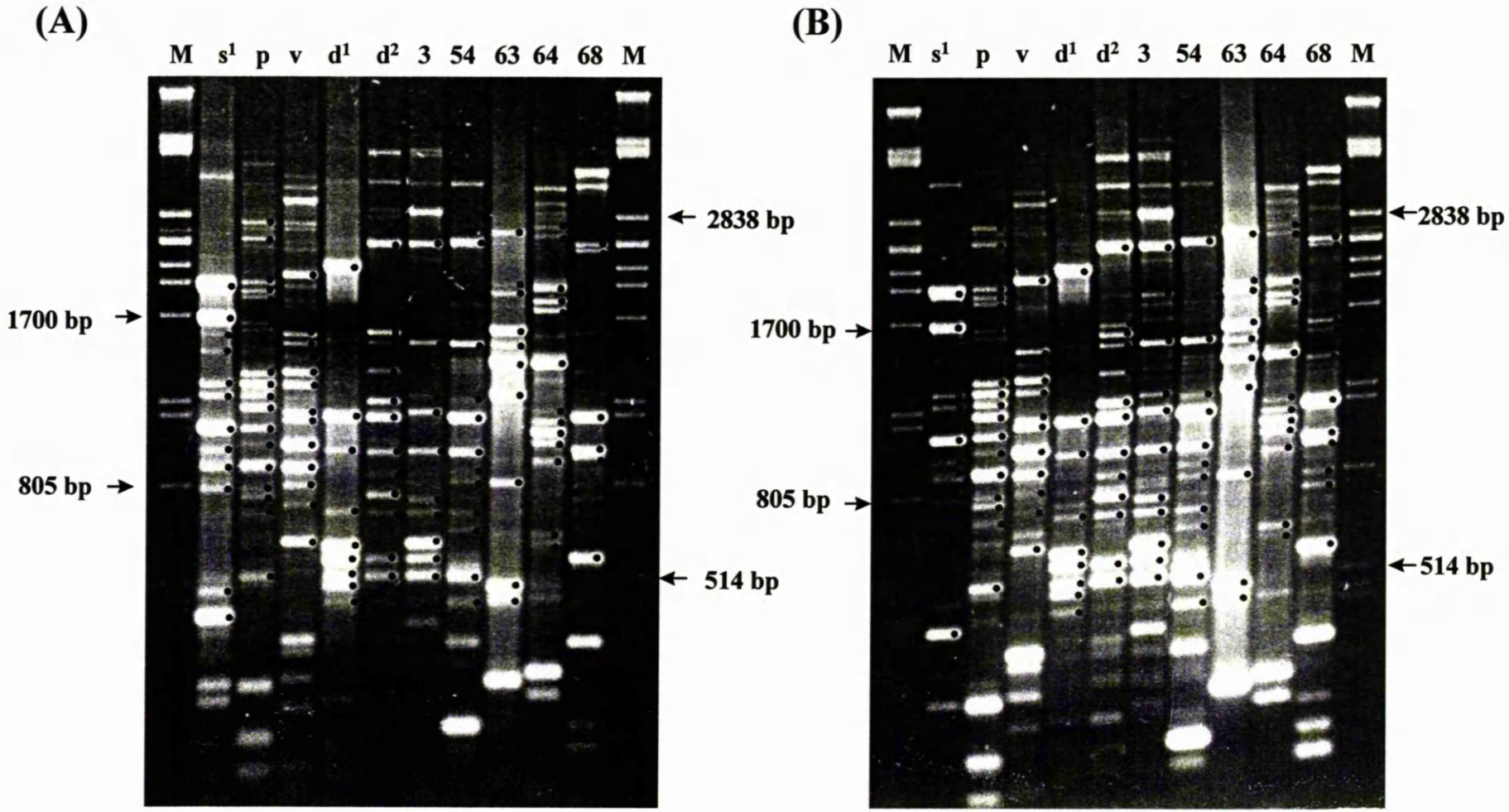

Figure 4.2 RAPD-PCR of *Treponema* strains with primer L10. **(A)** DNA fragments generated by each isolate after RAPD-PCR with primer L10. The fragments were run in 1.8 % agarose gels, stained with ethidium bromide and photographed under UV light (Section 3.2.1.i). **(B)** A second DNA extraction of each isolate subjected to RAPD-PCR on a separate occasion. Fifty-eight distinct bands marked '•' were scored, ranging from approximately 445 bp to 2730 bp. Key: *T. socranskii* subsp. *buccale* ATCC 35534 (**s¹**), *T. pectinovorum* ATCC 33768T (**p**), *T. vincentii* ATCC 35580 (**v**), *T. denticola* ATCC 35405 (**d¹**), *T. denticola* ATCC 33520 (**d²**), and patient isolates **3, 54, 63, 64** and **68**. Molecular weight markers λ*Pst*1 digest (**M**).

L10	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58
soc1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
pect	0	0	0	0	1	0	0	0	0	0	1	0	0	1	1	0	0	1	0	0	0	1	1	0	0	0	1	0	1	1	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	1	0	0	0	0	0	1	0	0	0	1
vin	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	1	0	0	0	0	1	0	1	0	1	0	0	0	1	1	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
den1	0	1	0	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
den2	0	0	0	0	1	1	0	0	0	0	1	0	0	1	0	1	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
3	0	0	0	0	1	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
54	0	1	0	0	1	0	0	0	0	1	1	0	0	0	1	0	1	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
63	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0
64	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	1	0	1	0
68	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0

Table 4.1 Binary matrix representing RAPD-PCR data from primer L10. Bands are numbered in order of increasing molecular size and are listed left to right. Presence of band = 1, absence = 0. Species are as follows: *T. pectinovorum* ATCC 33768^T (**pect**), *T. denticola* ATCC 33520 (**den2**), *T. denticola* ATCC 35405 (**den1**), *T. vincentii* ATCC 35580 (**vin**), *T. socranskii* subsp. *buccale* ATCC 35534 (**soc1**), and patient isolates **3** (isolated from a 44 year old patient with early-onset periodontitis), **54** (history unknown), **63** (isolated from a 55 year old patient with adult periodontitis), **64** (isolated from a 60 year old patient with adult periodontitis), and **68** (isolated from a 28 year old patient with ANUG).

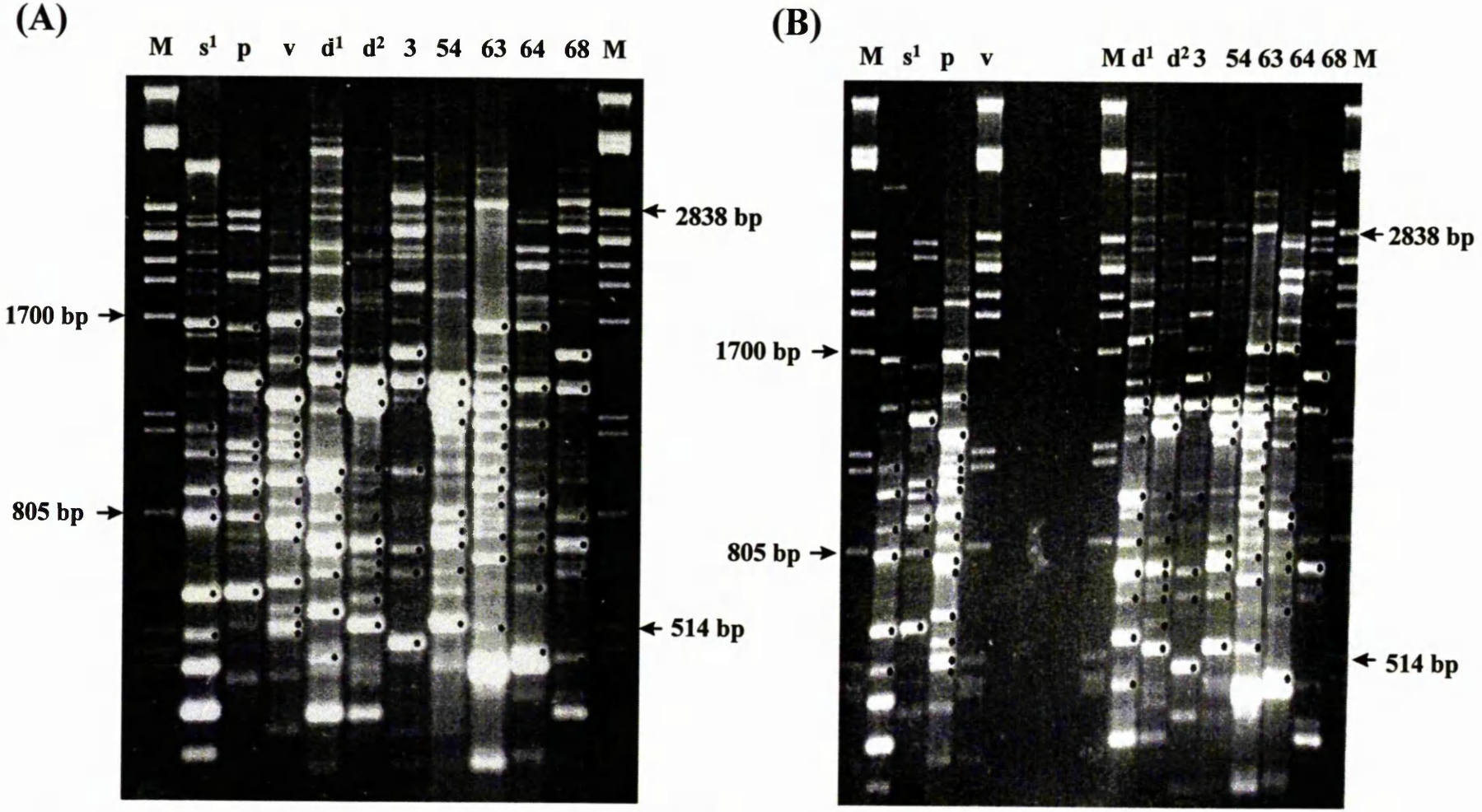

Figure 4.3 RAPD-PCR of *Treponema* strains with primer US. **(A)** DNA fragments generated by each isolate after RAPD-PCR with primer US. The fragments were run in 1.8 % agarose gels, stained with ethidium bromide and photographed under UV light (Section 3.2.1.i). **(B)** A second DNA extraction of each isolate subjected to RAPD-PCR on a separate occasion. Forty-six distinct bands marked '•' were scored, ranging from sizes 435 bp to 1800 bp. Bands above this size were not scored due to the lack of clear reproducibility in photograph 'B'. Key: *T. socranskii* subsp. *buccale* ATCC 35534 (**s¹**), *T. pectinovorum* ATCC 33768T (**p**), *T. vincentii* ATCC 35580 (**v**), *T. denticola* ATCC 35405 (**d¹**), *T. denticola* ATCC 33520 (**d²**), and patient isolates **3, 54, 63, 64** and **68**. Molecular weight markers λ*Pst*1 digest (**M**).

	US																																										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**46**
soc1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0
pect	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0	1	0	1	0	0	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0
vin	0	0	0	1	1	1	0	0	0	1	0	0	0	0	0	1	0	1	0	0	1	0	0	1	0	0	0	1	1	1	0	0	1	0	1	0	0	0	0	1	0	1	0	0	1	0
den1	1	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	1	0	0	1
den2	0	0	0	0	1	0	1	0	0	0	1	1	0	1	1	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0
3	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	1	0
54	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0
63	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	1	0	0	1	0	0	1	0	0	1	0	0	1	0	0	1	0	0	1	0	0	0	1	0
64	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0
68	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0

Table 4.2 Binary matrix representing RAPD-PCR data from primer US. Bands are numbered in order of increasing molecular size and are listed left to right. Presence of band = 1, absence = 0. Species are as follows: *T. pectinovorum* ATCC 33768^T (**pect**), *T. denticola* ATCC 33520 (**den2**), *T. denticola* ATCC 35405 (**den1**), *T. vincentii* ATCC 35580 (**vin**), *T. socranskii* subsp. *buccale* ATCC 35534 (**soc1**), and patient isolates **3** (isolated from a 44 year old patient with early-onset periodontitis), **54** (history unknown), **63** (isolated from a 55 year old patient with adult periodontitis), **64** (isolated from a 60 year old patient with adult periodontitis), and **68** (isolated from a 28 year old patient with ANUG).

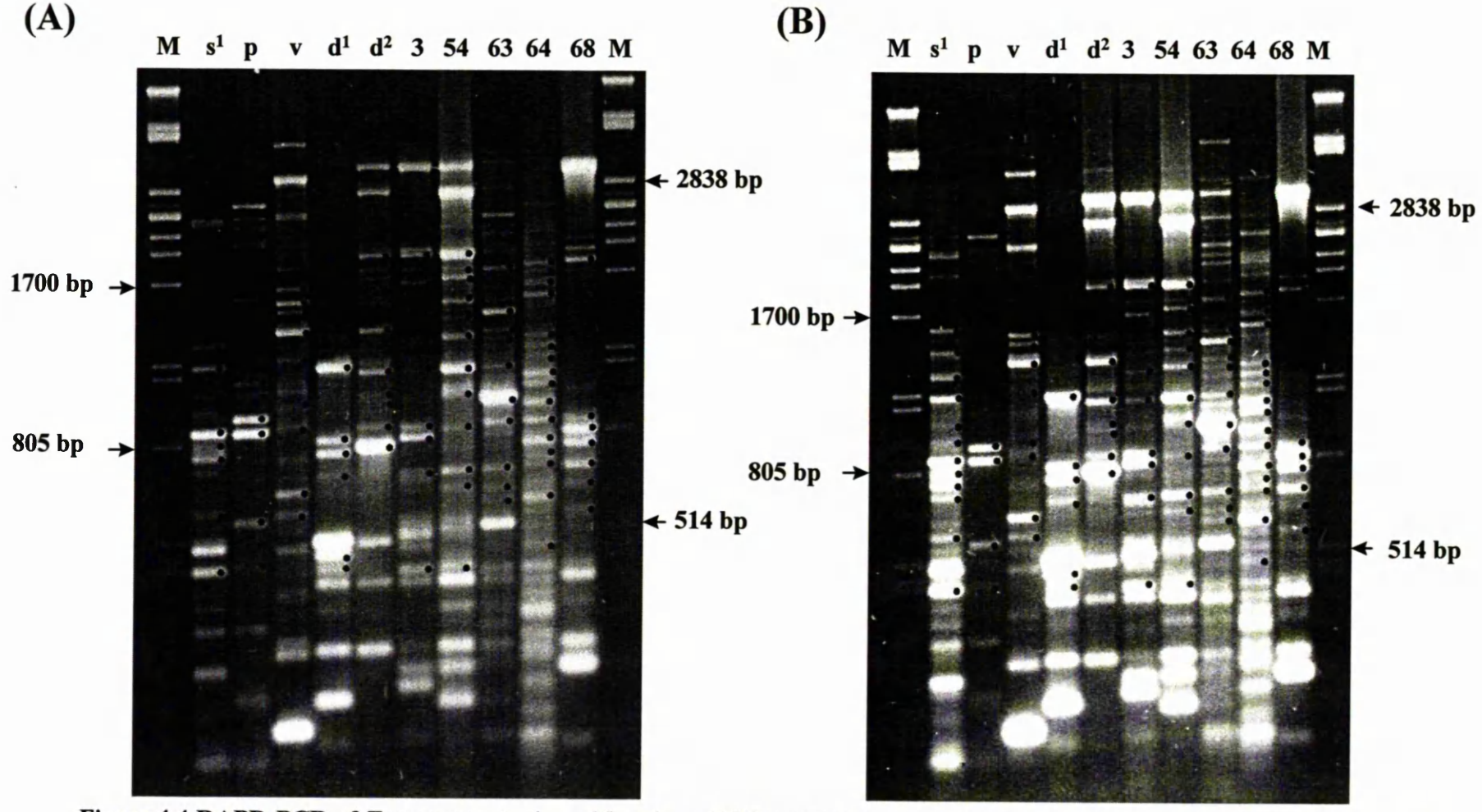

Figure 4.4 RAPD-PCR of *Treponema* strains with primer RSP. **(A)** DNA fragments generated by each isolate after RAPD-PCR with primer RSP. The fragments were run in 1.8 % agarose gels, stained with ethidium bromide and photographed under UV light (Section 3.2.1.i). **(B)** A second DNA extraction of each isolate subjected to RAPD-PCR on a separate occasion. Forty-nine distinct bands marked '•' were scored, ranging from approximately 400 bp to 2000 bp. Bands above this size were not scored due to the lack of clear reproducibility in photograph 'B'. Key: *T. socranskii* subsp. *buccale* ATCC 35534 (**s¹**), *T. pectinovorum* ATCC 33768T (**p**), *T. vincentii* ATCC 35580 (**v**), *T. denticola* ATCC 35405 (**d¹**), *T. denticola* ATCC 33520 (**d²**), and patient isolates **3, 54, 63, 64** and **68**. Molecular weight markers λ*Pst*1 digest (**M**).

RSP	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
soc1	1	0	0	0	1	0	0	0	1	1	0	1	0	1	0	0	1	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
pect	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
vin	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0
den1	1	0	1	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
den2	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1
3	1	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1
54	1	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	1	0	1	0	0	0	1	0
63	0	0	0	0	0	1	1	1	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0
64	0	0	1	0	0	1	0	1	1	0	1	0	0	1	0	0	1	0	0	1	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	1	0	0	0	0
68	0	0	0	1	0	0	1	1	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0

Table 4.3 Binary matrix representing RAPD-PCR data from primer RSP. Bands are numbered in order of increasing molecular size and are listed left to right. Presence of band = 1, absence = 0. Species are as follows: *T. pectinovorum* ATCC 33768^T (**pect**), *T. denticola* ATCC 33520 (**den2**), *T. denticola* ATCC 35405 (**den1**), *T. vincentii* ATCC 35580 (**vin**), *T. socranskii* subsp. *buccale* ATCC 35534 (**soc1**), and patient isolates **3** (isolated from a 44 year old patient with early-onset periodontitis), **54** (history unknown), **63** (isolated from a 55 year old patient with adult periodontitis), **64** (isolated from a 60 year old patient with adult periodontitis), and **68** (isolated from a 28 year old patient with ANUG).

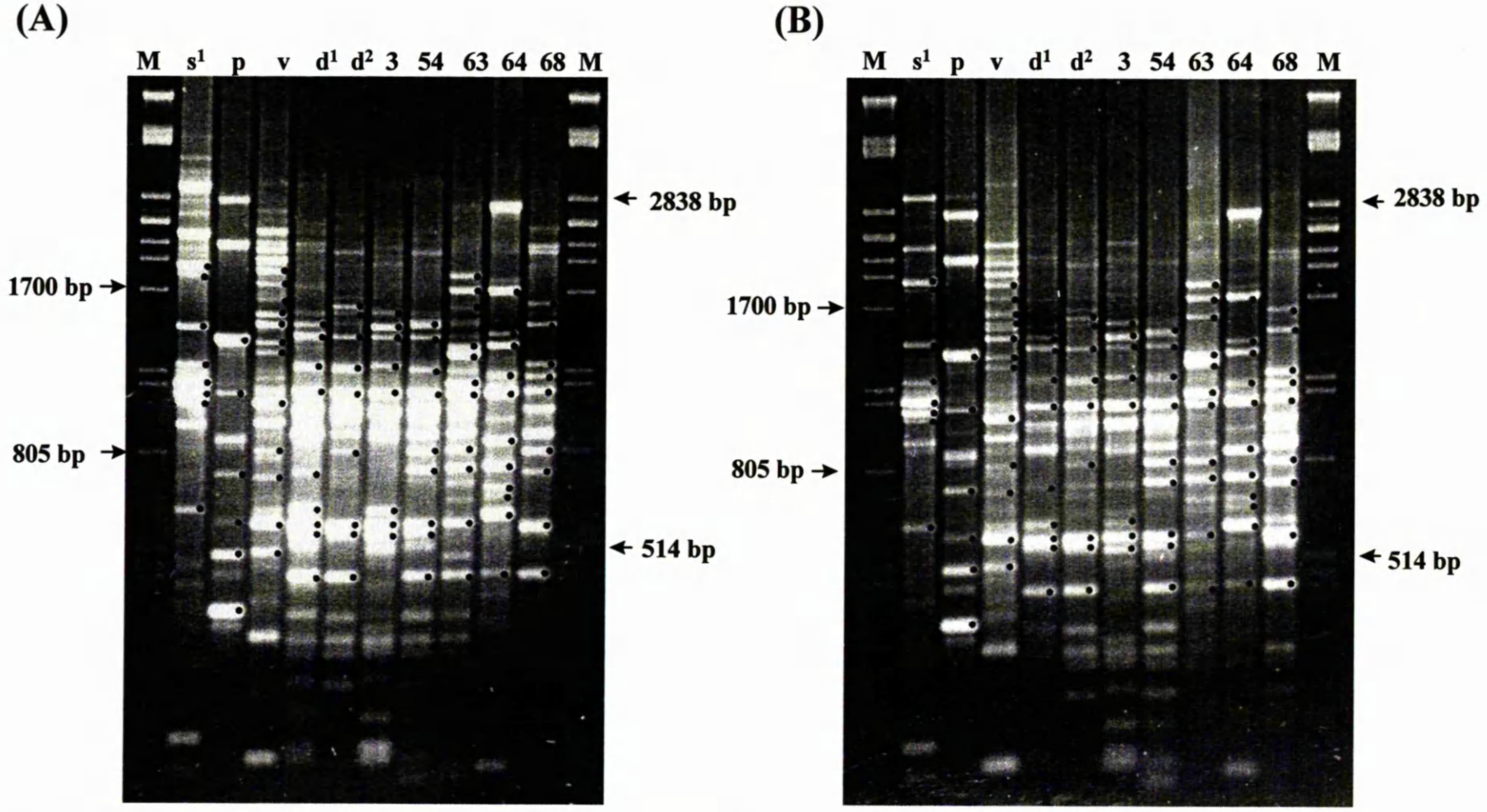

Figure 4.5 RAPD-PCR of *Treponema* strains with primer 970-11. **(A)** DNA fragments generated by each isolate after RAPD-PCR with primer 970-11. The fragments were run in 1.8 % agarose gels, stained with ethidium bromide and photographed under UV light (Section 3.2.1.i). **(B)** A second DNA extraction of each isolate subjected to RAPD-PCR on a separate occasion. Thirty-eight distinct bands marked '•' were scored, ranging from approximately 400 bp to 2200 bp. Key: *T. socranskii* subsp. *buccale* ATCC 35534 (**s¹**), *T. pectinovorum* ATCC 33768T (**p**), *T. vincentii* ATCC 35580 (**v**), *T. denticola* ATCC 35405 (**d¹**), *T. denticola* ATCC 33520 (**d²**), and patient isolates **3, 54, 63, 64** and **68**. Molecular weight markers λ*Pst*1 digest (**M**).

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	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**
soc1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1
pect	1	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
vin	0	0	0	1	0	0	1	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	1	1	0	0	0	1	0	1	0	0	1	0	1	0	1	0
den1	0	1	0	0	0	1	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
den2	0	1	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0
3	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0
54	0	1	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	1	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
63	0	1	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	0	1	0	1	0	1	0	0
64	0	1	0	0	0	0	0	1	0	1	1	0	1	0	1	0	0	1	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0
68	0	1	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	1	0	1	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0

Table 4.4 Binary matrix representing RAPD-PCR data from primer 970-11. Bands are numbered in order of increasing molecular size and are listed left to right. Presence of band = 1, absence = 0. Species are as follows: *T. pectinovorum* ATCC 33768^T (**pect**), *T. denticola* ATCC 33520 (**den2**), *T. denticola* ATCC 35405 (**den1**), *T. vincentii* ATCC 35580 (**vin**), *T. socranskii* subsp. *buccale* ATCC 35534 (**soc1**), and patient isolates **3** (isolated from a 44 year old patient with early-onset periodontitis), **54** (history unknown), **63** (isolated from a 55 year old patient with adult periodontitis), **64** (isolated from a 60 year old patient with adult periodontitis), and **68** (isolated from a 28 year old patient with ANUG).

4.3.3 Statistical analysis

Simple matching (SM) and Dice (D) similarity coefficients were determined from the binary matrix data from all four primers (Table 4.5).

Phenograms were drawn based on average linkage cluster analysis of the results, calculated using SPSS for Windows software (Figure 4.6).

The actual clustering depended on the similarity coefficient calculated, although the trends were the same. Both phenograms cluster three of the patient isolates (3, 54 & 68) with the two strains of *T. denticola*. The identities of isolates 63 and 64 are inconclusive, although they are probably not strains of *T. denticola*.

The average similarity between different species of *Treponema* is 63 % with the SM data (*16 % considering the D data*), with a range of 56 - 68 % *(7 - 25 %)*. The similarity between two strains of the same species (den1 & den2) is 77 % *(45 %)*. If isolates 3, 54 and 68 are *T. denticola*, then the average similarity between strains of the same species is 78 % *(44 %)* with a range of 74 - 83 % *(35 - 57 %)*. Isolates 63 & 64 do not show this level of similarity to any other strains or isolates, which suggests they could belong to a different species.

If the data for each primer are assessed separately, the phenograms produced with both SM and D coefficients are all slightly different from each other (Appendix 7.4.1, Figures 7.1 - 7.4). Primers L10, 970-11 and US all show the clustering of isolates 3, 54 and 68 with the *T. denticola* strains, albeit in a different order. Primer RSP closely clusters isolates 3 and 68 with den1. A looser cluster contains den2 and isolates 54 & 63.

The positions of soc1, pect, vin and isolates 63 & 64 differ when all the primers are analysed separately and when analysed together, and so little can be concluded as to the final position of these species and isolates to each other. The cluster of *T. denticola* strains with 3, 54 and 68 is constant, confirming the identity of isolates 3, 54 and 68 as *T. denticola*.

(A)

	soc1	pect	vin	den1	den2	3	54	63	64	68
soc1	1.00	.68	.61	.67	.62	.70	.63	.66	.64	.69
pect		1.00	.59	.67	.66	.67	.64	.67	.64	.67
vin			1.00	.57	.58	.59	.61	.65	.57	.62
den1				1.00	.77	.83	.76	.67	.63	.79
den2					1.00	.78	.80	.71	.60	.78
3						1.00	.74	.69	.64	.78
54							1.00	.72	.59	.74
63								1.00	.64	.68
64									1.00	.62
68										1.00

(B)

	soc1	pect	vin	den1	den2	3	54	63	64	68
soc1	1.00	.25	.20	.20	.12	.26	.12	.24	.24	.21
pect		1.00	.13	.18	.20	.16	.15	.24	.24	.16
vin			1.00	.07	.13	.09	.18	.30	.18	.16
den1				1.00	.45	.57	.41	.22	.20	.44
den2					1.00	.46	.53	.33	.17	.44
3						1.00	.35	.25	.20	.39
54							1.00	.33	.13	.35
63								1.00	.28	.23
64									1.00	.14
68										1.00

Table 4.5 Similarity coefficients calculated from binary matrix data.
(A) Simple Matching coefficients of similarity, based on the number of shared fragments and the number of common absences between pairs of isolates.
(B) Dice coefficients of similarity, based on the number of shared fragments between pairs of isolates.
Species are as follows: *T. pectinovorum* ATCC 33768^T (**pect**), *T. denticola* ATCC 33520 (**den2**), *T. denticola* ATCC 35405 (**den1**), *T. vincentii* ATCC 35580 (**vin**), *T. socranskii* subsp. *buccale* ATCC 35534 (**soc1**) and patient isolates **3, 54, 63, 64** and **68**.

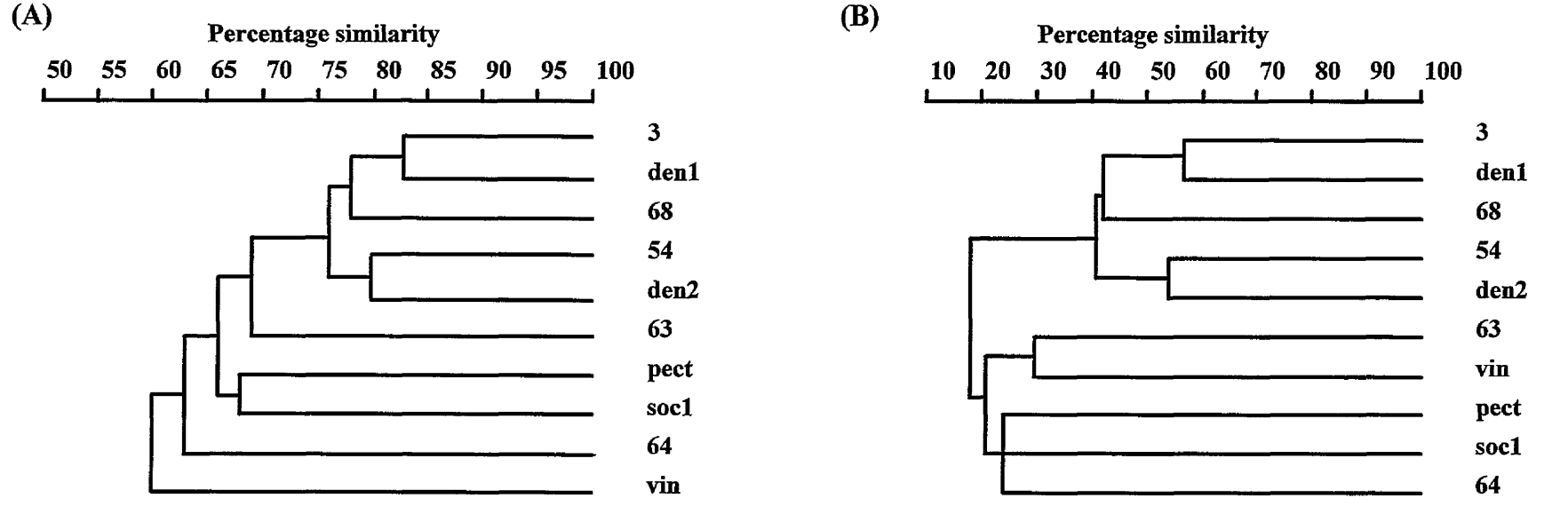

Figure 4.6 Phenograms of *Treponema* isolates, drawn after cluster analysis of **(A) Simple matching** and **(B) Dice** similarity coefficients from the RAPD-PCR binary matrix data, using SPSS for Windows software. Species are as follows: *T. pectinovorum* ATCC 33768^T (**pect**), *T. denticola* ATCC 33520 (**den2**), *T. denticola* ATCC 35405 (**den1**), *T. vincentii* ATCC 35580 (**vin**), *T. socranskii* subsp. *buccale* ATCC 35534 (**soc1**), and patient isolates **3** (isolated from a 44 year old patient with early onset periodontitis), **54** (history unknown), **63** (isolated from a 55 year old patient with adult periodontitis), **64** (isolated from a 60 year old patient with adult periodontitis), **68** (isolated from a 28 year old patient with ANUG).

4.4 Partial 16S rDNA sequencing

Partial 16S rDNA sequences were determined for soc1, soc2, soc3, vin, den1, and isolates 63, 64 and 68. The fragment sequenced corresponds to positions 8 to 536 in the 16S rRNA gene in *E. coli*. Four different methods were used to obtain the data as summarised in Table 4.6.

Initially, PCR fragments were cloned using two different vectors. The TA Cloning® Kit takes advantage of the nontemplate-dependent activity of *Taq* polymerase, where a single deoxyadenosine molecule is added to the 3' ends of duplex PCR fragments. These 3' A-overhangs can then be used to insert the PCR product into a vector such as pCR™II, which contains single 3' T-overhangs.

PCR products created by the action of *Pwo* enzyme are blunt-ended, due to the enzyme's extensive 3' - 5' exonuclease proof-reading activity. These fragments need to be phosphorylated prior to ligation in a blunt-ended vector, such as pGEX-2T.

Manual sequencing of cloned inserts, using the Sequenase™ version 2.0 T7 DNA Polymerase Sequencing Kit, was technically demanding and time-consuming, and revealed only partial sequence data from the PCR products. In order to increase the amount of sequence data, the PCR fragments were sequenced directly using Dynabeads® and also by cycle sequencing using the ABI Prism™ Dye Terminator Cycle Sequencing system. These methods were quicker to perform, resulting in more sequence information, in a shorter time, than the cloning methods.

Both forward and backward strands were sequenced when using each method. Sequences were aligned manually and any discrepancies between the two strands re-checked. Sequence data derived from the different methods were also compared, to arrive at a final consensus sequence for each isolate. The various sequencing methods did not result in differences in the sequences produced. However, the direct sequencing methods produced longer reads at one time, so that the experiments did not have to be repeated numerous times.

The sequences were entered into my directory on the computer at Daresbury, Warrington, Cheshire, UK. This was done using the Seqed program, which is part of the Genetics Computer Group (GCG) software package (GCG, Madison, Wisconsin, USA).

4.4.1 Statistical analysis

Appendix 7.4.2 shows the sequence data aligned with other *Treponema* species obtained from the EMBL DNA database. Sequences were available (accession number in brackets) for: *T. succinifaciens* ATCC 33096^T (M57738), *T. bryantii* ATCC 33254^T (M57737), *T. saccharophilum* ATCC 43261^T (M71238), *T. phagedenis* strain K5 (M57739), *T. pallidum* Nichols strain (M34266), *T. medium* G7201^T (D85437), *T. amylovorum* isolate HA2P (Y09959), *T. maltophilum* ATCC 51939^T (X87140), *T. pectinovorum* ATCC 33768^T (M71237) and *T. denticola* ATCC 33520 (M71236).

The unambiguously aligned sequences of soc1, vin, den1, isolates 63, 64 and 68 with den2 and pect were used to create a distance matrix with the Kimura correction (Table 4.7). A UPGMA phenogram was drawn and directly compared to the phenogram produced from the RAPD data (Figure 4.7), in order to relate the discriminatory abilities of RAPD-PCR and partial 16S rDNA sequencing.

(A) TA Cloning® Kit (Section 3.4.1)	(B) pGEX-2T (Section 3.4.2)	(C) Dynabeads® (Section 3.4.3)	(D) Automated sequencing (Section 3.4.4)	Total number of bases sequenced /bp
		soc1	soc1	480
			soc2	446
			soc3	428
		vin	vin	494
		den1	den1	452
63		63		469
64		64		469
	68		68	435

Table 4.6 Summary of sequencing strategies for soc1, soc2, soc3, vin, den1 & den2 and isolates 63, 64 & 68.

	64	63	soc1	den2	den1	68	vin	pect
64	.0000	.0026	.0267	.2441	.2396	.2391	.2441	.2347
63		.0000	.0294	.2406	.2396	.2426	.2405	.2312
soc1			.0000	.2436	.2391	.2386	.2472	.2444
den2				.0000	.0000	.0026	.0983	.1811
den1					.0000	.0000	.0980	.1804
68						.0000	.1007	.1833
vin							.0000	.1679
pect								.0000

Table 4.7 Distances between pairs of *Treponema* species and isolates as calculated using the EDNADIST program with the Kimura correction for nucleotide substitution. Species are as follows: *T. pectinovorum* ATCC 33768^T (**pect**), *T. denticola* ATCC 33520 (**den2**), *T. denticola* ATCC 35405 (**den1**), *T. vincentii* ATCC 35580 (**vin**), *T. socranskii* subsp. *buccale* ATCC 35534 (**soc1**) and patient isolates **63, 64** and **68.**

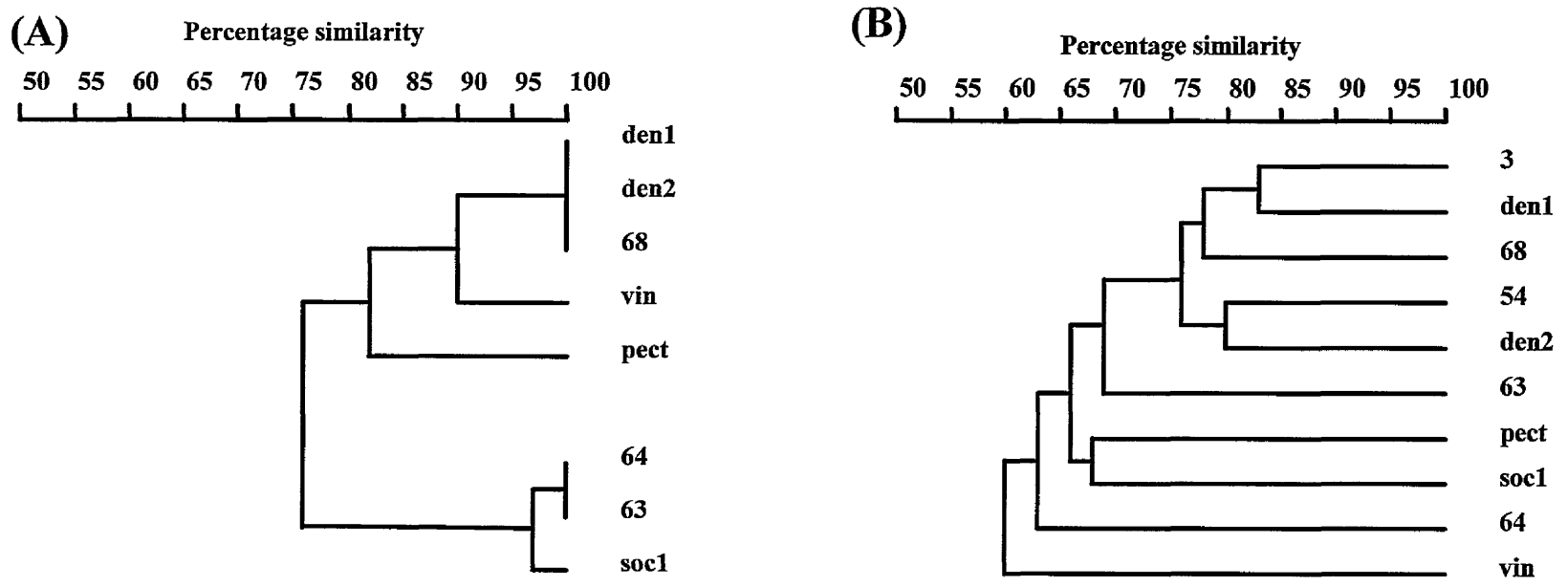

Figure 4.7 Phenograms showing % similarity between *Treponema* isolates, drawn after UPGMA cluster analysis using SPSS for Windows software derived from **(A) distance coefficients from the unambiguous alignment of 16S rDNA data**, and **(B) the simple matching similarity coefficients from the RAPD-PCR** binary matrix data. Species are as follows: *T. pectinovorum* ATCC 33768^T (**pect**), *T. denticola* ATCC 33520 (**den2**), *T. denticola* ATCC 35405 (**den1**), *T. vincentii* ATCC 35580 (**vin**), *T. socranskii* subsp. *buccale* ATCC 35534 (**soc1**), and patient isolates **3** (isolated from a 44 year old patient with early-onset periodontitis), **54** (history unknown), **63** (isolated from a 55 year old patient with adult periodontitis), **64** (isolated from a 60 year old patient with adult periodontitis), and **68** (isolated from a 28 year old patient with ANUG).

The sequencing phenogram shows two tight clusters. The first is of the near identical den1, den2 and isolate 68 (corresponding to only one base difference between isolate 68 and den2 at position 250, Appendix 7.4.2). The second is of *T. socranskii* and isolates 63 & 64 (only one base difference between isolates 63 and 64; 6 differences between soc2 and these isolates). In this region of the 16S rRNA gene, the mean similarity between species of *Treponema* is 81 % (range 75 - 90 %). From these data, patient isolate 68 is identified as *T. denticola* whilst patient isolates 63 and 64 are identified as *T. socranskii.*

4.4.2 Phylogenetic analysis

All strains sequenced (soc1, soc2, soc3, vin, den1, den2 isolates 63, 64 & 68) were aligned with ten other *Treponema* sequences retrieved from the EMBL DNA database. The complete alignment is shown in Appendix 7.4.2; only unambiguously aligned positions (385 bases) were included in the statistical analysis as indicated. The distance matrix method (Table 4.8) was used to estimate phylogenetic distances, using the Kimura correction for multiple nucleotide substitutions, and bootstrap analysis was performed as described in Section 3.4.6.

With the exception of *T. medium* and *T. vincentii*, the *Treponema* species are a diverse genus when comparing 16S rRNA sequences (Figure 4.8 A). The relative positions of the clinical isolates 63, 64 and 68 to *T. socranskii* and *T. denticola* respectively, confirms the identification of these isolates. The clustering was repeated using the alignments of whole 16S rDNA sequences that were available in the database (1350 bp). The resultant phylogenetic tree (Figure 4.8 B) showed similar, but not identical clustering, as the partial sequences.

	sacch	pect	amyl	den2	den1	68	vin	medium	phage	pallidum	malto	bryantii	64	63	soc2	soc1	soc3	succ
sacch	.0000	.1127	.1608	.1778	.1768	.1768	.1614	.1643	.1814	.1929	.1871	.2322	.2164	.2164	.2203	.2239	.2294	.2171
pect		.0000	.1336	.1811	.1804	.1833	.1679	.1676	.2054	.2130	.1666	.2007	.2347	.2312	.2352	.2444	.2286	.2511
amyl			.0000	.2147	.2138	.2168	.2156	.2151	.2157	.2307	.2173	.2177	.2006	.1973	.1977	.1943	.1835	.2211
den2				.0000	.0000	.0026	.0983	.0982	.0941	.1548	.1482	.2023	.2441	.2406	.2446	.2436	.2421	.2714
den1					.0000	.0000	.0980	.0978	.0938	.1539	.1474	.2014	.2396	.2396	.2401	.2391	.2375	.2665
68						.0000	.1007	.1006	.0938	.1539	.1474	.2044	.2391	.2426	.2396	.2386	.2370	.2660
vin							.0000	.0000	.0996	.1334	.1497	.2040	.2441	.2405	.2446	.2472	.2421	.2837
medium								.0000	.0994	.1332	.1494	.2036	.2436	.2401	.2441	.2467	.2416	.2868
phage									.0000	.1326	.1761	.2096	.2572	.2572	.2494	.2410	.2552	.2601
pallidum										.0000	.1852	.2449	.2469	.2469	.2428	.2454	.2449	.2834
malto											.0000	.2091	.2599	.2599	.2522	.2441	.2436	.2837
bryantii												.0000	.2760	.2723	.2643	.2657	.2815	.2793
64													.0000	.0026	.0079	.0267	.0240	.2155
63														.0000	.0105	.0294	.0267	.2190
soc2															.0000	.0240	.0267	.2124
soc1																.0000	.0268	.2089
soc3																	.0000	.2177
succ																		.0000

Table 4.8 Distances between pairs of *Treponema* species and isolates as calculated using the EDNADIST program with the Kimura correction for multiple nucleotide substitutions. Species are as follows: *T. saccharophilum* ATCC 43261^T (**sacch**), *T. pectinovorum* ATCC 33768^T (**pect**), *T. amylovorum* isolate HA2P (**amyl**), *T. denticola* ATCC 33520 (**den2**), *T. denticola* ATCC 35405 (**den1**), *T. vincentii* ATCC 35580 (**vin**), *T. medium* G7201^T (**medium**), *T. phagedenis* strain K5 (**phage**), *T. pallidum* Nichols strain (**pallidum**), *T. maltophilum* ATCC 51939^T (**malto**), *T. bryantii* ATCC 33254^T (**bryantii**), *T. socranskii* subsp. *paredis* ATCC 35535 (**soc2**), *T. socranskii* subsp. *buccale* ATCC 35534 (**soc1**), *T. socranskii* subsp. *socranskii* ATCC 35536 (**soc3**), *T. succinifaciens* ATCC 33096^T (**succ**) and patient isolates **63, 64** and **68**.

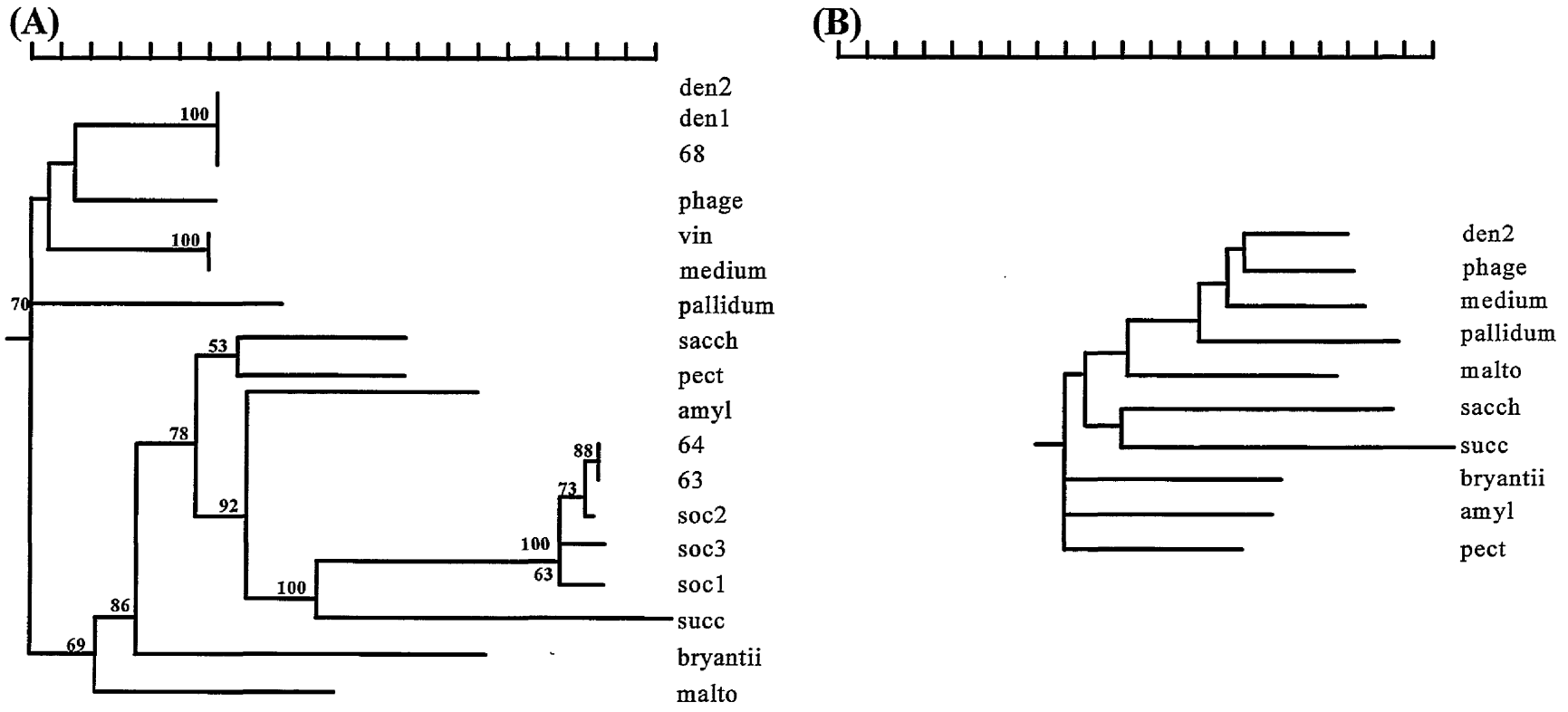

Figure 4.8 Phylogenetic trees of *Treponema* isolates Only unambiguously aligned positions were considered in the analysis. (A) partial 16S rDNA sequences (385 bp); (B) whole 16S rDNA sequences (1350 bp). One division of the scale bar represents 1 % difference in nucleotide sequence, as calculated by taking the sum of all the horizontal lines connecting two species. Bootstrap values over 50 % calculated from 100 trees are indicated in bold. Species are as follows: *T. saccharophilum* ATCC 43261^T (**sacch**), *T. pectinovorum* ATCC 33768^T (**pect**), *T. amylovorum* isolate HA2P (**amyl**), *T. denticola* ATCC 33520 (**den2**), *T. denticola* ATCC 35405 (**den1**), *T. vincentii* ATCC 35580 (**vin**), *T. medium* G7201^T (**medium**), *T. phagedenis* strain K5 (**phage**), *T. pallidum* Nichols strain (**pallidum**), *T. maltophilum* ATCC 51939^T (**malto**), *T. bryantii* ATCC 33254^T (**bryantii**), *T. socranskii* subsp. *paredis* ATCC 35535 (**soc2**), *T. socranskii* subsp. *buccale* ATCC 35534 (**soc1**), *T. socranskii* subsp. *socranskii* ATCC 35536 (**soc3**), *T. succinifaciens* ATCC 33096^T (**succ**) and patient isolates **63** (isolated from a 55 year old patient with adult periodontitis), **64** (isolated from a 60 year old patient with adult periodontitis), **68** (isolated from a 28 year old patient with ANUG).

4.5 Design of oligonucleotide probes and specific PCR primers

The probes and primers (SOC2, S2P & DEN2) were designed in this study to detect species of *Treponema* as outlined in Table 4.9; the positions and sequences of these, as well as the spirochaetal probe (TREP) and the PCR primers (TPU1 & RTU3) are shown in the alignment in Appendix 7.4.2. The probes were designed to hybridise to both rRNA and DNA. The TREP probe has a single base mismatch with some spirochaetes, including *T. socranskii*, at position 46 (Appendix 7.4.2), but has four or five mismatches with all other, non-spirochaetal bacteria.

4.5.1 Digoxigenin oligonucleotide 3' end labelling

Probes TREP, SOC2 and DEN2 were labelled, quantified and detected as described in Section 3.5.1. The concentrations of labelled probe were determined to be 12.5, 2.5 and 2.5 pmol μl^{-1} for TREP, SOC2 and DEN2 respectively. The value for TREP is obviously too high as only 5 pmol μl^{-1} was added to the labelling reaction. The concentration of this probe was determined using UV spectrophotometry. It is not unreasonable that the calculation underestimated the true concentration and thus a higher number of probe molecules was initially labelled.

4.6 Dot-blot hybridisation

The specificity and sensitivity of SOC2 and DEN2 were determined by dot-blot hybridisation, using differing amounts of DNA, and differing experimental conditions. Membranes were prepared as described in Section 3.6 with DNA amounts of 100, 25 & 5 ng from soc2, soc3, pect, vin, den1, den2, den3, den4, Li, Pi and patient isolates 3, 54, 63, 64 & 68. The poor growth of soc1 meant that the amount of extracted DNA was too low for this organism to be included in the dot-blot experiments. Membranes were also probed with TREP to reveal the presence of all DNA samples from the different species.

By decreasing the salt concentration in the washing solution, the effective T_m of the probe-target duplex is decreased, as estimated from Equation 4.1, and as demonstrated in Table 4.10. Thus, the specificity of the reactions could be increased by increasing the stringency of the washing conditions. A washing temperature

above the estimated T_m is regarded as a high stringency wash, whereas a temperature at lower than the T_m is regarded as low stringency.

Equation 4.1 $$T_m = 81.5 + 16.6 (\log M) + 0.41 (\% G+C) - \frac{600}{N}$$

Where N = length of oligonucleotide and M is the concentration of monovalent cations in the buffer (Sambrook *et al.*, 1989).

Probe/Primer	Target organisms
DEN2	*T. denticola* (including isolates 3, 54 & 68)
SOC2/S2P	*T. socranskii* (including isolates 63 & 64)
TREP	Spirochaetes
RTU3	All eubacterial organisms
TPU1	All eubacterial organisms

Table 4.9 Selectivity of probes and primers

Probe	Probe length (bp)	Probe % G+C	Washing solution	Theoretical T_m (°C)
SOC2	24	71	2.0 x	78.8
			1.0 x	73.8
			0.5 x	68.8
			0.1 x	57.2
DEN2	22	32	2.0 x	60.6
			1.0 x	55.6
			0.5 x	50.6
			0.1 x	39.0
TREP	18	44	2.0 x	59.4
			1.0 x	54.4

Table 4.10 The effect of decreasing the salt concentration on T_m values, as calculated using equation 4.1.

4.6.1 Detection of *T. denticola* isolates

All hybridisation and washing conditions used in this study are summarised in Table 4.11.

The membrane hybridised with DEN2 was washed twice for 15 min at 44.0°C, using 0.5 x washing solution (low stringency) and then detected as described in Section 3.5.1.ii. The developed film (Figure 4.9 A) shows DEN2 successfully detecting all four ATCC strains of *T. denticola* as well as patient isolates 3, 54 and 68. The detection of 5 ng is generally faint, but apparent. Cross-reactivity is seen with the highest amounts of DNA (100 ng) from soc2 and vin.

The washing conditions were made more stringent, raising the temperature to 47.5°C and lowering the salt concentration to 0.1 x washing solution (Figure 4.9 B). The cross-reactivity with soc2 and vin is still apparent.

The membrane was stripped as described in Section 3.6.1 and re-probed with DEN2 at a higher temperature of 45.0°C. The higher hybridisation temperature was chosen to try and reduce the cross-reactivity with soc2 and vin. The washing conditions were at 47.5°C with 0.1 x washing solution (high stringency) The developed film was blank. This was not a problem with the detection or film development steps as signals on another membrane were successfully detected at the same time.

DEN2 was re-labelled and re-quantified as described in Section 3.5.1. The membrane was re-probed at 42.0°C with this fresh aliquot of DEN2. The membrane was washed and detected exactly as before, but again the developed film was blank.

A new dot-blot was constructed from the same DNA samples as before, but this time at amounts of 25, 5 & 1 ng. The membrane was hybridised with DEN2 at 37.0°C for 3.5 hours. The membrane was washed twice at 45.0°C for 15 min, in 0.1 x washing solution and detected. The developed film was blank.

The membrane was stripped as described in Section 3.6.1, and re-probed with TREP at 37.0°C. The probed membrane was washed twice at 37.0°C in 2.0 x washing solution before being detected and the film developed (Figure 4.9 C). The film shows that DNA is present and detectable on the membrane.

This membrane was again stripped and re-probed with DEN2 with a larger amount of labelled probe (approx. 10 pmols). The membrane was then washed twice at the lower stringency conditions of 44.0°C, in 0.5 x washing solution. The membrane

was detected and the film developed in the standard manner. The film was still blank.

4.6.2 Detection of *T. socranskii* isolates

A third membrane was probed with TREP at 37.0°C. The membrane was washed twice at low stringency (Figure 4.10 A). Most strains, including Pi, were detected at 5 ng, the exception being isolate 63, which was only detected at 25 and 100 ng. The level of detection is slightly lower for *T. socranskii*, 63 and 64, as TREP is not 100 % complementary to these samples (Appendix 7.4.2); however, DNA on the membrane was confirmed as being present and detectable.

The membrane was stripped and then re-probed with SOC2. The probed membrane was then washed twice at 47.5°C in 2.0 x washing solution. This low stringency wash resulted in cross-reactivity with all other samples (except pect), at levels of 25 or 100 ng (Figure 4.10 B).

The membrane was washed with progressively more stringent conditions: up to 65.0°C and 0.1 x washing solution (Figure 4.10 C). Soc2, soc3 and patient isolates 63 and 64 were consistently detected at amounts of 5 ng. Cross-reactivity was only apparent at the highest amount of 100 ng with vin and faintly with den3.

A fresh dot-blot was prepared with 25, 5 & 1 ng of the same DNA samples. The new membrane was hybridised with SOC2. The membrane was then washed twice for 15 min at 60.0°C, in 0.1 x washing solution (high stringency). The developed film showed that soc2, soc3 and patient isolates 63 & 64 were successfully detected, with no cross-reactivity to other samples (Figure 4.11 A).

The membrane was stripped and re-probed with TREP, to show that the lack of reactivity of the other samples was not due to lack of DNA (Figure 4.11 B). Despite the membrane being stripped twice (which can also remove bound DNA), the presence of DNA was confirmed.

Probe	Hybridisation Temp. (°C)	Washing Temp. (°C)	Washing solution	Stringency[a]	Sensitivity (ng)	Cross-reactivity	Figure No.
[b]Membrane (1); 100, 25 & 5 ng DNA							
DEN2	37.0	44.0	2.0 x	low	5	soc2, vin (100ng)	4.9 A
DEN2	37.0	47.5	0.1 x	high	5	soc2, vin (100ng)	4.9 B
DEN2	45.0	47.5	0.1 x	high	no signal	no signal	
DEN2	42.0	47.5	0.1 x	high	no signal	no signal	
Membrane (2); 25, 5 & 1 ng DNA							
DEN2	37.0	45.0	0.1 x	high	no signal	no signal	
TREP	37.0	37.0	2.0 x	low	1	Pi (25 ng)	4.9 C
DEN2	37.0	44.0	0.5 x	low	no signal	no signal	
Membrane (3); 100, 25 & 5 ng DNA							
TREP	37.0	Room Temp.	2.0 x	low	5 (most samples) 25 (isolate 63)	Pi (5 ng)	4.10 A
SOC2	37.0	47.5	2.0 x	low	5	All (25 or100 ng) [c]	4.10 B
SOC2	37.0	65.0	0.1 x	high	5	vin, den3 (100 ng)	4.10C
Membrane (4); 25, 5 & 1 ng DNA							
SOC2	37.0	60.0	0.1 x	high	1	none	4.11 A
TREP	37.0	37.0	2.0 x	low	1	Pi (25 ng)	4.11 B

Table 4.11 Summary of dot-blot hybridisation results. **a**: as assessed by comparing the washing temperature with the estimated T_m. **b**: Each membrane was dotted with varying dilutions of each DNA sample as indicated. **c**: except pect.

Blank

Key for Figures 4.9 - 4.11

Strain	**s**[2]	*T. socranskii* subsp. *paredis*	ATCC 35534
	s[3]	*T. socranskii* subsp. *socranskii*	ATCC 35536
	p	*T. pectinovorum*	ATCC 33768
	v	*T. vincentii*	ATCC 35580
	d[1]	*T. denticola*	ATCC 35405
	d[2]	*T. denticola*	ATCC 33520
	d[3]	*T. denticola*	ATCC 33521
	d[4]	*T. denticola*	ATCC 35404
Patient isolate	**3**	isolated from a 44 year old patient with early-onset periodontitis	
	54	history unknown	
	63	isolated from a 55 year old patient with adult periodontitis	
	64	isolated from a 60 year old patient with adult periodontitis	
	68	isolated from a 28 year old patient with ANUG	
	Li	*Leptospira icterohaemorrhagiae*	
	Pi	*Prevotella intermedia*	

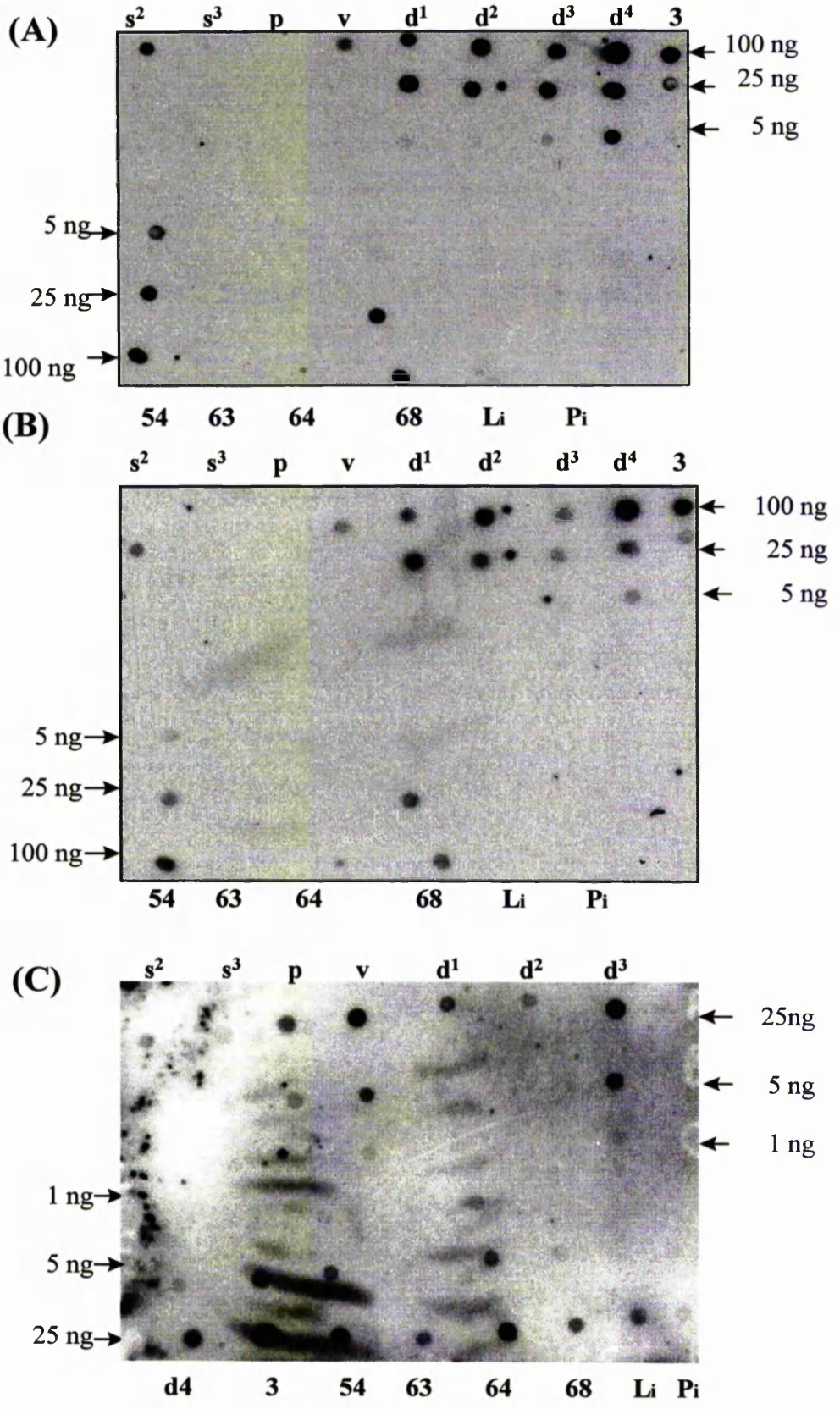

Figure 4.9. Dot-blots with various amounts of DNA, hybridised with either DEN2 or TREP probe (Section 3.6), with the following washing conditions:
(A) Probe DEN2. Low stringency (0.5 x washing solution at 44.0°C)
(B) Probe DEN2. High stringency (0.1 x washing solution at 47.5°C)
(C) Probe TREP. Low stringency (2.0 x washing solution at 37.0°C)

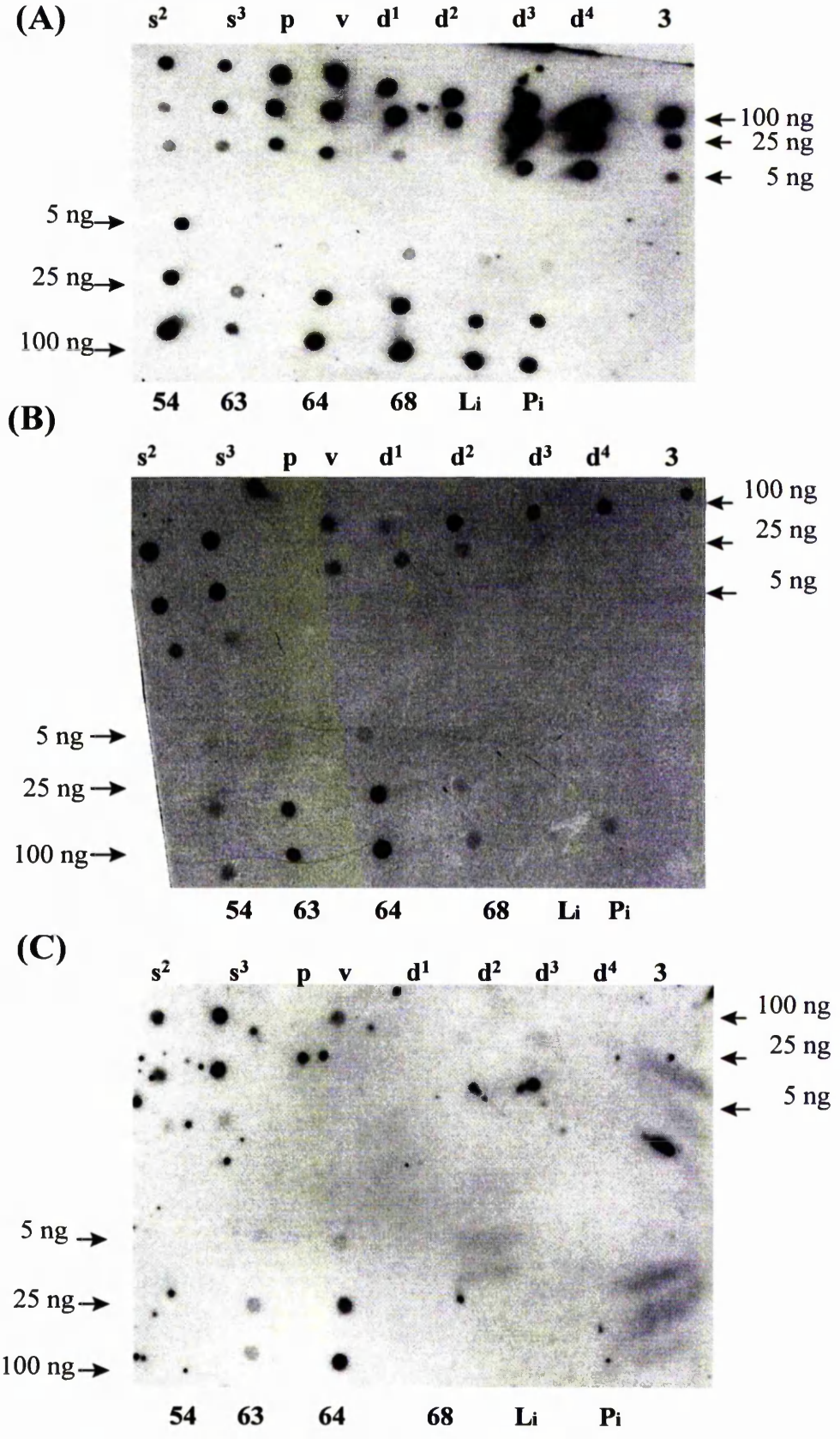

Figure 4.10. Dot-blots with various amounts of DNA, hybridised with either SOC2 or TREP probe (Section 3.6), with the following washing conditions:
(A) Probe TREP. Low stringency (2.0 x washing solution at room temperature)
(B) Probe SOC2. Low stringency (2.0 x washing solution at 47.5°C)
(C) Probe SOC2. High stringency (0.1 x washing solution at 65.0°C)

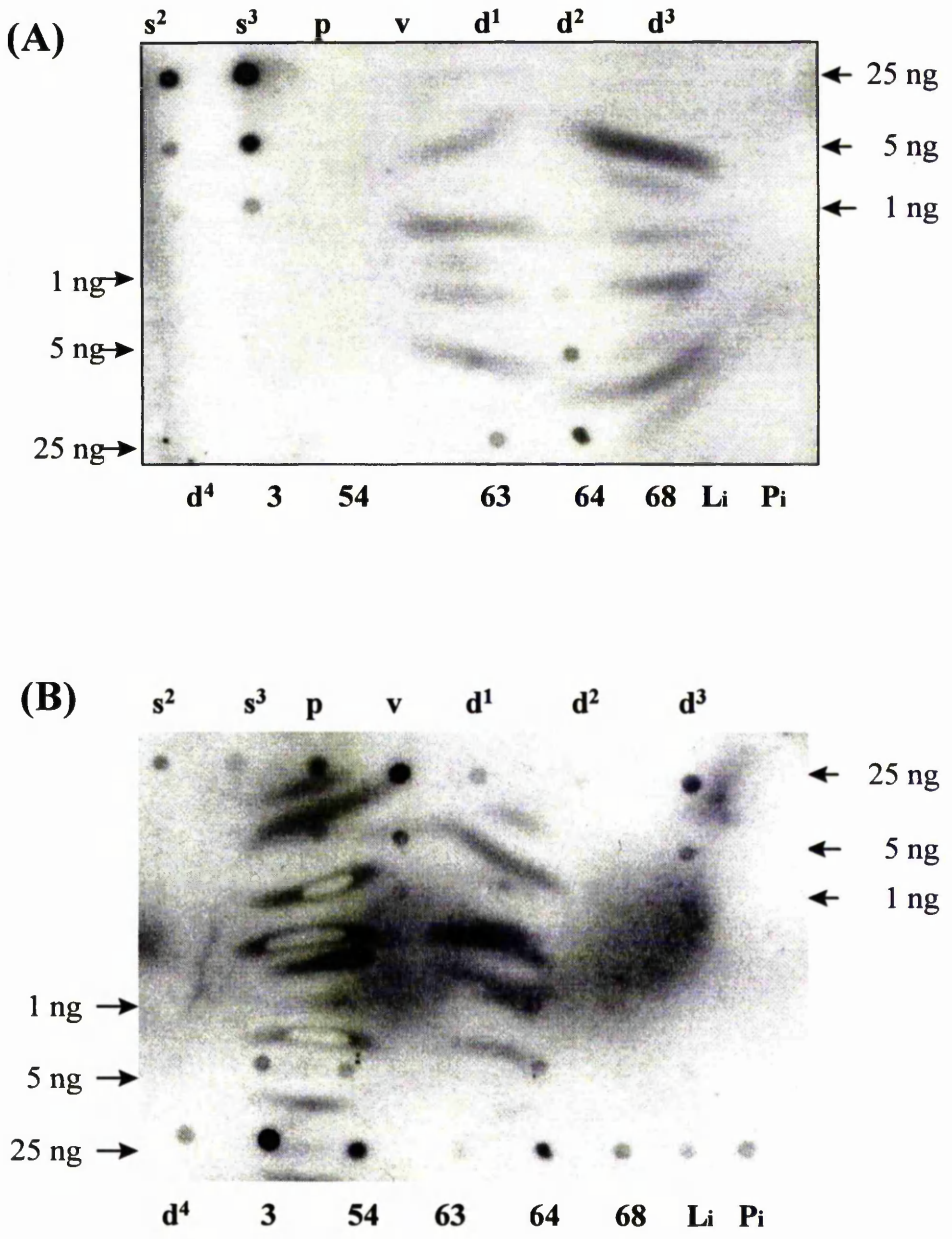

Figure 4.11. Dot-blots of various concentrations of DNA, hybridised with either SOC2 or TREP probe (Section 3.6), with the following washing conditions:
(A) Probe SOC2. High stringency (0.1 x washing solution at 60.0°C)
(B) Probe TREP. Low stringency (2.0 x washing solution at 37.0°C)

4.7 PCR detection of *T. socranskii* and *T. denticola* isolates

PCR was performed as described in Section 3.7 with approximately 25 ng of each DNA as template (soc2, soc3, pect, vin, den1, den2, den3, den4, Li, Pi, patient isolates 3, 54, 63, 64 & 68). PCR was performed with different primer pairs (S2P/RTU3, DEN2/TPU1 and TPU1/RTU3). The PCR was repeated for each primer pair with a different quantity of template DNA (100 ng) to ensure the results were reproducible. Reagent controls, containing all the components of the PCR except the DNA template, were always included in each PCR run. The controls were run in the agarose gels and were always blank.

S2P/RTU3 and DEN2/TPU1 were successful in the specific detection of strains of *T. socranskii* and *T. denticola* respectively. S2P/RTU3 produced a fragment of 465 bp with soc2, soc3 and patient isolates 63 & 64 (Figure 4.12 A). DEN2/TPU1 produced a fragment of 190 bp with den1, den2, den3, den4 and patient isolates 3, 54 & 68 (Figure 4.12 B). Neither primer pair produced a product with pect, vin, Li or Pi. The universal eubacterial primer pair TPU1/RTU3 produced a fragment of approximately 500 bp with all DNA templates as expected (Figure 4.12 C) showing that the absence of a band for pect, vin, Li and Pi with the other primer pairs was unlikely to be due to lack of DNA template.

Blank

Key for Figure 4.12

Strain	**s^2**	*T. socranskii* subsp. *paredis*	ATCC 35534
	s^3	*T. socranskii* subsp. *socranskii*	ATCC 35536
	p	*T. pectinovorum*	ATCC 33768
	v	*T. vincentii*	ATCC 35580
	d^1	*T. denticola*	ATCC 35405
	d^2	*T. denticola*	ATCC 33520
	d^3	*T. denticola*	ATCC 33521
	d^4	*T. denticola*	ATCC 35404
Patient isolate	**3**	isolated from a 44 year old patient with early onset periodontitis	
	54	history unknown	
	63	isolated from a 55 year old patient with adult periodontitis	
	64	isolated from a 60 year old patient with adult periodontitis	
	68	isolated from a 28 year old patient with ANUG	
	Li	*Leptospira icterohaemorrhagiae*	
	Pi	*Prevotella intermedia*	
	N	negative control PCR (no added DNA)	
	M^1	markers - lambda (*Pst*1 digest)	
	M^2	markers - ϕX174 (*Hae*III digest)	

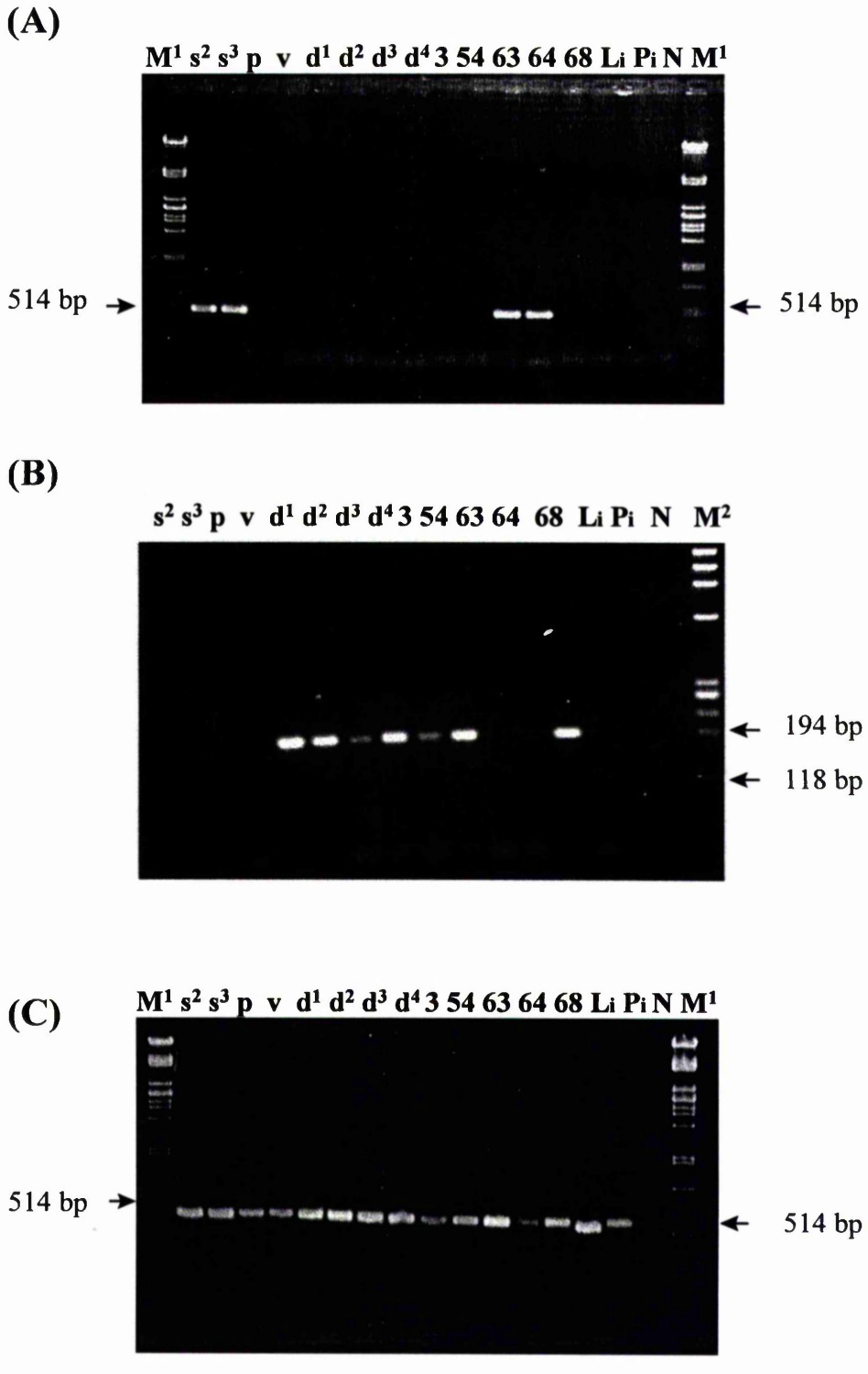

Figure 4.12. PCR detection of *Treponema* strains and isolates using the following primer pairs:
(A) S2P/RTU3; **(B)** DEN2/TPU1; **(C)** TPU1/RTU3
With the exception of the reagent controls, 100 ng DNA was used as template for each 50 µl PCR (Section 3.7). PCR product (5 µl) was subjected to electrophoresis in either 1 or 3 % agarose gels, stained in ethidium bromide solution (1 µg ml^{-1}) and photographed on Polaroid 667 film (Section 3.2.1.i.) Sizes of marker fragments are indicated in base-pairs.

4.7.1 Optimisation of PCR

In order to maximise the amount of product, whilst preventing cross-reactivity with other species, the PCR conditions were optimised for annealing temperature and magnesium concentration.

4.7.1.i Annealing temperature

PCR was performed with various annealing temperatures for each primer pair (Table 4.12).

Primer pair	PCR annealing temperatures (°C)
S2P/RTU3	55, 60, 62
DEN2/TPU1	50, 55, 57, 62
TPU1/RTU3	55, 57

Table 4.12 PCR annealing temperatures for each pair of primers

At temperatures below 62°C (S2P/RTU3) and 57°C (DEN2/TPU1), spurious products could be seen with some templates from different species. Specificity was established, without appreciable loss of product, by raising the annealing temperatures to 62°C for S2P/RTU3 and to 57°C for DEN2/TPU1. Raising the temperature further for DEN2/TPU1 resulted in loss of specific PCR product (Figure 4.13; compare lanes 2 & 4 and 1 & 3). The annealing temperature of 55°C for TPU1/RTU3 was adequate but was raised to 57°C with no obvious loss of product. This enabled the same PCR program to be used for TPU1/RTU3 and DEN2/TPU1.

4.7.1.ii Magnesium concentration

The concentration of magnesium (1.5 - 5.0 mM) had no appreciable effect on the amount of PCR product or the specificity of the primers S2P/RTU3 (Figure 4.14). No PCR product was generated if the magnesium concentration was below 1.5 mM and thus 1.5 mM magnesium was used in the subsequent experiments.

For primers DEN2/TPU1, an increase in the concentration of magnesium (1.0 - 5.0 mM) resulted in an increase in the amount of specific PCR product as assessed by agarose gel electrophoresis, although a faint band of approximately 400 bp was

visible for vin at concentrations over 3 mM. Figure 4.13 shows the increase in yield when the concentration is raised from 1.5 to 3 mM (compare lanes 1 & 2 and 3 & 4). A concentration of 3 mM was therefore used for these primers.

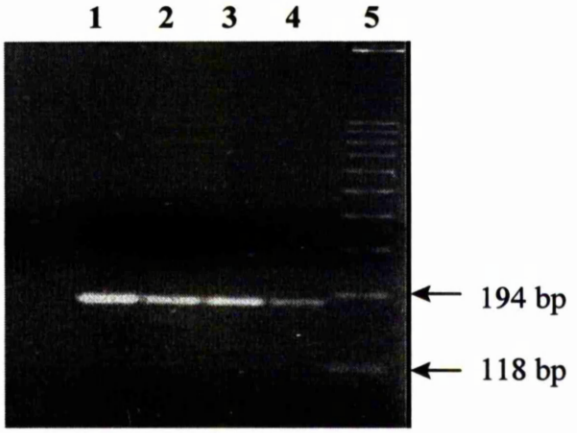

Figure 4.13. PCR detection of *T. denticola* ATCC 35405 using DEN2/TPU1. 100 ng DNA was used as template for each 50 µl PCR (Section 3.7). PCR product (5 µl) was subjected to electrophoresis in 3 % agarose gels, stained in ethidium bromide solution (1 µg ml^{-1}) and photographed on Polaroid 667 film (Section 3.2.1.i.) Sizes of marker fragments are indicated in base-pairs. The PCR was performed at two diffferent magnesium ion concentrations and with two different annealing temperatures:
Lane 1: 3 mM Mg^{2+}; 57°C
Lane 2: 1.5 mM Mg^{2+}; 57°C
Lane 3: 3 mM Mg^{2+}; 62°C
Lane 4: 1.5 mM Mg^{2+}; 62°C
Lane 5: ϕ X174 (*Hae*III digest)

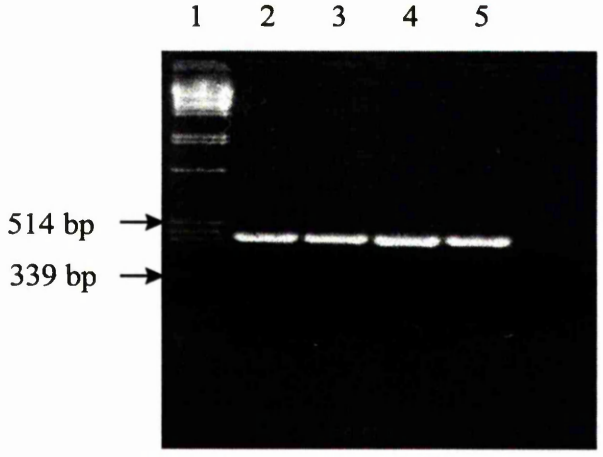

Figure 4.14. PCR detection of *T. socranskii* subsp. *socranskii* ATCC 35536 using S2P/RTU3. 100 ng DNA was used as template for each 50 µl PCR (Section 3.7). PCR product (5 µl) was subjected to electrophoresis in 1 % agarose gels, stained in ethidium bromide solution (1 µg ml^{-1}) and photographed on Polaroid 667 film (Section 3.2.1.i.) Sizes of marker fragments are indicated in base-pairs. The PCR was performed at various magnesium ion concentrations, with an annealing temperature of 62°C:
Lane 1: Lambda (*Pst*1 digest)
Lane 2: 5 mM Mg^{2+}
Lane 3: 3 mM Mg^{2+}
Lane 4: 2 mM Mg^{2+}
Lane 5: 1.5 mM Mg^{2+}

4.7.2 Sensitivity of PCR

Having optimised the conditions of the PCR, with respect to magnesium concentration and annealing temperature, the sensitivity of the PCR was investigated for S2P/RTU3 and DEN2/TPU1.

4.7.2.i DNA detection limits

PCR was performed as described in Section 3.7 using varying DNA concentrations of den1 & den2 with DEN2/TPU1 and soc2, soc3 & isolate 63 with S2P/RTU3. Initial concentrations of DNA were determined by agarose gel electrophoresis of stock DNA as described in Section 3.2.1. Five and ten-fold dilution series were used as templates in the subsequent reactions. Presence or absence of the expected band for each dilution was recorded (Tables 4.13 and 4.14). The intensity of the band was also scored on a scale from '+' meaning faintly present, '++' meaning fairly strong to '+++' for very strongly present. The difference between intensities is subjective and is not to be interpreted rigidly. On several occasions, gels were rerun to see if the visual appearance of the PCR differed. Those reactions scored '+' or 'X' were always scored the same. Occasionally, bands scored '++' were upgraded to '+++' but generally the first impression was accurate.

When optimising the conditions of the PCR, the quantity of DNA was either 100 or 25 ng and the result was always a strong band. The results where more variable when smaller quantities of DNA were used as template. For DEN2/TPU1, on two occasions, less than 1 pg of DNA was amplified, albeit faintly (Table 4.13, PCR nos 2 & 4). This range of DNA failed to amplify on two other occasions. The smallest amount of DNA to produce a strong positive reaction consistently was in the range of 10 pg. There was no appreciable difference in detection levels of den1 or den2.
The results were even more varied for S2P/RTU3. The smallest quantity of DNA amplified was in the range of 1 pg. This was strongly or fairly strongly amplified on three occasions but failed to be detected on four occasions (Table 4.14). The smallest quantity of DNA strongly amplified consistently was in the range of 0.2 ng DNA. Quantities down to 0.02 ng were strongly or fairly strongly detected on over 50 % of occasions. These results are less consistent than the DEN2 results; on four

occasions lower dilutions in a series failed where higher ones were successful, whereas this only happened on one occasion with the DEN2/TPU1 primers.

		Amount of DNA added to PCR (ng)									
PCR no.	**DNA**	**100**	**1**	**0.2**	**0.04**	**0.02**	**0.008**	**0.002**	**0.0016**	**0.00032**	**0.0002**
1	den1	+++	+++	+++	++	-	+	-	+	X	-
2	den1	+++	+++	+++	X	-	++	-	+	+	-
3	den1	-	-	+++	+++	+++	+	+	X	-	-
4	den2	-	-	+++	+++	+++	X	+	X	-	+
5	den2	-	-	+++	+++	+++	+++	+	+	-	X

Table 4.13 Limit of detection for PCR using DEN2/TPU1 and extracted DNA from den1 and den2
PCR was performed on 5 separate occasions (PCR nos 1 - 5), using DNA extracted from either *T. denticola* ATCC 35405 (den1) or from *T. denticola* ATCC 33520 (den2) as template. The amount of DNA added to each 50 µl PCR is shown. PCR product (5 µl) from each reaction was subjected to electrophoresis in 3 % agarose gels, stained in ethidium bromide solution (1 µg ml^{-1}) and photographed on Polaroid 667 film. The intensity of the band is scored on a scale from '+' indicating only faintly present, '++' indicating fairly strong to '+++' for very strongly present. 'X' indicates no band visible and '-' means the PCR for that dilution, on that occasion, was not performed.

PCR no.	DNA	Amount of DNA added to PCR (ng) 100	1	0.2	0.04	0.02	0.008	0.002	0.0016	0.0002
1	soc3	-	+++	+++	++	-	++	-	-	-
2	soc3	-	-	+++	++	-	X	-	++	-
3	soc3	+++	+++	+++	+++	-	+	-	+	-
4	soc3	+++	+++	+++	X	-	+	-	+	-
5	soc3	-	-	+++	X	++	X	X	X	-
6	soc3	-	-	+++	X	+++	+++	X	+++	-
7	soc2	-	-	++	+	++	X	X	X	X
8	soc2	-	-	+++	X	+	X	X	X	X
9	soc2	-	-	+++	++	++	++	++	++	-
10	63	-	-	+++	++	X	X	X	X	-

Table 4.14 Limit of detection for PCR using S2P/RTU3 and extracted DNA from soc2, soc3 and isolate 63
PCR was performed on 10 separate occasions (PCR nos 1 - 10), using DNA extracted from either *T. socranskii* subsp. *paredis* ATCC 35535 (soc2), *T. socranskii* subsp. *socranskii* ATCC 35536 (soc3) or from isolate 63 as template. The amount of DNA added to each 50 µl PCR is shown. PCR product (5 µl) from each reaction was subjected to electrophoresis in 1 % agarose gels, stained in ethidium bromide solution (1 µg ml^{-1}) and photographed on Polaroid 667 film. The intensity of the band is scored on a scale from '+' indicating only faintly present, '++' indicating fairly strong to '+++' for very strongly present. 'X' indicates no band visible and '-' means the PCR for that dilution, on that occasion, was not performed.

4.7.2.ii Whole cell counts

Whole cell counts for broth cultures of den2 and soc2 were performed as described in Section 3.7.1. These cultures were chosen to represent *T. denticola and T. socranskii*, as at the time of sampling, were both growing well. Four separate aliquots of each culture were subjected to the cell count (data not shown). The count values from each aliquot, for each sample, were all within the same order of magnitude, and were averaged to give the total count for each sample. The average count for den2 was 1 x 10^8 cells ml^{-1} and for soc2 was 4 x 10^8 cells ml^{-1}.

4.7.2.iii Cell detection limits

The cells were prepared for the PCR as described in Section 3.7.2. PCR was performed for varying amounts of cells as described in Section 3.7. Samples were always thoroughly boiled before adding to the PCR in order to release the DNA from cellular debris. If this was not done, the PCR failed unless the highest concentrations of cells were used. Results for detection of den2 by DEN2/TPU1 and soc2 by S2P/RTU3 are recorded as described in Section 4.7.2.i in Tables 4.15 and 4.16.

The lowest number of cells consistently amplified by DEN2/TPU1 was approximately 100 per 50 µl reaction although on three occasions a faint band was recorded with only 5 cells. S2P/RTU3 had more variable results with soc2. On one occasion 20 cells were detected but generally the level of detection was lower than that of DEN2/TPU1. Approximately 10^3 cells were consistently amplified, although if one particular set of results are discarded (Table 4.16, PCR nos. 3a & 3b), the detection limit is a factor of ten lower.

	Number of den2 cells added to PCR											
PCR no.	**10^6**	**10^5**	**5 x 10^4**	**10^4**	**5000**	**1000**	**500**	**100**	**75**	**50**	**10**	**5**
1a	+++	+++	+++	+++	-	-	-	-	-	-	-	-
1b	+++	-	+++	+++	-	-	-	-	-	-	-	-
2a	-	-	-	+++	+++	+++	+++	+++	-	-	-	-
2b	-	-	-	+++	+++	+++	+++	+++	-	-	-	-
3a	-	-	-	-	-	-	-	++	++	++	+	+
3b	-	-	-	-	-	-	-	++	+	+	X	+
4a	-	-	-	+++	-	+++	-	+++	-	-	+	+
4b	-	-	-	+++	-	+++	-	+++	-	-	+	X

Table 4.15 Limit of detection for PCR using DEN2/TPU1 and den2 cell suspensions
PCR was performed on 4 separate occasions, in duplicate (PCR nos 1a - 4b), using cell suspensions of *T. denticola* ATCC 33520 (den2) as template. The number of cells added to each 50 µl PCR is shown. PCR product (5 µl) from each reaction was subjected to electrophoresis in 3 % agarose gels, stained in ethidium bromide solution (1 µg ml^{-1}) and photographed on Polaroid 667 film. The intensity of the band is scored on a scale from '+' indicating only faintly present, '++' indicating fairly strong to '+++' for very strongly present. 'X' indicates no band visible and '-' means the PCR for that dilution, on that occasion, was not performed.

	Number of soc2 cells added to PCR																
PCR no.	**2 x 10^6**	**4 x 10^5**	**2 x 10^5**	**10^5**	**4 x 10^4**	**2 x 10^4**	**10^4**	**4000**	**2000**	**1000**	**400**	**200**	**100**	**40**	**20**	**10**	**4**
1	+++	+++	+++	+++	X	X	-	-	-	-	-	-	-	-	-	-	-
2a	+++	+++	+++	+++	++	++	-	-	-	-	-	-	-	-	-	-	-
2b	+++	+++	+++	+++	+++	++	-	-	-	-	-	-	-	-	-	-	-
3a	-	-	-	-	+++	+++	+++	+++	+	-	X	X	-	-	-	-	-
3b	-	-	-	-	+	++	+	++	X	-	X	X	-	-	-	-	-
4a	-	-	-	-	+++	+++	+++	+++	+++	-	++	-	-	-	-	-	-
4b	-	-	-	-	+++	+++	+++	+++	+++	-	+	-	-	-	-	-	-
5a	-	-	-	-	-	-	+++	+++	+++	-	+++	+++	++	+	-	-	-
5b	-	-	-	-	-	-	+++	+++	+++	-	+	++	++	++	-	-	-
6	-	-	-	-	-	-	-	-	+++	+++	+++	+++	X	++	+	X	X

Table 4.16 Limit of detection for PCR using S2P/RTU3 and soc2 cell suspensions

PCR was performed on 6 separate occasions, four of which were set up in duplicate (PCR nos 1 - 6), using *T. socranskii* subsp. *paredis* ATCC 35535 (soc2) cell suspensions as template. The number of cells added to each 50 µl PCR is shown. PCR product (5 µl) from each reaction was subjected to electrophoresis in 1 % agarose gels, stained in ethidium bromide solution (1 µg ml^{-1}) and photographed on Polaroid 667 film. The intensity of the band is scored on a scale from '+' indicating only faintly present, '++' indicating fairly strong to '+++' for very strongly present. 'X' indicates no band visible and '-' means the PCR for that dilution, on that occasion, was not performed.

5.0 Discussion

5.1 Growth of *Treponema*

Treponema are difficult organisms to cultivate due to their fastidious nutritional requirements and the anaerobic conditions needed to support growth (Koseki *et al.*, 1995; Wardle, 1997). No one medium supports the growth of all oral treponemes, as the species have different requirements (Section 1.1.2). The traditional medium recommended for *T. socranskii* and *T. pectinovorum* is a complex medium containing rumen fluid (Gherna *et al.*, 1992) and initially, pect and soc1 were grown in this. The dark colour of the medium, and the dark-staining bodies observed under Gram-stain made it difficult to confirm purity by light microscopic methods. Transmission electron microscopy of specimens negatively-stained using 3 % phosphotungstic acid, indicated a pure culture of spirochaetes (Wardle, 1997), but this is not a cost-effective or convenient method to establish purity. An alternative culture medium was developed by Helen Wardle, formerly of Hope Hospital, Salford, UK, based on the work of Caldwell & Bryant (1966). This medium consisted of 1367 medium supplemented with haemin and fatty acids, instead of the rumen fluid (Appendix 7.1.2). The advantage of using this medium was that it was more reproducible to prepare and paler in colour than 1367 medium, and was thus better to visualise the cultures in the light microscope. Soc1 and pect were successfully subcultured in this medium, although the growth was very sparse and slow when compared to the dense growth of *T. denticola* strains, taking at least three weeks to produce growth adequate enough to extract DNA.

Koseki *et al.* (1996) showed that growth of *T. socranskii* was enhanced if the serum-free 'enriched M10 medium' was used instead of the traditional 1367 medium, and thus the strains of *T. socranskii* obtained directly from ATCC (soc2 and soc3) were resuscitated in this medium. These two strains did grow well for the first few rounds of subculture, but like soc1, did not survive after a few rounds of subculture. Fiehn *et al.* (1995) also found that the strain *T. socranskii* subsp. *paredis* (soc2) did not survive repeated subculturing and remarked that other groups had also had this problem. The reason for the failure of some treponemes to thrive in broth culture is not known - perhaps slight variations in the composition of the media or changes to

the anaerobic environment may have some subtle effect on the ability of the culture to survive. However, *Treponema* species are not as strict anaerobes as sometimes claimed (Smibert, 1973). From my own experience, cultures are able to thrive if subcultured on the open bench, in the presence of oxygen. Fiehn (1989b) also found that *Treponema* cultures could survive transfer in aerobic conditions, but generally growth rates were slowed down when compared to anaerobic subculture.

Problems in maintaining *T. vincentii* in broth culture have not been reported in the literature. I found that *T. vincentii* did not grow well in the recommended serum-containing 1357 medium, producing sparse growth compared to *T. denticola*.

The patient isolates 3, 54 and 68 showed the same growth pattern as *T. denticola* strains - producing turbid growth within 5 days of subculture into fresh 1357 broth (approx. 1 x 10^8 cells ml^{-1}). Patient isolates 63 and 64 showed growth typical of *T. socranskii* strains, with isolate 64 not surviving repeated subculture.

The growth of *Treponema* strains *in vitro* is difficult - the media are time-consuming to prepare and the validation of pure cultures not easy. *T. denticola* strains survive repeated subculture in a relatively straight forward manner, but *T. socranskii, T. pectinovorum* and *T. vincentii* seem to be more sensitive to environmental conditions and are harder to culture successfully.

5.2 RAPD-PCR

My initial objective was to apply the technique of RAPD-PCR to five species of *Treponema,* obtained directly from ATCC, and also to five isolates of unknown species of *Treponema,* recently isolated from patients attending Mr Peter Hull's Periodontology clinic at the Turner Dental Hospital, Manchester (Appendix 7.2). The aim was to see if this technique could be used to distinguish the different species and to establish the identity of the unknown isolates. The isolates and strains all gave distinct patterns with each of the four primers. At a later date, I attempted to characterise four additional strains of *Treponema* by RAPD-PCR, to add to the original analysis. However, I was unable to produce reliable band profiles for these strains, due to the change in concentration of the *Taq* polymerase, and thus was unable to perform any RAPD-PCR analysis for these strains.

This highlights one of the main disadvantages of the technique - the requirement for consistency in reagents and equipment in order for the technique to be reliable enough to use as an identification or typing method. The problem of reproducibility of RAPD patterns from one run to another has been questioned by various workers (Penner *et al.*, 1993; Swaminathan & Barrett, 1995) although other groups have reported no problem with this (Hilton *et al.*, 1997). Problems I encountered with reproducibility were where the PCR failed completely or only small bands were present. These problems were overcome by using new aliquots of *Taq* polymerase or a fresh dilution of the DNA extraction. Some faint bands were not reproducible in some of the strains, and these bands were not scored in the subsequent analysis.

An advantage of RAPD-PCR is that the single primer required can be chosen at random, as no prior sequence knowledge is assumed. Two of the primers, (L10 and US) had been used successfully as RAPD-PCR primers on isolates of *Leptospira* (Corney *et al.*, 1993). Primer RSP had been used to differentiate isolates of *Borrelia burgdorferi* (Welsh *et al.*, 1992) and primer 970-11 had been used to distinguish isolates of *Porphyromonas gingivalis* (Ménard *et al.*, 1992). Many groups screen large numbers of primers in order to find ones that are adequately discriminatory for their strains and isolates (Chen & Slots, 1994), with some groups finding that certain primers produce very few or no bands at all (Caetano-Anollés *et al.*, 1992). The exact reason for the lack of amplification products is unclear, but is probably due to the rarity of target sites for the primer. Van Belkum (1994) stated that primers over

10 nucleotides long are less discriminatory than shorter primers and that the optimal length should be 8 bases as longer primer sequences do not increase the complexity of the patterns significantly (Caetano-Anollés *et al.*, 1992). Power (1996), however, found that longer primers (18 bases) yielded more information than shorter primers when typing isolates of *Enterococcus faecium*. The four primers I used adequately differentiated all isolates and species of *Treponema* in each RAPD-PCR, despite three of the primers being over 18 bp. The RAPD patterns were very distinct and generally contained many bands, ranging in size from approximately 200 to 3000 bp.

Optimising the band profiles

The concentration of the primers and magnesium ions can affect the band patterns and thus I optimised the concentrations of both.

For the primers, within the range 1 - 5 μM, the patterns were consistent, although no amplification occurred at lower concentrations. Bassam *et al.*, (1992) also found this to be the case at similar concentrations. Virk *et al.* (1995) commented that different sources and concentrations of primer can influence band profiles with some primers only reproducible at higher concentrations. The reason suggested is that the genomic DNA used as template probably contains an unusually high number of annealing sites for certain primers, and so the effective concentration available for amplification is reduced.

Increasing the concentration of magnesium from 1.5 mM to 4.0 mM can result in some bands being amplified more efficiently, whilst others are amplified less efficiently (Williams *et al.*, 1993). I found that sub-optimal concentrations (under 1.0 mM) resulted in no bands at all and that increasing the concentration from 1.0 mM to 5.0 mM only affected the intensity of the profiles. Over the this range of concentrations, other workers have also found this to be the case (Bassam *et al.*, 1992). The concentration of magnesium used in this study was kept at 1.5 mM for all RAPD-PCR reactions.

The band patterns were reproducible (from one dilution to another and in all PCR reaction replicates) from amounts 1 to 100 ng μl^{-1} DNA. This is in agreement with Bassam *et al.* (1992), who concluded that quantities of DNA should be at least 1 ng μl^{-1} for adequate reproducibility between PCR runs. The amount of template DNA used for the RAPD-PCR study was the excess amount of approximately 50 ng, in

order to avoid any problems with PCR amplification failure. Davin-Regli *et al.* (1995) performed RAPD-PCR on eight Gram-negative organisms of clinical origin, over a wide range of DNA quantities (10 fg to 100 ng μl^{-1}) and found that the amount of added DNA was crucial to the reproducibility of the band patterns. At higher amounts (between 100 pg - 100 ng μl^{-1}) strong bands were produced. The band profiles were reproducible at a given concentration, but differed from one dilution to another. At lower concentrations, most of the strong bands disappeared to be replaced by faint bands of different sizes. The strong bands probably corresponded to perfect annealing sites amplified with good efficiency or were the consequence of several bands of the same size, derived from different regions of the genome. The reason for the difference in band patterns is unclear but the conclusion offered is that priming events at high concentrations of DNA are inefficient at low concentrations and are replaced by amplification of rare molecular events, although this seems an unlikely explanation. In contrast, van Belkum (1994) showed that reducing the concentration of template DNA led to a decrease in staining intensity of the band patterns and not an actual difference in band patterns.

As *Treponema* do not grow as discrete colonies on the surface of agar-plates, it was not practical to pick the organisms directly off the plates for use in RAPD-PCR and a crude extraction method was performed instead. Highly purified DNA is not required for reproducible patterns. Ménard *et al.* (1992) showed that the quality of the template DNA (protein-rich, RNA-rich DNA mixtures) did not affect the patterns.

Analysis of the band profiles

The band patterns in this study were too complex for direct analysis and thus I opted for a band-matching method to compare the isolates, where reproducible bands were scored as present or absent.

Scoring the band patterns by eye was difficult and subjective, as many of the bands were either faint or ran very close together, making it impossible to score every band. For this reason, I had to develop a set of guidelines as to how to score the patterns, as outlined in Section 4.3.2. In retrospect, it would have been an improvement to run the agarose gels for longer, with less sample, at the expense of the bands smaller than 300 bp, in order to separate the fragments in the range 500 - 2000 bp. This would

probably have made the scoring slightly easier, removed the subjectivity and resulted in more information for the statistical analysis.

Various other methods to improve the visualisation of RAPD profiles have been discussed in the literature. Corney *et al.* (1997) describe a 'dry' method to load agarose gels instead of the conventional method of submerging under buffer. This leads to a clearer and sharper band pattern. Mondon *et al.* (1997) incorporated radioactivity in the PCR and separated DNA fragments in polyacrylamide gels for the same reason. However the use of radioactivity and acrylamide is not generally an advantage when considering personal safety. Bassam *et al.* (1992) also separated fragments in polyacrylamide gels and used a silver stain to visualise fragments. This again lead to greater clarity of the band patterns, although the expense of such a stain is a disadvantage.

After scoring bands and recording the results in a binary matrix, SM and D similarity coefficients were calculated. The SM coefficient, which takes into account both bands in common and bands absent in common, is perhaps the most useful when comparing RAPD band patterns. The common absence of a band in two given strains, that is present in another, could be a significant genetic similarity between the two strains and is recorded as such.

Virk *et al.* (1995) calculated four different similarity coefficients, including both SM and D, and found that the topologies of the dendrograms were virtually concordant. The SM and D dendrograms produced with my RAPD data were not completely identical but the trends were the same. The data from each primer, when analysed separately, were not identical to each other, but the overall trends were again similar. Other workers have found that the overall clustering is in concordance with each individual primer (Bandi *et al.*, 1995; Dugourd *et al.*, 1996) although there is no reason why this should be the case. It is perfectly possible for a given primer to amplify areas of the genome that are relatively conserved in two organisms, and for another primer to amplify regions of variability. The two primers would thus produce completely different patterns which would reflect the differing areas of the genome. After calculating the similarity coefficients and clustering the results, one primer would cluster the two strains whilst the other would not. For this reason it is recommended to use more than one primer and to analyse all the band data together

in one matrix. This will have the effect of (hopefully) sampling more of the genome and thus reduce the bias of one primer and increase the statistical significance of the analysis.

As the similarity between species is on average 63 % using the SM data, it is concluded that all the patient isolates are a different species to *T. pectinovorum, T. vincentii* and *T. socranskii.* Three of the patient isolates (3, 54 & 68) are *T. denticola.* The identities of 63 and 64 are uncertain, although they are probably not *T. denticola* isolates.

This is consistent with the isolation procedure adopted, where isolates 3, 54 and 68 grew best in 1357 medium, which is the recommended growth medium for *T. denticola.* Isolates 63 and 64 did not thrive in this medium, but grew better in modified 1367 medium. This is known to support the growth of *T. socranskii.*

Scoring RAPD bands as phenetic characters and using numerical taxonomy analysis is a valid method of analysing the data. Extending the analysis to draw phylogenetic conclusions is not rigorously proven with RAPD markers. Bands may coincidentally migrate to the same distance in an agarose gel but not be the same genomic sequence. Bayman & Cotty (1993) showed that some co-migrating bands in the RAPD were not homologous by Southern blot analysis of RAPD gels using DNA from isolated bands as probes. Wilkie *et al.* (1993) showed that co-migrating bands *were* homologous by the same method. Bandi *et al.* (1995) showed that there was consistency of RAPD clustering with classical methods of taxonomy and thus suggested that phylogenetic conclusions can be inferred from RAPD data. The capacity to make phylogenetic conclusions from RAPD data is probably a function of the primer and the template DNA and is not a given fact for all circumstances. The clustering exhibited from my statistical analysis are not meant to be interpreted as phylogenetic distances, more as a phenetic classification based on RAPD markers, hence the use of the word 'phenogram' to describe the resultant tree diagrams.

The conditions which promote RAPD-PCR as a reliable identification method for bacteria need to be determined for each organism. The choice of primers, reagent concentrations, cycling parameters and visualisation methods all affect the band profiles obtained for each strain. Only tentative conclusions as to the identity of the

patient isolates in this study can be drawn. RAPD-PCR as executed in this study, is perhaps too discriminatory for isolates of *Treponema*, with many differences revealed between strains of the same species, and for this reason is not a recommended method to characterise this genus. Modifications to the methodology *e.g.* altering the cycling parameters and running the agarose gels for longer could possibly have made the task of scoring the band profiles easier and perhaps have led to less potential error in the statistical analysis.

The error in the RAPD-PCR analysis is difficult to quantify and thus the results from such an analysis should be corroborated by additional techniques. I used another molecular method (partial 16S rDNA sequencing) to characterise the unknown isolates, and compared the results with those of the RAPD-PCR.

5.3 Partial 16S rDNA Sequencing

Partial 16S rDNA sequencing has been described as an adequate tool for the identification of bacteria (Ludwig & Schleifer, 1994), and thus approximately 300 bp of the 16S rRNA gene from the region 8 - 536 bp (*E. coli* numbering system) for isolates 63, 64 and 68 were initially cloned and manually sequenced. This region was chosen for two reasons: from the model of eubacterial secondary structure of 16S rRNA, as demonstrated in Gutell (1993), the 5' end of the molecule contains hypervariable sites. In addition, the universal primers used to amplify this region had been successful for oral treponemal species (Choi *et al.*, 1994), and were readily obtained for this project.

The sequences of 63 and 64 were virtually identical over this region, despite being only 70 % identical to isolate 68, den2 and pect. This was a surprising result as the two isolates were not similar by RAPD analysis. In order to discount the possibility that I had mistakenly cloned and sequenced the same isolate, the template DNA for both isolates 63 and 64 were again subjected to RAPD analysis. The band patterns were distinct from each other, and identical to the band patterns previously demonstrated for these isolates, confirming the DNA samples were unlikely to have been contaminated (data not shown).

The partial 16S rDNA sequences were again sequenced by the alternative method of direct sequencing of the PCR product using Dynabeads®. Each isolate was subjected to PCR and subsequently sequenced on different days, in order to discount the possibility of cross-contamination. In addition, soc1, vin and den1 were sequenced by this method. The sequences of isolates 63 and 64 differed in only one position in the 469 bases sequenced (Appendix 7.4.2, position 250, marked *) confirming the close relationship between these two isolates. Interestingly, the single base difference between isolates 63 and 64 is the position of the base difference between isolate 68, den1 and den2. It has been suggested that low level differences between strains of the same species reflects interoperon differences (Clayton *et al.*, 1995). Most bacteria have multiple rRNA operons (Mylvaganam & Dennis, 1992) which can differ in their base sequences. Van Wezel *et al.* (1991) found that two of the six *Streptomyces coelicolor* rRNA operons differed by 0.5 % (8 bases out of 1529 sequenced), whereas Mylvaganam & Dennis (1992) report that the two rRNA operons in the archeobacterium *Haloarcula marismortui* differ by as much as 5 %

(74 bases out of 1472). Some species of *Treponema* (*pallidum, phagedenis* and *denticola*) are known to have two rRNA operons (Fukanaga *et al.*, 1992; Macdougall & Saint Girons, 1995), and so this could explain the ambiguous base called in den1 at position 250. Additionally, if this level of difference exists between operons, the 6-12 differences between 63/64 and *T. socranskii* strains are probably not consequential.

5.3.1 Phylogenetic analysis

The relationship of the clinical isolates to other species of *Treponema* was investigated using phylogenetic methods. It is recommended that phylogenetic analysis is only performed with whole 16S rRNA sequences, in order to minimise bias (Ludwig & Schleifer, 1994). However, several groups have shown that a potentially meaningful interpretation can be made using the results from partial sequences as from complete sequences (Lane *et al.*, 1985; Schmidt *et al.*, 1991). Van Bruggen *et al.* (1993) performed phylogenetic analysis with 270 bp partial sequences from *Rhizomonas* species and confirmed the clustering observed by DNA-DNA hybridisation.

There are several different methods of phylogenetic analysis, but the importance of accurate sequence alignment in each method is paramount. Compared sequences must be aligned appropriately so that only truly homologous sequences positions are considered in the analysis (Lane *et al.*, 1985). The primary structure is first aligned by comparing conserved regions, and can usually be constructed with computer software. The computer will align regions of variability (that may contain deletions/insertions) in the best way possible. However, it is better to amend the alignments of these regions manually, by comparison with the secondary structure of the 16S rRNA, as the computer software can favour inserting gaps to increase the potential base matches. Gutell (1993) shows a schematic of 16S rRNA from *E. coli* to pinpoint areas that form hairpin loops *etc.* An alternative to making subjective decisions about sequence alignment, that may have an effect on the statistical analysis, is to delete regions of ambiguous alignment. It is better to have less information that is accurate in an analysis, than to have more information which could be potentially misleading. I opted for this latter strategy, and thus only the unambiguously aligned sequences were considered for the phylogenetic analysis.

The bases discounted from the alignment are indicated in Appendix 7.4.2, the resulting alignment consisting of 385 bases.

The distance matrix approach was used to infer phylogenetic relationships between species of *Treponema.* Although the Maximum Likelihood method is generally considered more robust than distant matrix methods (Li, 1997), the Neighbor-joining distance matrix method is easy to compute, and bootstrap analysis is quicker to calculate for these types of data. Neighbor-joining performs better than UPGMA for phylogenetic analysis (Hillis *et al.*, 1994; Li, 1997) and was thus used as the cluster method. The Kimura correction is more rigorous than the Jukes-Cantor correction (Section 1.3.2 xii.a), allowing for differences in transition and transversion rates, and so was the correction of choice. The 'maximum likelihood' correction, whilst being the most rigorous of the nucleotide substitution corrections, does not allow bootstrap analysis in a convenient time-span, and so was not chosen for this analysis.

The average similarity between the species of *Treponema* is approximately 81 %. This is slightly lower than the findings of Paster *et al.* (1991) who quote an interspecies similarity of 84 % for *Treponema.* This difference is explained by the fact that in the published study, whole (or nearly whole) 16S rDNA sequences were compared. The 3' end of the 16S rRNA gene is more conserved than the 5' end (Gutell, 1993), and as the partial 16S sequences in this study are from the 5' end of the molecule (from positions 8 to 536), the comparative similarities between the sequences are lower.

Isolates 63 and 64 cluster with *T. socranskii* strains and isolate 68 clusters with *T. denticola* strains. *T. vincentii* and *T. medium* form a very close cluster. The positions of most of the branches are supported by bootstrap values of over 50 %, despite the tendency of distance matrix methods of phylogenetic analysis to give conservative estimates of significance (Swofford *et al.,* 1996).

Due to the close similarity of isolates 63 & 64 and *T. socranskii* strains, and the near identity of isolate 68 with den1 and den2, it was concluded that isolates 63 & 64 are *T. socranskii* and isolate 68 is *T. denticola.* Umemoto *et al.* (1997) describe a new species of *Treponema* (*Treponema medium*) isolated from the periodontal pocket. *T. medium* has a very similar 16S rRNA gene sequence to *T. vincentii* (over 98 %

similarity which represents 8 base differences in 494 bp), but is distinguished from *T. vincentii* on the basis of morphology, % G+C content and by DNA reassociation studies (*T. medium* exhibits only 28 % DNA homology to *T. vincentii*). Thus the fact that isolates 63 and 64 exhibit similar partial 16S rDNA sequences to strains of *T. socranskii* is not enough to identify these isolates as *T. socranskii* without corroborating evidence.

There have been several published phylogenetic trees of *Treponema* species although most of them are from the same group (Paster *et al.*, 1991; Wyss *et al.*, 1997), with only one being published by other workers (Umemoto *et al.*, 1997). The usual method of analysis was the distance matrix approach with a Jukes-Cantor correction, followed by clustering using Neighbor-joining. Only one paper quotes bootstrapping values for the robustness of the phylogenetic tree (Defosse *et al.*, 1995), and this tree only includes a few *Treponema* species (*phagedenis, denticola, pallidum, bryantii, pectinovorum and socranskii*). The relationship between *T. denticola, T. phagedenis* and *T. pallidum* was supported by bootstrap values of 99 %, which is higher than the 70 % value in this study. In other published trees, the branching orders are not identical to the tree obtained in this study. The relatively tight cluster of *T. denticola, T. phagedenis, T. vincentii* and *T. pallidum* is consistent with this study, but the positions of all other *Treponema* species differs. This highlights the problem of performing phylogenetic analysis without bootstrapping, as the reliability of the branching points on the published trees cannot be assessed.

To check if the difference in clustering was due to the use of partial 16S rDNA sequences, as opposed to the nearly full sequences used in the published analyses, I constructed a similarity distance matrix using the full sequences obtained from the EMBL DNA database for *T. denticola, T. pectinovorum, T. phagedenis, T. pallidum, T. bryantii, T. succinifaciens, T. saccharophilum, T. maltophilum, T. medium* and *T. amylovorum*. Again, the positions of *T. denticola, T. medium, T. phagedenis* and *T. pallidum* were consistent with published trees, as were the positions of *T. succinifaciens* and *T. maltophilum*. The branching order of *T. bryantii, T. amylovorum, T. pectinovorum* and *T. saccharophilum* differed in the analysis with the full sequences, although bootstrap figures were not calculated. From this study and from the published papers, the evolutionary relationships of the various

Treponema species are generally unclear, with the exception of the *T. pallidum/T. medium/T. phagedenis/T. denticola* cluster.

Due to the relatively low 16S rRNA sequence similarity between most of the species of *Treponema,* the data supports the identification of isolates 63 and 64 as *T. socranskii* and confirms the identification of isolate 68 as *T. denticola.* However, identification should not be based solely on 16S rRNA similarity, with other corroborating evidence needed.

5.4 DNA probe detection of *Treponema denticola* and *Treponema socranskii*

Dot-blot hybridisation has been used successfully to detect various species of bacteria, in order to determine the composition of plaque isolated from diseased sites (Savitt *et al.*, 1990; Dix *et al.*, 1990). Probes must be both specific and sensitive enough to detect bacteria in mixed samples, which has led to the development of oligonucleotide probes designed to regions of the 16S rRNA gene. I designed probes to two species of *Treponema* and tested the specificity and sensitivity of the probes using DNA extracted from various species.

In this study, it was assumed that 16S rDNA identity is indicative of species identity, although this is not the case with *T. vincentii/T. medium*. Due to the lack of corroborative evidence for the identification of isolates 63 and 64 as *T. socranskii*, the primer/probe to detect these organisms (SOC2) should perhaps be more correctly referred to as detecting '*T. socranskii*-like' organisms. The probe was able to detect both ATCC strains of *T. socranskii* (subsp. *paredis* and *socranskii*) and also the two patient isolates 63 and 64, as predicted. The probe specifically detected 1 ng of DNA immobilised on the membrane, showing cross-reactivity with vin and faintly with den3 only at the higher level of 100 ng DNA. The probe to *T. denticola* species (DEN2) specifically detected all four ATCC strains of *T. denticola,* as well as the three patient isolates presumed to be *T. denticola* following RAPD analysis (isolates 3, 54 and 68). The lowest amount of DNA to be detected was 5 ng, with cross-reactivity to soc2 and vin only at the higher level of 100 ng DNA. However, due to the failure of subsequent experiments using 1 ng DNA, the actual limit of detection for this probe cannot be confidently quoted.

The reason for the DEN2 probe's failure to anneal to any DNA on the membrane after the first few experiments is not easily explained. This was not a problem with the quality of the target DNA (TREP probe was able to detect the DNA even after the rigorous stripping procedures) or the film development (another film was developed at the same time). Attempts to reproduce the initial experiments failed despite using several different strategies, including using washed-off probe from the first experiments, a fresh aliquot of probe, newly labelled probe, increasing the amount of probe added to the hybridisation mix and using a different membrane with fresh

DNA. The lack of reaction could be explained by competing unlabelled probe present in the hybridisation. Despite adding 5 pmol μl^{-1} to the labelling reaction, only 2.5 pmol μl^{-1} was successfully labelled, thus the fact that the hybridisation reactions worked for the first two experiments could have been fortuitous. However, SOC2 probe was labelled to the same extent and was consistently able to bind to the target DNA, and so the loss of reactivity for DEN2 cannot fully be explained by the competition between labelled and unlabelled probe. A more likely explanation for the failure, is that perhaps fewer DEN2 molecules were added to the labelling reaction than originally calculated. The DEN2 and SOC2 probes were ethanol-precipitated prior to the labelling reaction, and were not quantified afterwards by spectrophotometry to check the final concentration. Any loss of probe in this precipitation would have resulted in fewer molecules being labelled in the subsequent reaction, and this lower amount may not have been able to detect DNA immobilised on the membranes reproducibly.

This failure to reproduce the dot-blot experiments highlights one of the disadvantages of the technique. There are several steps in the procedure (preparation of target DNA, labelling of probe, preparation of membrane, hybridisation and washing steps, detection of bound probe) and thus it can take time to pinpoint exactly where problems arise.

The levels of detection quoted in this study are approximate, as using visual comparisons of known standards in agarose gels is not a precise method for the quantification of DNA. The difference in intensity of the dots for 63 and soc3 (Figure 4.10) is probably accounted for by a discrepancy in diluting the stock DNA to the appropriate levels. The intensity of soc3 is approximately five times as bright as isolate 63, suggesting the error in initial DNA quantification is in this range. If this is the case, then the level of detection for SOC2 is better quoted as 0.2 - 5 ng.

The probes are designed to bind to rRNA as well as DNA and thus the presence of rRNA in the DNA samples could potentially increase the signal. However, the slow-growing *Treponema* organisms probably do not contain high levels of rRNA (Choi *et al.*, 1994) and rRNA was not readily detectable by agarose gel electrophoresis, despite the extraction protocol not including a step to remove RNA. If the DNA samples had been quantified by UV spectrophotometry, the amount of RNA in the

sample would have been included in the absorbance reading, and thus, despite the errors associated with spectrophotometry, it may have been better to quantify DNA using this method in this experiment.

DNA probes are approx. 1000 times more sensitive than ELISA assays (Melvin *et al.*, 1994), and the level of detection recorded in this study is in accordance with the results of other groups using dot-blot hybridisation. Conrads & Brauner (1993) tested oligonucleotide probes directed against 16S rRNA to detect *Prevotella intermedia* and *Porphyromonas gingivalis* in plaque samples. The limit of detection for *Prevotella intermedia* was 0.5 ng and for *P. gingivalis* was between 0.5 - 5 ng. Moncla *et al.* (1990) specifically detected 500 ng of DNA using a similarly-derived oligonucleotide probe to *Porphyromonas gingivalis,* and a cloned genomic probe to the intestinal spirochaete *Serpulina hyodysenteriae* was used to detect approximately 8 ng of DNA in a dot-blot (Sotiropoulos *et al.*, 1993).

In a clinical setting, the probe must be able to detect nucleic acids in bacterial cells, rather than extracted DNA. Ideally, to perform a dot-blot with cell suspensions, all the strains should be harvested from approximately the same stage in the growth cycle. Due to the sluggish growth of some strains of *Treponema*, this was not practicable and thus I did not proceed with the dot-blot experiments using cell cultures. It is possible, however, to convert DNA amounts into approximate numbers of bacterial cells: the molecular weight of *Treponema pallidum* genomic DNA is 9 x 10^9 Da (Burstain *et al.*, 1991), which is equivalent to 1.49 x 10^{-14} g, (1 Da = 1.66033 x 10^{-24} g); therefore 1 cell corresponds to approximately 0.01 pg DNA. If it is assumed that oral *Treponema* have a similar molecular weight to *T. pallidum*, then the DNA in this dot-blot study can be converted to number of cells, with 1 ng of DNA corresponding to approximately 10^5 bacteria. This calculation is consistent with that of Conrads *et al.* (1996), who quote DNA amounts of 0.05 - 50 ng to be equivalent to 10^3 - 10^6 cells.

DiRienzo *et al.* (1991) designed randomly-cloned probes to detect species of oral *Treponema* in dot-blots. After exposing the membrane to X-ray film for 3 days, the limit of detection was 2 x 10^5 cells for the radioactively-labelled probes to *T. socranskii* and *T. denticola*. The time taken to develop the DIG-labelled probes in this study ranged from 10 - 45 min for the same limit of detection. Interestingly, in the study by DiRienzo *et al.*, the *T. denticola* probe cross-reacted with one strain of

T. vincentii (ATCC 35580) at the level of 100 ng DNA, in a similar fashion to this study, although the types of probe used were different (oligonucleotide probes versus randomly-cloned fragments).

Chromosomal probes have been developed by companies such as OmniGene Inc. (Cambridge, MA) that offer a commercial service to detect various periodontal organisms. Samples are sent to the company, where the principle of dot-blot hybridisation is employed using radioactively-labelled probes. The limit of detection offered by commercial services ranges from 6×10^3 cells to an upper limit of 6×10^5 (Söder *et al.*, 1992; Melvin *et al.*, 1994). Similar ranges of detection are also seen in studies using their own probes (6×10^3 - 6×10^5, Conrads & Brauner, 1993; $< 10^5$ - $> 10^6$, Papapanou *et al.*, 1997; 200 - 4×10^6, Dix *et al.*, 1990). The commercial probes and the studies mentioned quoted no cross-reactivity in the given ranges, but do not record if any occurs at levels outside these ranges.

The levels of detection for the treponemal probes in this study are thus consistent with those of probes available to other periodontal organisms. The cross-reactivity of SOC2 and DEN2 occurred only at levels of 100 ng DNA, which corresponds to approximately 10^7 cells. At levels lower than this (*i.e.* lower than 2.5×10^6 cells), no cross-reactivity was seen for either probe, suggesting that these probes could have potential use in a clinical situation.

The lower level of detection in this study is approximately 1 ng DNA or $\sim 10^5$ cells, although I did not attempt to detect lower levels. Levels of bacteria below 10^3 cells per site are not considered pathological (Conrads *et al.*, 1996) and it is possible to recover *Treponema* from periodontally healthy sites in low numbers (Loesche *et al.*,1990; Riviere *et al.*, 1995). It can be argued that the ability to detect organisms at levels below 10^3 is unnecessary, as it is not the presence of *Treponema* species *per se* that is indicative of the disease progression in an individual, rather a balance between the numbers and proportions of *Treponema*, the presence of other bacteria and host factors (Loesche, 1992a).

In this study, the technique of dot-blot hybridisation to detect DNA was unsatisfactory for the DEN2 probe as the reproducibility of the method was not high. However, the method was more successful for the SOC2 probe. To extend the method to clinical specimens, the conditions would have to be optimised so that cells

could be detected in mixed cultures and in plaque samples, with the levels of cross-reactivity to other species minimised. Alternative probes designed to other regions of the 16S rRNA gene may be more successful, especially for detecting *T. denticola.* Moter *et al.* (1998) designed probes based on 16S rRNA sequence to detect *T. socranskii, T. denticola, T. pectinovorum, T. vincentii* and *T. maltophilum.* The probe to *T. socranskii* was based on the same region of the 16S rRNA gene as the probe in this study (Appendix 7.4.2, positions 81 to 51), with the two sequences overlapping each other. However, the *T. denticola* probe was based on the region 493 to 470. This latter probe was successful in dot-blot hybridisation, although the sensitivity of the probe was not reported.

*Although these probes have only been tested with pure cultures, and not with clinical samples.

5.5 PCR detection of *T. denticola* and *T. socranskii*

The primers S2P/RTU3 were designed to detect *T. socranskii* (including soc1, soc2, soc3, patient isolates 63 and 64); DEN2/TPU1 were designed to detect *T. denticola* (including den1, den2, den3, den4, patient isolates 3, 54 and 68). The failure of soc1 to thrive in broth culture (Section 5.1) resulted in exclusion of this strain from the PCR analysis.

Both primer pairs were 100 % specific in their detection, with no cross-reactivity apparent to the other *Treponema* species, the oral bacterium *Prevotella intermedia* nor to the spirochaete *Leptospira icterohaemorrhagiae.*

The levels of detection were estimated, using as template extracted DNA and cell suspensions. As PCR is such a sensitive technique, very low amounts of DNA can be amplified and detected after agarose gel electrophoresis on one occasion, and on another, the PCR can fail. This was seen in this study where sometimes one dilution would fail in the PCR but a lower dilution would produce a strong band (Table 4.9, PCR no. 6). When this happened, the agarose gel was always re-run to ensure this was not an artefact of the electrophoresis. In addition, the PCR at various dilutions was repeated several times and the results compared. Variations between wells in the PCR machine or pipetting errors in setting up the reactions may account for the failure of some reactions. At very low amounts of DNA template, the probability of product formation occurring in the first few rounds of amplification is reduced and thus the PCR fails. In reactions that contain less than 10 cells, stochastic effects will come into play, making it possible that these reactions do not contain any cells at all, hence the reproducibility of the PCR at low amounts of template can vary.

The levels of detection quoted in this study are approximate. An initial estimation of the template was made by visualising DNA in agarose gels under UV light, or by counting cells in a haemocytometer, both methods of which are subject to error. Serially-diluting the samples more than ten times will inevitably entail errors. Gilson Pipetman® pipettes (Gilson, Villiers-le-Bel, France) were used in this study, which are subject to an error of ±0.8 - 5.0 %. The errors will be cumulative over the dilution series so that at higher dilutions, the calculated amount of template will be less accurate. It is difficult to quantify the error, given the number of dilutions performed, and so the detection levels quoted are estimations, and not exact limits.

The detection level for DEN2/TPU1 was in the order of 10 pg DNA (corresponding to 1000 cells if using the conversion discussed in Section 5.4), although on two occasions less than 1 pg of DNA (< 100 cells) was detected. For S2P/RTU3, the level was in the range 10 - 200 pg DNA (10^3 - 10^4 cells), with approx. 1 pg (~ 100 cells) detected on 5 out of 9 occasions.

These data do not tally with the detection ranges calculated from the actual cell suspensions. For DEN2/TPU1, 100 cells can be reliably detected, whilst the figures for S2P/RTU3 vary from 100 - 4000 cells. This discrepancy between the sensitivities can be accounted for by three possible explanations: either the size of the *T. pallidum* genome is not a good model for other *Treponema* strains, the method of quantifying DNA by agarose gel electrophoresis overestimates the true amount, or the haemocytometer method to calculate cells numbers underestimates the true figures. The latter explanations are the most likely. The usual method of calculating numbers of bacteria involves serial dilution of the broth culture, followed by a direct cell count. Unfortunately, due to the growth of *Treponema* as hazy subsurface colonies, rather than as discrete colonies on agar, it is not possible to do this with these bacteria. The visualisation of cells in the haemocytometer was not easy. The rich nutrient broth used to culture the bacteria produces visible precipitates and the concentration of *Treponema* cells was too high to visualise under the microscope without diluting the broth culture. *Treponema* species coil when stressed (*i.e.* in the presence of oxygen and almost certainly when being centrifuged), and thus could have been obscured from view in the haemocytometer. For this reason the cell count numbers are probably less accurate than the agarose gel method of quantifying DNA and thus the sensitivity is better calculated from the DNA data.

The sensitivities of the DEN2 and S2P primers were similar, and given the intrinsic error involved in DNA quantification and serial-dilution series, were not significantly different. The results from the S2P/RTU3 primers were less consistent than from DEN2/TPU1, and thus it was not possible to determine accurately the lower limit of detection for S2P/RTU3.

The detection of PCR product in agarose gels can be improved by Southern blotting the gel and hybridising with a labelled probe or by running the PCR product in polyacrylamide gels and using a silver stain. However, this added level of cost and complexity to the method would only be needed for situations where it was

absolutely necessary to detect levels as low as single organisms. The limit of PCR detection of oral bacteria by other groups ranges from 10 - 100 organisms (Ashimoto *et al.*, 1996; Conrads *et al.*, 1996; Watanabe & Frommel, 1996). The study by Conrads *et al.* (1996) probed healthy subjects and found that levels of *Porphyromonas gingivalis*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* were not detected above 100 cells per site. As this level of bacteria is not indicative of disease but represents the normal flora, it is of questionable value to be able to detect much lower than the level reached in this study.

The PCR primers must be tested against other bacteria, both oral and non-oral, and against human DNA in order to state with more confidence that the primers are fully 100 % specific. The ability to detect in mixed cultures of bacteria, and to detect DNA in plaque samples needs to be examined, in order to develop the primers to examine the microflora of diseased sites.

This PCR study shows higher levels of detection, with no cross-reactivity, than dot-blot hybridisation. The sensitivities are in the order of picograms rather than micrograms detected in dot-blot hybridisation. However, the sensitivity of the PCR technique will mean that dead cells and sub-pathogenic levels of bacteria will be amplified and could be classed as positive results. The combination of PCR to detect *Treponema* species in plaque samples can be combined with the dot-blot hybridisation to quantify the cell numbers of PCR-positive samples, in order to determine the presence of different *Treponema* species in periodontal disease.

5.6 General discussion

The procedure adopted for culturing oral *Treponema* from plaque samples results in a bias in those organisms that are isolated: the most fragile bacteria will probably not survive the transport back to the laboratory, nor the subsequent vortexing of the sample; the size of the membrane filter will exclude the largest spirochaetes; the nutritional composition of the medium will favour certain organisms over others, as will the anaerobic gas mixture of the environment.

By sequencing partial 16S rDNA sequences, amplified directly from plaque, Choi *et al.* (1994) demonstrated the presence of many diverse uncultivable *Treponema* species in subgingival plaque taken from patients with severe periodontitis, showing that the number of species could be increased even further, if and when culturing techniques are refined. Moter *et al.* (1998) found a similar trend in early-onset periodontal lesions using the technique of FISH (Amann & Ludwig, 1994; Section 1.3.2.x). The most recent *Treponema* species to be cultured and defined (*T. maltophilum*, *T. medium* and *T. amylovorum;* Wyss *et al.*, 1996, Umemoto *et al.*, 1997; Wyss *et al.,* 1997) were isolated using specially-enriched medium containing many different sugars, amino acids, fatty acids and salt compounds.

The five isolates characterised in this study were cultured by standard methods using an all-purpose spirochaete medium (Appendix 7.1), and thus it would have been surprising if they belonged to new, previously undescribed species. The growth characteristics and morphologies of three of the isolates (3, 54 and 68) were very similar to those of *T. denticola.* Isolates 3 and 68 were cultured from two distinct patients with differing diagnoses (early-onset periodontitis and ANUG respectively), the patient history of isolate 54 was not known, and isolates 63 and 64 were from two adult periodontitis patients. *T. socranskii* has been described as the treponeme most often recovered from the subgingival plaque of periodontitis sites (Smibert *et al.* 1984), and the same claim has also been made for *T. denticola* (Chan *et al.*, 1993). In reality, these two species are probably the easiest to culture, and their relative importance as aetiological agents has yet to be determined. The identifications of the clinical isolates as *T. denticola* and *T. socranskii* would be consistent with current knowledge.

Molecular methods are now available to characterise and identify bacteria. RAPD-PCR and partial 16S rDNA sequencing confirmed the identification of isolates 3, 54 and 68 as *T. denticola*. Isolates 63 and 64 are identified as *T. socranskii* on the basis of 16S rDNA sequences, but not on the basis of RAPD. Determining % G+C content and DNA homology values for isolates 63 and 64 would provide further evidence of their identity, although the limited growth of 64 precluded this possibility.

The genomic variation, as assessed by RAPD, is greater than that seen in the sequenced variable region of the 16S rRNA gene, but the overall clustering in the RAPD phenogram reveals the close relationship between isolates 3, 54, 68 and *T. denticola*. The same cannot be said for 'isolates' of *T. socranskii*. Despite having nearly identical 16S rDNA regions, the RAPD data does not cluster these isolates together.

It is possible that isolates 63 and 64 are *T. socranskii* despite the RAPD genomic data suggesting otherwise. The heterogeneity of *T. socranskii* strains is apparent from DNA homology studies, which reveal that *T. denticola* strains exhibit higher homology following S1 nuclease treatment than do strains of *T. socranskii*. These values for *T. denticola* have been estimated at between 76 - 82 % (Chan *et al.*, 1993). The homology values for different strains of *T. socranskii* are much more varied, ranging from 50 - 86 % (Smibert *et al.*, 1984). The homology values for both species are fairly low, but interspecies variation is even lower. For instance *T. socranskii* subsp. *socranskii* has only 3 % homology with *T. denticola* and only 10 % homology with *T. vincentii* (Smibert *et al.*, 1984). Ribotyping studies also confirm the relative heterogeneity of *T. socranskii* strains compared to *T. denticola* strains (Fiehn *et al.*, 1995). The explanation for this heterogeneity witnessed in *T. socranskii* strains is far from clear. Bacteria are assigned to the genus *Treponema* on the basis of gross morphology and habitat. Strains are assigned to the different species according to various criteria, including production of metabolic end products, growth requirements, morphology and % G+C content (Section 1. 1). The subspecies of *T. socranskii* are differentiated on the basis of agglutination of rabbit sera, differing abilities to ferment L-arabinose and rhamnose and DNA homology (homology between subspecies of *T. socranskii* is approximately 60 %, whereas for strains of the same subspecies, the level of homology is 80 %).

The distinguishing criteria are not easy to establish and can often be uninformative. *T. denticola* and *T. socranskii* produce the same acids as the end-products of fermentation (acetic, lactic, succinic and formic), although in different proportions (Section 1.1, Table 1.1). The growth of *Treponema* in broth culture can be difficult, as witnessed by this study and others (Wardle, 1997; Fiehn *et al.*, 1995), and thus establishing the proportion of acid end products and growth requirements for strains can be prone to error.

Electron microscopy of the organisms (courtesy of A. Curry & H. Cotterill, Public Health Laboratory, Manchester and H. Wardle formerly of Hope Hospital, Manchester) did not reveal consequential differences between species (data not shown) as has been found by other groups (Cheng *et al.*, 1985; Umemoto *et al.*, 1997). The axial filament configuration of isolate 64 is the same as for soc1 and for pect, revealing one filament anchored at each end of the organism, whilst isolate 63 has two filaments. However, the axial filament number does not remain constant (Listgarten & Socransky, 1964), with a temporary modification in the number and arrangement of filaments occurring during division, and thus this is not a reliable basis for deciding species differences.

Thus, the morphological variation of these bacteria does not provide an obvious or easy criterion on which to base identification. The % G+C figure however, is sufficiently different to distinguish *T. socranskii* (50 - 52 %), *T. denticola / pectinovorum* (39 %) and *T. vincentii* (44 %) species.

There is no obvious reason why strains assigned to the species *T. denticola* are less heterogeneous than strains assigned to *T. socranskii*, unless the ability to grow in serum-free medium and have a G+C content of over 50 % actually encompasses more than one species.

Given the low homology values for *Treponema* species, it is not surprising that whole genome characterisation methods such as RAPD-PCR, reveal many differences between strains. It would have been interesting to compare the RAPD-PCR patterns of soc2, soc3, den3 and den4 with the patterns in this study, to confirm the expected heterogeneity between strains of the same species. Due to this heterogeneity, RAPD is not a valid method to identify species of *Treponema*, a technique recommended to characterise very related organisms.

In order to detect and identify *Treponema* species in plaque samples directly, without having to isolate and culture the organisms, PCR primers and oligonucleotide probes were designed to variable regions of the 16S rRNA gene. These had to be initially validated and optimised against DNA extracts before they could be tested against plaque samples.

The dot-blot hybridisation experiments were only preliminarily successful for the *T. denticola* probe, although this was probably a problem with the technique rather than the probe itself. The probe to detect *T. socranskii* species was more successful, performing on a par with commercial probes in detecting DNA. The probe's ability to detect nucleic acids within whole cells in plaque samples now needs to be tested. This can be determined by further dot-blot hybridisation experiments, where the cell cultures are immobilised on membranes and made permeable to the probe. After establishing the conditions under which the probe can hybridise to whole cell samples, the technique of FISH can be attempted. This technique has recently been used to visualise different species of *Treponema* from the subgingival plaque of periodontitis patients.

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7.0 Appendix

7.1 Growth media

7.1.1 1357 NOS (New Oral Spirochaete) Broth/Agar (Leschine & Canale-Parola, 1980)

Constituent	Amount g l^{-1}
Brain Heart Infusion Broth	13.00
Tryptone	10.00
Yeast extract	2.50
Sodium thioglycolate	0.50
L-cysteine hydrochloride	1.00
L-asparagine	0.25
Glucose	2.00
Agar no. 1	7.00

The above constituents were added to 1 litre of distilled water and the pH adjusted to between 7.0 and 7.5 with 1M potassium hydroxide. After autoclaving and cooling to 40°C, the following filter-sterilised supplements were added :

Constituent	Amount ml l^{-1}
Rifampicin in DMSO (8 μg ml^{-1})	1.00
10 % (w/v) $NaHCO_3$	20.00
Rabbit serum	20.00
0.2 % (w/v) thiamine pyrophosphate	3.00
*Fatty acid mix	2.00

*Fatty acid mix : 0.5 ml of each acid (*iso*-butyric, DL-2-methylbutyric, *iso*-valeric and valeric) added to 100 ml of 0.1 M potassium hydroxide.

Broth medium was dispensed into 5 ml screw-capped, bijoux bottles. Both bottles of broth and agar plates were stored at 4°C. Before use, plates and bottles (with loosened lids) were pre-reduced overnight, at 37°C, in an anaerobic environment (Compact M, Don Whitley Scientific Ltd., Shipley, West Yorkshire, UK).

7.1.2 **Modified 1367 Medium** (Gherna *et al.*, 1992)

Constituent	**Amount g l^{-1}**
Polypeptone	5.00
Brain Heart Infusion Broth	5.00
Yeast extract	5.00
Pectin	0.80
Glucose	0.80
Starch	0.80
Maltose	0.80
Sodium pyruvate	0.80
Ribose	0.80
K_2HPO_4	2.00
NaCl	5.00
$MgSO_4$	0.10
L-cysteine hydrochloride	0.68

The above constituents were added to 900 ml of distilled water (or 500 ml water and 500 ml rumen fluid donated by André Rickers, University of Manchester). The pH was adjusted to 7.0 with 1M potassium hydroxide. After autoclaving and cooling to 40°C, the following filter-sterilised supplements were added :

Constituent	**Amount ml l^{-1}**
Rabbit serum	50.00
0.2 % (w/v) thiamine pyrophosphate	3.20

†0.05 % (w/v) haemin solution in 1M KOH	0.70
†*Fatty acid mix	2.00

† omitted if rumen fluid added

* as Section 7.1.1

Rifampicin See Section 7.1.1

Bottles and plates were dealt with as Section 7.1.1 prior to use.

7.1.3 **Modified M10 medium** (Koseki *et al.*, 1996)

Constituent	**Amount g l^{-1}**
Polypeptone	2.00
Yeast extract	10.00
Glucose	7.00
L-cysteine hydrochloride	1.00

The above constituents were added to 750 ml of distilled water. After autoclaving and cooling to 40°C, the following filter-sterilised supplements were added :

Constituent	**Amount ml l^{-1}**
+Salt solution 1	36.00
+Salt solution 2	36.00
+Salt solution 3	36.00
0.05 % (w/v) haemin solution in 1 M KOH	4.00
25 % (w/v) L-ascorbic acid	2.00
10 % (w/v) Na_2CO_3	100.00
*Fatty acid mix	3.00

* as Section 7.1.1

+All salt solutions were autoclaved and kept at 4°C prior to use :

Salt solution 1 : 0.78 % (w/v) K_2HPO_4

Salt solution 2 : 0.47 % (w/v) KH_2PO_4, 1.18 % (w/v) NaCl, 0.12 % (w/v) $CaCl_2$, 0.25 % (w/v) $MgSO_4$

Salt solution 3 : 1.2 % (w/v) $(NH_4)_2HPO_4$

The pH of the medium was adjusted to 7.00 with concentrated HCl. The medium was dispensed as Section 7.1.1.

7.2 Isolation of clinical isolates

Isolation and cultivation of spirochaetes from patient samples were performed by Helen Wardle, formerly of Hope Hospital, Salford, UK.

Patients attending Mr Peter Hull's Periodontology clinic at the Turner Dental Hospital, Manchester, UK were selected for sampling. Supragingival plaque was removed from the site and gently rinsed with distilled water. Subgingival plaque was collected with a curette or paperpoint and placed in a vial containing sterilised Reduced Transport Fluid (Syed & Loesche, 1972) and acid washed glass beads. As soon as possible, the samples were returned to the laboratory for processing. The vials were briefly vortexed, then in an anaerobic environment (Compact M Anaerobic Workstation, Don Whitley Ltd., Shipley, West Yorkshire, UK), an aliquot of each patient sample was dotted on the top of a 0.2 μm nitrocellulose filter that had been placed on a pre-reduced 1357 NOS agar plate. The plaque sample was also observed under darkground microscopy and Gram-stained.

The plates were left in the anaerobic cabinet at 37°C for 4 - 7 days. The filter was discarded and plugs of agar were removed. These were inoculated into semi-solid agar 1357 NOS medium. This process was repeated until only spirochaetes were consistently seen in samples as assessed by darkground microscopy and/or Gram-stain. When single colonies were obtained, the sample was subcultured into 1357 NOS broth.

7.3 Molecular Biology Kit contents and protocols

7.3.1 TA Cloning®Kit version 2.3

TA Cloning®vector pCR™II (25 ng μl^{-1})

10 x Ligation Buffer

T4 DNA Ligase

SOC medium (2 % Bactotryptone, 0.5 % Bactoyeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose)

0.5 M β-mercaptoethanol

One Shot™ competent *E. coli* cells

Ligation. PCR product (150 ng) was incubated with 50 ng pCR™II vector, 1 x Ligation buffer and 1 μl of T4 Ligase at 15°C, in a water bath, overnight. The ligation mix was then placed on ice.

Transformation. One vial (50 μl) of frozen One Shot™ competent cells were thawed on ice. Two microlitres of 0.5 M β-mercaptoethanol were mixed with the cells briefly. Ligation mix (1 μl) was added and the mixture incubated on ice for 30 min. The vial was then transferred to a 42°C water bath for exactly 30 s before being placed back on ice. Pre-warmed SOC medium (450 μl) was added to the vial which was then shaken at 37°C for 1 h at 225 rpm. The transformation mix (25 μl and 100 μl) was then spread on LB agar plates containing 50 μg ml^{-1} ampicillin and 25 μl of 40 mg ml^{-1} Bluo-Gal. The plates were then incubated at 37°C overnight.

7.3.2 Sequenase™ version 2.0 T7 DNA Polymerase Sequencing Kit

5 x Sequenase™ Reaction Buffer (200 mM Tris-Cl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl)

Enzyme Dilution Buffer (10 mM Tris-Cl pH 7.5, 5 mM DTT, 0.5 mg ml^{-1} BSA)

0.1 M DTT

5 x Labeling Mix (7.5 μM dGTP, 7.5 μM dCTP, 7.5 μM dTTP)

ddNTP Termination Mix - one for each dideoxynucleotide (80 μM dGTP, 80 μM dATP, 80 μM dTTP, 80 μM dCTP, 50 mM NaCl and 80 μM of either ddGTP, ddATP, ddTTP, ddCTP)

Stop Solution (95 % formamide, 20 mM EDTA, 0.05 % bromophenol blue, 0.05 % xylene cyanol FF)

Universal Sequencing primers (-40 forward, 23-mer, 2 μM, 2.0 pmol μl^{-1}) :

5'-GTTTTCCCAGTCACGACGTTGTA- 3'

(-50 reverse, 21 -mer, 2 μM, 2.0 pmol μl^{-1}) :

5' -TTGTGAGCGGATAACAATTTC- 3'

Sequenase™ T7 DNA polymerase (version 2.0)

Denaturation step. Buffer (200 mM NaOH, 200 μM EDTA pH 8.00) was added to template DNA (0.5 - 3 μg) in a total volume of 10 μl and incubated at 37°C for 30 min. 250 mM sodium acetate was added, followed by 4 volumes of ethanol. The mixture was placed at - 80°C for 15 min. After centrifuging for 15 min at 12 000 x *g*, the pellet was washed with 70 % ethanol and then dried completely.

Annealing step. The denatured pellet from step (1) was resuspended in 7 μl of water. Sequenase™ Reaction Buffer (2 μl) and 5 pmol of primer were added in a final volume of 10 μl. The mixture was incubated at 65°C for 5 min, then allowed to cool to room temperature over a period of 20 min. The mixture was then placed on ice.

Labelling reactions. The following were added to the annealed template-primer from step (2) : 1 μl DTT (100 mM), 2 μl Labeling Nucleotide Mix (1 x), 5 μCi [α - ^{35}S] dATP and 3 units of Sequenase™ enzyme (diluted 1:8 with Sequenase™ Dilution Buffer). The final volume was 15.5 μl. The reactions were thoroughly mixed and incubated at room temperature for 2 - 5 min. Termination Nucleotide Mix for each dideoxynucleotide (2.5 μl) were added to the wells of a microtitre plate (Agar Scientific, UK) and pre-warmed to approximately 40°C. On completion of the labelling reaction, 3.5 μl of labelling reaction were mixed with each individual termination mix and incubated at 37°C for 2 - 5 min. Care was taken to thoroughly mix the reactions, especially if the template was prepared using Dynabeads®, during the termination reactions. The reactions were stopped by the addition of 4 μl of Stop Solution into each well. Reactions were either heated to over 85°C for 5 min prior to loading on a gel or kept at - 20°C and loaded at a later date.

7.3.3 Wizard™ PCR Preps DNA Purification System

PCR Preps DNA Purification Resin

Direct Purification Buffer (50 mM KCl, 10 mM Tris-Cl (pH 8.8), 1.5 mM $MgCl_2$, 0.1 % Triton® X-100)

Wizard™ Minicolumns

The total PCR product was transferred to a clean tube, avoiding the mineral oil if present. Direct Purification Buffer (100 µl) was added to 50 µl of PCR product and vortexed. Resin (1 ml) was added and the mixture vortexed three times over a 1 min period. The barrel of a 5 ml disposable syringe was attached to a Wizard™ Minicolumn. The Resin/DNA mix was pipetted into the barrel and the plunger inserted slowly. The Minicolumn was washed by pipetting 2 ml of 80 % *iso*-propanol into the syringe barrel and plunging this through the Minicolumn. The syringe was detached and the Minicolumn centrifuged for 20 s at 12 000 x *g*. The Minicolumn was then inserted into a clean microcentrifuge tube. The PCR fragments were eluted by the addition of 50 µl of sterile water and centrifugation of the Minicolumn for 20 s at 12 000 x *g*.

7.3.4 Rapid DNA Ligation Kit

2 x T4 DNA Ligation Buffer

DNA Dilution Buffer

T4 DNA Ligase

Vector (10 ng) was mixed with 150 ng of phosphorylated PCR product and 4.5 µl of 1 x DNA Dilution Buffer. T4 DNA Ligation Buffer (10 µl of 2 x) was added and thoroughly mixed. T4 DNA Ligase (5 units) was added and the mixture incubated at room temperature for 5 min. The mixture was stored on ice prior to the transformation protocol.

7.3.5 DIG Oligonucleotide 3' -End Labeling Kit

5 x Reaction Buffer pH 6.6 (1 M potassium cacodylate, 125 mM Tris-Cl, 1.25 mg ml $^{-1}$ BSA)

2 mM $CoCl_2$

1 mM Digoxigenin -11- ddUTP (2', 3' - dideoxyuridine -5'- triphosphate, coupled to digoxigenin via an 11 -atom spacer arm) in redistilled water

50 U μl^{-1} Terminal Transferase pH 6.5 (200 mM potassium cacodylate, 1 mM EDTA, 200 mM KCl, 0.2 mg ml $^{-1}$ BSA, 50 % (v/v) glycerol)

Control DIG-ddUTP labelled oligonucleotide (5'-pTTG GGT AAC GCC AGG GTT TTC CCA GTC ACG OH -3') 20 pmol μl^{-1}

Probes were labelled in a final volume of 20 µl containing: 1 x Reaction Buffer, 5 mM $CoCl_2$ solution, 5 pmol µl $^{-1}$ oligonucleotide, 0.05 mM DIG -ddUTP and 2.5 units µl ^{1} Terminal Transferase. The components were mixed on ice. The reaction was incubated at 37°C for 15 min and then placed on ice. The reaction was terminated by the addition of 1 µl of 200 mM EDTA disodium salt, pH 8.0.

7.4 Results

7.4.1 RAPD-PCR phenograms

SM and D similarity coefficients were determined from the binary matrix data from each individual primer. Phenograms were drawn based on average linkage cluster analysis of the results using SPSS for Windows software (Figures 7.1 - 7.4).

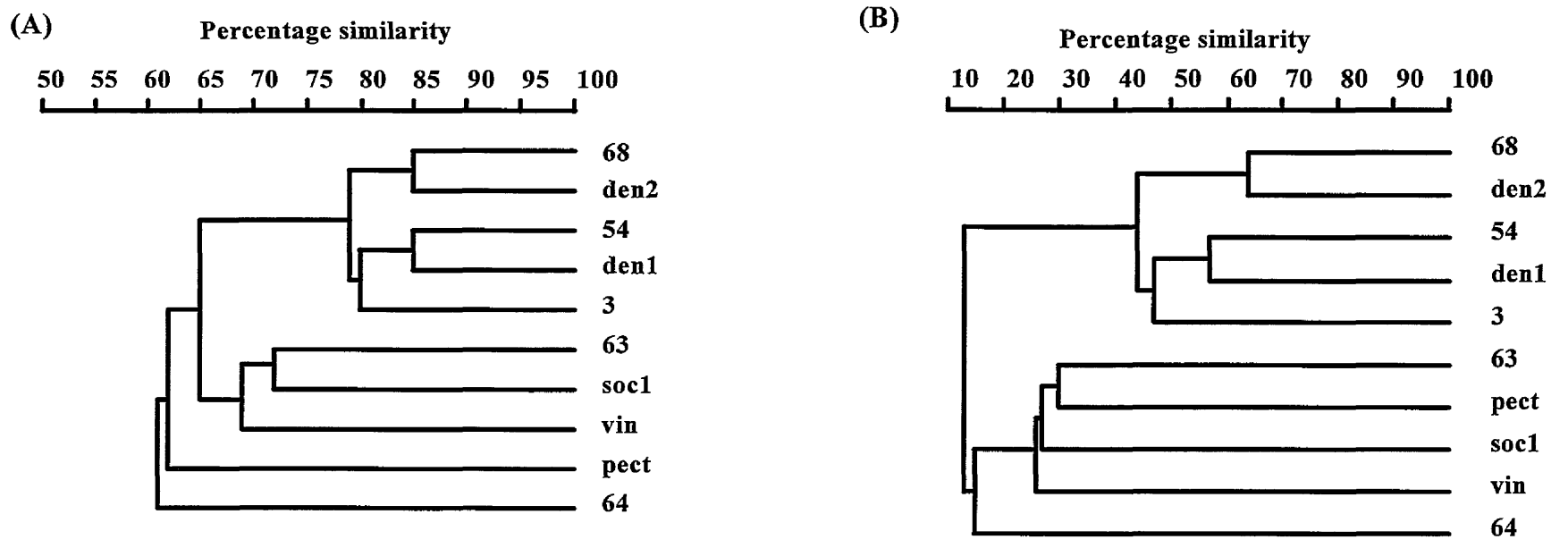

Figure 7.1 Phenograms of *Treponema* isolates, drawn after cluster analysis of **(A) Simple matching** and **(B) Dice** similarity coefficients from the RAPD-PCR binary matrix data with **primer L10** only, using SPSS for Windows software. Species are as follows: *T. pectinovorum* ATCC 33768^T (**pect**), *T. denticola* ATCC 33520 (**den2**), *T. denticola* ATCC 35405 (**den1**), *T. vincentii* ATCC 35580 (**vin**), *T. socranskii* subsp. *buccale* ATCC 35534 (**soc1**), and patient isolates **3** (isolated from a 44 year old patient with early onset periodontitis), **54** (history unknown), **63** (isolated from a 55 year old patient with adult periodontitis), **64** (isolated from a 60 year old patient with adult periodontitis), **68** (isolated from a 28 year old patient with ANUG).

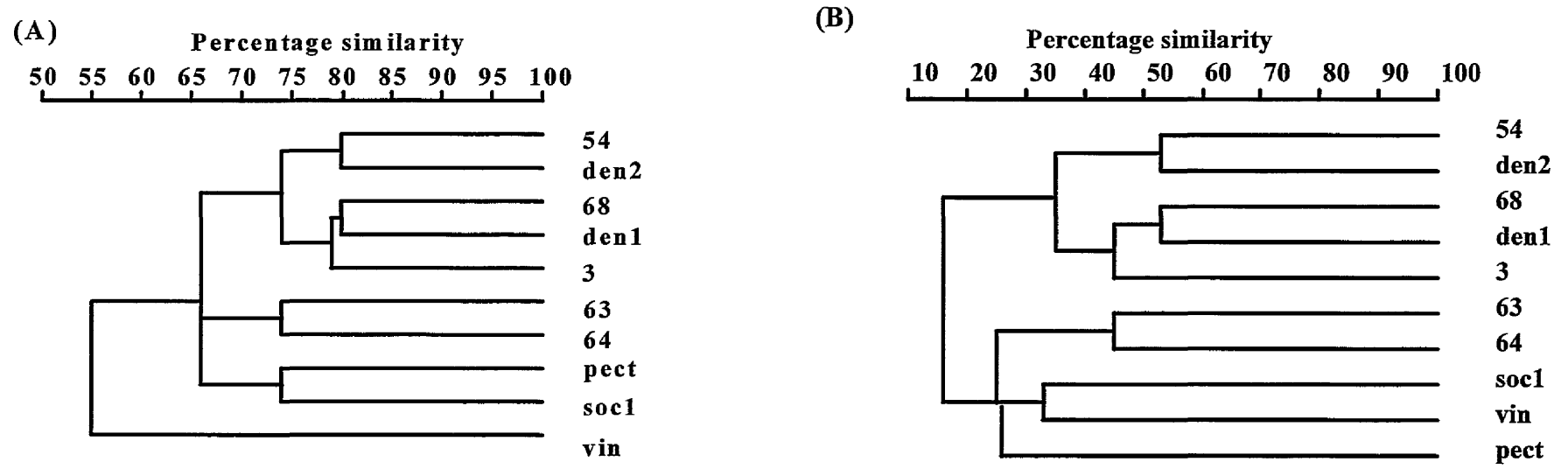

Figure 7.2 **Phenograms** of *Treponema* isolates, drawn after cluster analysis of **(A) Simple matching** and **(B) Dice** similarity coefficients from the RAPD-PCR binary matrix data with **primer US** only, using SPSS for Windows software. Species are as follows: *T. pectinovorum* ATCC 33768^T (**pect**), *T. denticola* ATCC 33520 (**den2**), *T. denticola* ATCC 35405 (**den1**), *T. vincentii* ATCC 35580 (**vin**), *T. socranskii* subsp. *buccale* ATCC 35534 (**soc1**), and patient isolates **3** (isolated from a 44 year old patient with early onset periodontitis), **54** (history unknown), **63** (isolated from a 55 year old patient with adult periodontitis), **64** (isolated from a 60 year old patient with adult periodontitis), **68** (isolated from a 28 year old patient with ANUG).

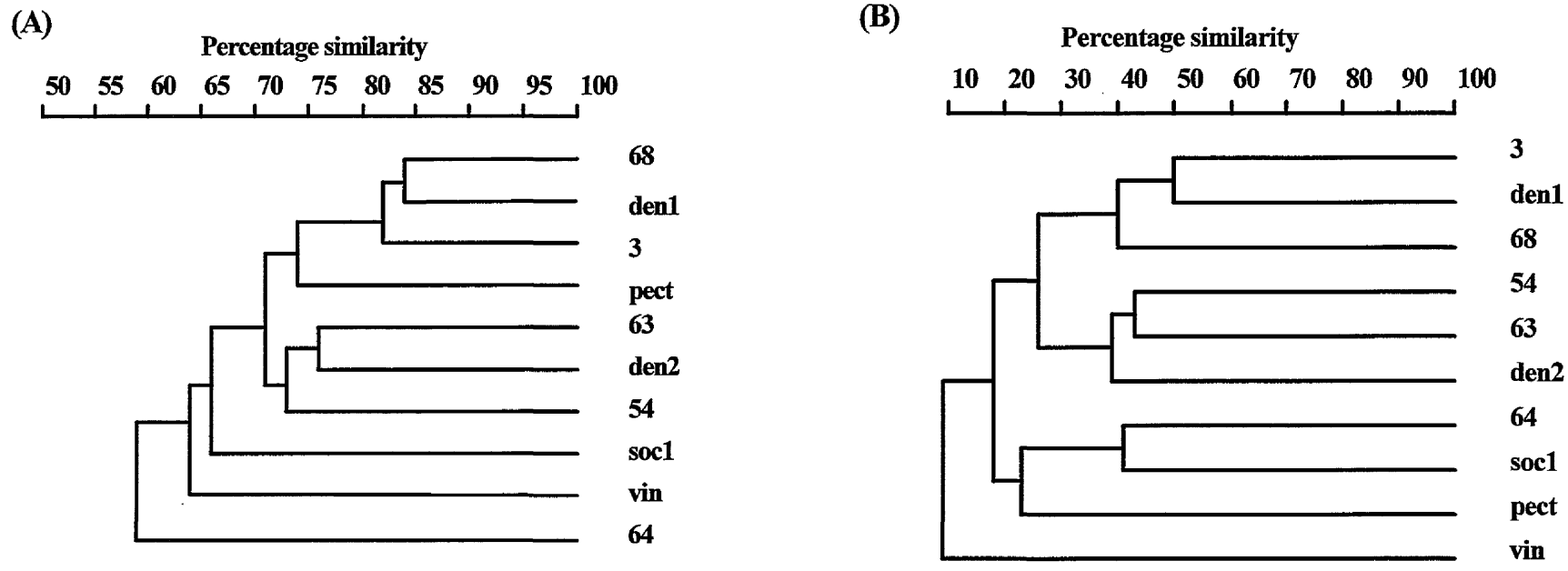

Figure 7.3 Phenograms of *Treponema* isolates, drawn after cluster analysis of **(A) Simple matching** and **(B) Dice** similarity coefficients from the RAPD-PCR binary matrix data with **primer RSP** only, using SPSS for Windows software. Species are as follows: *T. pectinovorum* ATCC 33768^T (**pect**), *T. denticola* ATCC 33520 (**den2**), *T. denticola* ATCC 35405 (**den1**), *T. vincentii* ATCC 35580 (**vin**), *T. socranskii* subsp. *buccale* ATCC 35534 (**soc1**), and patient isolates **3** (isolated from a 44 year old patient with early onset periodontitis), **54** (history unknown), **63** (isolated from a 55 year old patient with adult periodontitis), **64** (isolated from a 60 year old patient with adult periodontitis), **68** (isolated from a 28 year old patient with ANUG).

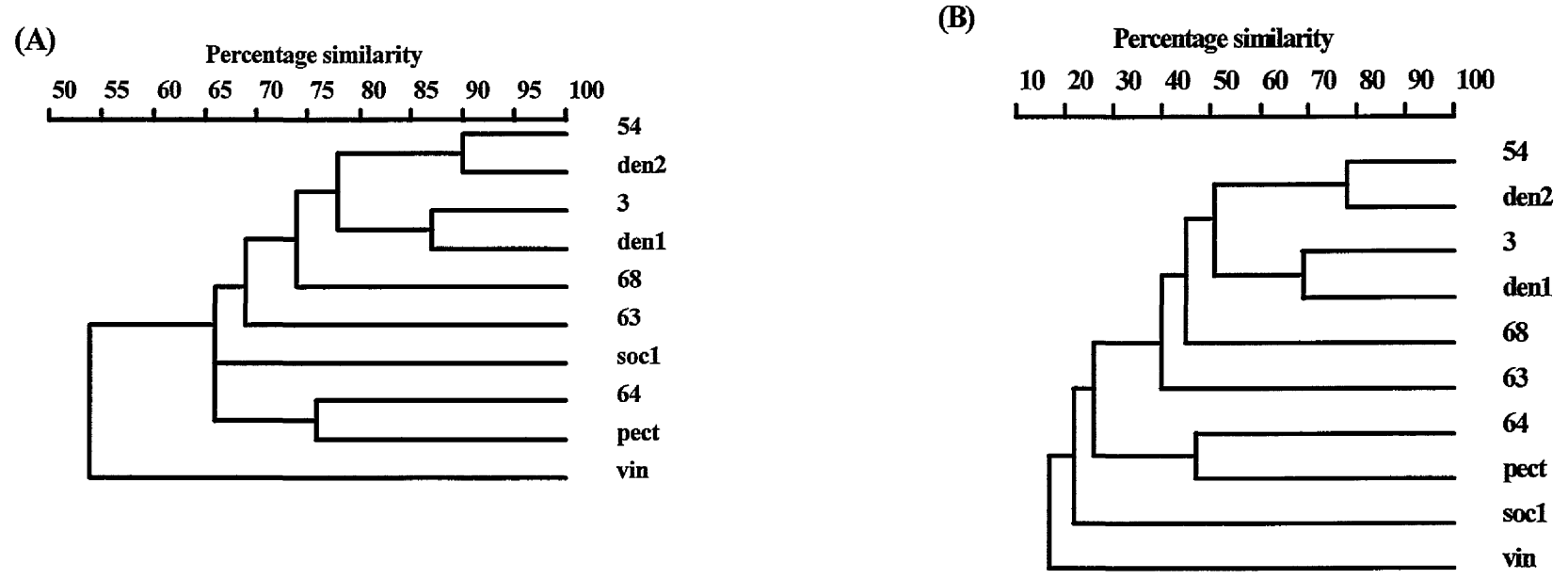

Figure 7.4 **Phenograms** of *Treponema* isolates, drawn after cluster analysis of **(A) Simple matching** and **(B) Dice** similarity coefficients from the RAPD-PCR binary matrix data with **primer 970-11** only, using SPSS for Windows software. Species are as follows: *T. pectinovorum* ATCC 33768^T (**pect**), *T. denticola* ATCC 33520 (**den2**), *T. denticola* ATCC 35405 (**den1**), *T. vincentii* ATCC 35580 (**vin**), *T. socranskii* subsp. *buccale* ATCC 35534 (**soc1**), and patient isolates **3** (isolated from a 44 year old patient with early onset periodontitis), **54** (history unknown), **63** (isolated from a 55 year old patient with adult periodontitis), **64** (isolated from a 60 year old patient with adult periodontitis), **68** (isolated from a 28 year old patient with ANUG).

7.4.2 Alignment of *Treponema* species

Partial 16S rDNA sequences from the *Treponema* species soc1, soc2, soc3, pect, vin, den1, patient isolates 63, 64 and 68 were aligned with ten *Treponema* sequences available in the EMBL DNA database (December, 1997), using the program 'PILEUP' (Wisconsin Package, Version 8.1-UNIX, Genetics Computer Group, Madison, Wisconsin, USA). Regions of ambiguous alignment were removed from the analysis using the program 'LINEUP' (Wisconsin Package). The alignment was displayed using the program 'PRETTY' (Wisconsin Package). The consensus sequence, indicated by capital letters, was constructed when at least 14 out of the 18 sequences had a conserved base at that position.

Key:

Arrow brackets indicate the regions of the alignment used in the data analysis

The asterisk (*) at position 250 indicates the one base difference between isolates 63 & 64, and between strains of *T. denticola* (Section 4.4.1)

(positions 65 to 84; 171 to 199 and 204 to 207).

Primer or probe sequences (5' - 3'):

TPU1	positions 1 to 20
TREP	positions 56 to 38
S2P	positions 61 to 95
DEN2	positions 194 to 171
RTU3	positions 537 to 520

```
                    1                                                  50
T. saccharophilum   agagtttgat cctggctcag aatgaacgct ggcggcgggT NTTAAgCATG
T. pectinovorum     agagtttgat cctggctcag aataaacgct ggcggcgtgT CTTAAgCATG
T. bryantii         agagtttgat cctggctcag aacgaacgct ggcggcgcgT CTTAAgCATG
T. vincentii        .......... .........g aacgaacgct ggcggcgcgT CTTAAgCATG
T. medium           .......... .......... .......gct ggcagtgcgT CTTAAgCATG
T. denticola 2      agagtttgat cctggctcag aacgaacgct ggcggcgcgT CTTAAgCATG
T. denticola 1      .......... .......... .......... .......cgT CTTAAgCATG
Isolate 68          .......... .......... .......... .......... .TTAAgCATG
T. phagedenis       agagtttgat cctggctcag aacgaacgct ggcggcgcgT CTTAAgCATG
T. pallidum         .......gat cctggctcag aacgaacgct ggcggtgcgT TTTAAgCATG
T. maltophilum      .......... .......... aacgaacgct ggcggcgcgT CTTAAaCATG
Isolate 64          .......... .......... .......... .........T CTTAAcCATG
Isolate 63          .......... .......... .......... .........T CTTAAcCATG
T. socranskii 2     .......... .......... .......... .......... ..TAAcCATG
T. socranskii 3     .......... .......... .......... .......... ...AAcCATG
T. socranskii 1     .......... .........g aacgaacgct ggcggcgcgT CTTAAcCATG
T. amylovorum       .......... .......... .......... .........T CTTAAgCATG
T. succinifaciens   agagttcgat cctggctcag aacgaacgct ggcggcgcgT CTTAAgCATG
          Consensus ---------- ---------- ---------- ---------T CTTAA-CATG
```

```
                    51                                                  100
T. saccharophilum CAANTCGGaC GGcA                          CCtAGA GtGGCGGACT
T. pectinovorum   CAAGTCGAaC GGcA                          CCtAGA GtGGCGGACT
T. bryantii       CAAGTCGAgC GGtA                          CCtAGA GcGGCGGACT
T. vincentii      CAAGTCGRaC GGcA                          CCtAGA GtGGCGGACT
T. medium         CAAGTCGAaC GGcA                          CCtAGA GtGGCGGACT
T. denticola 2    CAAGTCGAaC GGtA                          CCtAGA GtGGCGGACT
T. denticola 1    CAAGTCGAaC GGtA                          CCtAGA GtGGCGGACT
Isolate 68        CAAGTCGAaC GGtA                          CCtAGA GtGGCGGACT
T. phagedenis     CAAGTCGAaC GGcA                          CCtAGA GtGGCNGACT
T. pallidum       CAAGTCGAaC GGcA                          tCtAGA GtGGCGGACT
T. maltophilum    CAAGTCGAaC GGcA                          ttgAGA GtGGCGGACT
Isolate 64        CAAGTCGAgC GGcAgGca.....GCaa.....tgCCgAGA GcGGCGGACT
Isolate 63        CAAGTCGAgC GGcA                          CCgAGA GcGGCGGACT
T. socranskii 2   CAAGTCGAgC GGcA                          CCgAGA GcGGCGGACT
T. socranskii 3   CAAGTCGAgC GGcA                          CCgAGA GcGGCGGACT
T. socranskii 1   CAAGTCGAgC GGcA                          CCgAGA GcGGCGGACT
T. amylovorum     CAAGTCGAaC GGcA                          CCtAGA GtGGCGGACT
T. succinifaciens CAAGTCGGgC GGgA                          gtgAGA GcGGCGGACT
        Consensus CAAGTCGA-C GG-A-G---- --GC------ ----CC-AGA G-GGCGGACT
```

```
                    101                                                 150
T. saccharophilum GGTGAGgAAC aCGTtGgTgA CgTaCCCTtt gGAtgGGGAt AGCcggTAGA
T. pectinovorum   GGTGAGTAAC aCGTGGgTgA CgTaCCCNtt gGAcgGGGAt AGtcggTAGA
T. bryantii       GGTGAGTAAC gCGTGGgTgA CgTaCCtTtg tGAcgGGGAc AGCtccTAGA
T. vincentii      GGTGAGgAAC aCGTGGgTaA tcTaCCCTta aGAtgGGGAt AGCtgcTAGA
T. medium         GGTGAGgAAC aCGTGGgTaA tcTaCCCTta aGAtgGGGAt AGCtgcTAGA
T. denticola 2    GGTGAGTAAC gCGTGGgTgA CcTgCCCTga aGAtgGGGAt AGCtagTAGA
T. denticola 1    GGTGAGTAAC gCGTGGgTgA CcTgCCCTga aGAtgGGGAt AGCtagTAGA
Isolate 68        GGTGAGTAAC gCGTGGgTgA CcTgCCCTga aGAtgGGGAt AGCtagTAGA
T. phagedenis     GGTGAGTAAC gCGTGGgTgA tcTaCCCTta aGAtgGGGAt AGCtccTAGA
T. pallidum       GGTGAGTAAC gCGTGGgTaA tcTgCCtTtg aGAtgGGGAt AGCctcTAGA
T. maltophilum    GGTGAGTAAC aCGTGGgTgA CaTaCCtTtt aGttgGGGAt AGCtatTAGA
Isolate 64        GGTGAGTAAC aCGTGGaTaA CgTaCCCCgc tGAccGGGAc AGCctgTAGA
Isolate 63        GGTGAGTAAC aCGTGGaTaA CgTaCCCCgc tGAccGGGAc AGCctgTAGA
T. socranskii 2   GGTGAGTAAC aCGTGGaTaA CgTaCCCCgc tGAccGGGAc AGCctgTAGA
T. socranskii 3   GGTGAGTAAC aCGTGGaTaA CgTaCCCCga tGtccGGGAc AGCctgTAGA
T. socranskii 1   GGTGAGTAAC aCGTGGaTaA CgTaCCCCga tGcccGGGAc AGCctgTAGA
T. amylovorum     GGTGAGTAAC gCGTGGaTgA CgTaCCCTcg tGAcgGGGAt AGCcggTAGA
T. succinifaciens GGcGAGTAAC aCGTGGgcgA CgcgCCCTcc gGAcgGGaAt AGCctgcAGA
        Consensus GGTGAGTAAC -CGTGG-T-A C-T-CCCT-- -GA--GGGA- AGC---TAGA
```

```
                   151                                           200
T. saccharophilum  AATAccgGgT AATACCGaAT [redacted]G
T. pectinovorum    AATAccgGgT AATACCGgAT [redacted]G
T. bryantii        AATAggaGaT AATACCGgAT [redacted]G
T. vincentii       AATAgcaGgT AATACCGaAT [redacted]G
T. medium          AATAgcaGgT AATACCGaAT [redacted]G
T. denticola 2     AATAttaGaT AATACCGaAT gtgctca..t Ttacataaag gtaa[redacted]G
T. denticola 1     AATAttaGaT AATACCGaAT [redacted]G
Isolate 68         AATAttaGaT AATACCGaAT [redacted]G
T. phagedenis      AATAggaGgT AATACCGaAT [redacted]G
T. pallidum        AATAgggGgT AATACCGaAT [redacted]G
T. maltophilum     AATAgtaGgT AATACCGaAT [redacted]G
Isolate 64         AATAgagGgT gATACCGgAT [redacted]G
Isolate 63         AATAgagGgT gATACCGgAT [redacted]G
T. socranskii 2    AATAgagGgT gATACCGgAT [redacted]G
T. socranskii 3    AATAgagGgT AATACCGgAT [redacted]G
T. socranskii 1    AATAgagGgT gATACCGgAT [redacted]G
T. amylovorum      AATAccgGaT AATACCGgAT [redacted]G
T. succinifaciens  AATgcagGgT AATgCCGgAT [redacted]G
        Consensus  AATA---G-T AATACCG-AT ---------- T------A-- ------G-GG
```

```
                   201                                              250
T. saccharophilum  aAA[redacted]GCT TCGGCcGCGC cgaaGGAacG GcCtGCGtCC CATcA.GCTt
T. pectinovorum    aAA[redacted]GCT AtGGCcACGC cgaaGGAacG GcCtGCGgCC tATcA.GCTA
T. bryantii        gAA[redacted]tCT TttGatTCGC acgaaGAgcG GcCcGCGtaC tATtA.GCTA
T. vincentii       aAA[redacted]GCT ACGGCtTCGC ttgaGGAtga GcttGCGtCC CATtA.GCTA
T. medium          aAA[redacted]GCT ACGGCtTCGC ttgaGGAtga GcttGCGtCC CATtA.GCTA
T. denticola 2     aAA[redacted]GCT ACGGCtCNNC ttcaGGAtgG GcCcGCGtCC CATtA.GCTA
T. denticola 1     aAA[redacted]GCT ACGGCtCCGC ttcaGGAtgG GcCcGCGtCC CATtA.GCTR
Isolate 68         aAA[redacted]GCT ACGGCtCCGC ttcaGGAtgG GcCcGCGtCC CATtA.GCTG
T. phagedenis      aAA[redacted]GCT GCGGCcNNGC ttgaGGAtga GcCcGCGtCC CATtA.GCTt
T. pallidum        aAA[redacted]GCT NCGGCcTCGC tcagaGAtga GcCtGCGaCC CATtA.GCTt
T. maltophilum     aAA[redacted]GCT AaGGCtTCGC taaaaGAatG GctcGCGgCC CATtAtGCTt
Isolate 64         gAA[redacted]GCT TCGGCtCCGC gacgGGAtcG GtCtGCGgCC CATcA.GCTG
Isolate 63         gAA[redacted]GCT TCGGCtCCGC gacgGGAtcG GtCtGCGgCC CATcA.GCTA
T. socranskii 2    gAA[redacted]GCT TCGGCtCCGC aacgGGAtcG GtCtGCGgCC CATcA.GCTG
T. socranskii 3    gAA[redacted]GCT TCGGCtCCGC gacgGGAtcG GtCtGCGgCC CATcA.GCTG
T. socranskii 1    gAA[redacted]GCT TCGGCtCCGC gccgGGAacG GtCtGCGgCC CATcA.GCTG
T. amylovorum      aAA[redacted]GCg TatGCtGCGC aggaGGAacG GttcGCGgCC tATcA.GCTA
T. succinifaciens  gAA[redacted]cCc ACGGggGCGC cggaGGAgcG GcCcGCGgCC CATcA.GCTG
        Consensus  -AA-G--GCT -CGGC-CCGC ----GGA--G G-C-GCG-CC CAT-A-GCTA
                                                                 *
```

```
                    251                                                 300
T. saccharophilum GttGGcGgGG TAAaNGCCCa CCaaGGCaAt GAcGGGTATC CGGCCTGAGA
T. pectinovorum   GttGGtGaGG aAAaGGCCCa CCaaGGCgAt GAcaGGTATC CGGCCTGAGA
T. bryantii       GttGGtGaGG TAAcGGCCCa CCaaGGCaAc GAtaGtTAcC CGGCCTaAGA
T. vincentii      GttGGtGaGG TAAaGGCCCa CCaaGGCgAc GAtGGGTATC CGGCCTGAGA
T. medium         GttGGtGaGG TAAaGGCCCa CCaaGGCgAc GAtGGGTATC CGGCCTGAGA
T. denticola 2    GttGGtGaGG TAAaGGCCCa CCaaGGCaAc GAtGGGTATC CGGCCTGAGA
T. denticola 1    GttGGtGaGG TAAaGGCCCa CCaaGGCaAc GAtGGGTATC CGGCCTGAGA
Isolate 68        GttGGtGaGG TAAaGGCCCa CCaaGGCaAc GAtGGGTATC CGGCCTGAGA
T. phagedenis     GttGGtGaGG TAAcGGCtta CCaaGGCtAc GtatGGNATC CGGCCTGAGA
T. pallidum       GttGGtGgGG TAAtGGCCta CCaaGGCgtc GAtGGGTATC CGaCCTGAGA
T. maltophilum    GttGGtGaGG TAAcGGCCCa CCaaGGCgAt GAtGGGTATC CGGCCTGAGA
Isolate 64        GacGGcGgGG TAAaAGCCCg CCgtGGCgAg GAcGGGTATC CGGCCTGAGA
Isolate 63        GacGGcGgGG TAAaAGCCCg CCgtGGCgAg GAcGGGTATC CGGCCTGAGA
T. socranskii 2   GacGGcGgGG TAAcGGCCCg CCgtGGCgAg GAcGGGTATC CGGCCTGAGA
T. socranskii 3   GacGGcGgGG TAAaGGCCCg CCgtGGCgAg GAcGGGTATC CGGCCTGAGA
T. socranskii 1   GacGGcGgGG TAAcGGCCCg CCgtGGCgAg GAcGGGTATC CGGCCTGAGA
T. amylovorum     GttGGcagGG TAAaGGCCta CCaaGGCgAc GAcGGGTATC CGGCCTGAGA
T. succinifaciens GtaGGcGgtG cAAgGGaCCa CCtaGGCtAc GAcGGGTAcC CGGCNTaAGA
        Consensus G--GG-G-GG TAA-GGCCC- CC--GGC-A- GA-GGGTATC CGGCCTGAGA
```

```
                    301                                                 350
T. saccharophilum GGGTGagCGG ACACATTGGG ACTGAGATAC GGCCCNgACT CCTACGGGAG
T. pectinovorum   GGGTGaaCGG ACACATTGGG ACTGAGATAC GGCCCNgACT CCTACGGGAG
T. bryantii       GGGTGatCGG gCACATTGGG ACTGAGATAC GGCCCAgACT CCTACGGGAG
T. vincentii      GGGTGatCGG ACACATTGGG ACTGAGATAC GGCCCAaACT CCTACGGGAG
T. medium         GGGTGatCGG ACACATTGGG ACTGAGATAC GGCCCAaACT CCTACGGGAG
T. denticola 2    GGGTGaaCGG ACACATTGGG ACTGAGATAC GGCCCAaACT CCTACGGGAG
T. denticola 1    GGGTGaaCGG ACACATTGGG ACTGAGATAC GGCCCAaACT CCTACGGGAG
Isolate 68        GGGTGaaCGG ACACATTGGG ACTGAGATAC GGCCCAaACT CCTACGGGAG
T. phagedenis     GGGTGgaCGG ACACATTGGG ACTGAGATAC GGCCCAaACT CCTACGGGAG
T. pallidum       GGGTGacCGG ACACAcTGGG ACTGAGATAC GGCCCAgACT CCTACGGGAG
T. maltophilum    GGGTGaaCGG ACACATTGGG ACTGAGATAC GGCCCAgACT tCTACGGGAG
Isolate 64        GGGCGgaCGG ACACATTGGG ACTGAGATAC GGCCCAgACT CCTACGGGAG
Isolate 63        GGGCGgaCGG ACACATTGGG ACTGAGATAC GGCCCAgACT CCTACGGGAG
T. socranskii 2   GGGCGgaCGG ACACATTGGG ACTGAGATAC GGCCCAgACT CCTACGGGAG
T. socranskii 3   GGGYGgaCGG ACACATTGGG ACTGAGATAC GGCCCAgACT CCTACGGGAG
T. socranskii 1   GGGCGgaCGG ACACATTGGG ACTGAGATAC GGCCCAgACT CCTACGGGAG
T. amylovorum     GGGTGgaCGG ACACATTGGG ACTGAGATAC GGCCCAgACT CCTACGGGAG
T. succinifaciens GGGCGgaCGG gCACATTGGG ACTGAGATAC GGCCCAgACT CNTACGGGAG
        Consensus GGGTG--CGG ACACATTGGG ACTGAGATAC GGCCCA-ACT CCTACGGGAG
```

```
                    351                                                 400
T. saccharophilum GCAGCAGcTA AGAATATTCC aCAATGGgcG aAAgCCTNAt GGAGCcACGC
T. pectinovorum   GCAGCAGcTA AGAATATTCC GCAATGGacG aAAgTCTGAC GGAGCaACGC
T. bryantii       GCAGCAGcTA AGAATATTCC GCAATGGacG aAAgTCTGAC GGAGCGACGC
T. vincentii      GCAGCAGcTA AGAATATTCC GCAATGGacG gAAgTCTGAC GGAGCGACGC
T. medium         GCAGCAGcTA AGAATATTCC GCAATGGacG gAAgTCTGAC GGAGCGACGC
T. denticola 2    GCAGCAGcTA AGAATcTTCC GCAATGGacG aAAgTCTGAC GGAGCGACGC
T. denticola 1    GCAGCAGcTA AGAATcTTCC GCAATGGacG aAAgTCTGAC GGAGCGACGC
Isolate 68        GCAGCAGcTA AGAATcTTCC GCAATGGacG aAAgTCTGAC GGAGCGACGC
T. phagedenis     GCAGCAGcTA AGAATATTCC GCAATGGacG gAAgTCTGAC GGAGCGACGC
T. pallidum       GCAGCAGcTA AGAATATTCC GCAATGGgcG aAAgCCTGAC GGAGCGACaC
T. maltophilum    GCAGCAGtTA AGAATATTCC GCAATGGacG aAAgTCTGAC GGAGCGACGC
Isolate 64        GCAGCAGgTA AGAATATTCC GCAATGGggG aAAcCCTGAC GGAGCGACGC
Isolate 63        GCAGCAGgTA AGAATATTCC GCAATGGggG aAAcCCTGAC GGAGCGACGC
T. socranskii 2   GCAGCAGgTA AGAATATTCC GCAATGGggG aAAcCCTGAC GGAGCGACGC
T. socranskii 3   GCAGCAGgTA AGAATATTCC GCAATGGggG gAAcCCTGAC GGAGCGACGC
T. socranskii 1   GCAGCAGgTA AGAATATTCC GCAATGGggG gAAcCCTGAC GGAGCGACGC
T. amylovorum     GCAGCAGcTA AGAATATTCC GCAATGGggG gAAcCCTGAC GGAGCGACGC
T. succinifaciens GCAGCAGcTA AGAATATTCC GCAATGGggG gAAcNNTGAC GGAGCGACGC
        Consensus GCAGCAG-TA AGAATATTCC GCAATGG--G -AA-TCTGAC GGAGCGACGC
```

```
                    401                                                 450
T. saccharophilum CGCGTGgAcG AtGAAGGCCG NAAGGTTGTA AAgTcCTTTa gcaagtGAgG
T. pectinovorum   CGCGTGgAtG AtGAAGGtCG GAAGaTTGTA AAaTcCTTTT attattGAaG
T. bryantii       CGCGTGgAtG AtGAAGGCCG GAAGGTTGTA AAaTcCTTTT atgactGAgG
T. vincentii      CGCGTGgAtG AaGAAGGCtG AAAaGTTGTA AAaTcCTTTT gttgatGAaG
T. medium         CGCGTGgAtG AaGAAGGCtG AAAaGTTGTA AAaTcCTTTT gttgatGAaG
T. denticola 2    CGtGTGaAtG AaGAAGGCCG AAAGGTTGTA AAaTtCTTTT gcagatGAaG
T. denticola 1    CGtGTGaAtG AaGAAGGCCG AAAGGTTGTA AAaTtCTTTT gcagatGAaG
Isolate 68        CGtGTGaAtG AaGAAGGCCG AAAGGTTGTA AAaTtCTTTT gcagatGAaG
T. phagedenis     CGCGTGgAcG AaGAAGGCCG AAAGGTTGTA AAgTtCTTTT gccgatGAaG
T. pallidum       CGCGTGgAtG AgGAAGGtCG AAAGaTTGTA AAgTtCTTTT gccgacGAaG
T. maltophilum    CGCGTGgAtG AaGAAtGCCG AAAGGTTGTA AAaTcCTTTT aagcctGAtG
Isolate 64        CGCGTGaAcG AaGAAGGCCG GAAGGTTGTA AAgTtCTTTT ctgtccGAgG
Isolate 63        CGCGTGaAcG AaGAAGGCCG GAAGGTTGTA AAgTtCTTTT ctgtccGAgG
T. socranskii 2   CGCGTGaAcG AaGAAGGCCG GAAGGTTGTA AAgTtCTTTT ctgtccGAgG
T. socranskii 3   CGCGTGaAcG AaGAAGGCCG GAAGGTTGTA AAgTtCTTTT ctgtccGAgG
T. socranskii 1   CGCGTGaAcG AaGAAGGCCG GAAGGTTGTA AAgTtCTTTT ctgtccGAgG
T. amylovorum     CGCGTGgAcG AtGAAGGtCG GAAGaTTGTA AAgTcCTTTT atacatGAaG
T. succinifaciens CGCGTGggcG AgGAAGGCCG GAAGGTTGTA AAgccCTTTT gcgcgcGAgG
        Consensus CGCGTG-A-G A-GAAGGCCG GAAGGTTGTA AA-T-CTTTT ------GA-G
```

```
                  451                                                  500
T. saccharophilum AATAAGcggg acAGGgAATG gtctcgtGgT GACtGTAG.c ttgaGAATAA
T. pectinovorum   AATNAactgt acAGGgAATG gtatagaGgT GACaGTAG.g taatGAATAA
T. bryantii       AtaAgcagag tGAGagAAac gctttgtGgT GACtGTAG.g tcatGAATAA
T. vincentii      AATAAGggtg aGAGGgAATG ctcatctGaT GACgGTAatc ga.cGAATAA
T. medium         AATAAGggtg aGAGGgAATG ctcatctGaT GACgGTAatc ga.cGAATAA
T. denticola 2    AATAAGaaga aGAGGgAATG cttctttGaT GACgGTAGtc atgcGAATAA
T. denticola 1    AATAAGaaga aGAGGgAATG cttctttGaT GACgGTAGtc atgcG.....
Isolate 68        AATAAGaaga aGAGGgAATG cttctttGaT GA..... .....
T. phagedenis     AATAAGagga tGAGGgAATG cgtccttGaT GACgGTAGtc gagcGAATAA
T. pallidum       AATGAGgacg gGAGGgAATG cccgtttGaT GACgGTAGtc gtgcGAATAA
T. maltophilum    AATAAGcaca atAGGgAATG attgtgcGgT GACcGTAG.g gcttGAATAA
Isolate 64        AATAAGtgtt gGAGGaAATG ccgacatGgT GACgGTAG.g gcagGAATAA
Isolate 63        AATAAGtgtt gGAGGaAATG ccgacatGgT GACgGTAG.g gcagGAATAA
T. socranskii 2   AATAAGtgtt gGAGGaAATG ccgacatGgT GACgGTAG.g gcagGAATAA
T. socranskii 3   AATAAGttty gGAGGaAATG ccgggacGgT GA..... .....
T. socranskii 1   AATAAGtgta gGAGGaAATG cctgcatGgT GACgGTAG.g acagGAATAA
T. amylovorum     AAgAAGctaa gaAGGaAATG gaatagcGgT GACtGTAG.t gtatGAATAA
T. succinifaciens AATNAGggga gGAGGgAATG ccttcccGgT GACtGTAG.c gcgcGAATAA
        Consensus AATAAG---- -GAGG-AATG -------G-T GAC-GTAG-- ----GAATAA
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                  501                                          540
T. saccharophilum GggtCGGCTA Attacgtgcc agcagccgcg gtnacacgta
T. pectinovorum   GcacCGGCTN Attacgtgcc agcagccgcg gtnacacgta
T. bryantii       GcaaCGGCTA Attacgtgcc agcagccgcg gtaacacgta
T. vincentii      GcccCGGCTA Attac..... .......... ..........
T. medium         GcccCGGCTA Attacgtgcc agcagccgcg gtaacacgta
T. denticola 2    GcccCGGCTA Attacgtgcc agcagccgcg gtaacacgta
T. denticola 1    .......... .......... .......... ..........
Isolate 68        .......... .......... .......... ..........
T. phagedenis     GcccCGGCTA Attacgtgcc agcagccgcg gtaacacgta
T. pallidum       GcccCGGCTA Attacgtgcc agcagccgcg gtaacacgta
T. maltophilum    GcacCGGCTA Attacgtgcc agcagccgcg gtaacacgta
Isolate 64        GcacCGGCTA Attacgtgcc .......... ..........
Isolate 63        GcacCGGCTA Attacgtgcc .......... ..........
T. socranskii 2   .......... .......... .......... ..........
T. socranskii 3   .......... .......... .......... ..........
T. socranskii 1   GcacCGGCTA A......... .......... ..........
T. amylovorum     GcacCGGCTA Attacgtgcc agcagccgcg gtaacacgta
T. succinifaciens GcgcCGGCTA Attacgtgcc agcagccgcg gtnacacgta
        Consensus G---CGGCTA A--------- ---------- ----------
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